*chl*B Requirement for Chlorophyll Biosynthesis under Short Photoperiod in *Marchantia polymorpha* L.

Minoru Ueda[1], Ayumi Tanaka[2], Kazuhiko Sugimoto[1], Toshiharu Shikanai[1], and Yoshiki Nishimura[1,*]

[1]Department of Botany, Graduate School of Science, Kyoto University, Japan

[2]Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan

*Corresponding author: E-mail: yoshiki@pmg.bot.kyoto-u.ac.jp.

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Data deposition: The nucleotide sequence data reported in this article have been deposited at the DDBJ/EMBL/GenBank database under the accessions *MpCHL27*, AB889743; *MpFLU,* AB889744; *MpHEMA*, AB889745; *MpLPOR*, AB889746, and *MpDELLA1,* AB889747.

Abstract

Chlorophylls (Chls) play pivotal roles in energy absorption and transduction and also in charge separation in reaction centers in all photosynthetic organisms. In Chl biosynthesis steps, only a step for the enzymatic reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) is mediated by both nuclear- and chloroplast-encoded genes in land plants. Many plants encode the genes for light-dependent Pchlide reductase (LPOR) and light-independent Pchlide reductase (DPOR) in the nucleus and chloroplast genome, respectively. During the diversification of land plants, the reduction step of Pchlide to Chlide has become solely dependent on LPOR, and the genes for DPOR have been lost from chloroplast genome. It remains unclear why DPOR persists in some land plants, how they were eliminated from chloroplast genomes during the diversification of land plants, and under what environmental conditions DPOR was required. We demonstrate that DPOR is functional in liverwort (*Marchantia polymorpha* L.) and plays an important role in Chl biosynthesis. Having established a plastid transformation system in liverwort, we disrupted *chl*B, which encodes a subunit of DPOR in the *M. polymorpha* chloroplast genome. Morphological and Chl content analysis of a *chl*B mutant grown under different photoperiods revealed that DPOR is particularly required for Chl biosynthesis under short-day conditions. Our findings suggest that an environmental condition in the form of photoperiod is an important factor that determines the loss or retention of chloroplast-encoded genes mediating Pchlide reduction to Chlide.

Key words: plastid transformation, chloroplast genome evolution, bryophyte, chlorophyll metabolism.

Introduction

Chloroplasts are the main site for photosynthesis and are considered to be descendants of cyanobacteria that were engulfed by an ancestral eukaryotic cell. After this engulfment event, cyanobacterial genes were either lost or transferred into the nuclear genome. Alternatively, genes of different origin or de novo genes in the nuclear genome replaced organelle-encoded genes (Mereschkowski 1905; Gray 1992; Martin and Kowallik 1999; Ueda and Kadowaki 2012). In this study, we focused on genes involved in chlorophyll (Chl) synthesis to elucidate the driving force of chloroplast genome evolution.

Chls play pivotal roles in energy absorption and transduction and also in charge separation in reaction centers. In angiosperms represented by *Arabidopsis thaliana*, there are 15 steps in the pathway from glutamyl-tRNA to Chls *a* and *b*, and the genes involved in these steps have been identified (Beale 2005; Nagata et al. 2005). Among them, Pchlide oxidoreductase, which is responsible for the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide), is characteristic, given that genes for the enzymes are encoded in both nuclear and chloroplast genomes in most plants (Armstrong 1998; Schoefs and Franck 2003).

There are two distinct Pchlide oxidoreductases: light-dependent NADPH-Pchlide oxidoreductase (LPOR), which requires photoenergy for catalysis and is encoded in the nucleus, and light-independent Pchlide oxidoreductase (DPOR), which requires no photoenergy and whose subunits are encoded in the chloroplast genome. LPOR is a monomeric protein, whereas DPOR is a multimeric protein consisting of

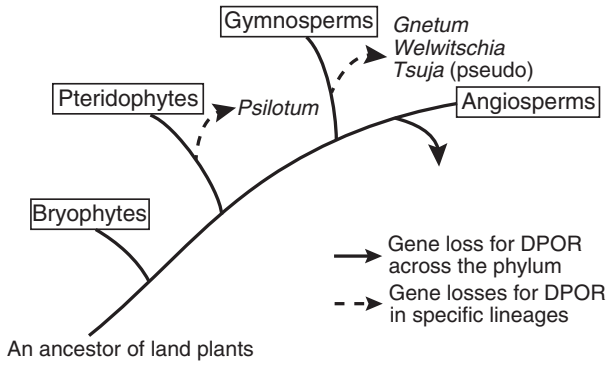

Fig. 1.—Schematic diagram illustrating the distribution of DPOR in land plants. The distribution of DPOR among land plant chloroplast genomes is described by Armstrong (1998), Wicke et al. (2011), and Wu et al. (2013). *Tsuja* species have lost the greening ability in darkness, although they appear to have conventional open reading frames for genes of DPOR subunits (Kusumi et al. 2006).

three subunits encoded by the *chl*B, *chl*L, and *chl*N genes (Reinbothe et al. 2010).

