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A prokaryotic origin for light-dependent chlorophyll biosynthesis of plants

(protochlorophyllide reductase/bacteriochlorophyll/*Synechocystis*/ *Rhodobacter capsulatus*)

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ABSTRACT **Flowering plants require light for chlorophyll synthesis. Early studies indicated that the dependence on light for greening stemmed in part from the light-dependent reduction of the chlorophyll intermediate protochlorophyllide to the product chlorophyllide. Light-dependent reduction of protochlorophyllide by flowering plants is contrasted by the ability of nonflowering plants, algae, and photosynthetic bacteria to reduce protochlorophyllide and, hence, synthesize (bacterio)chlorophyll in the dark. In this report, we functionally complemented a light-independent protochlorophyllide reductase mutant of the eubacterium *Rhodobacter capsulatus* with an expression library composed of genomic DNA from the cyanobacterium *Synechocystis* sp. PCC 6803. The complemented *R. capsulatus* strain is capable of synthesizing bacteriochlorophyll in the light, thereby indicating that a chlorophyll biosynthesis enzyme can function in the bacteriochlorophyll biosynthetic pathway. However, under dark growth conditions the complemented *R. capsulatus* strain fails to synthesize bacteriochlorophyll and instead accumulates protochlorophyllide. Sequence analysis demonstrates that the complementing *Synechocystis* genomic DNA fragment exhibits a high degree of sequence identity (53–56%) with light-dependent protochlorophyllide reductase enzymes found in plants. The observation that a plant-type, light-dependent protochlorophyllide reductase enzyme exists in a cyanobacterium indicates that light-dependent protochlorophyllide reductase evolved before the advent of eukaryotic photosynthesis. As such, this enzyme did not arise to fulfill a function necessitated either by the endosymbiotic evolution of the chloroplast or by multicellularity; rather, it evolved to fulfill a fundamentally cell-autonomous role.**

One of the more extensively characterized steps in the chlorophyll biosynthetic pathway involves reduction of the fourth ring of the Mg-tetrapyrrole intermediate, protochlorophyllide (1–3) (Fig. 1). Interest in this step of the pathway owes to the dependence of angiosperms on light for protochlorophyllide reduction. Protochlorophyllide reduction has been thought to play an important regulatory role in angiosperm development, since it functionally acts as a gate in the biosynthetic pathway that allows chlorophyll synthesis only when the plant is illuminated. Enzymatic studies have indicated that light-dependent reduction of protochlorophyllide is catalyzed by an enzyme known as NADPH–protochlorophyllide oxidoreductase (E.C. 1.3.1.33) or "light-dependent protochlorophyllide reductase" (reviewed in ref. 1). Light-dependent protochlorophyllide reductase is one of only two enzymes known to require light for catalysis, the other being photolyase (4).

In contrast to angiosperms' dependence on light for protochlorophyllide reduction, nonflowering land plants, algae, and photosynthetic bacteria have a distinct enzyme known as light-independent protochlorophyllide reductase that catalyzes the reduction of protochlorophyllide (and hence promotes photopigment biosynthesis) in the dark. Recent studies with the bacteriochlorophyll-producing, purple, nonsulfur, photosynthetic bacterium *Rhodobacter capsulatus* have resulted in the identification of three genes, *bchN*, *bchB*, and *bchL*, that code for putative subunits of the light-independent protochlorophyllide reductase (5–7). The studies with *R. capsulatus* have also led, in part, to the identification of one or more corresponding light-independent protochlorophyllide reductase genes (*chlN*, *chlB*, *chlL*) in cyanobacteria (8–10) and in the chloroplast genomes of numerous dark-greening photosynthetic eukaryotes, including the green alga *Chlamydomonas reinhardtii* (11–14) and several gymnosperms (14–18). However, light-independent protochlorophyllide reductase genes are conspicuously absent from chloroplast genomes of angiosperms, which correlates with the dependence of this lineage of plants on light for greening (14, 15, 17–19).

