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Mini Review

Protochlorophyllide Reduction: a Key Step in the Greening of Plants

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The reduction of Protochlorophyllide (Pchlide) is a major regulatory step in the biosynthesis of chlorophyll (Chl) in oxygenic phototrophs. Two different enzymes catalyze this reduction: a light-dependent enzyme (LPOR), which is unique as a consequence of its direct utilization of light for catalysis; and a light-independent Pchlide reductase (DPOR). Since the reduction of Pchlide in angiosperms is catalyzed exclusively by LPOR, they become etiolated in the absence of light. LPOR, a major protein in etioplast membranes, consists of a single polypeptide and it exists as a ternary complex with its substrates, Pchlide and NADPH. By contrast to the copious information about LPOR, limited information about DPOR has been reported. Recent molecular genetic analyses in a cyanobacterium and a green alga have revealed that at least the three genes, namely, *chlL*, *chlN* and *chlB*, encode proteins essential for the activity of DPOR. These genes are widely distributed among phototrophic organisms with the exception of angiosperms and Euglenophyta. This distribution seems to be well correlated with light-independent greening ability. These genes might have been lost during the evolution of gymnosperms to angiosperms. The similarities among the deduced amino acid sequences of the three gene products and the subunits of nitrogenase suggest an evolutionary relationship between DPOR and nitrogenase. The identification of genes for the reduction of Pchlide provides the groundwork for investigations of the mechanism that regulates the synthesis of Chl, which is closely coordinated with greening in plants.

Key words: *chlB* — *chlL* — *chlN* — Chlorophyll biosynthesis — *por* — Protochlorophyllide reduction.

Chl *a* is a tetrapyrrole pigment that is essential for photosynthesis. In spite of the obvious biological importance of Chl, limited information is available about the biosynthesis of Chl and its regulation. In oxygenic phototrophs, Chl is synthesized from glutamate via a complex pathway that consists of at least fifteen enzymatic steps. The first nine steps from glutamate to protoporphyrin IX are shared with the biosynthetic pathway to heme. The Chl-specific biosynthetic route, known as the Mg-branch, consists of the following six steps: insertion of a magnesium ion into the porphyrin ring, methylation of the C-15-propionyl group, formation of an isocyclic ring, reduction of ring D, reduction of the 8-vinyl group and esterification of the C-17 propionyl group with phytol (e.g., Castelfranco and Beale 1983, Beale and Weinstein 1991, Kannangara 1991, Bauer et al. 1993, Richard 1993, von Wettstein et al. 1995). On the Mg-branch, reduction of ring D, namely, the conversion of protochlorophyllide (Pchlide) to chlorophyllide (Chlide), is known to be a rate-limiting reaction in the biosynthesis of Chl. The integration of the newly synthesized Chl into developing thylakoids is tightly coupled with the biogenesis of the photosynthetic apparatus and the development of chloroplasts (e.g., Mullet 1988, Hachtel and Friemann 1993). Thus, the conversion of Pchlide to Chlide is an initial regulatory step in the overall greening process in plants.

In phototrophic organisms two different enzymes catalyze the reduction of Pchlide: a light-dependent enzyme, NADPH:Pchlide oxidoreductase (LPOR; E.C. 1.3.1.33), which is a unique enzyme in that it utilizes light directly for catalysis; and a light-independent Pchlide reductase (DPOR). Recent molecular genetic studies have led to some important findings in both enzymatic systems. This review focuses on recent progress in the analysis of the two Pchlide-reduction systems in oxygenic phototrophs.

Figure 1 shows the structures of Pchlide and Chlide, including an outline of the biosynthesis of Chl. The Pchlide pool in phototrophic organisms consists of a mixture of monovinyl (MV) and divinyl (DV) forms of Pchlide (Mg-3-vinyl-8-ethyl phaeoporphyrin a_5, Mg-3, 8-divinyl phaeoporphyrin a_5, respectively; Carey and Rebeiz 1985, Shioi and Takamiya 1992, Rebeiz et al. 1994). In this review the term Pchlide refers to an unspecified mixture of the MV and DV forms of Pchlide, unless otherwise noted.

Light-dependent reduction of Pchlide

NADPH:Pchlide oxidoreductase—When seedlings of

Abbreviations: Bchl, bacteriochlorophyll; Chlide, chlorophyllide; ctDNA, chloroplast DNA; DPOR, light-independent protochlorophyllide reductase; DV, divinyl; IPTG, isopropyl thio-β-D-galactopyranoside; LPOR, light-dependent protochlorophyllide reductase; MV, monovinyl; ORF, open reading frame; Pchlide, protochlorophyllide; URF, unidentified open reading frame.