DPOR has been lost and the reduction step of Pchlide to Chlide has become solely dependent on LPOR in angiosperms, several gymnosperms, and some Pteridophytes (fig. 1). Based on LPOR and DPOR distribution in the plant, eubacterial, and archaebacterial kingdoms and the oxygen sensitivity of DPOR, a model has been proposed to explain the gene transfer of LPOR and the substitution of LPOR for DPOR during land plant evolution: 1) oxygen-sensitive DPOR initially emerged when the atmosphere of Archaean Earth was anaerobic, presumably from nitrogenase-like genes (Fujita et al. 1991) and 2) LPOR, which is an oxygen-insensitive and light- and NADPH-dependent enzyme belonging to a short-chain dehydrogenase/reductase superfamily, evolved during the transition from an anaerobic to aerobic atmosphere (Reinbothe et al. 1996, 2010). Both enzymes seem to have emerged before the endosymbiosis of cyanobacteria, which are believed to be the ancestors of chloroplasts, given that Chlides are biosynthesized using both DPOR and LPOR in modern cyanobacteria (Schoefs and Franck 2003). 3) Upon the establishment of chloroplasts, it is likely that LPOR was transferred to the nucleus, whereas the genes for DPOR were retained in chloroplast genomes. Despite their apparent functional redundancy, both genes have been strictly retained in several algae, lower plants, and gymnosperms. 4) As a final step of "gene replacement," DPOR loss independently occurred in land plants.

Why are LPOR and DPOR preserved together in a large number of plants, and what was the force driving the loss of DPOR during the evolution of land plants? Previous studies suggest that DPOR plays a key role in greening of algae and vascular plants under heterotrophic growth conditions, such as in chemoheterotrophic-grown *Leptolyngbya boryana* (formerly *Plectonema boryanum*), dark-grown *Chlamydomonas reinhardii* cell cultures, and dark-grown gymnosperm seedlings (Suzuki and Bauer 1992; Li et al. 1993; Fujita et al. 1996; Armstrong 1998). These reports suggest that the light environment is one of the critical factors that determine the fate of DPOR.

The liverwort *Marchantia polymorpha* L. may be a key organism for elucidating the aforementioned questions. For studying the early evolution of land plants, particularly the water-to-land transition that dramatically changed the light environment, *M. polymorpha* is emerging as a premier model system because it is considered to be the earliest lineage of land plants derived from green algal ancestors. In particular, liverworts occupy a critical position in land plant evolution, forming the sister group to all extant land plants (Crandall-Stotler et al. 2008).

Gene losses from the chloroplast genome have repeatedly occurred even after the divergence of land plants (Martin et al. 2002; Jansen et al. 2007; Wicke et al. 2011). The *Marchantia* chloroplast genome has the largest number of genes of all land plants (Ohyama et al. 1986; Martin et al. 2002; Ohyama et al. 2009), suggesting that liverworts retain ancestral characters in terms of the gene content of chloroplast genome. This peculiarity raises the possibility of identifying roles of genes encoded in the liverwort chloroplast genome, that is, genes for DPOR in this study, which have been lost from the chloroplast genomes of other land plants.

Liverworts possess both LPOR and DPOR (Suzuki et al. 2001). Having established the plastid transformation system in *M. polymorpha* (Chiyoda et al. 2007; Ueda et al. 2012), we prepared a DPOR-deficient mutant (a *chl*B knockout mutant). We obtained evidence that Chl biosynthesis operates in the dark through DPOR in *M. polymorpha*. Mutant analysis revealed that DPOR is still required for Chl biosynthesis in *M. polymorpha*, particularly under short-day conditions. Our results suggest that DPOR is required for at least *M. polymorpha* to adapt to environmental conditions (photoperiod) that may lead to the persistence of DPOR in the *M. polymorpha* plastid genome. The establishment of a regulation system for Chl biosynthesis without DPOR during the evolution of land plants is discussed, with reference to the comparison of genes, involving the suppression of Chl biosynthesis between *A. thaliana* and *M. polymorpha*.

Materials and Methods

Plant Materials and Growth Conditions

Male accession Takaragaike-1 (Tak1) and female accession Takaragaike-2 (Tak2) liverworts (*M. polymorpha* L.) were asexually maintained and propagated via gemmae as described previously (Chiyoda et al. 2008). Plants were usually grown on 1/2 Gamborg's B5 media, containing 1% sucrose in a

growth chamber at 20 °C under continuous light. Fourteen-day-old plants grown under a 14-h light (L) and 10-h dark (D) cycle (70–90 μmol photons $m^{-2}s^{-1}$) at 20 °C were used for immunoblotting. To monitor morphological difference and measure Chl content and Pchlide under different photoperiod, plants were grown in a Biomulti incubator with cold cathode fluorescent lamps (170–200 μmol photons $m^{-2}s^{-1}$) at 20 °C for 2 weeks (Nihon-ika, Osaka, Japan).

