January 1997

Genetic Analysis of Chlorophyll Biosynthesis

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Annual Reviews Inc.
Annu. Rev. Genet. 1997. 31:61–89

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ABSTRACT

During this decade, there have been major advancements in the understanding of genetic loci involved in synthesis of the family of Mg-tetrapyrroles known as chlorophylls and bacteriochlorophylls. Molecular genetic analysis of Mg-tetrapyrrole biosynthesis was initiated by the performance of detailed sequence and mutational analysis of the photosynthesis gene cluster from *Rhodobacter capsulatus*. These studies provided the first detailed understanding of genes involved in bacteriochlorophyll *a* biosynthesis. In the short time since these studies were initiated, most of the chlorophyll biosynthesis genes have been identified by virtue of their ability to complement bacteriochlorophyll *a* biosynthesis mutants as well as by sequence homology comparisons. This review is centered on a discussion of our current understanding of bacterial, algal, and plant genes that code for enzymes in the Mg-branch of the tetrapyrrole biosynthetic pathway that are responsible for synthesis of chlorophylls and bacteriochlorophylls.

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INTRODUCTION

Photosynthetic organisms ranging from eubacteria to plants synthesize a variety of Mg-tetrapyrroles used for light harvesting and energy-generating charge separation. Cyanobacteria and chloroplasts of algae and plants, which evolve oxygen as a byproduct of photosynthesis, typically synthesize chlorophyll *a* or *b* (8). Purple and green photosynthetic bacteria, which do not evolve oxygen, synthesize a variety of related tetrapyrroles, termed bacteriochlorophylls (103). All of these Mg-tetrapyrroles contain a similar five-membered ring structure, with variations in side chains and/or hydration states of the ring structure. Alterations in the ring structure allow photosynthetic organisms to harvest light at different wavelengths, depending on the type of chlorophylls that are synthesized.

The two types of Mg-tetrapyrroles discussed in detail in this review are bacteriochlorophyll *a* and chlorophyll *a* for which thorough genetic understanding of their biosynthetic pathways exists (Figure 1). As shown in Figure 1, both pathways utilize common intermediates from Mg-protoporphyrin IX through chlorophyllide *a*. Indeed, most of the various bacteriochlorophylls and chlorophylls that are synthesized by photosynthetic organisms appear to utilize similar early metabolic intermediates, which suggests that the various endproducts arose as variants of an evolutionarily conserved biosynthetic pathway (6, 8, 103). Common ancestry of these pathways has been convincingly demonstrated by the presence of extensive sequence homology among enzymes that catalyze similar steps in both chlorophyll *a* and bacteriochlorophyll *a* biosynthetic pathways; details of these are discussed below.

GENETIC DISSECTION OF THE BIOSYNTHETIC PATHWAY

The first genetic analyses of the Mg-tetrapyrrole branch were studies by Granick in 1948 on chlorophyll *a* biosynthesis in the green alga *Chlorella* (41–45). This

initial work was followed in the early 1960s by genetic and biochemical analyses of bacteriochlorophyll *a* biosynthesis with the purple bacterium *Rhodobacter sphaeroides* (see Reference 55 for a review of these initial studies). These early studies demonstrated that the bacteriochlorophyll *a* and chlorophyll *a* pathways utilized a common set of intermediates from Mg-protoporphyrin IX through chlorophyllide *a*. These studies were followed by the cloning, sequencing and directed mutational analyses of genes for the bacteriochlorophyll *a* biosynthetic pathway in the bacterium *Rhodobacter capsulatus*. The *Rb. capsulatus* studies have significantly advanced our understanding of bacteriochlorophyll *a* biosynthesis genes, which in many cases exhibit an ancestral relationship with chlorophyll *a* biosynthesis genes that catalyze similar reactions. The sections below discuss what is known about individual steps of the Mg-tetrapyrrole pathway in bacterial, algal, and plant systems that have been studied.

Bacterial Genetics

Molecular genetic analysis of the Mg-branch of the tetrapyrrole biosynthetic pathway was initiated by Marrs and coworkers with the bacterium *Rb. capsulatus* (73). A combination of generalized transduction (124), R′ mobilization (73), plasmid-based complementation/marker rescue (91), and transposon mutagenesis (10, 128) techniques demonstrated that all of the known loci essential for bacteriochlorophyll *a* biosynthesis were tightly linked to a 45-kb region of the chromosome termed the "photosynthesis gene cluster." Sequence analysis of the entire photosynthesis gene cluster (2, 3, 7, 12, 16, 120, 127), coupled with the construction of defined sets of insertion mutations within each of the open reading frames (14, 15, 123, 125), provided the first comprehensive molecular understanding of genes involved in specific steps in the biosynthetic pathway (Figure 1).

Genetic analysis of the chlorophyll *a* biosynthetic pathway in cyanobacteria was advanced first by cloning *nifH*-like genes from *Plectonema boryanum, Synechococcus* sp. PCC 7002, and *Synechocystis* sp. PCC 6803 (18, 33, 35, 81). Subsequent mutational analysis indicated that the *nif*-like gene was actually involved in protochlorophyllide reduction (34). In a functional approach, several chlorophyll biosynthesis genes from *Synechocystis* 6803 were obtained by complementation of bacteriochlorophyll biosynthesis mutants of *Rb. capsulatus* (102, 109). A major advance in this field was obtained by complete sequencing of the *Synechocystis* 6803 genome from which numerous chlorophyll biosynthesis genes have been identified based on sequence homology to *Rb. capsulatus* bacteriochlorophyll biosynthesis genes (56, 57). Because of the high degree of sequence identity that exists between the cyanobacterial and plant homologs, it should be possible to obtain many plant genes by direct hybridization using cyanobacterial chlorophyll (*chl*) genes as probes (18, 108).