Despite the fact that cyanobacteria, algae, and gymnosperms clearly have the capability of synthesizing chlorophyll in the dark, genetic studies also indicate that these diverse oxygenic, photosynthetic organisms appear to contain both the light-independent and light-dependent versions of protochlorophyllide reductase (8–21). While it is clear from DNA sequence analysis that the plant light-independent protochlorophyllide reductase is of bacterial origin, the nature of the light-dependent protochlorophyllide reductase from cyanobacteria has not been examined at a molecular level. In this study, we utilized complementation analysis to clone and sequence the light-dependent protochlorophyllide reductase gene from the cyanobacterium *Synechocystis* sp. PCC 6803.‡ Our results indicate that *Synechocystis* contains a homolog of a plant-type, light-dependent protochlorophyllide reductase gene. These results, as well as those of previous genetic studies (5, 7–10), indicate that both the light-dependent and the light-independent protochlorophyllide reductase enzymes are of prokaryotic origin. The evolutionary implications pertaining to the acquisition and loss of light-dependent and light-independent enzymes are discussed.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *R. capsulatus* strains ZY5 (*bchL*::*km*), JDA (*bchB*::*km*), and JDB (*bchN*::*km*), which contain mutations that disrupt light-independent protochlorophyllide reductase, have been described previously (5, 22, 23). Wild-type *Synechocystis* sp. PCC 6803 was kindly provided by Wim Vermaas, Arizona State University.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. L37783).

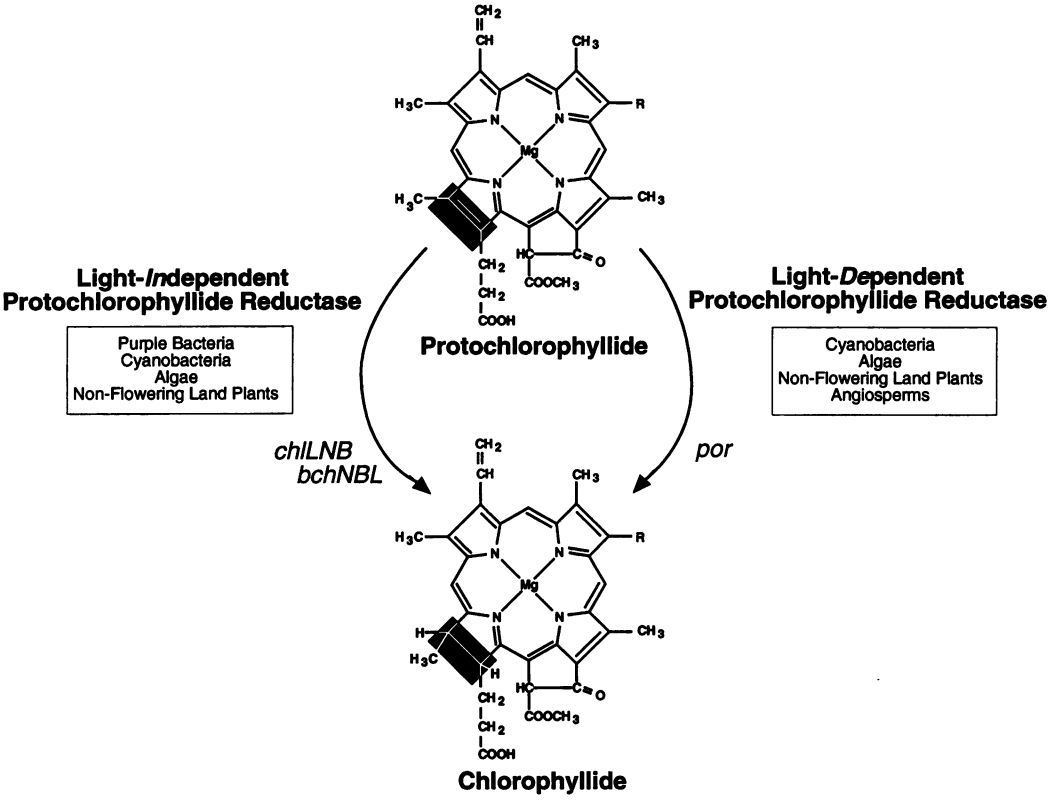

FIG. 1. Latter common steps of the chlorophyll and bacteriochlorophyll biosynthetic pathways.

R. capsulatus strains were routinely grown in RCV 2/3 PY medium (24). For photosynthetic growth, cells were propagated in screw-cap tubes that were completely filled with medium and illuminated with a bank of 60-W incandescent Lumiline lamps emitting approximately 5000 lx. For semianaerobic growth conditions, cells were grown in 30 ml of RCV 2/3 PY or RCV (+) medium (5, 24) in a 50-ml Erlenmyer flask that was gently shaken at approximately 90 rpm.