Cloning of a Plastid Genome Fragment Containing *chl*B for Plastid Transformation

Total DNA was isolated from thalli of plants using Isoplant II (Nippon gene, Tokyo, Japan). Genomic polymerase chain reaction (PCR) to amplify the *chl*B fragment for the preparation of the plastid transformation vector was performed using KOD-plus-Neo DNA polymerase (TOYOBO, Osaka, Japan) with P1 and P2 primers. All the primers used in this article are shown in supplementary tables S1 and S2, Supplementary Material online. The resulting products were cloned into the pTAC-2 vector (BioDynamics Laboratory, Tokyo, Japan) after adenosine addition, according to the manufacturer's instructions, and the clones were sequenced using universal or randomly designed primers. The *aadA* cassette, which confers spectinomycin resistance to chloroplasts (Shikanai et al. 1998), was inserted into the *Bcl*I site present in the *chl*B exon, so that the gene was inactivated (fig. 2*A*). The resulting plasmid (pCS*chl*B vector) was sequenced again and digested with *Not*I to linearize them for plastid transformation.

Plastid Transformation to Knock Out *chl*B

Spores used for plastid transformation were prepared as described by Chiyoda et al. (2008). Plastids were transformed using 7-day-old sporelings, as described previously (Chiyoda et al. 2007; Ueda et al. 2012). Homoplasmic transplastomic lines were obtained by asexual reproduction via gemmae on selective 0-M51C agar medium (Takenaka et al. 2000), containing 300 μg/ml spectinomycin without sucrose, for 3 months. The consistent difference in Chl and Pchlide accumulation between the *chl*B_5 and *chl*B_6 mutants that are plastid transformants responsive to day length may be because of genetic differences between male (Tak-1) and female (Tak-2) lines in *M. polymorpha*. This is because *M. polymorpha* is a dioecious plant and has sex chromosomes from which it is difficult to prepare genetically pure lines.

Genotyping of Transplastomic Plants

Total DNA was isolated from thalli using conventional methods and diluted in 200 μl of Tris–EDTA Buffer (10 mM Tris–HCl [pH 8.0], 1 mM ethylenediaminetetraacetic acid [pH 8.0]). To find homotransplastomic plants, 1 μl of the extract was used for a genomic PCR with primers P3 and P4. Primer positions and primer combinations for genotyping both types are shown in figure 2*A*. PCR was performed with KOD-FX DNA polymerase (TOYOBO).

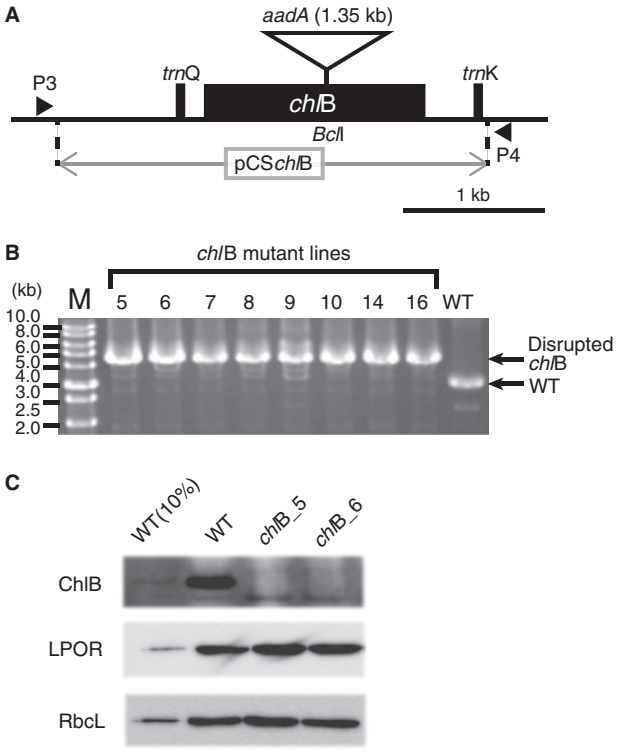

Fig. 2.—Preparation of *chl*B mutant in *Marchantia polymorpha*. (*A*) Gene structure around *chl*B in the *M. polymorpha* chloroplast genome. Black boxes and horizontal line indicate exons and intergenic region, respectively. Solid triangles show primer positions designed for the confirmation of the *aad*A cassette insertion. A restriction enzyme (*Bcl*I) site within the *chl*B coding sequences shown with a triangle is the position of the *aad*A cassette insertion. Black angles and the triangle are not to scale. (*B*) Genotyping for the confirmation of homoplasmic transplastome in the *chl*B mutant. P3 and P4 primer pairs detected WT plastome and/or transplastome defective in the *chl*B (3,165 bp in WT and 4,520 bp in *chl*B mutant). (*C*) Immunoblotting analysis of ChlB protein accumulation. Samples containing 0.5 μg chlorophyll (Chl) crude chloroplast fraction proteins were loaded. In the case of WT, 0.05 μg Chl crude chloroplast fraction proteins (10% WT) were loaded to confirm the limit of detection by each antibody. The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the blots were probed with specific antibodies against ChlB (a subunit of DPOR), LPOR, and rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit).