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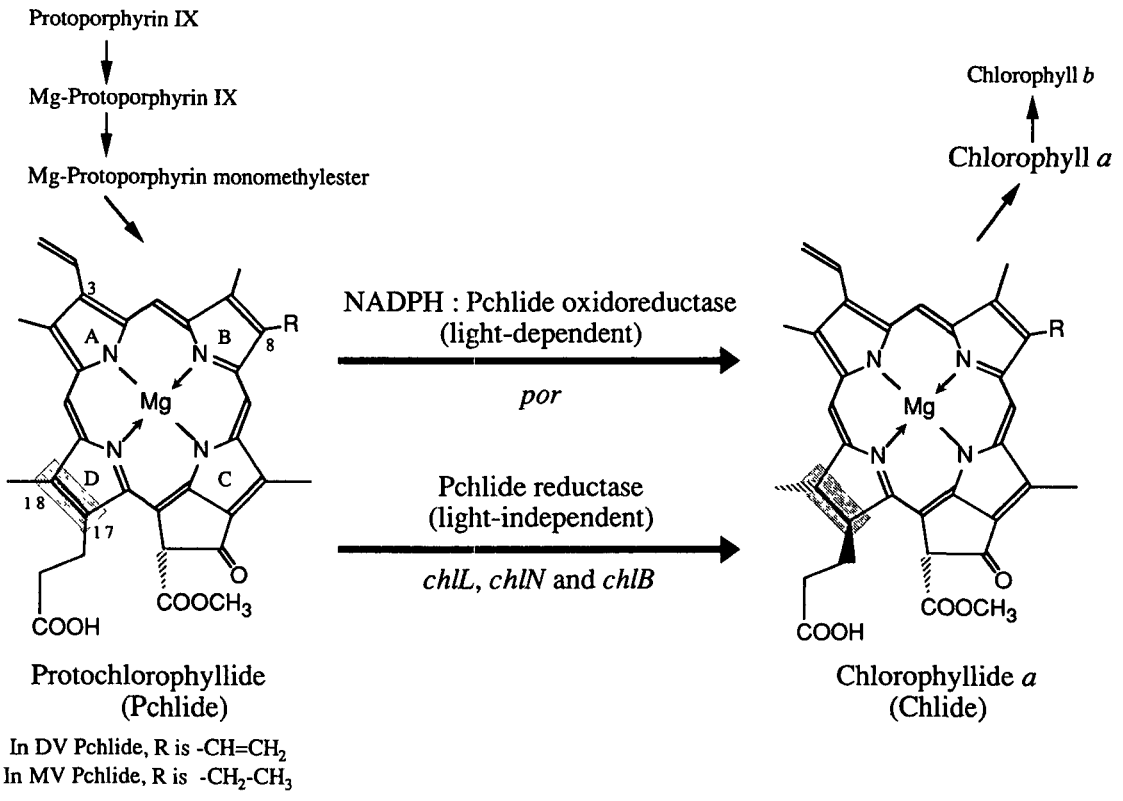

Fig. 1 The reduction of Pchlide on the Mg-branch that leads to the of biosynthesis of Chl. The double bond of ring D of Pchlide (indicated by shade) is stereospecifically reduced by two different reductase systems: the light-dependent reductase encoded by the *por* gene and the light-independent reductase that is probably encoded by three genes, *chlL, chlN* and *chlB*.

angiosperms grow in darkness, the synthesis of Chl is arrested at the Pchlide-reduction step, with resultant etiolation since, in angiosperms, Pchlide is reduced by LPOR exclusively. LPOR, a major protein in etioplast membranes, consists of a single polypeptide and exists as a ternary complex with its two substrates, Pchlide and NADPH. The Pchlide molecule bound to LPOR also functions as a photoreceptor for this reduction. Upon absorption of light energy by Pchlide, the Pchlide molecule is excited and the double bond of ring D is reduced by NADPH to yield Chlide. In this reduction, a pair of hydrogens from NADPH and the medium (or the conserved tyrosine residue in LPOR) are stereospecifically added to C-18 and C-17 of the Pchlide, respectively. This enzymatic process can be monitored spectroscopically in vivo and in vitro since both the substrate (Pchlide) and the product (Chlide) have distinct spectral properties (for reviews, see Griffiths 1991, Schulz and Senger 1993). Quinacrine and trifluoroperazine, inhibitors of flavoproteins, inhibited the activity of LPOR, and FAD co-purified with the LPOR protein (Walker and Griffiths 1988). These observations suggest that LPOR might be a flavoprotein. However, the role of FAD in the reaction remains unknown.

LPOR in plants and algae is encoded by the nuclear genome. Complementary DNAs encoding LPOR have been cloned from a variety of plants, namely, barley (Schulz et al. 1989), oat (Darrah et al. 1990), *Arabidopsis* (Forreiter et al. 1990), pea (Spano et al. 1992a), pine (Spano et al. 1992b, Forreinter and Apel 1993), wheat (Teakle and Griffiths 1993) and cucumber (Kuroda et al. 1995). Genomic DNA fragments encoding LPOR have also been cloned from pine (Spano et al. 1992b) and the green alga *Chlamydomonas reinhardtii* (Li and Timko 1996). Eukaryotic LPOR is synthesized as a longer precursor protein with an amino-terminal extension that functions as a transit peptide for transport of LPOR into plastids. Thus, for example, the LPOR precursor of pea consists of a transit peptide of 86 amino acid residues and a mature region of 313 amino acid residues (Spano et al. 1992a). The extent of the homology at the amino acid level among the mature regions of various LPORs is very high (more than 79%).

Expression of gene(s) for LPOR(s)—LPOR is present at high levels in the etioplasts of dark-grown seedlings of angiosperms. When monocots are illuminated, LPOR activity starts to decrease dramatically, with the concomitant disappearance of mRNA for LPOR and the LPOR polypeptide. When Chl accumulates at the maximal rate during the greening process, LPOR activity drops to below

the limit of detection (Apel 1981, Santel and Apel 1981, Forreiter et al. 1990). A recent detailed reinvestigation in barley with LPOR-specific antiserum resolved this paradox. Two distinct isozymes of LPOR, POR-A and POR-B, were identified (Holtorf et al. 1995). The respective cDNAs were also cloned. The overall amino acid sequence identity was found to be 75% while the extent of sequence identity was only 45% in the transit peptides. In etioplasts, POR-A accumulated in large amounts. Upon exposure of seedlings to light, POR-A disappeared within 24 h. By contrast, POR-B remained at a constant level throughout the greening of etiolated seedlings. The transcription of POR-A ceased within 8 h but that of POR-B remained constant after illumination. Moreover, a photoactive POR complex with properties distinct from those of LPOR in etiolated leaves was detected in greening leaves of barley (Franck and Strzalka 1992), and it might correspond to the POR-B complex. These observations suggest functional differences between the two isozymes. This suggestion is also supported by differences in properties related to import into plastids (see below).