Synechocystis cultures were grown in BG-11 medium (25) supplemented with 5 mM glucose. Cells were grown at 30°C under continuous light and aeration (provided by bubbling). For genomic DNA isolation (below), cells from a nearly saturated 4-liter culture of *Synechocystis* were isolated and stored at −80°C.

Construction of the *Synechocystis* sp. PCC 6803 Expression Library. A *Synechocystis* cosmid expression library was constructed by ligating unfractionated *Sau*3AI partial digests of *Synechocystis* genomic DNA that was isolated basically as described by Williams (26) into the *Sal* I site of the cosmid vector pPUFHC. Cosmid vector pPUFHC is a derivative of the *R. capsulatus* expression cosmid vector pPUFP1 (5) which exhibits a high copy number in *Escherichia coli*. A 3443-bp *Eco*RI–*Bam*HI fragment from the high copy number plasmid pCop7 (kindly provided from the laboratory of B. Polisky by T. Fitzwater, Indiana University) was blunt end ligated into the unique *Not* I site in pPUFP1, thereby constructing pPUFHC. To prevent the cloning of multiple inserts, digested genomic and vector DNA ends were modified by being partially filled in, as described by Zabarovsky and Allikmets (27). The cosmids were packaged by using Gigapack II XL packaging extract (Stratagene) and subsequently transfected into *E. coli* strain XL1-Blue MR (Stratagene) containing the mobilizing element pDPT51 (28). A total of 12,852 colony-forming units of the XL1-Blue MR/pDPT51 strain library, representing an estimated 19 genome equivalents, were pooled and subsequently stored at −80°C.

Library Matings and Complementing Plasmid Isolation. Bipartite matings were performed by mixing 1.5 ml of a culture of the library-containing *E. coli* cells with the same volume of *R. capsulatus* cells and then drawing the cells down onto sterile Nalgene 0.45-μm filter discs by vacuum filtration. Filters were subsequently placed on RCV (29) plates and incubated for 12 h at 34°C. The RCV plates were then placed in an anaerobic Gas-PAK jar (BBL) and illuminated with a bank of incandescent Lumiline 60-W lamps (emitting approximately 5000 lx) for 4–7 days. Cosmids p6803-1 and p6803-2 were rescued from complemented (photosynthetically competent) ZY5 (*bchL*$^-$) and JDB (*bchN*$^-$) colonies, respectively, and back-mated to ZY5 and JDB as well as to the *bchB* mutant strain JDA to confirm complementation. Based on restriction and Southern hybridization analyses, it was determined that cosmids p6803-1 and p6803-2 contained an identical genomic DNA insert of approximately 1.5 kb.

Sequence Analysis. A *Pst* I restriction fragment ≈2100 bp in size, containing the *puf* promoter region, as well as the *Synechocystis* genomic DNA insert, was subcloned into M13 cloning vectors mp18 and mp19 (Promega). Dideoxynucleotide sequencing reactions from both strands of the insert region were performed from single stranded templates (30) with a Sequenase sequencing kit as described (United States Biochemical) with ^{33}P-dATP as the label (Amersham). Sequence analysis was performed by using the BESTFIT or XPILEUP programs of the University of Wisconsin Genetics Computer Group sequence analysis software package.

Spectral Analyses. Whole-cell absorption spectral analyses were performed on cells grown under either white light (1.25 W from a 65-W, 130-V flood lamp at a distance of approximately 30 cm) or dark growth conditions. For optimum pigment production, strains were grown semianaerobically at 34°C for 2 days in RCV 2/3 PY medium and then subcultured into RCV (+) medium for an additional 2 days. For cosmid maintenance, relevant cultures contained streptomycin at 0.5 μg/ml. For whole-cell spectral analysis, the cell pellet from a

1.5 ml culture was resuspended in 50 μl of RCV medium and mixed with 1.0 ml of 30% bovine serum albumin (5), and the cell suspension was scanned with a Beckman DU-50 recording spectrophotometer.