Chl Measurement

Extraction and measurement of Chl contents were performed according to the method by Arnon (1949). A sample for measurement of Chl contents was extracted from frozen thalli ground to a fine powder with a mortar and pestle in liquid nitrogen. The average value for each experiment was obtained from four independent samples.

Immunoblot Analysis

Crude chloroplast proteins were loaded onto a sodium dodecyl sulfate–polyacrylamide gel on an equal Chl basis. Samples of 0.5 μg Chl proteins from the crude chloroplast fraction were used for immunoblotting. Immunoblotting was performed with anti-ChlB (Fujita et al. 1996), anti-RbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) (Agrisera, Vännäs, Sweden), and anti-L-POR (Agrisera) antibodies. Signals were detected with an ECL Plus Western Blotting Detection Kit (GE Healthcare UK Ltd, Buckinghamshire, UK) and visualized with an LAS3000 chemiluminescence analyzer (Fuji Film, Tokyo, Japan). The results presented represent two independent experiments.

Pchlide Measurement

Pchlide measurement was performed by high pressure liquid chromatography (HPLC), according to Nagata et al. (2007). Plants grown under different light/dark cycles were frozen with liquid nitrogen at end of light and dark periods, respectively. Sampling was performed in a darkroom with safety light (Nagatani laboratory, Kyoto Univ.).

Database Analysis in *M. polymorpha*

All similarity searches were conducted using the default parameters of the stand-alone Basic Local Alignment Search Tool (Blast) available through the National Center for Biotechnology Information as described in the previous study (Ueda et al. 2012).

Quantitative Reverse-Transcription PCR

The transcript levels were assayed using the Mx30000p qPCR instrument (Stratagene, La Jolla, CA) and FastStart Universal SYBR Green Master (Rox) reaction mix (Roche Diagnostics GmbH, Mannheim, Germany). The error bars show the standard deviations calculated from three repeated experiments. To standardize the data, the ratio of the absolute transcript level of each gene to the absolute transcript level of elongation factor 1-a (*EF-1a*) was calculated for each sample. The transcript abundance of genes is shown as the expression relative to the wild-type (WT) plant. The primer sequences and scores in quantitative reverse-transcription PCR (qRT-PCR) experiments are listed in supplementary tables S2 and S3, Supplementary Material online.

Results

Knockout of the Plastid *chl*B Gene in *M.* polymorpha

To determine whether *chl*B is functional in *M. polymorpha*, we took advantage of the established technique of plastid transformation to knock out the gene (Chiyoda et al. 2007; Ueda et al. 2012). The pCS*chl*B vector encodes a 3.0-kb region including *trn*Q, *chl*B, and *trn*K genes cloned from the *M. polymorpha* plastid genome, and *chl*B was disrupted by the insertion of a chimeric *aadA* cassette (fig. 2*A*). To confirm the homoplasmic state of the genome, in which *chl*B had completely segregated out, the chloroplast DNA was analyzed using genomic PCR. The primer pair of P3 and P4 distinguishes transplastomic DNA from WT DNA. The 4.5-kb fragment that originated in the transplastomic copy was preferentially amplified, and the 3.2-kb fragment that originated in the WT copy was not detected (fig. 2*B*), indicating that *chl*B was knocked out in the mutant lines.

To evaluate the impact of the *chl*B knockout on protein accumulation, a protein blot of crude chloroplast extracts from WT plants and two independent *chl*B mutant lines was probed with antisera against ChlB, LPOR, and RbcL. Consistent with the knockout of the gene (fig. 2*B*), ChlB protein was below the detection limit in the *chl*B mutants. The absence of ChlB did not affect LPOR accumulation (fig. 2*C*).

*chl*B Contributes to Chl Synthesis Particularly under Short-Day Conditions

The *chl*B mutant showed no visible phenotype under continuous light conditions (fig. 3*A*). DPOR most likely functions in the dark because of the inhibition of its enzymatic activity by oxygen produced in photosystem II in the light (Armstrong 1998; Reinbothe et al. 2010). In a continuous-light condition, DPOR may be inactive and Chl synthesis does not depend on *chl*B in *M. polymorpha*. We accordingly grew the *chl*B mutant under different day-length conditions and observed its morphological variation. An obvious morphological phenotype (pale green and slow growth) was observed in the *chl*B mutants, particularly when they were grown under 10-h L/14-h D or 8-h L/16-h D conditions (fig. 3*A*).

