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MINIREVIEW

Genetic Analyses of Photopigment Biosynthesis in Eubacteria: a Guiding Light for Algae and Plants

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INTRODUCTION

A wide variety of macrocyclic tetrapyrrole compounds are synthesized by cells for use in diverse cellular processes. As shown in Fig. 1, this pathway includes the synthesis of closed tetrapyrrole compounds that contain metals, such as cobalt-containing cobalamin (vitamin B_{12}), nickel-containing methanogenesis coenzyme F430, copper-containing pigment turacin, iron-containing heme and siroheme, and magnesium-containing chlorophylls and bacteriochlorophylls. Also synthesized by this pathway are a variety of open macrocyclic compounds such as the family of bilins that are used as a source of light-harvesting pigments in cyanobacteria, red algae, and cryptophytes as well as the chromophore that is covalently attached to phytochrome apoproteins in plants. Several comprehensive reviews which focus on the biochemistry and enzymology of the various branches of the tetrapyrrole biosynthetic pathway have recently been published (6, 22, 51).

Genetic analysis of the tetrapyrrole biosynthesis pathway is an active area of study in a number of laboratories. Over the past decade, there has been significant progress in obtaining molecular genetic information on loci involved in the early part of the pathway leading from 5-aminolevulinic acid to heme (33). Loci involved in vitamin B_{12} synthesis have also been characterized at the molecular genetic level (33). Molecular genetic information on loci involved in the Mg-tetrapyrrole branch of the biosynthesis pathway has, however, only recently been obtained. It is this latter area that this minireview will cover, particularly the recent use of bacterial sequence information to aid in defining the roles and evolutionary relationships of similar loci from plant and algal systems.

GENETIC ANALYSIS OF BACTERIOCHLOROPHYLL BIOSYNTHESIS

The first genetic analysis of the Mg-tetrapyrrole branch was a study by Granick in 1948 (27) of chlorophyll *a* biosynthesis in the green alga *Chlorella vulgaris*. This analysis was followed by several studies in the early 1960s on the genetic and biochemical analysis of bacteriochlorophyll *a* biosynthesis in the bacterium *Rhodobacter sphaeroides* (for a review of these initial studies, see reference 32). The results of these early studies indicated that both bacteriochlorophyll *a* and chlorophyll *a* biosynthesis utilized a common set of intermediates from Mg-protoporphyrin IX through chlorophyllide *a*, the structures of which are diagrammed in Fig. 2. The similarity of these pathways is highlighted by the observation that photopigment intermediates from eubacteria also serve as substrates for corresponding plant enzymes (29). Not shown in Fig. 2 are numerous additional bacteriochlorophyll structures that are synthesized by various green and purple eubacteria (bacteriochlorophylls *b*, *c*, *d*, *e*, *f*, and *g*); these additional Mg tetrapyrroles have yet to be characterized by genetic means. Structural features of these additional eubacterial photopigments indicate that they are undoubtedly produced from the same common core of intermediates (Mg-protoporphyrin IX→chlorophyllide) as are bacteriochlorophyll *a* and chlorophyll *a* (52).

Molecular genetic analysis of the loci involved in the Mg branch of the tetrapyrrole biosynthesis pathway was initiated by Marrs and coworkers with the bacterium *Rhodobacter capsulatus*. These workers utilized a combination of generalized transduction (63), R′ mobilization (41), and plasmid-based complementation/marker rescue techniques (55) to demonstrate that all of the known essential loci involved in bacteriochlorophyll *a* biosynthesis were tightly linked to a 45-kbp region of the chromosome that was termed the photosynthetic (photosynthesis) gene cluster. A physical map of this region, which has been obtained primarily by the work of Hearst and coworkers, has been derived by performing transposition mutagenesis (67) coupled with the recently completed sequence analysis of the entire photosynthesis gene cluster (2, 9, 11, 30, 60, 62, 66). The physical map has also recently been refined by the construction of defined sets of insertion mutations within each of the open reading frames of this region (8, 25, 62, 65). Collectively, these analyses have allowed the determination of which individual open reading frames are involved in specific steps in the biosynthetic pathway (Fig. 2). A note of caution should be made: even though it is likely that most, if not all, of the bacteriochlorophyll (*bch*) loci in the photosynthesis gene cluster code for subunits of enzyme complexes, catalytic activity has yet to be demonstrated for polypeptides encoded by these genes.