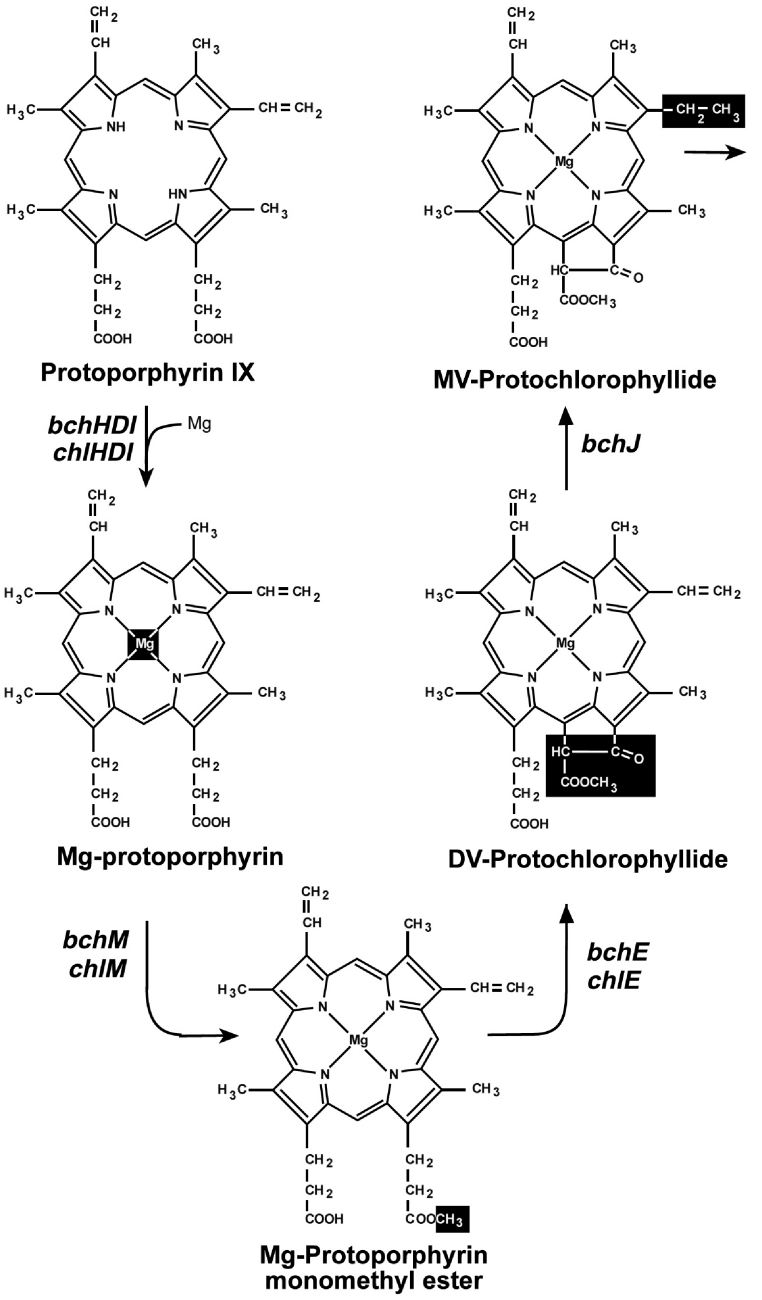

Figure 1 (*Continued*)

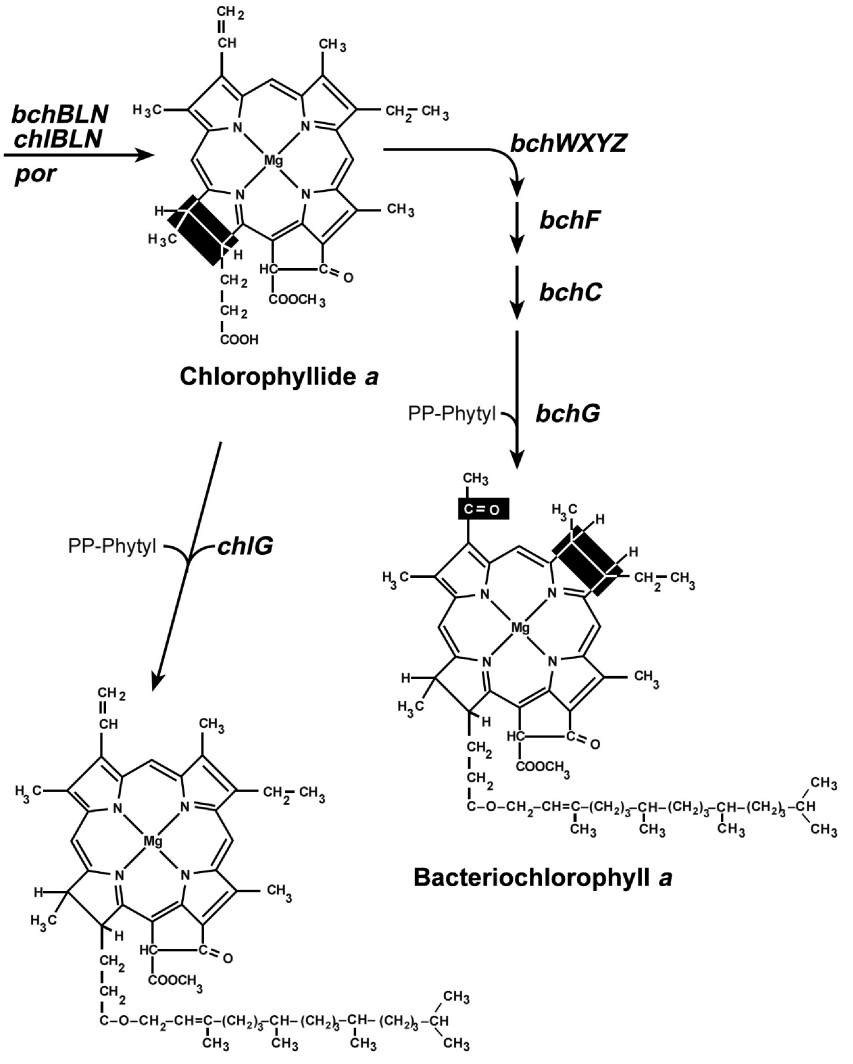

Figure 1 Mg-branch of the bacteriochlorophyll *a* and chlorophyll *a* biosynthetic pathways. Both pathways share common intermediates up to the synthesis of chlorophyllide, at which point the tetrapyrrole ring in the bacteriochlorophyll *a* pathway undergoes additional modification. Modifications of the tetrapyrrole ring at various stages of the pathway are highlighted with a black box. Genetic loci that affect individual steps of the pathway are also indicated above the arrows.

Alternatively, polymerase chain reaction can be used to amplify plant genes by designing primers that hybridize to regions that are conserved among *Rb. capsulatus* and *Synechocystis* 6803 homologs (18).

Algal Genetics

Genetic analysis of the chlorophyll *a* biosynthetic pathway in algae was initiated in the mid-1940s by Granick, who isolated chlorophyll-deficient strains of *Chlorella* (41–45). These studies were complemented by the characterization of additional *Chlorella* mutants by Ellsworth & Arnoff in the late 1960s (20–23). Work with the green alga, *Chlamydomonas reinhardtii,* began in 1955 (95), and it has become the model organism for genetic analysis of chlorophyll *a* biosynthesis in algae.

One of the first detailed studies of a chlorophyll biosynthesis mutant of *C. reinhardtii* was performed by Ruth Sager, who used the mutant *y-1* to help define the nuclear inheritance patterns of this organism (95, 96). *y-1* mutants exhibit a "yellow in the dark" phenotype as a result of dark accumulation of protochlorophyllide, although these cells are still capable of synthesizing chlorophyll in the light. Despite many attempts over the years, no additional loci with a similar phenotype were identified, until Ford & Wang described a series of temperature-sensitive mutants that were generated by UV mutagenesis (25–27). Their work led to the identification of six more nuclear loci with similar "yellow in the dark" phenotypes, as described for *y-1*. A set of brown mutants (*br*), also isolated by Wang et al (117), accumulate protoporphyrin IX that presumably contains a defect in Mg-chelatase.

The development of mutagenesis techniques that allow the creation of nuclear insertion mutations in *C. reinhardtii* should facilitate the cloning of some of these nuclear-encoded *y* and *br* loci in the future. The utility of such an approach is highlighted by the cloning of nuclear genes from *C. reinhardtii* that are involved in flagellar biosynthesis (111).

Plant Genetics

Mutants characterized for chlorophyll deficiency have been isolated from barley, wheat, rice, maize, and Arabidopsis. Mutant classes described in these plants include *albina, xan, chlorina, vir*, and *chlb. albina* mutants lack detectable tetrapyrrole intermediates, whereas *xan, chlorina*, and *vir* are defective at biosynthetic steps from magnesium insertion to the synthesis of protochlorophyllide. *chlorina* mutants, which are defective in Mg-chelation, are usually recessive lethal. *vir* mutants, which are a phenotype that describes various pigment types, typically appear conditionally such as under low temperatures.