Consistent with the morphological phenotype, Chl *a* accumulation in the *chl*B mutant was drastically lower in short-day conditions than in the WT; Chl *a* accumulation was reduced to 37.8% (*chl*B_5 mutant line) and 44.6% (*chl*B_6 mutant line) in *chl*B mutant lines compared with WT plants, when they were grown under a 8-h L/16-h D cycle (fig. 3*B* and *C*). In contrast to short-day conditions, drastic differences in Chl *a* accumulation between the *chl*B mutant and WT plants were not observed under long-day periods. *chl*B mutants showed Chl *a* accumulations of 74.3% (*chl*B_5 mutant line) and 84.7% (*chl*B_6 mutant line) under a 16-h L/8-h D cycle and 93.2% (*chl*B_5 mutant line) and 98.8% (*chl*B_6 mutant line) under continuous light compared with the respective WT plants (fig. 3*B* and *C*).

These results suggest that the *M. polymorpha* chloroplast genome retains DPOR as necessary for Chl biosynthesis, particularly under short-day conditions.

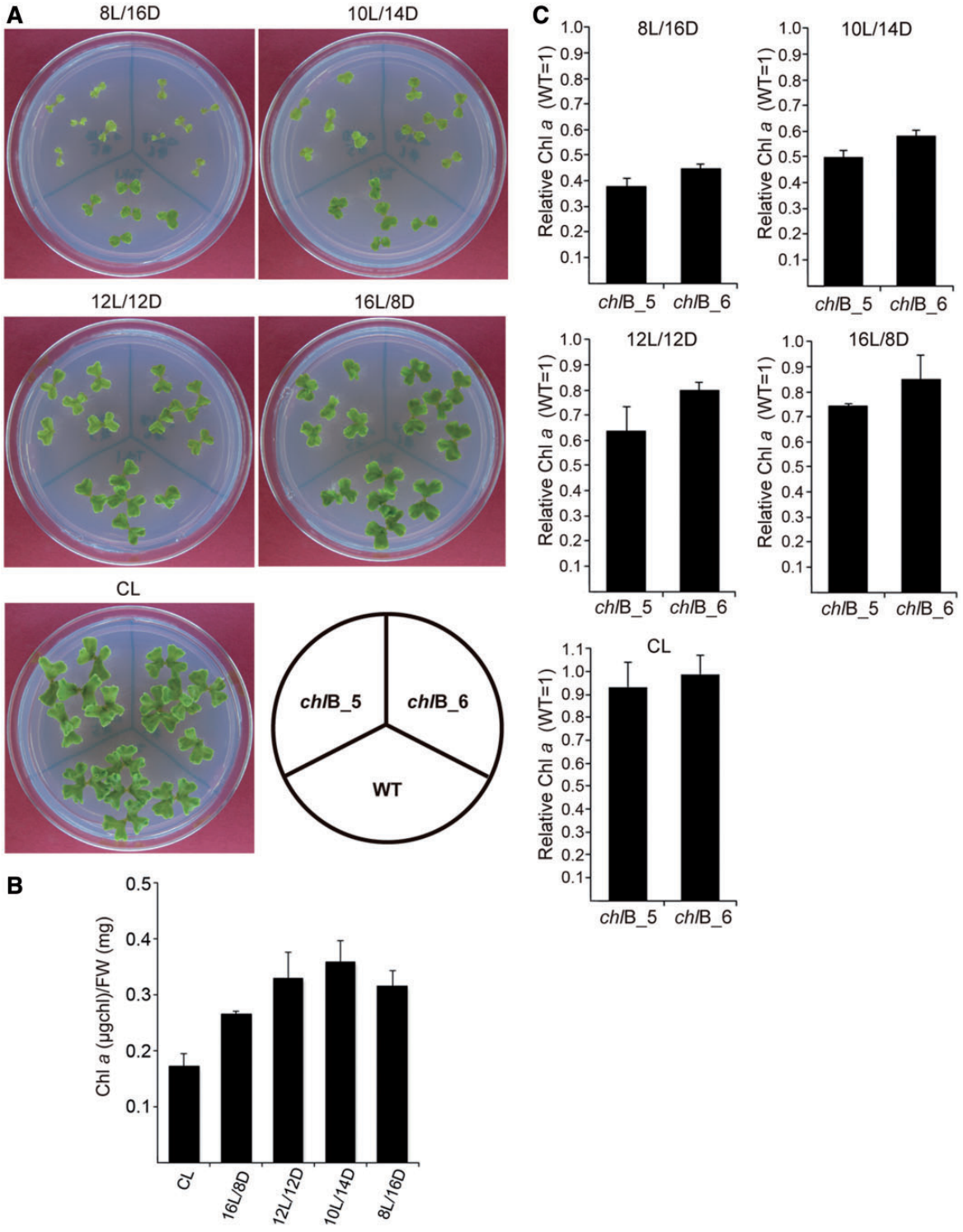

Fig. 3.—Morphological observation and comparison of Chl content in *chl*B mutants grown under different day-length conditions. (*A*) Morphological difference between WT plants and *chl*B mutants grown in plastic petri dish (9 cm diameter). Plants were grown on 1/2 Gamborg's B5 media without sucrose in a Biomulti incubator with cold cathode fluorescent lamps (170–200 μmol photons $m^{-2} s^{-1}$) at 20 °C (Nihon-ika, Osaka, Japan). (*B*) Quantification of Chl *a* content in WT plants under different day-length conditions. Chl *a* contents (μg of Chl per fresh weight mg) were measured according to Arnon (1949). Two-week-old plants were used under different day-length conditions in these experiments. Standard deviations ($n = 3$) are indicated by lines extending from the bars. (*C*) Levels of Chl *a* accumulation in the *chl*B mutants. Standard deviations ($n = 3$) are indicated by lines extending from the bars. Each mean represents the ratio of Chl *a* accumulation level in the *chl*B mutants to that of WT plants.