A curious result has been obtained from parallel studies which have demonstrated that the tightly compacted linkage order of photosynthesis genes observed for *R. capsulatus* is also conserved among such diverse species of purple photosynthetic eubacteria as *R. sphaeroides* (14), *Rhodospirillum centenum* (64), and *Rhodospirillum rubrum* (47, 48) (Fig. 3). In each case, photopigment biosynthesis genes were observed to be in the same order, and in several cases, these pigment biosynthesis genes were also shown to be flanked by light-harvesting and reaction center genes which code for the pigment-binding structural polypeptides that form the backbone of the photosystem. Retention of the photosynthesis

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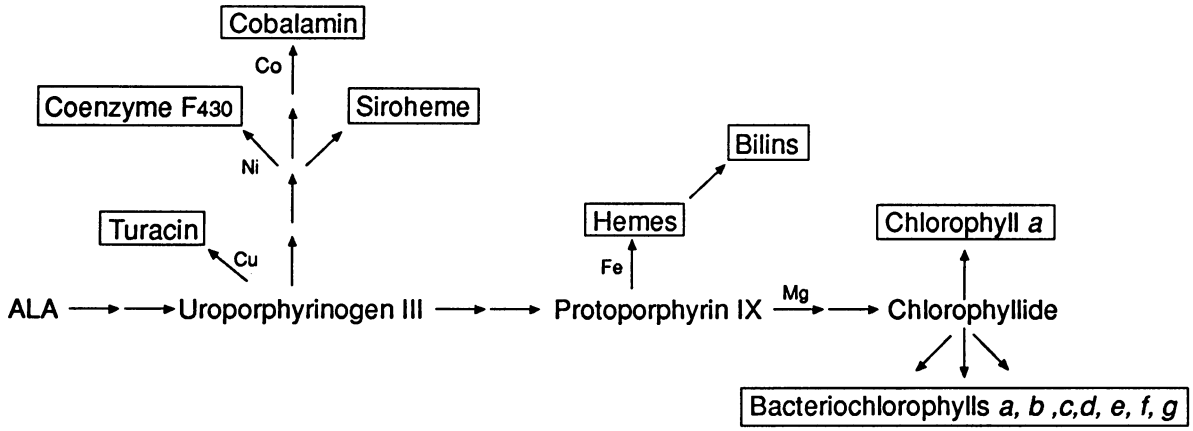

FIG. 1. Tetrapyrrole biosynthesis pathway. ALA, 5-aminolevulinic acid.

gene cluster arrangement among such diverse species indicates either that there has been recent lateral transfer of photosynthesis genes among these species or that there is selective pressure to retain this linkage order. An argument for the latter has been made (64) which is based on observations that in *R. capsulatus*, there appears to be functionally significant transcriptional coupling of light-harvesting and reaction center genes with bacteriochlorophyll biosynthesis genes (4, 59, 62, 65).

GENETIC ANALYSIS OF CHLOROPHYLL *a* BIOSYNTHESIS

Despite the critical role chlorophyll biosynthesis plays in oxygenic photosynthesis, and the success of early studies by Granick (27), there has been surprisingly little genetic information available for the Mg branch of the chlorophyll *a* biosynthesis pathway. This is especially true for the chlorophyll *a*-synthesizing cyanobacteria, for which there have been no significant genetic studies on chlorophyll biosynthesis. The lack of genetic analysis of this group of eubacteria is presumably due to the difficulty (with a few exceptions [see reference 1]) of growing cyanobacteria under nonphotosynthetic (heterotrophic) conditions. The lack of appropriate conditions for an alternative bioenergetic growth mode obviously complicates the isolation and cultivation of mutant strains that are affected in chlorophyll synthesis. A similar argument, i.e., difficulty in selection and cultivation, can be made for the lack of extensive genetic analysis of chlorophyll biosynthesis in plants, an area for which there has been only a few reports of mutants that affect chlorophyll biosynthesis (26, 34, 36, 37). Theoretically, it should also be possible to utilize the technology of reverse genetics to clone loci that code for enzyme subunits that catalyze chlorophyll biosynthesis reactions. However, only one enzyme involved in the Mg branch of the pathway, light-dependent NADPH:protochlorophyllide oxidoreductase, has been purified to a degree of homogeneity that has allowed the production of antibodies for use in cDNA expression library screening and subsequent cloning of the gene from plants (15, 50). The difficulty in isolating these enzymes appears to stem, in part, from the low cellular levels of these enzymes coupled with their association with membrane fractions.