Pigment-deficient seedling lethal mutants of grasses attain a relatively advanced level of growth morphologically, even in the absence of light. This

property allows for easy visual identification of mutants as well as providing for sufficient plant material for biochemical analysis. To date, systematic screens for chlorophyll biosynthesis mutants of barley have yielded five loci thought to encode structural genes for chlorophyll biosynthesis at two biosynthetic steps. The loci *xan-f, xan-h,* and *xan-g* are mutants in subunits of the enzyme for protoporphyrin IX Mg-chelatase, whereas *xan-l* and *vir-k* are thought to be components of the cyclase reaction (discussed in detail below).

Runge et al (93) recently attempted to isolate mutants in structural genes for chlorophyll biosynthesis on a systematic basis in Arabidopsis. Three classes of mutants were screened for: (*a*) mutants for steps prior to protoporphyrin IX, (*b*) mutants for steps between magnesium chelation and protochlorophyllide reduction, and (*c*), mutants at steps following protochlorophyllide reduction. In their screen for mutants, the authors utilized the fact that loss of chlorophyll is often a consequence of chloroplast dysfunction correlating with nutritional deficiencies rather than the cause of chloroplast dysfunction. A fraction of mutants initially isolated as chlorophyll deficient could be eliminated based on their ability to produce chlorophyll when sucrose was added to the medium. As observed in other systems, no mutants in the first class were isolated.

A study by Kruse et al (63) addressed why this and other studies in barley failed to result in mutants at biosynthetic steps prior to protoporphyrin IX. In their analysis, they assayed the effect of reducing expression of enzymes early in the pathway using antisense inhibition. Using an antisense coproporphyrinogen oxidase gene behind the strong constitutive cauliflower mosaic virus promoter in tobacco, the authors found growth-inhibition phenotypes similar to those in plants treated with the diphenyl ether–based herbicides, which disrupt the same enzyme. Thus depression of the activities of certain enzymes can result in the accumulation of photoactive tetrapyrrole intermediates that cause photooxidative damage. Similar experiments using a glutamate-1-semialdehyde aminotransferase antisense gene (an enzyme involved in synthesis of ALA) yielded plants with varying degrees of pigment loss, which coincided with the effectiveness of the antisense gene at inhibiting expression (48). Completely inviable seeds and white plantlets, which could not be kept alive, were sometimes obtained and presumably represent clones containing complete repression of enzyme synthesis. Based on these results, it appears that mutants that accumulate intermediates prior to protoporphyrin IX have pleiotropic effects on germination and growth that may explain why this class of mutants is difficult to obtain. Pleiotropic effects, such as lethality caused by defects at enzymatic steps prior to protoporphyrin IX formation, would also be expected if heme and chlorophyll are indeed derived from the same pool of intermediates.

The tagging of genes by random insertion of *Agrobacterium* T-DNA, or with transposable mobile elements, has proven useful in isolating at least two

chlorophyll genes in plants (50, 61). Coincidentally, they are both mutants that disrupt the Mg-chelatase step. T-DNA can be utilized in any plant that *Agrobacterium* can infect (62). Pigment mutants are found among T-DNA transformed lines of Arabidopsis that are maintained at the Ohio Biological Resource Center, USA, but most have not been characterized as to their primary defect. The use of transposon mutagenesis has the advantage that the mobile elements can be found in a wide variety of model plants. For example, the *Ac* and *Ds* transposons of maize are mobile in a number of other plants such as *Nicotiana tabacco, Lycopersicon esculentum* (tomato), and Arabidopsis (40), which widens the use of this mutagenesis technique. On the other hand, many mutants involved in chloroplast development and biogenesis have defects in chlorophyll synthesis, which makes it difficult to determine the primary lesion.

Random sequence analysis of expressed sequence tagged (EST) cDNAs from dicots Arabidopsis, *Brassica campestris,* and Castor bean; from monocots maize and rice; and the conifer *Pinus taeda* has recently yielded putative chlorophyll biosynthesis homologs of genetically characterized bacteriochlorophyll genes. Isolates of *chlG* (chlorophyll synthetase) and *chlP* from Arabidopsis and rice have been detected (69, 100). As shown in Table 1, four of the seven loci listed yielded plant homologs. The various cDNA sequencing projects are thus useful clone pools for identifying chlorophyll biosynthesis genes from plants. As the plant genome sequencing databases become more complete, most if not all of the higher plant chlorophyll biosynthesis loci will likely be identified by homology searches.

COMMON STEPS IN Mg-TETRAPYRROLE PATHWAYS

Mg-Chelation

Mutational analysis of the *Rb. capsulatus* photosynthesis gene cluster initially revealed that three sequenced genes, *bchH, bchD*, and *bchI*, are involved in Mg-chelation (12, 14, 123, 128). Identification of BchH as the probable magnesium binding subunit of the Mg-chelatase was reasoned based on homology to the divalent cation binding subunit of the cobalto-chelatase enzyme from *Pseudomonas denitrificans* (50). Subsequent expression of the *Rb. sphaeroides* and *Synechocystis* 6803 homologs in *Escherichia coli* has definitively proven the requirement for all three subunits for catalytic activity (39, 53). The reaction is shown in vitro to involve the formation of an ATP-binding BchI homodimer that interacts with the 550-kDa BchD aggregate. Mg insertion appears to occur on the 140-kDa BchH subunit that binds protoporphyrin IX on a 1:1 molar ratio. ATP activation is consistent with studies by Walker & Weinstein (118).

Brown mutations of *C. reinhardtii,* described by Wang et al (117), accumulate elevated protoporphyrin IX, which suggests that they affect Mg-chelatase.

These authors isolated mutants representing two different loci: Strains bearing mutations in the br_c locus were able to make chlorophyll in the light, but not in the dark, whereas strains having mutations in the br_s, locus were unable to synthesize chlorophyll regardless of the light conditions. The mutant *y-y* has been described that accumulates protoporphyrin IX at much lower levels than are observed with the *br* mutants (78, 79). The reason for this difference as well as the relationship between these genetic loci and the *Rhodobacter* subunits remain unclear. Sequence analysis of chloroplasts from a number of alga (*Porphyra purpurea, Cyanophora paradoxa, Olisthodiscus luteus, Cryptomonas phi, Odontella sinensis*; see Table 1) has revealed the presence of *bchI* homologs (*chlI*). Homologs of *bchI* in plant chloroplasts are, however, absent. Homologs of *bchH* and *bchD* are also absent in sequenced chloroplasts.