Table 1
Pchlide Accumulation in Plants Grown under Different Day-Length Conditions at End of Light and Dark Periods

Day Length	Plant Materials	MV-Pchlide/Chl a
Continuous light	WT	0
	*chl*B_5	0
	*chl*B_6	0
16-h light/8-h dark (16L/8D) EL	WT	0
	*chl*B_5	0
	*chl*B_6	0
16L/8D ED	WT	0
	*chl*B_5	0.0030
	*chl*B_6	0.0027
8-h light/16-h dark (8L/16D) EL	WT	0
	*chl*B_5	0
	*chl*B_6	0
8L/16D ED	WT	0
	*chl*B_5	0.015
	*chl*B_6	0.017

NOTE.—The results presented represent three independent experiments. Pchlide consists of a mixture of MV and divinyl forms. We measured MV-Pchlide at ends of light and dark periods under different light cycles. EL, end of light period; ED, end of dark period.

The *chl*B Mutant Accumulates Monovinyl-Pchlide a in the Dark

DPOR mediates the reduction of Pchlide (a phototoxic Chl precursor) to Chlide in the dark. The *chl*B mutant may accumulate Pchlide in the dark. To test this possibility, the ratio of Pchlide to Chl *a* was evaluated using HPLC at the ends of both light and dark periods in plants grown under different photoperiods. Pchlide consists of a mixture of monovinyl (MV) and divinyl forms. We measured MV-Pchlide *a*.

At the end of the light period, no MV-Pchlide *a* accumulation was observed in either WT plants or the *chl*B mutant under any light/dark cycle condition (table 1), suggesting that LPOR primarily reduces Pchlide to Chlide in the light. In contrast, at the end of the dark period, MV-Pchlide *a* accumulation in the *chl*B mutant was detected (table 1). WT plants did not accumulate MV-Pchlide *a*, suggesting that DPOR-dependent reduction of MV-Pchlide *a* operates in the dark (table 1). MV-Pchlide *a* levels were approximately 5-fold higher under 8-h L/16-h D than in 16-h L/8-h D plants (table 1). Chl *a* content in plants grown under an 8-h L/16-h D cycle accumulates to at least 1.2-fold relative to those grown under a 16-h L/8-h D cycle (fig. 3*B*), suggesting that MV-Pchlide *a* accumulation increased with longer dark period from a 16-h L/8-h D to a 8-h L/16-h D cycle.

The reduction in Chl *a* content and MV-Pchlide *a* accumulation in the dark suggests that DPOR is functional and contributes to Chl biosynthesis in the dark and that its function is particularly important under short-day conditions in *M. polymorpha*.

DPOR-Deficiency Influenced Interconnection of Regulatory Control Circuits for the Chl Biosynthesis Pathways

There are negative and positive feedback regulations in Chl synthesis to avoid photo-oxidative damage caused by Chl intermediates in plants (fig. 4*A*, see Discussion). To check the impact of DPOR deficiency on the feedback regulations in mRNA level, we measured the relative transcript abundance of five genes (*MpHEMA*, *MpDELLA*, *MpFLU*, *MpCHL27*, and *MpLPOR*) involved in Chl biosynthesis in the WT plant and the *chl*B mutants grown under an 8-h L/16-h D cycle.

Gene expression of *MpDELLA* was decreased in the *chl*B mutants at the end of the light and dark conditions (fig. 4*B* and *C*). In addition, gene expression of *MpLPOR* was mildly reduced only in darkness in the *chl*B mutants (fig. 4*C*). These results suggest that DPOR deficiency influenced the regulatory control circuits for the Chl biosynthesis pathway or at least the mRNA expression level in both dark and light periods under short-day conditions.

Discussion

Plants accumulating free tetrapyrroles are particularly prone to photo-oxidative damage. Chl intermediates cause photo-oxidative damage to the cell. In particular, free Pchlide acts as a strong photosensitizer and produces singlet oxygen, leading to cell death (Kim et al. 2008). Tight control of this pathway is essential for survival, particularly for land plants that must tolerate intense and rapidly fluctuating light environments, which may have been the basis for the evolution of several different mechanisms that achieve this goal in land plants.