RESULTS

Cloning Chlorophyll Biosynthesis Genes by Heterologous Complementation of *R. capsulatus* Mutants. In a previous study, we observed intergenus complementation of *R. capsulatus* bacteriochlorophyll biosynthesis mutants with bacteriochlorophyll biosynthesis genes from the related purple photosynthetic bacterium *Rhodospirillum centenum* (23). In this and in a related study (31), we extended this result by isolating chlorophyll biosynthesis genes by functional complementation of *R. capsulatus* bacteriochlorophyll biosynthesis mutants with an expression library that was constructed from the genome of the more distantly related cyanobacterium *Synechocystis* sp. PCC 6803. Prior studies indicated that bacteriochlorophyll and chlorophyll biosynthesis pathways are related in that they are known to share common intermediates (3, 6). There are also structural similarities that exist between the few chlorophyll biosynthesis genes that have been characterized and those of the more extensively characterized bacteriochlorophyll biosynthesis loci (6, 11–15). We therefore reasoned that functional complementation may occur upon overcoming the obvious problems of expression caused by different promoter structures and codon usages between these genera. These problems were overcome in part by constructing a genomic library that placed *Synechocystis* genes under the control of the strong *R. capsulatus puf* operon promoter in the cosmid vector pPUFHC.

We used three strains of *R. capsulatus* which contained stable kanamycin-resistance (Km^r) insertion mutations in genes that coded for putative subunits of light-independent protochlorophyllide reductase for our attempt at heterologous complementation. (*R. capsulatus* strains containing kanamycin-resistance insertion mutations were chosen over strains containing point mutations since we have observed no detectable spontaneous reversion of the insertion mutants.) For complementation, we used strain ZY5 (22) which contained a Km^r insertion in the light-independent protochlorophyllide reductase subunit BchL, strain JDB (5, 23) which contained a Km^r insertion mutation in the light-independent protochlorophyllide reductase subunit BchN, and strain JDA (5, 23) which contained a Km^r insertion mutation in the third light-independent protochlorophyllide reductase subunit BchB. Each of these strains was mated with the *E. coli* strain harboring the *Synechocystis* library and selected for growth under photosynthetic conditions (anaerobic in the light). From the mating with strain ZY5, we obtained a single photosynthetically competent (PS+) exconjugate. Similarly, a single PS+ colony was obtained from a mating with strain JDB. Restriction and Southern hybridization analyses of the cosmids that were rescued from these exconjugates (p6803-1 and p6803-2, respectively) indicated that they contained identical genomic insertions of approximately 1.5 kb (data not shown). As indicated in Table 1, these cosmids were subsequently shown to also complement the *bchB*-disrupted strain JDA.

Sequence Analysis of the Complementing Cosmid. As discussed above, it was determined that cosmids p6803-1 and p6803-2 contained identical genomic inserts of approximately 1.5 kb, which is well below the sum total length (3.843 bp) of the *bchN*, *bchB*, and *bchL* genes that these cosmids complement. This strongly suggested that complementation of these mutants was occurring via a nonhomologous genetic element. Indeed, sequence analysis of the cosmid insert in p6803-1 revealed an open reading frame that coded for a 318-amino acid polypeptide having no sequence similarity to light-independent protochlorophyllide reductase subunits (data not shown). Instead, the *Synechocystis* open reading frame was found to be 56% identical and 74% similar to the sequence of the light-dependent protochlorophyllide reductase of the angiosperm *Arabidopsis thaliana* (32) (Fig. 2 and Table 2). The *Synechocystis* light-dependent protochlorophyllide reductase homolog was located 63 bp downstream of the cosmid cloning site, suggesting utilization of the *R. capsulatus puf* promoter (data not shown). No other recognizable complete open reading frames were deduced from the genomic DNA fragment.

Table 1. Cyanobacterial cosmid complementation of *R. capsulatus* mutants

Mutant strain	Gene	Gene size, bp	Cosmid	
			p6803-1	p6803-2
JDA	*bchB*	1539	+	+
JDB	*bchN*	1392	+	+
ZY5	*bchL*	912	+	+

Complementation of *R. capsulatus* light-independent protochlorophyllide reductase mutants with cosmids from a cyanobacterial (*Synechocystis* sp. PCC 6803) genomic library. + denotes complementation to photosynthetic competence.

