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Chlorophyll Biosynthesis

Diter von Wettstein,[1] Simon Gough, and C. Gamini Kannangara

Department of Physiology, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark

INTRODUCTION

A leaf with 70 million cells houses ~5 × 10^9 chloroplasts, each containing ~600 million molecules of chlorophyll (Simpson and Knoetzel, 1995). These 10^{18} chlorophyll molecules, all of which are bound to proteins of the photosynthetic membranes, harvest the sunlight. Approximately 250 to 300 of them transfer the absorbed light energy through neighboring pigments to the "special pair" chlorophylls in a reaction center. These special pair chlorophylls in photosystems I and II are the primary electron donors that drive the conversion of light into chemical energy to be conserved in $NADPH_2$ and ATP.

In chlorophyll, four pyrrole rings (designated I to IV) are ligated into a tetrapyrrole ring with a magnesium atom in the center (Figure 1). Ring IV is esterified with phytol. Chlorophyll *a* has a methyl group in position 3, but higher plants and algae use for light harvesting an additional form of chlorophyll, chlorophyll *b*, that has a formyl group instead of a methyl group at this position. The porphyrin ring with its conjugated double bonds is assembled in the chloroplast from eight molecules of 5-aminolevulinic acid, a highly reactive nonprotein amino acid (5-amino, 4-keto pentanoic acid). The bonds highlighted in red in the chlorophyll structure of Figure 1 delineate the locations of the atoms derived from the eight 5-aminolevulinic acid carbon skeletons. An intact 5-aminolevulinate molecule can be recognized in pyrrole ring IV: the nitrogen coordinated with the magnesium atom originates from the amino group; it is followed by four carbon atoms and the carboxyl-group, which is esterified to phytol.

Biosynthesis of chlorophyll comprises a number of challenging topics. Because mutants play an increasing role in exploring the biochemistry and molecular biology of this pathway and its regulation, we first highlight recent results on the use of mutants. The subsequent sections deal with the biosynthesis of 5-aminolevulinate, the synthesis of the pyrrole ring, the synthesis of the tetrapyrrole chain, and the formation of the tetrapyrrole ring. We then discuss trimming of the acetate and propionate side chains and establishment of the conjugated double bond system; insertion of the magnesium, formation of ring V, and synthesis of protochlorophyllide; and reduction to chlorophyllide and phytylation. Next, we discuss chlorophyll synthesis in chloroplast development and end with a discussion of chlorophyll binding proteins.

[1] To whom correspondence should be addressed.

EXPLORING CHLOROPHYLL SYNTHESIS WITH MUTANTS

When barley grains are germinated in the dark, the seedlings have yellow leaves because they lack chlorophyll and contain only small amounts of protochlorophyllide (Figure 2A). If dark-germinated seedlings are supplied with 5-aminolevulinate in the dark, they will green in the course of a few hours due to the accumulation of protochlorophyllide, the next-to-last precursor for chlorophyll that in barley and other angiosperms requires light and binding to protochlorophyllide reductase to be converted into chlorophyllide, which is then phytylated to chlorophyll *a* (see later discussion). The accumulation of large amounts of protochlorophyllide reveals that all enzymes required to convert 5-aminolevulinate into chlorophyll are present in the plastids of dark-grown leaves. The formation of 5-aminolevulinate thus is limiting in the etioplasts. Mutations in four barley genes (*tigrina-b*, *-d*, *-n*, and *-o*) remove this limitation and accumulate two to 10 times the wild-type amount of protochlorophyllide in the dark (Nielsen, 1974). The homozygous mutant seedlings develop a 10-cm-high primary leaf but die after 10 to 15 days, when the nutrient supply from the grain endosperm is exhausted. One of these mutants, *tigrina-d*^{12} (Figure 2B), has no other defect than unregulated 5-aminolevulinate formation and therefore excessive protochlorophyllide synthesis in the dark. Homozygous *tigrina-d*^{12} mutants are fully viable and fertile if grown in continuous light but are dark sensitive. The dark sensitivity accounts for the name of these mutants: in light/dark cycles, the seedlings are green–white banded (Figures 2D, *tigrina-b*^{19} and 2E, *tigrina-d*^{12}). The white, often necrotic leaf domains are caused by the accumulation of protochlorophyllide in the dark. Only a normal amount of protochlorophyllide reductase is available to convert the protochlorophyllide into chlorophyllide, and when the light turns on, the excess of protochlorophyllide causes photodynamic damage to the plastids. With short light pulses, the accumulated protochlorophyllide can be converted successively into chlorophyll, and the *tigrina-d*^{12} mutant can be saved. This is not so with other mutants, such as *tigrina-b*^{19}, which accumulate ζ-carotene in addition to protochlorophyllide (Nielsen and Gough, 1974). Mutations in the *tigrina-n* and *-o* genes deregulate the synthesis of β-carotene as well as protochlorophyllide, with the result that lycopenic pigments accumulate and chromoplasts form.