The one system for which genetic analysis of chlorophyll biosynthesis has more extensively been undertaken are the studies by Wang and coworkers with the green alga *Chlamydomonas reinhardtii*, which has the capability of growing under heterotrophic conditions (18–20, 56, 58). In their analyses, they were able to obtain mutations in two nuclear loci involved in the conversion of protoporphyrin IX to Mg-protoporphyrin IX as well as mutations at seven nuclear loci that affected the dark reductive conversion of protochlorophyllide to chlorophyllide. To date, none of the nucleus-encoded loci have been cloned or sequenced, so their relationship, if any, to bacterial loci is unclear. However, in the section below, we discuss evidence that the *C. reinhardtii* chloroplast genome actually codes for what appear to be subunits of the light-independent protochlorophyllide reductase enzyme complex. This evidence implies that some of the nuclear loci identified by Wang and coworkers which disrupt dark protochlorophyllide reduction may be affecting the synthesis or assembly of this enzyme complex.

USE OF BACTERIOCHLOROPHYLL LOCI TO CHARACTERIZE CHLOROPHYLL BIOSYNTHESIS GENES IN ALGAE AND PLANTS

As noted above, reduction of the double bond in ring D of protochlorophyllide is one step of the Mg-tetrapyrrole branch of the pathway that has been genetically characterized in both eubacterial and eukaryotic (algal and plant) studies. This step of the pathway has generated much interest owing to the observation that angiosperms (flowering plants), which as a group are the most dominant and recently evolved land plants, contain an enzyme (light-dependent NADPH:protochlorophyllide oxidoreductase) that requires the absorption of light to catalyze ring D reduction. Hence, the observation that these organisms exhibit light-mediated regulation of chlorophyll biosynthesis at this step in the pathway (6). In contrast, bacteria, algae and nonflowering land plants (including gymnosperms) were thought to utilize a different protochlorophyllide (ring D) reduction mechanism, since these organisms are capable of producing chlorophyll in the dark. Until recently, it was not known whether dark ring D reduction involved modification or accessory factors of the light-dependent enzyme or was due to the existence of a separate light-independent enzyme. However, mutational, hybridization and sequence analyses (discussed below) from such diverse dark-greening organisms as cyanobacteria (23), algae (12, 18, 40, 54), and gymnosperms (39, 53) have indicated not only the existence of a distinct light-independent enzyme but also the coexistence of the light-dependent version. These results suggest that both the light-dependent and light-independent enzymes