By contrast to the negative effects of light on the level of expression of the gene for POR-A that have been commonly observed in monocots such as barley, the effects of light in dicots seem to be dependent on the plant species. In pea, the level of the LPOR mRNA remained constant in 6-d-old dark-grown seedings for at least 48 h upon exposure to light (He et al. 1994), suggesting that expression of the gene for LPOR might be regulated mainly by endogenous factor(s) related to the developmental stage rather than by light (He et al. 1994). In cucumber, illumination of dark-grown seedlings induced a rapid decrease in the level of LPOR within 2 h, which was followed by a transient submaximal peak and a slow decrease. The changes in the level of LPOR seem to be correlated with the accumulation of Chl during greening (Yoshida et al. 1995). Another study on LPOR in cucumber seedlings demonstrated an elevated level of the transcript after illumination (Kuroda et al. 1995).

Two isozymes of LPOR have been detected not only in monocots but also in dicots, such as *Arabidopsis thaliana* (Armstrong et al. 1995) and pea (He et al. 1994). Thus, the synthesis of Chl might be controlled by the differential actions of the two distinct isozymes of LPOR in angiosperms, with the nature of such regulation depending on the species.

Import into plastids and stability of LPOR—It was demonstrated recently that at least two processes, in addition to transcription, are involved in regulation of the ratio of the levels of the two LPOR isozymes in plants. The first of these two processes is import of the gene product into plastids (Reinbothe et al. 1995c). As mentioned above, LPOR is a nucleus-encoded plastid protein that is imported posttranslationally into plastids, namely, etioplasts or chloroplasts. The import of POR-A has a novel feature distinct from that of POR-B. The precursor to POR-A (pPOR-A), synthesized in vitro, was imported into etioplasts but not into chloroplasts. The ability of chloroplasts to take up pPOR-A was restored by raising the endogenous level of Pchlide by the addition of exogenous Pchlide or by feeding of δ-aminolevulinic acid. This result indicates that the import of pPOR-A into plastids is dependent on the presence of Pchlide in the plastids. By contrast, the precursor to POR-B was imported into chloroplasts irrespective of the presence or absence of Pchlide (Reinbothe et al. 1995c).

The second additioinal process is the selective degradation of POR-A in plastids (Reinbothe et al. 1995a). POR-A, which is abundantly present in etioplasts, is selectively digested by a light-induced protease after illumination. It remains unknown, however, why the protease selectively attacks POR-A and not POR-B in vivo. The level of POR-B in plastids appears to be kept constant by the continuous supply of newly synthesized POR-B, which is due to maintenance of a constant level of the transcript. LPOR was shown to be localized in the envelope of chloroplasts after complete greening (Joyard et al. 1990, 1991, Pineau et al. 1993). These observations suggest that a difference in localization might be the cause of the selective degradation of POR-A. Thus, POR-B is localized in the envelope of plastids and is protected from degradation, while POR-A is present in prolamellar bodies in etioplasts and is exposed to the protease (Griffiths 1978, Dehesh and Ryberg 1985).

Prokaryotic origin of LPOR—Since cyanobacteria, which are prokaryotic oxygenic phototrophs, are able to turn green in a light-independent manner, it was proposed that they might reduce Pchlide only via the action of DPOR, as do anoxygenic photosynthetic bacteria and, moreover, that the LPOR protein evolved at an early stage of the evolution from prokaryotic to eukaryotic phototrophs. Contrary to this hypothesis, a conditional Chl-less mutant of a cyanobacterium *Plectonema boryanum* has provided the first evidence for the coexistence of both LPOR and DPOR in cyanobacteria (Fujita et al. 1992). The *chlL*-disrupted mutant YFC1004, which lacks DPOR, is incapable of synthesizing Chl and accumulates Pchlide in darkness. In the light, by contrast, this mutant synthesizes Chl normally, suggesting the presence of LPOR in this cyanobacterium (Fujita et al. 1992). The cyanobacterial gene for LPOR (*por*) was recently cloned for the first time from a unicellular strain, *Synechocystis* sp. strain PCC6803, by in vivo complementation of mutants of *Rhodobacter* (see below, Suzuki and Bauer 1995b). The *por* gene was also cloned from the filamentous cyanobacterium *P. boryanum* by PCR using degeneate primers (Fujita et al. 1995). The cyanobacterial LPORs lack a transit peptide and exhibit approximately 52–56% homology to the mature regions of plant LPORs at the amino acid level (Table 1). The homology between the cyanobacterial

LPORs is 71%. LPOR activity was detected in the membrane fraction prepared from another filamentous strain *Phormidium laminosum*. The activity was very low as compared with that associated with the mature chloroplasts of higher plants (Rowe and Griffiths 1995).

A model of catalysis—Three cysteine residues are completely conserved among all the known amino acid sequences of LPORs. One of the three, Cys-256 of barley LPOR, was specifically protected from modification by a thiol-specific reagent, N-phenylmaleimide, in the presence of Pchlide, suggesting that Cys-256 might be involved in the binding of Pchlide (Oliver and Griffiths 1981). Site-directed mutagenesis should allow us to determine the essential nature and the function of these cysteine residues. For the evaluation of the activity of mutated forms of LPOR, two heterologous expression systems have recently become available. One such expression system is an *E. coli* system. Barley LPOR can be expressed in *E. coli* cells that have been lysogenized by a recombinant λgt11 that carries the cDNA. Exogenously added Pchlide is converted to Chlide by an extract of these *E. coli* cells in the presence of light and NADPH (Schulz et al. 1989). The substrate specificity of LPOR has been examined using this system. Both the MV and DV forms of Pchlide were converted in a light-dependent manner to Chlides, an indication that LPOR does not discriminate between Pchlide molecules with vinyl and ethyl groups at C-8 (Knaust et al. 1993). The other expression system is a system involving the anoxygenic bacterium *Rhodobacter capsulatus*, which reduces Pchlide in a reaction catalyzed only by DPOR to yield bacteriochlorophyll (Bchl). Three mutants lacking genes for DPOR are unable to synthesize Bchl, with the resultant accumulation of Pchlide. A shuttle vector between *E. coli* and *R. capsulatus*, carrying pea cDNA for LPOR connected to a strong promoter, restored the capacity for the synthesis of Bchl to all of these mutants, indicating the expression of a functional pea LPOR protein in *R. capsulatus* (Wilks and Timko 1995).