Alignment of the deduced *Synechocystis* light-dependent protochlorophyllide reductase peptide sequence with that of various land plants (Fig. 2) demonstrated a high degree of sequence conservation with very few gaps along the entire length of the enzyme. The start site of the cyanobacterial peptide is within six amino acids of the putative processing site for the barley chloroplast transit peptide (32, 35) and ends at the same relative position as all the eukaryotic homologs. Since light-dependent protochlorophyllide reductase is highly conserved among plants (Table 2), it is difficult to assess the relative structural/functional importance of individual amino acids and domains among these sequences. However, inclusion of the cyanobacterial sequence in the sequence comparison clearly divides this protein into modular domains of identically conserved residues. For example, strong blocks of identity of the *Synechocystis* enzyme to plant homologs are found from residue 5 through residue 34, a region proposed to resemble a nucleotide-binding fold and a suggested binding site of the enzyme's cofactor, NADPH (34). In addition, of the six cysteine residues present in the cyanobacterial sequence, three are universally conserved among the plant sequences. One or more of these cysteines have been implicated to be in the active site (1).

Light-Dependent Bacteriochlorophyll Biosynthesis by Complemented *R. capsulatus*. To assess the question of functional homology, we grew the complemented *R. capsulatus* strain ZY5/p6803-1 in the presence or absence of light. As shown in Fig. 3, when strain ZY5/p6803-1 is grown under white light illumination, the cells synthesize bacteriochlorophyll as indicated by the presence of light harvesting and reaction center pigment–protein complexes that absorb light at 800–850 nm. In contrast, when the same strain is grown in the dark, it accumulates a Mg-tetrapyrrole intermediate that exhibits a major absorption peak at 636 nm, a spectrum that is indicative of protochlorophyllide. Thus, complementation of an *R. capsulatus* light-independent protochlorophyllide reductase mutant with the *Synechocystis* light-dependent protochlorophyllide reductase gene confers a dependence on light for bacteriochlorophyll biosynthesis analogous to that observed for chlorophyll biosynthesis in angiosperms.

Since *R. capsulatus* and related anoxygenic photosynthetic bacteria contain no apparent functional homolog of the light-dependent enzyme, the observed light-dependent nature of the complementing cyanobacterial clone reiterates biochemical evidence that the capacity is afforded by this single protein component (35). Light-dependent complementation also im-