Plant homologs of the *Rhodobacter* Mg-chelatase complex have been identified in several species (Table 1). T-DNA tagging resulted in the disruption of a homolog of *bchI* (*ch-42*) in Arabidopsis (61), whereas Tam transposition mutagenesis resulted in the disruption of a *bchH* homolog (*oli*) of *Antirrhinum majus* (50). So far, *oli* and *ch-42* are the only examples of tagged genes that have been identified as being involved in chlorophyll biosynthesis. *xan* mutants of barley, which are defective in Mg-chelation, were evaluated to ascertain the basis of their mutant phenotype using antibody probes to CH42 of Arabidopsis and OLI of *A. majus* (54). Some, but not all, *xan-f* alleles showed a loss of OLI antibody crossreactivity, indicating a defect in synthesis of a BchH homolog (Xan-H or ChlH), whereas *xan-h* mutants appeared defective in synthesis of a BchI homolog (Xan-I or ChlI) (54). Loss of accumulation of ChlI did not necessarily affect the accumulation of ChlH and vice versa. Accumulation of both ChlI and ChlH could be observed in *xan-g* mutants purported to be defective in a homolog of BchD (ChlD) (54, 58). These studies showed that the different mutants in Mg-chelation did not necessarily prevent the accumulation of other subunit proteins involved in the same step. Although the *xan* mutants had defects in Mg-chelation, all could perform the subsequent enzymatic step of methylation in vitro when Mg-protophorphyrin IX was included in the reaction mix, indicating that loss of chelatase activity did not abolish activity of a later step.

xan-h and *xan-f* were cloned through the use of degenerate PCR primers that were designed to hybridize to conserved regions of known sequences (54). Templates for the PCR amplification primers were single-strand cDNA from greening barley leaves. The deduced XAN-H (ChlI) protein sequence of barley was found to be 85% identical to Ch42 of Arabidopsis, 49–69% identical to the algal and *Euglena* sequences, and 49% identical to BchI of *Rb. capsulatus.* The XAN-F protein (ChlH) was found to be 82% identical to the *A. majus* OLI, 66% to ChlH of *Synechocystis* 6803, and 34% to BchH of *Rb. capsulatus.*

Table 1 Genbank submitted genes common to the bacteriochlorphyll *a* and chlorophyll *a* biosynthetic pathways

Enzyme gene	Organism	Alternate name	Accession no.
Mg-Chelatase			
bchH/chlH	*Rhodobacter capsulatus*		Z11165, M74001
	Synechocystis		D90902, X96599
	Antirrhinum majus	*oli* (*olive*)	X73144
	Arabidopsis thaliana	*chlH*	D68495, Z68495
	Hordeum vulgare	*xan-f*	U26916
	Arabidopsis thaliana		H76104
	Oryza sativa		D47916
bchI/chlI	*Rhodobacter capsulatus*		Z11165
	Synechocystis		D90904
	Anabaena variabilis		D49426
	Porphyra purpurea		U38804
	Cyanophora paradoxa		U30821
	Olisthodiscus luteus		Z21959
	Cryptomonas phi		Z21976
	Odontella sinensis		Z67753
	Arabidopsis thaliana	*ch-42* (*chlorata*)	Z11165
	Glycine max		D45857
	Hordeum vulgare	*xan-h*	U26545
	Arabidopsis thaliana		AA042226
	Zea maize		W49410
bchD/chlD	*Rhodobacter capsulatus*		Z11165
	Synechocystis		X96599
	Anabaena variabilis		D49426
Mg-Protoporphyrin methyl transferase			
bchlM/chlM	*Rhodobacter capsulatus*		Z11165
	Synechocystis		D64006, L47125
Cyclase			
bchlE/chlE	*Rhodobacter capsulatus*		Z11165
	Rhodobacter sphaeroides		L37197
	Synechocystis		D64003, L47125
Protochlorophyllide reductase (light-dependent)			
por	*Synechocystis*		L37783, D64004
	Chlamydomonas reinhardtii		U36752
	Hordeum vulgare	*porA, porB*	X15869, 84738
	Avena sativa		X17067
	Oryza sativa		D46584
	Zea maize		T27547, W49454
	Arabidopsis thaliana	*porA, porB*	U29699,U29785, AA042730
	Pisum sativum	*pcr*	X63060
	Cucumis sativa	*npr*	S78381
	Pinus strobus	*lpcr*	
	Pinus taeda	*lpcr*	X66727, H75261
	Pinus mugo	*porA*?, *porB*?	S63824, S63825

Table 1 (*Continued*)

Enzyme gene	Organism	Alternate name	Accession no.
Protochlorophyllide reductase (light-independent)			
bchL/chlL	*Rhodobacter capsulatus*		Z11165
	Synechocystis		D90916
	Synechococcus		X67694
	Plectonema boryanum		D00665
	Chlamydomonas reinhardtii frxc		X60490
	Porphyra purpurea		U38804
	Marchantia polymorpha	*frxC*	X04465
	Pinus thunbergii	*chlL*	D17510
	Pinus contorta	*frxC*	X56200
bchB/chlB	*Rhodobacter capsulatus*		Z11165
	Synechocystis		U36144, D64000
	Plectonema boryanum		D78208
	Chlamydomonas reinhardtii		U02526
	Porphyra purpurea		U38804
	Marchantia polymorpha	ORF513	X04465
	Ginkgo biloba		U01531, U04440
	Pinus thunbergii		D17510
	Pinus strobus		U02533
	Larix eurolepis		X98680, X98681
	Pinus sylvestris		X98683, X98684
	Picea abies		X98686, X98687
bchN/chlN	*Rhodobacter capsulatus*		Z11165
	Synechocystis		D90916
	Synechococcus		X67694
	Plectonema boryanum		D12973
	Chlamydomonas reinhardtii		
	Porphyra purpurea		U38804
	Marchantia polymorpha	ORF465	X04465
	Pinus thunbergii		D17510
	Pinus contorta	*gidA*	X56200
8-vinyl reductase activity			
bchlJ	*Rhodobacter capsulatus*		Z11165
Chlorophyll synthetase			
bchG/chlG	*Rhodobacter capsulatus*		Z11165
	Chloroflexus aurantiacus		U43963
	Synechocystis		U36144
	Arabidopsis thaliana	g^4	U19382, Z34566
	Oryza sativa		D48639
Phytol synthesis (from HMG-CoA)			
bchP/chlP	*Rhodobacter capsulatus*		Z11165
	Synechocystis		X97972
	Arabidopsis thaliana	*Gghyl*	T13808
	Oryza sativa	*Gghyl*	D47484

Cloning of the *bchD* homolog thought to correspond to *xan-g* has not yet been reported.

Probes to *xan-f* and *xan-h* were used to monitor expression patterns during greening of etiolated seedlings (54). Results indicated that the steady state level of *xan-h* mRNA was tenfold lower than that observed for *xan-f* mRNA. During greening, both *xan-h* and *xan-f* mRNA levels increased to maximum levels, with *xan-f* increasing 2–3-fold and *xan-f* increasing 18–20-fold at 4 h. Mg-chelatase activity showed a 3- to 4-fold induction during this period, with a peak at 6 h and a drop to dark levels at 24 h. These levels were maintained up to 20 h in continuous light for *xan-f*, but declined at 8 h and to the dark level at 24 h. *xan-f* also showed a diurnal pattern of induction with plants grown on a 8:00–24:00-h light period. *xan-f* expression peaked at 11:00, with light-harvesting chlorophyll binding gene expression (*lhcb2*) exhibiting a peak at 13:00. Subsequent growth of plants in continuous light showed that both *xan-f* and *lhcb2* maintained a circadian rhythm in transcript accumulation that was not reflected by changes in chelatase activity.