A detailed comparison of the amino acid sequence of LPOR with sequences in protein databases led to the finding that LPOR is a member of the family of short-chain alcohol dehydrogenases (Baker 1994, Labesse et al. 1994). A model of catalysis by LPOR has been presented that is based on the known catalytic mechanism of such dehydrogenases. According to the model, the proton for the C-18 position of Pchlide is transferred from Tyr-275 (numbering in pea LPOR), while Lys-279 functions to lower the pKa of the tyrosine residue to facilitate the deprotonation. The Tyr-275 and Lys-279 residues are completely conserved not only in LPORs but also in the other members of this family of dehydrogenases. The essential nature of the two residues was confirmed by site-directed mutagenesis in combination with in vivo complementation analysis in *R. capsulatus* (see above; Wilks and Timko 1995). Elucidation of the three-dimensional structure of LPOR is needed, in addition to more extensive site-directed mutagenesis, for a better understanding of the catalytic mechanism of LPOR.

Light-independent reduction of Pchlide

Unidentified open reading frames in chloroplast DNA—In contrast to the etiolation of angiosperms in darkness, the greening of some gymnosperms, mosses, green algae and cyanobacteria is possible even in darkness. Some green algal mutants are known to accumulate Pchlide in darkness but produce Chl normally when exposed to light (Schulz and Senger 1993). These observations suggest the presence of an alternative system for the reduction of Pchlide that is operative in darkness. Very little information is available about DPOR, while LPOR has been studied extensively (see above). Studies of the functions of unidentified open reading frames (URFs) in the chloroplast DNA (ctDNA) of the liverwort *Marchantia polymorpha* led to the identification of a gene that is essential for the light-independent reduction of Pchlide (Fujita et al. 1992).

The chlL (frxC) gene—The *frxC* (named after ferredoxin) gene was first found in liverwort ctDNA as a URF (Ohyama et al. 1988, Kohchi et al. 1988). Homologs of *frxC* were then found in the ctDNAs from many plants (Lidholm and Gustafsson 1991, Suzuki and Bauer 1992, Huang and Liu 1992, Yamada et al. 1992a, b, Yamada and Yamamoto 1992, Reith and Munholland 1993, Wakasugi et al. 1994) and genomic DNAs from cyanobacteria (Fig. 2; Fujita et al. 1991, Ogura et al. 1992) but not in the ctDNAs from angiosperms (Table 1). The *frxC* gene of *P. boryanum* encodes a polypeptide of 286 amino acid residues with a molecular weight of 31,218 (Fujita et al. 1991). In attempt to reveal the function of *frxC*, a mutant of the cyanobacterium *P. boryanum*, in which *frxC* had been disrupted by an interposon, was isolated (Fujita et al. 1992). Similar mutants of the green alga *Chlamydomonas reinhardtii* were also obtained (Suzuki and Bauer 1992). The phenotypes of all the mutants, a "*yellow-in-the-dark*" phenotype ("*blue-in-the-dark*" in the cyanobacterial mutant, YFC1004), were essentially the same: the synthesis of Chl was arrested, concomitant with the anomalous accumulation of Pchlide in darkness, in contrast to the normal accumulation of Chl in the light. Thus, the *frxC* gene was concluded to be essential for the action of DPOR and it was renamed the *chlL* gene after a homolog of *frxC* '*bchL*' that is involved in the synthesis of Bchl in *R. capsulatus* (Yang and Bauer 1990). The ChlL protein was detected in soluble fraction of liverwort chloroplasts by an immunochemical technique (Fujita et al. 1989).

The chlN gene—Some "*yellow-in-the-dark*" mutants of *C. reinhardtii* were shown to be caused by chloroplast-related mutations (Roitgrund and Mets 1990). A second gene, *chlN*, essential for the action of DPOR was identified in a wild-type ctDNA fragment that complemented one

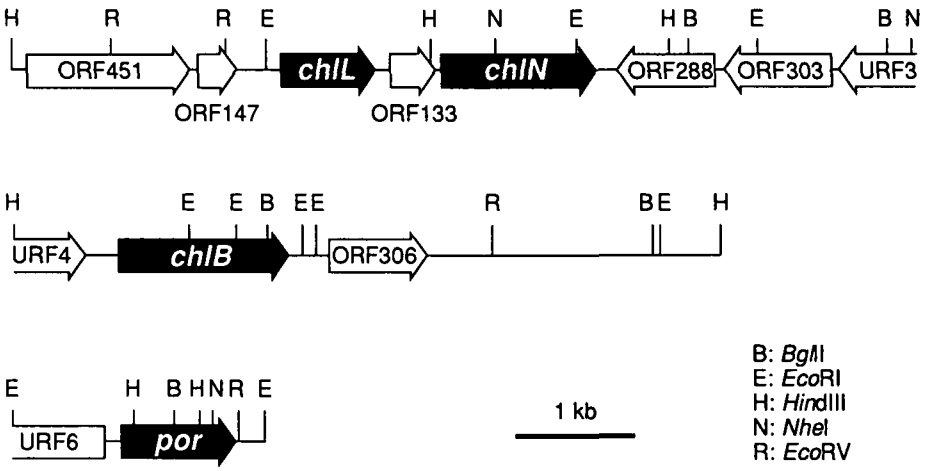

Fig. 2 Organization of the four genes for the reduction of Pchlide in the chromosomal DNA of the cyanobacterium *P. boryanum*. The *chlL* and *chlN* genes form an operon (Fujita et al. 1993). The *chlB* and *por* genes are found on different chromosomal fragments (Fujita et al. 1995, 1996).