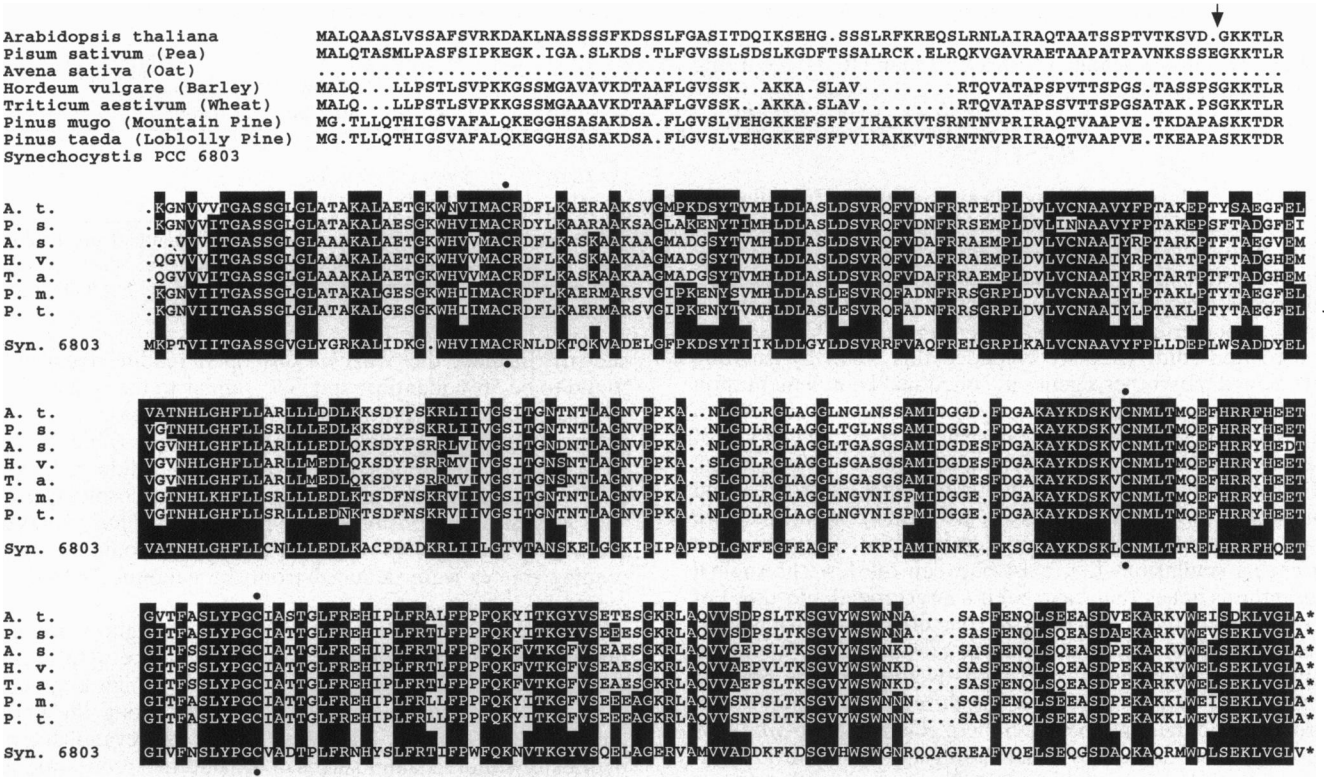

FIG. 2. Amino acid sequence alignment of the deduced *Synechocystis* open reading frame of cosmid p6803-1 with the deduced amino acid sequences of the light-dependent protochlorophyllide reductases from the following land plants: *Arabidopsis thaliana* (32), *Pisum sativum* (33), *Avena sativa* (34), *Hordeum vulgare* (35), *Triticum aestivum* (36), *Pinus mugo* (20), *Pinus taeda* (21), and *Synechocystis* sp. PCC 6803 (this work). Boxes represent cyanobacterial residues conserved identically in the plant homologs. Dotted lines represent gaps in sequence alignment. The oat sequence is missing information in the region corresponding to the transit peptide. The arrow denotes a proposed chloroplast transit peptide processing site, as proposed for the barley sequence (32). Conserved cysteine residues are marked by dots above and below the sequences. Alignment within the boxed region was achieved in part by using the XPILEUP program of the Wisconsin Genetics Computer Group sequencing software package.

plies that elements for proper enzymatic function, such as protochlorophyllide, NADPH, and perhaps FAD (37), are sufficiently accessible to the enzyme and that the product of the reaction, chlorophyllide, can be successfully passed on to subsequent steps. This observation should be taken in the context of models which suggest that bacteriochlorophyll biosynthesis may involve Mg-tetrapyrrole intermediates that are associated with a membrane-bound carrier polypeptide (38, 39).

DISCUSSION

Besides flower development, one of the distinctive features of angiosperms is the requirement of light for greening. *In vivo* spectral analysis by Shibata (40) suggested that angiosperms may have evolved a distinct enzyme that controlled chlorophyll biosynthesis at the point of protochlorophyllide reduction in response to the presence or absence of light. However, recent genetic and molecular genetic studies have indicated that the control of chlorophyll biosynthesis at the step of protochlorophyllide reduction by angiosperms is, in reality, not fundamentally different from that which occurs in other phototrophs. Indeed, the results of this and additional molecular genetic studies (5, 6, 8–21) have indicated that both light-dependent and light-independent versions of protochlorophyllide reductase are present in cyanobacteria, algae, and gymnosperms. Angiosperms, therefore, seem to be a lineage that has simply lost the genes that code for light-independent protochlorophyllide reductase.

As the light-dependent function of pigment biosynthesis is apparently absent from anoxygenic purple photosynthetic bacteria, two questions that arise from this study are why did a light-dependent enzyme evolve and why is it maintained, when a light-independent form of protochlorophyllide reductase was already available? One possible clue may come from the striking evolutionary relationship of the light-independent protochlorophyllide reductase subunits to the subunits of the nitrogenase enzyme complex (41, 42). Since it is well known

Table 2. Percent sequence identity/similarity among light-dependent protochlorophyllide reductase homologs

	Synechocystis	*P. taeda*	*P. mugo*	*T. aestivum*	*H. vulgare*	*A. sativa*	*P. sativum*
P. taeda	54/74						
P. mugo	54/74	98/99					
T. aestivum	53/72	79/89	79/89				
H. vulgare	53/73	79/89	79/89	99/99			
A. sativa	52/73	80/90	80/90	97/98	97/98		
P. sativum	56/74	86/95	85/95	84/91	84/91	85/93	
A. thaliana	56/74	86/94	85/94	82/91	82/91	83/92	90/95

The plant sequences used to generate percent identity/similarity in this table have been truncated to the same relative position as the +2 lysine residue in the *Synechocystis* peptide (see Fig. 2).