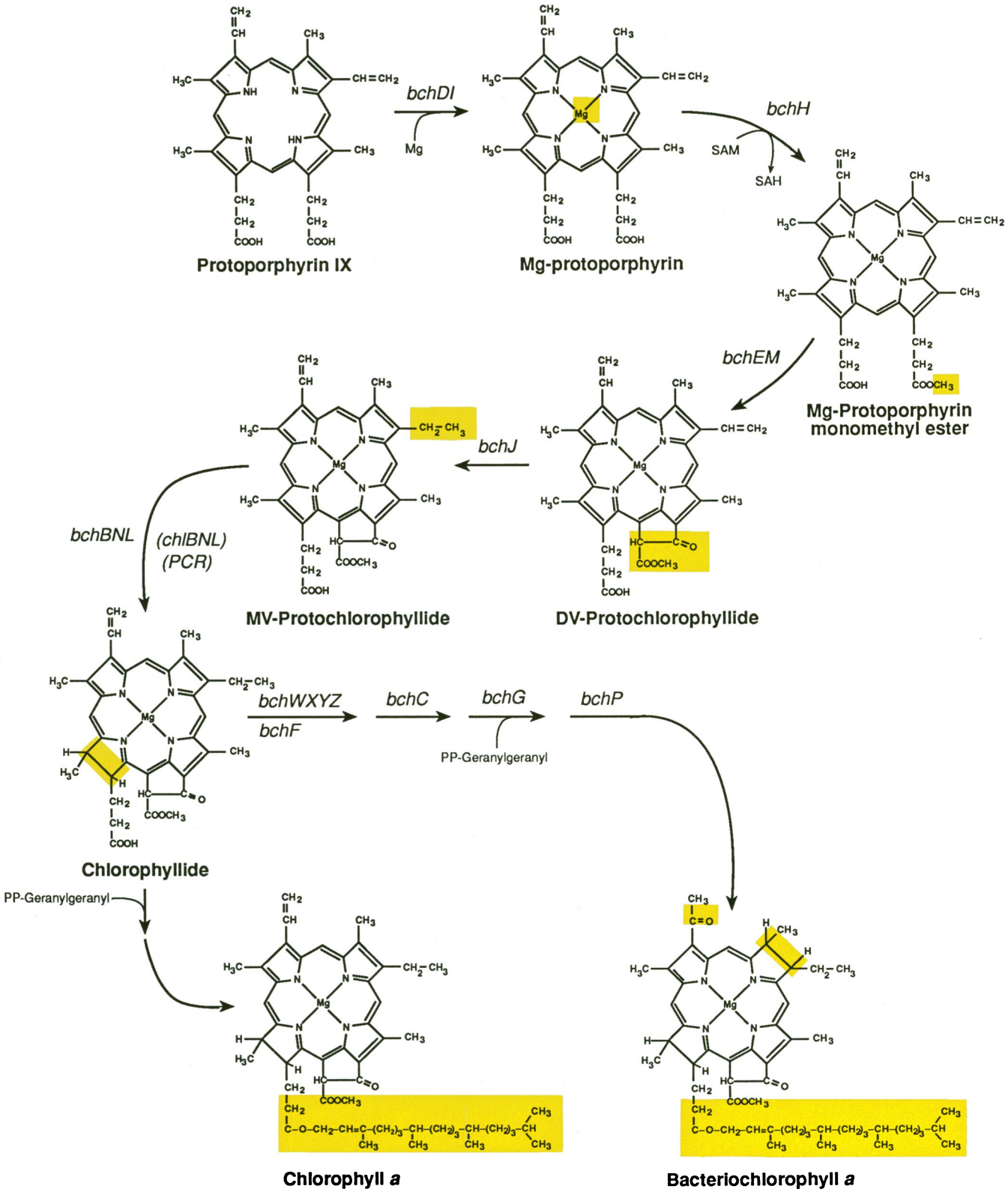

FIG. 2. Mg branch of the bacteriochlorophyll *a* and chlorophyll *a* biosynthetic pathways. Both pathways share common intermediates up to the synthesis of chlorophyllide, at which point the tetrapyrrole ring in the bacteriochlorophyll *a* pathway undergoes additional modification. Modification of the tetrapyrrole ring at various stages of the pathway is indicated by a yellow box. Genetic loci that affect individual steps of the pathway are also indicated above the arrows.