such mutant (Choquet et al. 1992). A homolog of *chlN* (ORF465) was found just downstream of *chlL* in liverwort ctDNA (Kohchi et al. 1988). All of the *chlN* genes found to date in land plants are located next to a *chlL* gene to form an operon in the ctDNA (Lidholm and Gustafsson 1991, Wakasugi et al. 1994, Yamada, K. personal communication), while the green algal *chlN* is located about 84 kb from *chlL* in the ctDNA (Choquet et al. 1992). The cyanobacterial homolog of *chlN* (ORF467) was also found downstream of *chlL* (Fig. 2; Fujita et al. 1993, Ogura et al. 1992). The cyanobacterial *chlN* gene encodes for a protein of 467 amino acids with a molecular weight of 52,795. There is a small ORF (ORF133) between *chlL* and *chlN* (Fujita et al. 1993), but its function remains unknown: an ORF133-disrupted mutant exhibited no detectable change in phenotype (Fujita, Y. unpublished result). Northern blot analysis indicated that polycistronic transcription occurs from *chlL* to *chlN* in the cyanobacterium (Mikami, A., Takahashi, Y., Fujita, Y. unpublished result) and in the liverwort *Marchantia paleacea* (Takio and Satoh 1995). The *bchN* gene is a homolog required for the synthesis of Bchl in *R. capsulatus*, and this gene is present in a large operon together with the other several Bchl-biosynthetic genes, including *bchL* and *bchB* (Burke et al. 1993b).

The chlB gene—A third gene, *bchB*, essential for the activity of DPOR in the synthesis of Bchl in *R. capsulatus* was identified together with *bchL* and *bchN* by interposon mutagenesis (Burke et al. 1993b). The *bchB* gene was found to exhibit significant similarity to ORF513, one of the URFs in liverwort ctDNA (Umesono et al. 1988). This finding led to the investigation of the homolog of ORF513 in the ctDNA of *C. reinhardtii* (Li et al. 1993, Liu et al. 1993). Algal mutants with disruption of the homolog of ORF513 showed a "*yellow-in-the-dark*" phenotype indistinguishable from that of *chlL*- or *chlN*-disrupted mutants. Thus, it was concluded that the homolog of ORF513 is the third gene, designated *chlB*, that is essential for the action of DPOR in chloroplasts. The green algal *chlB* is located about 10 kb upstream of *chlL* (Li et al. 1993). The *chlB* gene of land plants is also located far from the *chlLN* operon in the ctDNA (Ohyama et al. 1988, Wakasugi et al. 1994). In the cyanobacterium *P. boryanum*, the *chlB* gene has been identified at a genomic locus different from that of the *chlLN* operon (Fig. 2; Fujita et al. 1996). The cyanobacterial *chlB* gene encodes a polypeptide of 508 amino acid residues with a molecular weight of 56,819 (Fujita et al. 1996). The *chlB* gene was also found in the chromosomal 1-Mb region of the cyanobacterium *Synechocystis* sp. strain PCC6803 (Kaneko et al. 1995). The ChlB protein was detected immunochemically in a membrane fraction of cyanobacterial cells, suggesting that either the cytoplasmic or the thylakoid membranes are the site of the light-independent reduction of Pchlide (Fujita et al. 1996).

Nuclear genes—In addition to the three chloroplast genes, genetic analyses in *C. reinhardtii* resulted in the mapping of six nuclear loci (*y1, y5, y6, y7, y8* and *y10*) that affect DPOR activity (Ford and Wang 1980a, b, 1982, Ford et al. 1983). The *y* mutants are capable of greening in the light, resembling the wild type, but they do not accumulate Chl in darkness. However, the functions of the *y* loci are unclear. They might be involved either in regulation of the expression of the three genes in the chloroplast genome or in the biosynthesis of some cofactor(s), if present, for DPOR, rather than of the structural parts of the reductase.

Similarities to nitrogenase—The amino acid sequences encoded by the three chloroplast genes for DPOR show significant similarities to those of the three subunits of nitrogenase (Fujita et al. 1991, 1993, 1996, Burke et al. 1993c).

Nitrogenase, a multi-subunit enzyme that catalyzes the reduction of dinitrogen to ammonia, consists of two separable components, the MoFe-protein ($\alpha_2\beta_2$ structure, consisting of the products of the *nifD* and *nifK* genes) and the Fe-protein (γ_2 structure, consisting of the product of the *nifH* gene). The Fe-protein specifically transfers electrons to the MoFe-protein in a reaction that is coupled with the hydrolysis of Mg-ATP. The MoFe-protein catalyzes the reduction of dinitrogen directly (for reviews, see Burris 1991, Dean et al. 1993, Peters et al. 1995). ChlL and ChlB are homologous to NifH (about 35%) and NifK (about 19%), respectively (Fujita et al. 1991, 1996). ChlN is homologous to both NifD and NifK (about 19%, Fujita et al. 1993). The ATP-binding motif (GXXXXGKS) of NifH is completely conserved in ChlL, as are the four Cys residues involved in the chelation of the Fe-S cluster (Fujita et al. 1989, 1991, Burke et al. 1993c). In addition, some similarity (about 16%) between ChlN and ChlB is also apparent, as it is between NifD and NifK (Fujita et al. 1996). These similarities between subunits of DPOR and those of nitrogenase suggest that DPOR has a molecular architecture similar to that of nitrogenase. In the DPOR complex, ChlL might function as a specific donor of electrons to the other component, consisting of ChlN and ChlB, which might directly catalyze the reduction of Pchlide.