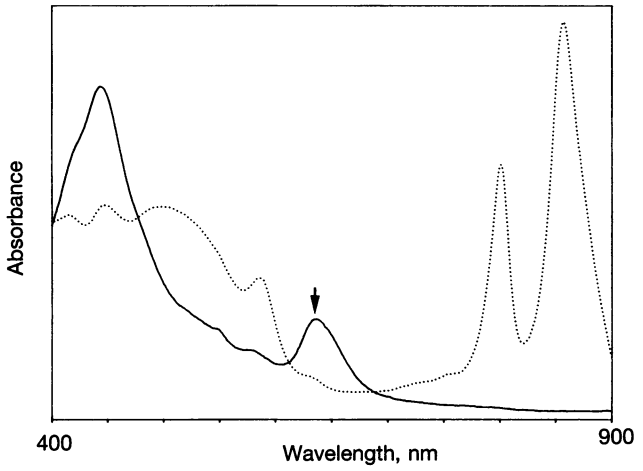

FIG. 3. Whole cell absorption spectrum of strain ZY5 containing cosmid p6803-1 in the presence (dotted line) or absence (solid line) of white light. The arrow denotes the accumulation of protochlorophyllide.

that nitrogenase is sensitive to oxygen (43), it is possible that light-independent protochlorophyllide reductase may also harbor some sensitivity to molecular oxygen. Although this hypothesis will have to be substantiated by enzymatic studies, if true, it would explain why anoxygenic purple photosynthetic bacteria, which synthesize a photosystem only under anaerobic conditions, appear to contain only a light-independent form and why cyanobacteria, which produce oxygen as a consequence of photosynthesis, appear to have evolved a distinct enzyme. A comprehensive survey for the existence of the light-dependent and light-independent protochlorophyllide reductases in oxygenic and anoxygenic prokaryotic phototrophs should give clues to the origins of these enzymes and to their physiological relevance. The results of recent studies also suggest that the light-independent and light-dependent enzymes discriminate between different pools (monovinyl versus divinyl) of protochlorophyllide (44, 45), thereby adding another level of distinction between these two enzymes. Such questions pertaining to the physiological functions of light-dependent versus light-independent protochlorophyllide reductase can now be addressed by using *Synechocystis* sp. PCC 6803, since it is now known to possess both light-dependent and light-independent protochlorophyllide reductases (10) and is amenable to genetic manipulation (26).