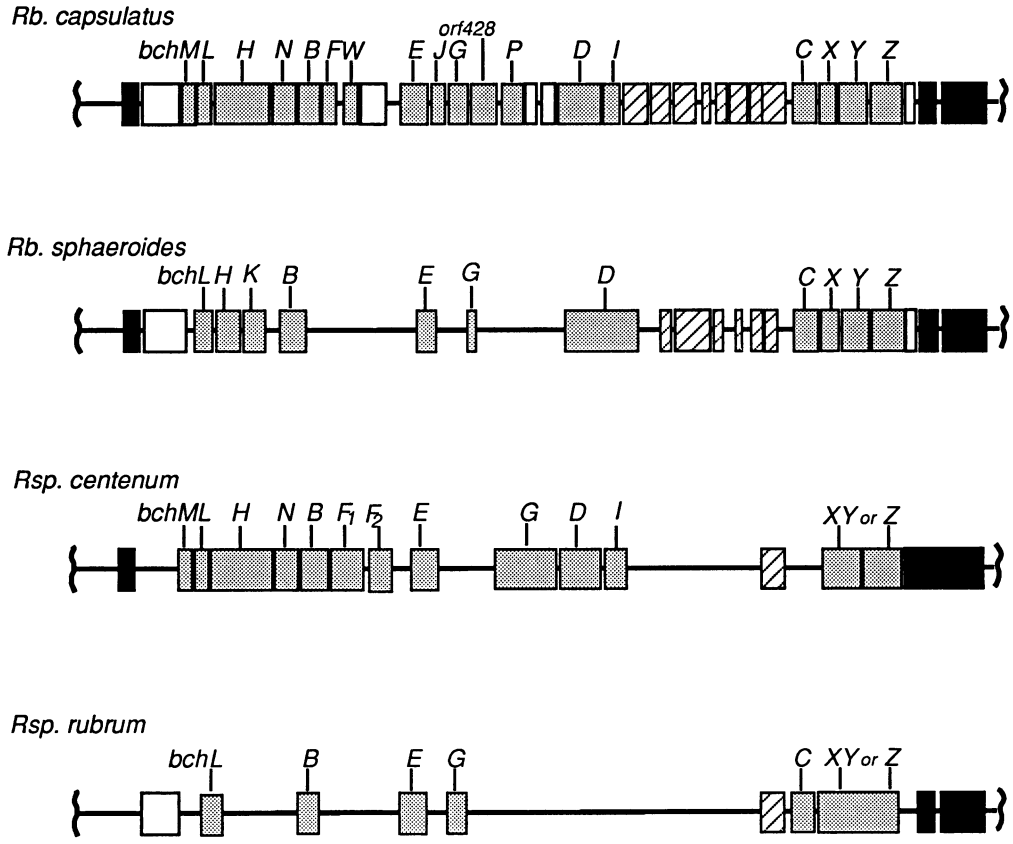

FIG. 3. Photosynthesis gene clusters of various purple eubacterial species. The *R. capsulatus* map is derived from high-resolution sequence and mutational analyses as described in the text. The *R. sphaeroides* (14, 42), *R. centenum* (64), and *R. rubrum* (47, 48) maps are based primarily on lower-resolution mutational analyses. Solid boxes denote light-harvesting and reaction center structural genes, hatched boxes denote carotenoid genes, and stippled boxes denote bacteriochlorophyll biosynthesis genes. Loci that are not involved in these processes are indicated by open boxes.

are likely derived from eubacteria and that angiosperms have simply lost the capability of synthesizing the light-independent enzyme.

The relationship of the light-independent protochlorophyllide reaction among diverse dark-greening organisms was recently advanced by molecular genetic characterization of loci that are involved in this reduction from eubacterial, algal, and plant systems. Mutational analysis initially demonstrated that there are three loci, *bchL*, *bchB*, and *bchN*, which are required for light-independent protochlorophyllide reduction in *R. capsulatus* (11, 30, 62). One of these genes, *bchL*, was known to exhibit a high degree of sequence identity (51%) to a previously sequenced *Marchantia polymorpha* chloroplast gene (*frxC*) which had an unknown function (44). To test whether the chloroplast did indeed code for a functional homolog of *bchL*, Suzuki and Bauer cloned and subsequently disrupted, via particle gun-mediated transformation, a *bchL* or *frxC* homolog present in the chloroplast of *C. reinhardtii* (54). The resulting transformants were subsequently shown to be deficient in the dark conversion of protochlorophyllide to chlorophyllide, thereby demonstrating that the chloroplast does code for a gene that has a similar role in chlorophyll biosynthesis. Concurrently, Choquet et al. (12) used a similar analysis to show that the *C. reinhardtii* chloroplast also contains a light-independent protochlorophyllide reductase gene that exhibits significant sequence identity to *bchN* of *R. capsulatus*. More recently, a chloroplast-encoded *bchB* homolog has also been demonstrated by mutational and sequence analysis to be located within *C. reinhardtii* (40) and *M. polymorpha* (11) chloroplasts, thereby demonstrating that all three homologs of the eubacterial enzyme are encoded by the chloroplast. Sequence and hybridization analyses have also demonstrated that homologs of *bchN* and *bchL* are present in the chloroplasts of a wide variety of dark-greening eukaryotes, such as algae, ferns, and gymnosperms. Importantly, all DNAs tested from angiosperms also do not exhibit cross-hybridization with plant chloroplast homologs of the *bchL* gene (54, 61). This is consistent with their requirement of light for greening.

The evolutionary relationship between the eubacterial and eukaryotic Mg-tetrapyrrole biosynthetic pathways was also confirmed and extended by sequence information for the light-independent protochlorophyllide reductase genes from the cyanobacterium *Plectonema boryanum* (23). These results demonstrated that the *P. boryanum bchL* homolog exhibits a much higher degree of sequence identity to chloroplast-encoded *bchL* homologs (for example, 82% sequence identity to the Pine homolog!) than it does to *bchL* of *R. capsulatus*, to which it exhibits only 51% sequence identity (23, 39, 54). Phylogenetic analyses have indicated that chloroplast genomes are derived from a "recent" endosymbiosis of an eubacterium closely related to the cyanobacterial lineage (17, 24, 28, 43). Since the cyanobacterial

bchL homolog is more closely aligned to the chloroplast-encoded homolog than to the purple eubacterial homolog, the chloroplast genetic loci for light-independent protochlorophyllide reduction are most likely derived from a cyanobacterium-like ancestor.