Chlide reductase, which catalyzes the reduction of chlorin ring B in the synthesis of Bchl, is another enzyme that is structurally related to DPOR. Three genes, *bchX*, *bchY* and *bchZ*, which are essential for the reduction, are homologous to *chlL*, *chlN* and *chlB*, respectively (Burke et al. 1993a). The extent of the identity at the amino acid level to cyanobacterial ChlL, ChlN and ChlB is 29%, 18% and 21%, respectively. Thus, there exist three structurally related enzyme complexes, namely, nitrogenase, DPOR and Chlide reductase. The similarities suggest an evolutionary relationship among the three enzymes. A detailed phylogenetic analysis of "the *nifH* family", *nifH*, namely *chlL* and *bchX*, including the *vnfH* and *anfH* genes for alternative nitrogenases, suggests that the *chlL/bchX* subfamily diverged initially from the common ancestor of the family before the divergence of the nitrogenase subfamily, *nifH*, *vnfH* and *anfH* (Ueda et al. 1995).

Attempts to characterize DPOR—In spite of the genetic identification, there is no direct evidence in support of the hypothesis that the three chloroplast genes do indeed encode subunits of DPOR. The activity of DPOR in vitro has been detected in only two cases. One case involved plasma membranes from the cyanobacterium *Anacystis nidulans* (*Synechococcus* sp. strain PCC6301). Conversion of endogenous Pchlide to Chlide was observed by spectroscopic and HPLC analysis (Peschek et al. 1989b). The activity was dependent on NADPH and was enhanced by menadione, an efficient mediator of electron transport between NADPH and membrane-bound enzymes. Calcium ions stimulated the activity (Peschek et al. 1989a). After discontinuous sucrose density gradient centrifugation, this activity was recovered in a light-green fraction that might have been derived from 'thylakoid centers', putative sites of contacts between cytoplasmic and thylakoid membranes. This localization is consistent with the idea that thylakoid centers are the sites at which lipids and pigments synthesized in cytoplasmic membranes are transferred to thylakoid membranes (Hinterstoisser et al. 1993). The other case involved a cell-free extract of dark-grown seedlings of the pine *Pinus mugo*. In this case, the conversion of [^{14}C]Pchlide to [^{14}C]-Chlide was monitored by HPLC. The activity was again dependent on NADPH. Attempts to purify the activity were not successful since the activity was lost during fractionation of the extract for unknown reasons (Forreinter and Apel 1993).

The molecular architecture of DPOR, as deduced from the similarity in sequence to that of nitrogenase, seems to contradict in some respects of the characteristics of this enzyme as revealed by above-described assays. Although ChlL has an ATP-binding motif that is completely conserved in NifH (Fujita et al. 1989, 1991), which suggests a requirement for ATP of the catalysis, such a requirement for ATP was not observed in the assays of DPOR described above (Peschek et al. 1989b, Forreiter and Apel 1993). The ChlL and ChlB proteins were detected immunochemically in soluble and membrane fractions of liverwort chloroplasts (Fujita et al. 1989) and cyanobacterial cells (Fujita et al. 1996), respectively. The immunochemical localization of ChlL and ChlB seems to suggest that the activity might require both soluble and membrane fractions of cyanobacterial cells. However, the activity was, in fact, detected only in plasma membranes of the cyanobacterium (Peschek et al. 1989b). Further characterization of DPOR, including purification of each subunit and reconstitution of the activity, is needed to verify the nitrogenase-like model.

Indirect evidence for the substrate specificity of DPOR was reported recently (Suzuki and Bauer 1995a). A *bchJ*-disrupted mutant of *R. capsulatus* synthesized Bchl at reduced levels, concomitant with the accumulation of only the DV form of Pchlide. By contrast, a double mutant of *bchJ* and *bchL* accumulated Pchlide as a mixture of the MV and DV forms at a ratio of the MV to the DV form much lower than that in a single mutant of *bchL*. Suzuki and Bauer (1995a) proposed that BchJ might be involved in the 8-vinyl reduction of Pchlide rather than in the catalytic activity of DPOR. DPOR might prefer the MV form of Pchlide as a substrate to the DV form. On the basis of this hypothesis, they explained the phenotype of the *bchJ*-disruptant as follows. DPOR with such substrate-specificity catabolizes MV Pchlide selectively from the mixed Pchlide pool, with resultant formation of a biased pool of Pchlide. However, this interpretation remains to be confirmed by use of an assay system in vitro.

Evolutionary and physiological aspects of the two Pchlide reduction systems

Distribution of genes—The distribution of the three genes for DPOR, *chlL, chlN* and *chlB*, and the gene (*por*) for LPOR among phototrophic organisms is summarized in Table 1, together with the extent of sequence homologies to cyanobacterial genes. The three genes for DPOR are distributed in the ctDNAs from a wide variety of plants (gymnosperms, Lidholm and Gustafsson 1991, Wakasugi et al. 1994; Pteridophyta, Yamada et al. 1992b; Bryophyta, Ohyama et al. 1988) and algae (Chlorophyta, Suzuki and Bauer 1992, Choquet et al. 1992, Huang and Liu 1992, Li et al. 1993, Liu et al. 1993; Rhodophyta, Reith and Munholland 1993), and in the chromosomal DNAs from cyanobacteria (Fujita et al. 1991, 1993, 1996, Ogura et al. 1992) and anoxygenic photosynthetic bacteria (Yang and Bauer 1990, Burke et al. 1993b). However, no homolog has been reported in ctDNAs from angiosperms (rice, Hiratsuka et al. 1989; tobacco, Shinozaki et al. 1986; and beechdrop, Wolfe et al. 1992) and Euglenophyta (*Euglena gracilis*, Hallick et al. 1993), all of which become etiolated in darkness. No hybridization signals have been obtained by Southern blot analysis of nuclear DNA from angiosperms with the liverwort *chlL* gene as a probe (Suzuki and Bauer 1992), suggesting the absence of genes for DPOR in angiosperms. This distribution of genes for DPOR agrees