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1. Griffiths, W. T. (1991) in *Chlorophylls,* ed. Scheer, H. (CRC, Boca Raton, FL), pp. 433–450.
2. Sironval, C. & Brouers, M., eds. (1984) *Protochlorophyllide Reduction and Greening* (Nijhoff/Junk, The Hague).
3. Beale, S. I. & Weinstein, J. D. (1991) in *New Comprehensive Biochemistry: Biosynthesis of Tetrapyrroles*, ed. Jordan, P. M. (Elsevier, Amsterdam), Vol. 9, pp. 155–235.
4. Sancar, G. B. & Sancar, A. (1987) *Trends Biochem. Sci.* **12,** 259–261.
5. Bollivar, D. W., Suzuki, J. Y., Beatty, J. T., Dobrowolski, J. M. & Bauer, C. E. (1994) *J. Mol. Biol.* **237,** 622–640.
6. Bauer, C. E., Bollivar, D. W. & Suzuki, J. Y. (1993) *J. Bacteriol.* **175,** 3919–3925.
7. Burke, D. H., Alberti, M. & Hearst, J. E. (1993) *J. Bacteriol.* **175,** 2414–2422.
8. Fujita, Y., Matsumoto, H., Takahashi, Y. & Matsubara, H. (1993) *Plant Cell Physiol.* **34,** 305–314.
9. Fujita, Y., Takahashi, Y., Chuganji, M. & Matsubara, H. (1992) *Plant Cell Physiol.* **33,** 81–92.
10. Ogura, Y., Takemura, M., Oda, K., Yamato, K., Ohta, E., Fukuzawa, H. & Ohyama, K. (1992) *Biosci. Biotech. Biochem.* **56,** 788–793.
11. Li, J., Goldschmidt-Clermont, M. & Timko, M. (1993) *Plant Cell* **5,** 1817–1829.
12. Liu, X.-Q., Xu, H. & Huang, C. (1993) *Plant Mol. Biol.* **23,** 297–308.
13. Choquet, Y., Rahire, M., Girard-Bascoe, J., Erickson, J. & Rochaix, J.-D. (1992) *EMBO J.* **11,** 1697–1704.
14. Suzuki, J. Y. & Bauer, C. E. (1992) *Plant Cell* **4,** 929–940.
15. Burke, D. H., Raubeson, L. A., Alberti, M., Hearst, J. E., Jordan, E. T., Kirch, S. A., Valinski, A. E. C., Conant, D. S. & Stein, D. B. (1993) *Plant Syst. Evol.* **187,** 89–102.
16. Tsudzuki, J., Nakashima, K., Tsudzuki, T., Hiratsuka, J., Shibata, M., Wakasugi, T. & Sugiura, M. (1992) *Mol. Gen. Genet.* **232,** 206–214.
17. Yamada, K., Matsuda, M., Fujita, Y., Matsubara, H. & Sugai, M. (1992) *Plant Cell Physiol.* **33,** 325–327.
18. Lidholm, J. & Gustafsson, P. (1991) *Plant Mol. Biol.* **17,** 787–798.
19. Ohyama, K., Kohchi, T., Sano, T. & Yamada, Y. (1988) *Trends Biochem. Sci.* **13,** 19–22.
20. Forreiter, C. & Apel, K. (1993) *Planta* **190,** 536–545.
21. Spano, A. J., He, Z. & Timko, M. (1992) *Mol. Gen. Genet.* **236,** 86–95.
22. Yang, Z. & Bauer, C. E. (1990) *J. Bacteriol.* **172,** 5001–5010.
23. Yildiz, F. H., Gest, H. & Bauer, C. E. (1992) *Mol. Microbiol.* **6,** 2683–2691.
24. Young, D. A., Bauer, C. E., Williams, J. C. & Marrs, B. L. (1989) *Mol. Gen. Genet.* **218,** 1–12.
25. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979) *J. Gen. Microbiol.* **111,** 1–61.
26. Williams, J. G. K. (1988) *Methods Enzymol.* **167,** 766–778.
27. Zabarovsky, E. R. & Allikmets, R. L. (1986) *Gene* **42,** 99–123.
28. Taylor, D. P., Cohen, S. N., Clark, W. G. & Marrs, B. L. (1983) *J. Bacteriol.* **154,** 580–590.
29. Weaver, P. F., Wall, J. D. & Gest, H. (1975) *Arch. Microbiol.* **105,** 207–216.
30. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987) *Current Protocols in Molecular Biology* (Wiley, New York).
31. Suzuki, J. Y. (1994) Ph.D. thesis (Indiana University, Bloomington).
32. Benli, M., Schulz, R. & Apel, K. (1991) *Plant Mol. Biol.* **16,** 615–625.
33. Spano, A. J., He, Z., Michel, H., Hunt, D. F. & Timko, M. P. (1992) *Plant Mol. Biol.* **18,** 967–972.
34. Darrah, P. M., Kay, S. A., Teakle, G. R. & Griffiths, W. T. (1990) *Biochem. J.* **265,** 789–798.
35. Schulz, R., Steinmüller, K., Klaas, M., Forreiter, C., Rasmussen, S., Hiller, C. & Apel, K. (1989) *Mol. Gen. Genet.* **217,** 355–336.
36. Teakle, G. R. & Griffiths, W. T. (1993) *Biochem. J.* **296,** 225–230.
37. Walker, C. J. & Griffiths, W. T. (1988) *FEBS Lett.* **239,** 259–262.
38. Lascelles, J. (1966) *Biochem. J.* **100,** 175–183.
39. Bauer, C. E. & Marrs, B. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7074–7078.
40. Shibata, K. (1957) *J. Biochem. (Tokyo)* **44,** 147–173.
41. Fujita, Y., Takahashi, Y., Shonai, F., Ogura, Y. & Matsubara, H. (1991) *Plant Cell Physiol.* **32,** 1093–1106.
42. Burke, D., Hearst, J. & Sidow, A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7134–7138.
43. Fay, P. (1992) *Microbiol. Rev.* **56,** 340–369.
44. Whyte, B. J. & Griffiths, W. T. (1993) *Biochem. J.* **291,** 939–944.
45. Suzuki, J. Y. & Bauer, C. E. (1995) *J. Biol. Chem.* **270,** 3732–3740.