The conclusion that plant chlorophyll biosynthesis genes are most likely derived from a cyanobacterial endosymbiont has also recently been solidified by the observation that *Arabidopsis thaliana* contains a nucleus-encoded gene, *CH42*, that contains approximately 49% sequence identity to *bchI* of *R. capsulatus* (36, 37, 45). Mutations in CH42, like that of *bchI* from *R. capsulatus*, result in the accumulation of protoporphyrin IX (37), thereby suggesting that it is a true homolog of the bacterial locus. Additional homologs of *bchI* have also recently been reported to be located in the chloroplasts of *Euglena gracilis* (45), *Cryptomonas* Φ (17), and the red alga *Porphyra purpurea* (46). The presence of a *bchI* homolog in the chloroplasts of these additional species provides additional evidence for the argument that the nucleus-encoded *bchI* homolog of *A. thaliana* was most likely transferred from the chloroplast. Presumably, additional sequence information of plant loci involved in the Mg branch of the pathway will show that they have also been obtained in a similar manner.

CONCLUDING REMARKS AND PROSPECTS

Recent genetic and molecular genetic analysis of bacteriochlorophyll biosynthesis in *R. capsulatus* has allowed the identification of specific open reading frames that are involved in individual steps of the bacteriochlorophyll *a* biosynthetic pathway. Even though it is likely that these loci code for enzymes or subunits of enzymes involved in these reactions, it remains to be demonstrated that these gene products actually exhibit catalytic activity. Overexpression of these genes in heterologous systems, coupled with assaying for enzyme activity, should help define which loci actually code for enzyme subunits. It should be noted that many of the steps in the Mg branch of the pathway are still poorly characterized biochemically; thus, heterologously expressing *bch* loci could also help advance future biochemical studies of these reactions.

Sequence information derived from *R. capsulatus* studies has also been useful in identifying chloroplast-encoded homologs of bacterial loci that are involved in light-independent protochlorophyllide reduction. Phylogenetic analysis has established that at least some of the genetic loci involved in the Mg branch of the pathway were most likely acquired from an endosymbiont and that it is feasible to obtain chlorophyll biosynthesis genes from plants via an evolutionary walk from eubacteria. It seems likely, therefore, that future studies of additional Mg-tetrapyrrole loci from eubacteria will provide the necessary information needed to clone and further characterize chlorophyll biosynthesis genes from plant systems.

One area not covered in this minireview is the mechanism whereby the Mg branch of the tetrapyrrole biosynthetic pathway is regulated, a topic about which there is little known at a molecular genetic level. Studies of *R. capsulatus* indicate that bacteriochlorophyll genes are weakly expressed and that expression is regulated via an unknown mechanism in response to changes in oxygen tension and light intensity (4, 62, 65). There is also evidence for post-transcriptional control of the Mg branch, models of which invoke a carrier polypeptide that functions to tether tetrapyrrole intermediates to the membrane (5, 38). Direct evidence for a carrier polypeptide is, however, lacking (10). It has been suggested that in plants and algae, chlorophyll precursors may regulate the expression of chlorophyll biosynthesis genes (31) as well as activities of enzymes involved with chlorophyll synthesis (34, 57). Light is also reported to modulate the level and stability of the light-dependent protochlorophyllide reductase enzyme (7, 21, 35) as well as expression of the *bchI* homolog from *A. thaliana* (36). It is also clear that chlorophyll biosynthesis in plants is regulated by tissue-specific expression, developmental patterns (13, 16), and light (21, 53). This regulation occurs, in part, via phytochrome (3), which is a red-light photoreceptor that contains a covalently attached linear tetrapyrrole. Interestingly, aligned phytochrome apoprotein sequences from a number of plants exhibit blocks of sequence similarity to bacterial sensor kinases, thereby suggesting that phytochrome may function in signal transduction in a manner analogous to bacterial sensor kinases (49). Hopefully, characterization of these and additional loci involved in Mg-tetrapyrrole production in eubacteria and eukaryotes will help to further our understanding of how these diverse photosynthetic organisms control synthesis of this vital pigment.

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