A cDNA coding for a homolog of *bchH* (*CHL H*) has also been cloned and sequenced from Arabidopsis (38). When grown in a light/dark cycle *CHL H* transcripts exhibited a diurnal variation with maximal levels at the end of the dark cycle, increasing slightly during the onset of light and then decreasing to minimal levels until the end of the light period. In situ hybridization experiments reveal that cytosolic messages for *CHL H* and *ch-42* (a homolog of *bchI*) are found associated with the surface of the chloroplast (38, 72).

The availability of the *chlI* gene from Arabidopsis has led to the isolation of a plant homolog from soybean by cross species hybridization with the Arabidopsis gene (77). Among the structural features conserved in the plant genes is an ATP binding motif, which is consistent with the requirement of ATP for in vitro activity. In cell cultures of soybean, increased transcript accumulation is observed in response to light stimulation similar to the observation in Arabidopsis and barley (77).

Methyl Transferase

After some initial confusion about the nature of the intermediate accumulated by *bchM* mutants of *Rb. capsulatus*, it was demonstrated that this locus most likely codes for the enzyme *S*-adenosyl-methionine:Mg-protoporphyrin IX methyl transferase, which is responsible for methylating a propionate side chain on ring 3 in protoporphyrin IX (14). The BchM polypeptide was unequivocally shown to catalyze this reaction by demonstrating that *E. coli* extracts containing heterologous expressed BchM could undertake this reaction in vitro (13, 37).

A homolog of BchM (ChlM) was cloned from *Synechocystis* 6803 by functional complementation of a *bchM* mutant of *Rb. capsulatus* with a *Synechocystis* expression library (102) (Table 1). Sequence analysis indicates that the

Synechocystis ChlM polypeptide exhibits 29% sequence identity with that of *Rb. capsulatus* BchM. As yet, no plant gene for this step has been isolated.

Isocyclic Ring Formation

A fifth ring of the tetrapyrrole is synthesized by a complex multistep process involving the 6-methyl propionate group (the biochemistry of this reaction is reviewed in Reference 8). To date, only the *bchE* gene of *Rb. capsulatus* has been genetically demonstrated to be involved in this reaction (14, 128). The *Synechocystis* 6803 genome sequencing project has demonstrated that this species contains a homolog of *bchE* (56) (Table 1). The existence of an identifiable chlorophyll biosynthesis homolog of *bchE* is notable, given that the cyclization reaction is fundamentally different in these two pathways. Specifically, the addition of a hydroxyl during the closure reaction in chlorophyll biosynthesis involves a mixed function oxidase reaction that utilizes dioxygen (O_2) as a substrate (119). In contrast, the hydroxyl group in the anaerobic reaction that occurs during bacteriochlorophyll biosynthesis is obtained from H_2O (84). This would indicate either that two BchE homologs have evolved to use separate substrates or that there are unrelated polypeptide components of this reaction that are yet to be identified.

In barley, the *xan-l* and *vir-k* mutants may contain defects in the cyclase activity since these mutants exhibit both Mg-chelatase and methyltransferase activity but do not produce protochlorophyllide. It is not known whether these loci code for structural components of this enzyme or for regulatory components. There have been no reports of the cloning and sequence analysis of these genes, nor of any other plant or algal genes in this step of the pathway.

Vinyl Reductase(s)

Most of the early common intermediates in bacteriochlorophyll and chlorophyll biosynthesis (from protoporphyrin IX through Mg-protoporphyrin IX) contain vinyl groups at the 2 and 4 positions of the ring and are thus appropriately called divinyl intermediates (8). However, the 4-vinyl group is reduced to an ethyl group in both pathways, giving rise to monovinyl chlorophyll and bacteriochlorophyll as the final product. Even though the final product in the pathway is exclusively monovinyl, reduction of the 4-vinyl group occurs incompletely at several locations of the pathway. As a result, there are mixed pools of monovinyl and divinyl intermediates. A number of studies show that protochlorophyllide and chlorophyllide are found in varying monovinyl/divinyl ratios depending on species, environmental condition, or plant age (discussed in Reference 110). The significance of these observations is not known.

A mixed pool of monovinyl and divinyl intermediates indicates either that there is a single enzyme with a reduced substrate specificity or that there are multiple 4-vinyl reductases that are responsible for reducing the 4-vinyl group

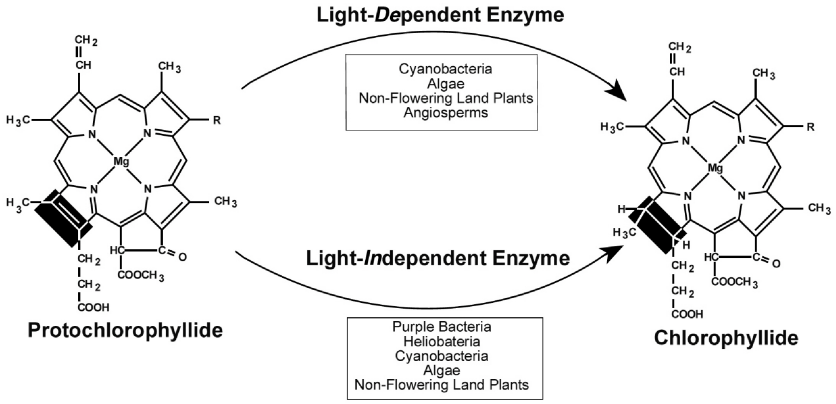

Figure 2 Light-dependent versus light-independent protochlorophyllide reduction.

on different intermediates. The existence of a nonenzymatic route or possibly multiple enzymes is supported by genetic studies of *Rb. capsulatus* in which it was observed that disruption of *bchJ* resulted in the accumulation of a larger pool of divinyl protochlorophyllide (110). However, there still remained a significant fraction of monovinyl protochlorophyllide in *bchJ* mutants indicating that the cells were still capable of 4-vinyl reduction at some level. No homolog of *bchJ* has been observed in any other photosynthetic organism including *Synechocystis*. Thus chlorophyll biosynthesis either has an alternative mechanism of reducing the 4-vinyl group or a chlorophyll biosynthesis homolog of *bchJ* has diverged to a point where no readily identifiable sequence similarity remains.

Protochlorophyllide Reduction

There are two mechanisms for reducing the double bond in the fourth ring of protochlorophyllide (Figure 2) (recently reviewed in 31, 85, 86). One enzyme complex functions irrespective of the presence or absence of light and is thus termed "light-independent protochlorophyllide reductase." The second is a light-dependent reaction that utilizes the enzyme NADPH-protochlorophyllide oxidoreductase (POR). Until recently, the POR enzyme was thought to be unique to the angiosperm lineage of plants, which require light for chlorophyll synthesis. However, we now know that angiosperms have simply lost the light-independent enzyme complexes and that all other photosynthetic organisms ranging from gymnosperms to cyanobacteria, as well as some species of anoxygenic photosynthetic bacteria, contain both light-dependent and light-independent protochlorophyllide reductase enzyme complexes. Below is a discussion of these two enzymes.