In our study, *M. polymorpha*, lacking DPOR, showed pale green and slow growth phenotypes under the 8-h L/16-h D condition, probably due to aberrant Pchlide accumulation during the dark period (fig. 3; table 1). This result indicates that DPOR in *M. polymorpha* plays an important role in Pchlide reduction and prevents Pchlide accumulation under short-day conditions. This behavior may have been one of the major driving forces for DPOR retention in the of *M. polymorpha* chloroplast genome. In general, bryophytes, including *M. polymorpha,* prefer shady conditions (Glime 2007). From an ecological perspective, bryophytes occupy light-limited regions (short-day conditions) such as the Arctic and show a pattern of increasing abundance with increasing latitude (Vitt and Pakarinen 1977; Jagerbrand et al. 2006). DPOR retention may also be important for *M. polymorpha* to adapt to these natural habitats.

One of the interesting aspects of the genus *Marchantia* is that it grows all over the world from tropical to polar regions. Therefore, in the future, it would be possible to dissect their strategies to adapt to various ecological environments by analyzing various ecotypes of *Marchantia*. At present, chloroplast genome sequences are only available for Japanese isolates. Further analysis of chloroplast genomes from various

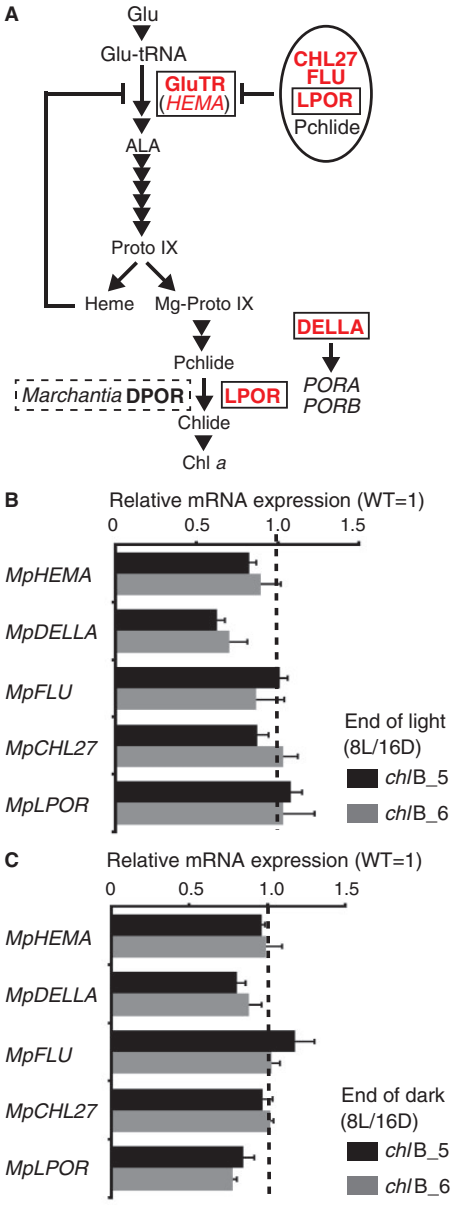

Fig. 4.—Influence of DPOR deficiency on interconnection of regulatory control circuits for the Chl biosynthesis pathways. (*A*) Simplified schematic representation of regulation in Chl biosynthesis steps in *Arabidopsis thaliana*. Genes duplicated during the diversification of land plants are surrounded by a box. Genes involving the regulation of Chl biosynthesis in the dark are shown in red letters. Their gene IDs are listed in table 2. *LPORA* and *LPORB* expressions are positively regulated in darkness. DPOR is encoded in the *Marchantia polymorpha* chloroplast genome but not in *A. thaliana*. FLU bound to the CHL27–POR–Pchlide complex is able to inhibit glutamyl-tRNA reductase (GluTR) activity. (*B*) The levels of transcripts in the *chl*B mutants at the end of the light period. (*C*) The levels of transcripts in the *chl*B mutants at end of the dark period. The standard deviations ($n = 3$, *n* stands for technical replicates) are indicated by lines extending from the bars. Each mean represents the ratio of the expression level of transcripts in the *chl*B mutants compared with that of WT plants. The means are depicted by black and gray bars for the *chl*B_5 and the *chl*B_6 mutant lines, respectively.

Table 2

Homologous Genes Involved in Regulation of Chl Biosynthesis in the Dark in *Marchantia polymorpha*

Gene Name in *Arabidopsis thaliana*	Gene ID in *A. thaliana*	Gene ID of Homologs in *M. polymorpha*
CHL27	At3g56940	AB889743
FLU	At3g14110	AB889744
HEMA	At1g58290 (HEMA1)	AB889745
	At1g09940 (HEMA2)	
LPOR	At5g54190 (PORA)	AB889746
	At4g27440 (PORB)	
	At1g03630(PORC)	
DELLA	At2g01570 (RGA)	AB889747
	At1g14920 (GAI)	
	At1g66350 (RGL1)	
	At3g03450 (RGL2)	
	At5g17490 (RGL3)	

Note.—Gene IDs of LPOR and DELLA in *A. thaliana* refer to Cheminant et al. (2011).

ecotypes, particularly focusing on *chl*B, *chl*L, and *chl*N, may shed light on their adaptational strategies to various light environments. In particular, drought-tolerant bryophytes could survive in sunny locations, where DPOR requirement would be less, suggesting the possibility that *chl*B, *chl*L, and *chl*N genes on their chloroplast genomes may initiate pseudogenization if day length is the main selection pressure for this gene disposition. The molecular evolutionary analysis of genes for DPOR subunits on their chloroplast genomes may prove our hypothesis presented in this study.