Table 1 Distribution of the four genes for the reduction of Pchlide among phototrophic organisms and the extent of homologies (%) to cyanobacterial homologs[a]

Taxon		Representative species	Light-dependent	Light-independent		
			por	*chlL*	*chlN*	*chlB*
Prokaryotes	Purple non-sulfur bacteria	*Rhodobacter capsulatus*	none	47[b] (*bchL*)	34[b] (*bchN*)	27[b] (*bchB*)
	Cyanobacteria	*Plectonema boryanum*	100[c]	100[d]	100[e]	100[f]
Eukaryotes	Chlorophyta	*Chlamydomonas reinhardtii*	53 (n)[g]	86 (c)[h]	62 (c)[i]	61 (c)[j]
	Rhodophyta	*Porphyra purpurea*	?	(c)[k]	(c)[k]	?
	Euglenophyta	*Euglena gracilis*	+ (n)[l]	none[l]	none[l]	none[l]
	Bryophyta	*Marchantia polymorpha*	?	83 (c)[m]	72 (c)[m]	73 (c)[n]
	Pteridophyta	*Adiantum capillus-veneris*	?	78 (c)[o]	70 (c)[p]	+ (c)[p]
	Gymnosperms	*Pinus thunbergii*	A: 54 (n)[q] B: 53 (n)[r]	82 (c)[s]	69 (c)[s]	65 (c)[s]
	Angiosperms	*Nicotiana tabacum*	A: 53 (n)[t] B: 53 (n)[u]	none[v]	none[v]	none[v]

(c) The gene is present in the ctDNA.
(n) The gene is present in the nuclear genome.
[a] Values in the Table show percent homology to the cyanobacterial homolog at the amino acid level.
[b] Yang and Bauer 1990, Burke et al. 1993b; Z11165.
[c] Fujita, Y., Takagi, H., Hase, T. in preparation.
[d] Fujita et al. 1991; D00665.
[e] Fujita et al. 1993; D12973.
[f] Fujita et al. 1996; D78208.
[g] Compared with the mature part of *CRlpcr-1* (Li and Timko 1996; U36752).
[h] Suzuki and Bauer 1992; X60490.
[i] Choquet et al. 1992; P29683.
[j] Li et al. 1993; U02526.
[k] Reith and Munholland 1993.
[l] There is no homolog in the ctDNA whose nucleotide sequence has been determined (Hallick et al. 1993).
[m] Kohchi et al. 1988.
[n] Umesono et al. 1988.
[o] Yamada et al. 1992b.
[p] Yamada, K. personal communication.
[q] Compared with the mature part of Pm1 from *Pinus mugo* (Forreiter and Apel 1993; S63824).
[r] Compared with the partial sequence of Pm2 from *Pinus mugo* (Forreiter and Apel 1993; S63825).
[s] Wakasugi et al. 1994; D17510.
[t] Compared with the mature part of POR-A from barley (*Hordeum vulgare*, Schulz et al. 1989; X15869).
[u] Compared with the mature part of POR-B from barley (*Hordeum vulgare*, Holtorf et al. 1995; X84738).
[v] There is no homolog in the ctDNA whose nucleotide sequence has been determined (Shinozaki et al. 1986).

well with that of greening ability in darkness.

Although there is no evidence for the presence of LPOR in Pteridophyta and Bryophyta, the gene for LPOR seems to be distributed ubiquitously among oxygenic phototrophs from cyanobacteria (Suzuki and Bauer 1995b, Rowe and Griffiths 1995, Fujita et al. 1995) to angiosperms, but not in anoxygenic photosynthetic bacteria. Thus, the distribution of DPOR and LPOR implies that the two systems coexisted throughout evolution from the cyanobacteria to the gymnosperms, and that the genes for DPOR were lost during the evolution from gymnosperms to angiosperms, with the resultant loss of light-independent greening ability.

However, there are some gymnosperms that provide evidence against this evolutionary scenario. *Ginkgo biloba* and *Larix kaempferi* are not able to green in darkness and become etiolated (Mukai et al. 1992, Chinn and Silverthorne 1993) even though they have the genes for DPOR in their ctDNAs (Yamada and Yamamoto 1992, Boivin et al. 1994, Richard et al. 1994). These gymnosperms might represent intermediate species on the way to the complete loss of DPOR. It would be of interest to examine whether their genes are pseudogenes or whether may encode active proteins by complementation analysis in the cyanobacterium *P. boryanum* (see below).

Physiological aspects of the two PORs—Both LPOR and DPOR coexist in almost all oxygenic phototrophic organisms except the angiosperms. These organisms might regulate their Chl contents by using the two systems differentially in response to environmental signals, such as light intensity and nutrition conditions, and in response to developmental stages, as in the case of the two isozymes of LPOR in angiosperms (Holtorf et al. 1995). The functional differentiation of the two Pchlide-reduction systems is poorly understood. However, there are some fragmentary results suggestive of functional differentiation. In the cyanobacterium *P. boryanum*, transcripts for *chlL* and *chlN* were detected at higher levels in photomixotrophically grown cells than in photoautotrophically grown cells (Mikami, A., Takahashi, Y., Fujita, Y. unpublished result). Pines have light-independent greening ability during the development of cotyledons. However, after their development the reduction of Pchlide depends exclusively on LPOR (Bogorad 1950, Bogdanovíc 1973). A green algal mutant, *pc-1*, lacking LPOR activity, produced 32% as much Chl as the wild type in the light (Ford et al. 1981), indicating that DPOR was operative even in the light but that the activity was not high enough to compensate for the loss of LPOR. However, in darkness, the *pc-1* mutant accumulated only 56% as much Chl as the wild type (Ford et al. 1981). This result suggests that LPOR has not only an enzymatic role but also some regulatory role(s) in green algal cells.