THE LIGHT-INDEPENDENT REACTION Our understanding of light-independent protochlorophyllide reductase stems from genetic studies initiated in *Rb. capsulatus*, where it was demonstrated that three genes, *bchL, bchB*, and *bchN*, were involved in protochlorophyllide reduction (7, 105, 123, 128) (Table 1). Subsequent sequence analysis revealed the surprising finding that the three open reading frames exhibit significant sequence similarity to the three subunits of nitrogenase (32, 33, 35, 108). It has thus been proposed that light-independent protochlorophyllide reductase and nitrogenase share a common evolutionary ancestor (17, 31, 33, 108). In vitro light-independent activity has, however, been difficult to observe. Thus, biochemical proof that these open reading frames code for catalytic subunits awaits future studies. Based on similarities of this enzyme to nitrogenase, which has oxygen-labile metal centers, it has been proposed that the light-independent enzyme might similarly be sensitive to oxygen (71, 108). Purification and assays for activity under anaerobic conditions may be a fruitful approach to this problem.

Algal and plant homologs of light-independent protochlorophyllide reductase were identified by piecing together clues from sequence data of plant and algal chloroplasts that contained open reading frames with sequence similarity to the bacterial subunits (Table 1). A study with *C. reinhardtii* initially uncovered a locus that caused the loss of protochlorophyllide reduction in the dark which was appropriately termed *gidA* (*g*reen *i*n the *d*ark) (90). In 1992, the *gidA* locus (subsequently been renamed *chlN*) was revealed to be a homolog of the *bchN* gene of *Rb. capsulatus* (19). Sequence analysis of the chloroplast genome of *Marchantia polymorpha* revealed that the chloroplast contained a gene, termed *frxC*, with a high degree of sequence identity to the *nifH* gene of nitrogenase, as well as to the *bchL* gene present in the photosynthesis gene cluster of *Rb. capsulatus* (60). Clues to the function of this open reading frame came from work by Yang & Bauer (123), who showed that mutations in the *bchL* gene resulted in the loss of light-independent protochlorophyllide reduction. Suzuki & Bauer (108) confirmed that the chloroplast gene *frxC* (renamed *chlL*) was involved in light-independent protochlorophyllide reduction by isolating a homologous gene from *C. reinhardtii* using the *M. polymorpha* gene as probe. Subsequent disruption of *chlL* by particle-gun mediated transformation resulted in a "yellow in the dark phenotype" caused by the inability of the transformants to reduce protochlorophyllide in the dark. Similarly, directed mutations in *chlN* confirmed earlier evidence for the role of this gene in light-independent protochlorophyllide reduction in *C. reinhardtii* (19). Li et al (66) and Liu et al (68) discovered and disrupted a third chloroplast-encoded gene in *C. reinhardtii* that is homologous to *bchB* in *Rb. capsulatus*.

One or more of the protochlorophyllide reductase subunit genes have been detected in the chloroplast genome of all algae and nonflowering land plants

tested with the exception of *Psilotum* and *Welwitchia* (18, 108). The sequences are notably absent from the sequenced chloroplasts of Euglena (46), rice (47), tobacco (107), maize (70), and beechdrop (122), which lack light-independent chlorophyll synthesis. Thus, the distribution of these genes appears to correlate well with the ability of nonflowering plants, algae, and bacteria to synthesize chlorophyll in the dark. More recently, *chlB* has been utilized to study the phylogenetic relationship among nonflowering plants based on structural differences in the *chlB* gene from various phyla (11). Interestingly, *chlB, chlL,* and *chlN* are unlinked in the *C. reinhardtii* chloroplast (19, 66, 68, 108), whereas *chlN* and *chlL* are linked in all other chloroplast genomes of nonflowering plants (60, 67) and nongreen algae (87) so far sequenced, as well as in the genome of *Synechocystis* 6803 (81) and *Plectonema boryanum* (33–35). The *chlB* gene is unlinked to the other loci in all cases so far investigated.

Except for the initial studies, expression/function of genes for light-independent protochlorophyllide reduction has not been extensively examined in vivo. In one study, transcripts for *chlB* in synchronously grown cells of *Chlamydomonas moewusii* were observed mainly between the tenth hour of light and fourth hour of darkness when grown on a 12-h light /dark cycle (92), a finding that correlates with a function for this gene in dark chlorophyll synthesis.

Genetic selection for strains of *C. reinhardtii* defective in light-independent protochlorophyllide reduction has resulted in the isolation by Sager, Wang, and coworkers (25–27, 95, 97) of mutations in seven different nuclear loci, *y-1, y-5, y-6, y-7, y-8, y-9,* and *y-10*. Most of these loci were represented by temperature-sensitive mutations, indicating that they encode polypeptides. This leaves in question the role that the nuclear loci play in the synthesis or assembly of the light-independent protochlorophyllide enzyme: several reasonable hypotheses bear consideration. The "yellow in the dark" mutants have an unusual chloroplast morphology (82, 83, 97), suggesting that some *y* loci may encode genes involved in regulation of chloroplast development. Another reasonable hypothesis is that these loci are involved in coordinating gene expression between the chloroplast and the nucleus, either as nuclear-encoded chloroplast transcription factors or as translational regulators. Recently, RNA editing has been reported for the *chlB* gene of conifers (59), although it is not known how widespread this phenomenon is, or whether other genes such as *chlL* and *chlN* are affected or if editing affects gene function. In the pine *Pinus thunbergii*, editing of chloroplast genes has the potential of creating start and stop codons (116).

THE LIGHT-DEPENDENT REACTION The biochemistry of the light-dependent protochlorophyllide reductase enzyme (POR) is interesting in that it is one of only two enzymatic activities requiring light for catalysis (reviewed in

Reference 8); the other activity is a reaction carried out by photolyase. The polypeptide binds both protochlorophyllide and NADPH. Reduction of the double bond does not occur until protochlorophyllide absorbs light at 628–630 nm. The enzyme reaction then undergoes several intermediate states that can be observed by spectral shifts in the tetrapyrrole, ultimately resulting in the release of chlorophyllide and NADP.

The first eukaryotic chlorophyll biosynthesis gene successfully cloned was that of POR. Isolation of the *por* gene was achieved by purification of the enzyme from barley in quantities large enough to allow the production of antibodies for cloning *por* by immunodetection from cDNA expression libraries (99). The *por* gene has subsequently been isolated and sequenced from a wide variety of plants such as barley, wheat, oat, pea, Arabidopsis, and pine (see 85 and references therein) as well as from *C. reinhardtii* (65) and *Synechocystis* 6803 (109) (Table 1). Molecular analysis of POR expression has recently been discussed in several reviews (31, 85, 114).

It was initially thought that the light-dependent enzyme may have evolved to fulfill a functional role for light-dependent chlorophyll synthesis in angiosperms. However, genetic analysis of the light-independent enzyme revealed that algal and cyanobacterial cells also contain a gene that codes for a copy of the light-dependent POR enzyme (27, 34, 95–97). The presence of POR in cyanobacteria was confirmed by cloning the *Synechocystis por* gene by functional complementation of light-independent protochlorophyllide reductase mutants of *Rb. capsulatus* (109). Sequence analysis demonstrated that the *Synechocystis* POR polypeptide exhibited 53–56% sequence identity with deduced mature peptides of plant homologs. This clearly demonstrates a direct ancestral relationship of the bacterial and plant enzymes. Thus, it appears that both enzymes are of bacterial origin and that their co-presence is a widely disseminated feature among photosynthetic organisms. The exceptions are purple bacteria, which appear to have retained only the light-independent enzyme, and angiosperms and Euglena, which have retained only the light-dependent enzyme.