In contrast, it is likely that land plants, including angiosperms, several gymnosperms, and some Pteridophytes have established additional and more elaborate regulatory systems for Chl biosynthesis to inhibit the aberrant Pchlide accumulation that have led to the complete elimination of DPOR from their chloroplast genomes. In case of *A. thaliana*, the regulation of Chl biosynthesis has been attributed to metabolic feedback control of glutamyl-tRNA reductase, the first enzyme committed to tetrapyrrole synthesis, by heme and another negative regulator, FLU protein. The FLU protein contains a coiled-coil and tetratricopeptide repeat (TPR) domains, both implicated in protein–protein interactions. The TPR domain of FLU is required for interaction with the C-terminus of GluTR. Given that GluTR inhibition by heme requires the N-terminal region, heme and FLU seem to act independently on the same target to regulate Chl biosynthesis (fig. 4*A*) (Meskauskiene et al. 2001; op den Camp et al. 2003; Kauss et al. 2012). In addition to FLU, LPOR expression (PORA and PORB paralogs in *A. thaliana*) is positively regulated in the dark by DELLA, which is considered to be a transcriptional factor having unclear DNA-binding domain and plant-specific GRAS domain, and LPOR proteins bound to Pchlide to form a prolamellar body in

chloroplasts, circumventing the photodamage caused by free Pchlide accumulation (Cheminant et al. 2011).

In *M. polymorpha*, our genome analysis revealed the presence of homologs for the genes involved in the control of Chl biosynthesis (table 2), suggesting the possibility that control systems analogous to those of *A. thaliana* are present and functional. However, we hypothesize that this condition is unlikely, given that pale green phenotype and Pchlide accumulation could not be prevented in the *chl*B mutant of *M. polymorpha* under short-day conditions (fig. 3*A*: table 1). Another reason that would support this speculation is the simplicity of the *M. polymorpha* genome. Only one homologous gene has been observed for each of the gene families, *LPOR*, *DELLA*, and *HEMA* (the gene encoding GluTR). Gene duplication must have played a major role in the evolution of elaborate mechanisms for controlling Chl biosynthesis in land plants (table 2). In *A. thaliana*, genes encoding LPOR have been duplicated, and the light-response expression among the duplicated genes has diversified. *PORA* and *PORB* expressions are primarily induced in darkness. Their expression in the dark can alleviate photodamage during the dark-to-light transition, with complex formation containing Pchlide (fig. 4*A*). On the other hand, *PORC* expression is strongly induced in light. Given that the ethylene signaling pathway involves *PORC* expression, duplicated *LPOR* genes are subjected to different hormone signaling pathways (Runge et al. 1996; Sperling et al. 1997; Bae and Choi 2008; Kauss et al. 2012). *HEMA* is also duplicated in angiosperms. They show different tissue expression patterns and distinct interaction ability with FLU among them (references cited in Tanaka R and Tanaka A 2007).

The genome organization of *M. polymorpha* is much simpler than that of *A. thaliana* and shows lower gene redundancy, resulting in that gene functions tend to be in the primitive stage of subfunctionalization in *M. polymorpha* (Ueda et al. 2013). In the case of *A. thaliana*, LPOR accumulation with complex formation containing Pchlide in darkness can alleviate photodamage during the dark-to-light transition. The induction of *MpLPOR* expression in the *chl*B mutants should be beneficial to protect against photodamage because DPOR deficiency increases Pchlide accumulation. However, qRT-PCR analysis revealed that *MpLPOR* expression in the *chl*B mutants was not induced in darkness (fig. 4*C*), which suggests that the response of *LPOR* expression to Pchlide accumulation is absent or incomplete in *M. polymorpha*. Under this circumstance, it would be difficult to establish an elaborate regulatory system for Chl biosynthesis, which is comparable with that of *A. thaliana*. To investigate the actual function of the genes homologous to *LPOR*, *HEMA* (the gene encoding GluTR), and *DELLA*, it would be interesting to perform gene disruption experiments by exploiting the genetic tractability of *M. polymorpha*.

Development of a regulatory system for Chl biosynthesis and its harmonization to the land environment may eliminate genes for DPOR from the chloroplast genome. Our study provides insights for the detailed process, by which genes for DPOR have been eliminated from the chloroplast genome, and further analysis will shed light on the diversification between the nuclear and chloroplast gene expression networks during the evolution of land plants.

Supplementary Material

Supplementary tables S1–S3 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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