Why did the angiosperms lose DPOR?—In our efforts to understand the evolution of the genes for DPOR and LPOR, we should consider how the expression of Chl-binding proteins, such as the core proteins of photosystems I and II and light-harvesting Chl *a/b*-binding proteins, is regulated in accord with the supply of Chl. Many Chl-binding proteins are known to be very unstable in the absence of Chl (Bennett 1981, Mullet et al. 1990, Kim et al. 1994), and Chl is required for the translation of certain plastid-encoded Chl-binding proteins (D2, CP43, CP47, PsaA and PsaB; Eichacker et al. 1990, 1992). In addition, the accumulation of Chl or its intermediates, such as Pchlide, in a free form and not bound to proteins, causes lethal damage to photosynthetic cells via the formation of highly reactive oxygen radicals and singlet oxygen upon illumination. Thus, phototrophic organisms need to synthesize Chl in synchrony with the synthesis of Chl-binding proteins. Indeed, the expression of Chl-binding proteins is dependent on light in angiosperms. By contrast, many lower plants and algae that have DPOR produce Chl-binding proteins in a light-independent manner (Mukai et al. 1992, Forreinter and Apel 1993). During the evolution from gymnosperms to angiosperms, the loss of DPOR might have led to a dramatic change in the regulation of the expression of Chl-binding proteins. Use of LPOR as the sole Pchlide-reduction system seems to give angiosperms several advantages, as follows. (1) Angiosperms do not need to produce Chl-binding proteins to generate photosystems that are useless in darkness, because Chl is no longer provided in a light-independent manner as a result of the absence of DPOR. (2) The highly concentrated LPOR protein, together with Pchlide and NADPH, in etioplasts ensures not only that Chl is synthesized as quickly as possible upon illumination but it also maintains Pchlide, a highly toxic compound, in a harmless state in the absence of illumination.

Concluding remarks—LPOR is one of only two known enzymes that require light for catalysis. The other enzyme is DNA photolyase, which is involved in the repair of pyrimidine dimers in DNA (Sancar and Sancar 1987). The three-dimensional structure of DNA photolyase was reported recently (Park et al. 1995). Heterologous systems for expression of LPOR in *E. coli* (Schulz et al. 1989) and *R. capsulatus* (Wilks and Timko 1995) have opened a path not only to assignment of functions of the conserved amino acid residues by site-directed mutagenesis but also to preparation of LPOR protein in large amounts sufficient for crystallography. A combination of molecular genetic and biochemical approaches, including spectroscopic analysis of the reaction, will be necessary to characterize the details of the catalytic mechanism of LPOR. It has been suggested that the photoreduction of Pchlide results in a major conformational change (Reinbothe et al. 1995b). Thus, a comparison of three-dimensional structures of Pchlide-LPOR and Chlide-LPOR complexes will also be important in our efforts to understand the mechanism of catalysis.

The discovery of the two isozymes in angiosperms provided evidence that not only transcription but also import into plastids and stability in plastids play important roles in the regulation of the synthesis of Chl (Reinbothe et al. 1995a, c). Results obtained in barley suggest differences in the functions of the two isozymes, POR-A and POR-B, in plants (Holtorf et al. 1995). Isolation of transgenic plants in which expression of each gene is modified to allow selective suppression or induction should provide interesting insights into this possibility.

Assignment of the three gene products, ChlL, ChlN and ChlB, as authentic subunits of DPOR is of pressing importance in studies of the light-independent system. Co-expression of the three genes in *E. coli* or mixing of extracts from *E. coli* cells that express each gene should be carried out to examine this possibility, as was done in the case of magnesium chelatase. The activity of magnesium chelatase was recently demonstrated by mixing extracts from three strains of *E. coli* that expressed *bchD, bchH* and *bchI*, respectively (Gibson et al. 1995).

Regulation of the reduction of Pchlide in oxygenic phototrophic organisms other than angiosperms is more complicated than that in angiosperms because they contain DPOR together with LPOR. For example, pines (*Pinus mugo* and *P. taeda*) have two LPOR isozymes, as do angiosperms, in addition to DPOR (Spano et al. 1992b, Forreiter and Apel 1993). Very little is known about the regulation of the two different Pchlide-reduction systems in any organism. The cyanobacterium *P. boryanum* provides an excellent model system with which to address this regulatory mechanism since the *por* gene and the three genes for DPOR have been cloned and a shuttle vector pPBH201 is available (Walton et al. 1993). Using this shuttle vector, we successfully complemented a *chlB*-disrupted mutant with a *chlB* gene under control of the *trc* promoter (Takagi, H., Fujita, Y., Hase, T. unpublished result). This complementation system should allow us to examine whether chloroplast genes for DPOR encode functional proteins. A new shuttle vector carrying the $lacI^q$ gene and *trc* promoter was recently constructed. The level of expression of a gene under control of the *trc* promoter in cyanobacterial cells could be regulated by changes in the concentration of IPTG (Matsumura, T., Fujita, Y., Hase, T. unpublished result).

Photosystems and light-harvesting systems consist not only of many structural polypeptides but also of photosynthetic pigments, such as Chls and carotenoids (e.g., Chitnis and Nelson 1991, Vermaas and Ikeuchi 1991). Although these pigments are essential for the biogenesis of photosynthetic apparatus, most previous studies have focused on aspects of polypeptides, such as their transcription (Fluhr et al. 1986), translation (Slovin and Tobin 1982, Eichacker et al. 1992), stability (Bennett 1981, Mullet et al. 1990, Kim et al. 1994) and assembly (Dreyfuss and Thornber 1994a, b), ignoring the roles of pigments in these processes except some reports (Herrin et al. 1992). Further studies on the synthesis of Chl, including the reduction of Pchlide will help us to integrate the mechanism of regulation of the synthesis of Chl by including the contribution of pigments to the biogenesis of the photosynthetic apparatus in plants.

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