The activity and expression pattern of the light-dependent enzyme have been studied in a variety of land plants. Two *por* genes with distinct expression patterns, *porA* and *porB*, have recently been discerned in both the monocot barley and the dicot Arabidopsis (4, 49). Noticeable differences exist between the predicted protein precursors of PORA and PORB in barley (75% overall a.a. identity; 388 a.a., 395 a.a., respectively), particularly in the putative transit peptide region (46% a.a. identity) (49) but less so in Arabidopsis (88% overall a.a. identity; 405 a.a., 401 a.a., respectively) (4). In barley, PORA (36 kDa) is abundant in dark-grown tissue and disappears upon exposure of seedlings to light, whereas the minor form PORB persists in equal abundance throughout

(49). In Arabidopsis, a 36-kDa form of POR (which corresponds to PORB; Reference 94) is both more abundant and persistent in the light compared to a 37-kDa form (PORA). The *porA* gene is expressed specifically in etiolated tissue and its transcript levels decrease after exposure to white light, whereas *porB* is expressed more or less constitutively irrespective of light conditions in both types of plants (4). Germination of Arabidopsis seedlings under red light causes a light-grown phenotype, and the specific depletion of PORA, but not PORB. These seedlings when transferred to white light photoreduce protochlorophyllide precursors with PORB. However, while light-harvesting complexes are formed, there are defects in reaction center formation demonstrating one distinguishable unique function of PORA in germinating seeds in Arabidopsis (94).

A transient increase in transcripts corresponding to *por* genes in response to exposure of dark-grown seedlings to light has been reported in *Cucumis sativa* (cucumber) and *Curcurbita maxima* cv, Houkouaokawaguri (pumpkin), which are both members of the *Curcurbitaceae* (64, 126). In pea, no effect of light was observed (105). (However, as the studies in barley and Arabidopsis show, analysis of individual members of *porA* or *porB*-like genes, if they exist, is a prerequisite before any conclusion can be drawn from those studies.) At least in the case of pea, levels of the enzyme decrease upon exposure of etiolated seedlings to light, indicating that proteolytic mechanisms to control enzyme levels in pea are also present.

In dark greening conifers, *Pinus strobus* (104) and *Pinus mugo*, there is also evidence for at least two genes for light-dependent protochlorophyllide reductases (28). The relevant genes in *P. mugo* exhibit expression properties similar to barley *porA* and *porB*. In addition, proteins of 38 kDa and 36 kDa are recognized (similar to the two forms found in flowering plants), with the latter being most abundant in dark-grown cotyledons, but disappearing upon exposure to light, whereas the larger peptide persists at unchanged levels similar to the situation observed in barley. Only one light-dependent protochlorophyllide reductase gene is found in the alga *C. reinhardtii* (65), and its expression pattern has not yet been thoroughly investigated.

Regarding the mechanism of regulation of *por* expression, phytochrome mediates negative light regulation of expression of *porA* in monocots (5, 75). Since *porA* and *porB* genes are regulated differently, it would be interesting to know how their promoter regions differ. However, to date, genomic sequence analysis on *por* has been reported only for *Pinus taeda* and *Pisum sativum* (104).

Structural differences in the barley enzymes apparently account for differences in transport properties of POR into the organelle. For translocation into etioplasts or chloroplasts, the barley PORA protein requires ATP for initial

binding to a protease-sensitive component in addition to its substrate protochlorophyllide; both of these are required at the surface of the organelle (87, 88). Detectable levels of protochlorophyllide are normally associated with the cotyledons of dicots or the primary leaf blades of grasses of germinated seedlings in the period prior to greening, coinciding with the high expression of the gene for PORA. By contrast, the barley PORB protein can be transported into organelles without protochlorophyllide, thereby allowing it to function in the entire period following greening during which protochlorophyllide does not accumulate to significant levels at the surface of the organelle (87, 88).

PORA and PORB levels are also regulated by a light-activated protease(s) that is nuclear encoded and synthesized in the chloroplast, but not in etioplasts (89). Studies have indicated that the PORA apoprotein, as well as PORA bound to any combination of substrates (NADPH and/or protochlorophyllide), is resistant to degradation. In contrast, POR noncovalently bound to chlorophyllide was sensitive to digestion. Western analysis indicates that the POR protein levels show no fluctuations during light/dark cycles during the greening period. However, diurnal fluctuations of the barley *porB* gene in plants grown on a 12 h light/12 h dark cycle occurred with a maximum at 4 h into the light cycle and at 16 h (4 h into the dark cycle) similar to that of the chlorophyll binding protein gene *lhcb1*. This fluctuation in levels is proposed to compensate for decreased levels of protein that would result from proteolysis. Similar diurnal fluctuations in expression of the *porB* gene of Arabidopsis are also observed (4). These findings help to explain the long-debated paradox of decreased expression of this gene in a variety of land plants in the light during the period when its function is required (29, 71, 106).

Recently, Wilks & Timko (121) devised a simple method to identify residues critical for enzyme function of plant POR proteins by heterologous complementation of protochlorophyllide reductase mutants of *Rb. capsulatus*. By virtue of homology to members of the short-chain alcohol dehydrogenase family, two residues, Tyr-275 and Lys-279, of the pea enzyme that are universally conserved among members of this family were mutated to Phe or Cys and Ile or Arg, respectively. These mutant forms of the enzyme were found to be defective in light-dependent complementation of *Rb. capsulatus* protochlorophyllide reductase mutants.

Phytol Addition

The phytol tail of chlorophyll *a* and bacteriochlorophyll *a* is derived from an esterification reaction that utilizes phytyl diphosphate as a substrate. Mutational analysis of *Rb. capsulatus* initially indicated that *bchG* coded for the enzyme bacteriochlorophyll synthase that catalyzed this reaction (14, 15, 112, 128). A

chlorophyll synthase homolog of *bchG* (*chlG*) has been identified in the *Synechocystis* genome sequence database (36% sequence identity to *bchG*) (57, 59), as well as in the EST database from Arabidopsis and rice (36, 69) (Table 1). Recently, bacteriochlorophyll synthase from *Rb. capsulatus* and chlorophyll synthase from *Synechocystis* have been overexpressed in *E. coli.* Cell-free extracts demonstrated enzymatic activity with remarkable substrate specificity (113). Specifically, bacteriochlorophyll synthase would esterify bacteriochlorophyllide *a* but not chlorophyllide *a*, whereas chlorophyll synthase would esterify chlorophyllide *a* but not bacteriochlorophyllide *a*. Both enzymes also exhibited a marked preference for phytol diphosphate over geranylgeraniol diphosphate. Two *bchG* homologs have also been cloned and sequenced from the anoxygenic bacterium *Chloroflexus aurantiacus,* which synthesizes bacteriochlorophyll *a* as well as bacteriochlorophyll *c* (69). It has been proposed that one of the *bchG* homologs is involved in synthesis of bacteriochlorophyll *a*, whereas the other in synthesis of bacteriochlorophyll *c*.

There is an interesting dependence of chlorophyll biosynthesis on the carotenoid biosynthetic pathway, which is involved in providing intermediates for the synthesis of phytyl diphosphate. The carotenoid pathway generates several isoprenoid intermediates such as isopentylpyrophosphate, farnesyl pyrophosphate, and geranylgeraniol pyrophosphate (100). Phytyl diphosphate is thought to be produced from geranylgeranyl pyrophosphate via multiple step reduction catalyzed by a reductase that is coded by the *bchP* gene, which was identified by mutational analysis of the *Rb. capsulatus* photosynthesis gene cluster (15, 113). Recently, a homolog of *bchP* from *Synechocystis* 6803 was isolated and demonstrated to complement *bchP* mutants in *Rb. sphaeroides* (1). Using the cyanobacterial amino acid sequence as a query, EST homologs of *bchP* have also been identified in both rice and Arabidopsis. Reduction of the pool of geranylgeraniol pyrophosphate in tomato, by overexpressing a gene involved in carotenoid synthesis, results in significant reduction in chlorophyll content as well as the phytohormone gibberellin (30). This indicates that there is a delicate partitioning of early isoprenoid intermediates into divergent pathways and that this partitioning can be dramatically altered by subtle changes in expression of an enzyme in one of these pathways.

DIVERGENT BRANCHES OF THE PATHWAY

Bacteriochlorophyll a Specific Steps

Detailed interposon mutagenesis of the sequenced *Rb. capsulatus* photosynthesis gene cluster has given a fairly complete understanding of genes unique to this pathway (See Reference 14). Products of the *bchC* and *bchF* genes are thought to be involved in conversion of the 2-vinyl group to 2-acetyl. The *bchX, bchY,*

and *bchZ* genes are responsible for reducing ring 2 in a reaction that appears to be similar to reduction of ring 4 by the light-independent POR (16). Because of a high degree of sequence similarity between BchX and BchL (34% identity), it has been proposed that BchL and BchX both function as electron donors to BchB-N and BchY-Z catalytic subunits, respectively (17). BchB-N and BchY-Z are presumed to have specificities for different areas of the tetrapyrrole ring that they reduce (ring 4 versus ring 2). As is the case for BchBNL, biochemical activity for BchXYZ polypeptides has not been demonstrated.

Other Bacteriochlorophylls

Very little is known about the genes involved in the synthesis of alternative bacteriochlorophylls such as bacteriochlorophylls *b, c, d, e*, and *g*. Each of these pathways most likely utilizes common intermediates from Mg-protoporphyrin IX through chlorophyllide (103). The only definitive information is that bacteriochlorophyll *c* synthesis appears to utilize a *bchG* gene product that is distinct from that utilized in bacteriochlorophyll *a* production (noted above).

Recently, we have cloned numerous bacteriochlorophyll biosynthesis genes from the bacteriochlorophyll *g* synthesizing species *H. mobilis* and the bacteriochlorophyll *c* synthesizing organism *Chlorobium tepidum* by complementation of *Rb. capsulatus* mutants (K Inoue & C Bauer, unpublished results). Preliminary studies indicate that bacteriochlorophyll biosynthesis genes are clustered in *H. mobilis*, which should facilitate sequence analysis of genes involved in this pathway.

There has also been a report of a Zn-containing variant of bacteriochlorophyll *a* (115). Characterization of the Zn-chelatase would be of interest to see if it is structurally related to Mg-chelatase.

Chlorophylls b, c, and d

The structure of chlorophyll *b* differs from that of chlorophyll *a* by the substitution of a formyl group for a methyl group in ring 2. The pathway for chlorophyll *b* synthesis is thought to occur by direct modification of chlorophyll *a* to chlorophyll *b* via a hydroxymethyl intermediate (80, 98).

Enzymatic studies in cucumber and barley have characterized an activity converting chlorophyll *b* to chlorophyll *a* via a hydroxymethyl intermediate (51, 52). Conventional thought is that the reaction is unidirectional from chlorophyll *a* to *b*. This new finding provides an elegant scenario on how the chlorophyll *a*/*b* ratios can be altered in response to environmental changes. The interconverting enzyme(s) would thus play a major role in the regulation of photosynthesis efficiency by altering light-harvesting and reaction center complex formation. This interconversion process has been dubbed the "chlorophyll cycle." No genetic models are available among the chlorophyll *a* only

cyanophytes, nor have any organisms been reported that only accumulate chlorophyll *b*. In light of the possible physiological relevance of activities at this step, further characterization and purification of this enzyme should be of high priority.

Chlorophyll *b* minus mutants such as *chlorina f2* mutants of barley (9) and *ch1* of Arabidopsis (76) have major defects in the formation of their light-harvesting complexes due to the instability of their chlorophyll *a*/*b* binding proteins (Lhcb) in the absence of pigments. Ten independently isolated chlorophyll *b*-deficient mutants of barley were found to belong to a single complementation group. This suggests that only a single locus may be involved in its synthesis (101). Studies with other *chlorina* mutants of wheat and barley show that mutants generally characterized as chlorophyll *b*-deficient are actually defective in overall chlorophyll biosynthesis due to partial blocks at the magnesium chelation step (23, 24). It is hypothesized that chlorophyll *b* is synthesized only from chlorophyll *a* that is leftover after formation of reaction center complexes.

Chlorophylls c_1, c_2, and c_3 are used as accessory light-harvesting pigments in many algae. They differ from chlorophyll *a* and *b* in that ring 4 is not reduced, which indicates that they are most likely derived from protochlorophyllide. Chlorophyll c_2 appears to be derived from divinyl protochlorophyllide, whereas c_1 is derived from monovinyl protochlorophyllide (8). Chlorophyll *d* has recently been identified as a major light-harvesting pigment of a prochlorophyte-like (oxychlorobacteria-like) prokaryote *Acarychloris marina* Miyashita et Chihara gen. et sp. Nov. (74). This pigment, which differs from chlorophyll *a* by substitution of a 2-formyl group for 2-vinyl, shows an interesting absorbance in the red region (716 nm). No mutants or genes involved in chlorophyll *c* or *d* production have been identified.

CONCLUDING REMARKS

Since the first chlorophyll biosynthesis gene was cloned and sequenced in 1989 (99), sequence information has been obtained for most of the enzymes in the Mg-branch of the tetrapyrrole biosynthetic pathway that give rise to chlorophyll *a* and bacteriochlorophyll *a*. Given the rate at which this field has developed of late, most of the "missing genes" in chlorophyll *a* biosynthesis in plants will likely be identified based on sequence homology to the bacterial genes. Areas still needing to be addressed include the rigorous establishment that genetically identified genes in specific steps of the pathway actually code for catalytic subunits of enzymes. This hypothesis is currently being addressed in several laboratories by the heterologous expression of genes in *E. coli* coupled with in vitro assays for enzyme activity. The issue of regulation of bacteriochlorophyll

and chlorophyll biosynthesis at the transcriptional and posttranscriptional levels also needs to be expanded beyond the few experimental systems, and steps of the pathway, that have been studied to date. Genes involved in synthesis of additional bacteriochlorophyll and chlorophyll end products must also be identified and characterized. A thorough understanding of these additional pathways, particularly in deeply divergent green sulfur and nonsulfur bacteria, could give significant insight into the evolutionary relationships of these pathways.

ACKNOWLEDGMENTS

We thank Jin Xiong and Sylvie Elsen for comments regarding the manuscript. We also apologize to those whose work was not directly cited owing to space limitations. Research in this area is supported by National Institutes of Health grants GM 539040 and GM 00618 to CEB.

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