Another class of mutants with disrupted chlorophyll biosynthesis is the yellow *xantha* mutants of barley. Using

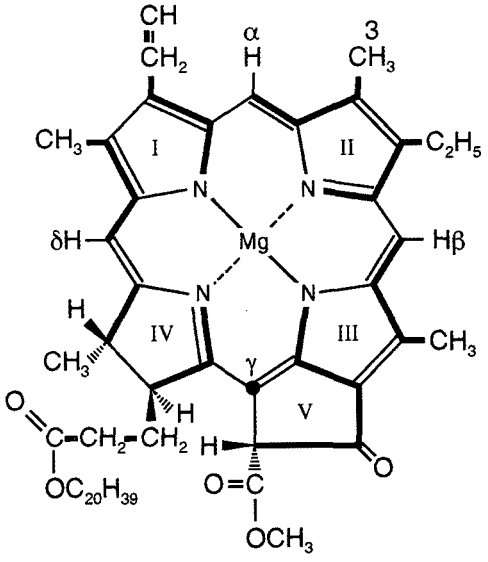

Figure 1. The Chemical Structure of Chlorophyll *a*.

Every chlorophyll molecule is synthesized in the chloroplast from eight molecules of 5-aminolevulinic acid. The eight red heavy lines indicate the location of the atoms derived from these molecules of 5-aminoluevulinic acid in the finished molecule. Position 3, which is occupied by a methyl group in chlorophyll *a* and a formyl group in chlorophyll *b*, is indicated.

complementation tests, five *xantha* genes have been identified that upon mutation block the pathway between 5-aminolevulinate and protochlorophyllide (see later discussion). When dark-grown leaves of the *xantha-f*[35] mutant are fed 5-aminolevulinate, they accumulate a brown-red pigment (Figure 2C) that is a mixture of Mg-protoporphyrin and its monomethyl ester (von Wettstein et al., 1971; Henningsen et al., 1993). This mutant is leaky because it accumulates some protochlorophyllide. To obtain a mutant allele that is completely blocked in the conversion of Mg-protoporphyrin to protochlorophyllide, we exploited the observation (von Wettstein et al., 1974) that plants homozygous for both the *tigrina-f*[35] and *tigrina-d*[12] mutations accumulate Mg-protoporphyrin and its monomethyl ester, just like *xantha-f*[35] seedlings fed 5-aminolevulinate. Therefore, we mutagenized a large number of *tigrina-d*[12] mutant caryopses. In the M_2 generation, Mg-protoporphyrin accumulaters were screened out by in vivo spectrophotometry, and the nonleaky allele *xantha-f*[81] was obtained (Kahn et al., 1976). The *viridis-k*[23] mutant also accumulates Mg-protoporphyrins when fed 5-aminolevulinate and thus defines another protein required for the conversion of Mg-protoporphyrin into protochlorophyllide. In *Rhodobacter*, disruption of the *bchE* open reading frame prevents the cyclization of ring V of chlorophyll and leads to secretion of Mg-protoporphyrin monomethyl ester. Using probes from the *Rhodobacter* gene, efforts are currently under way to clone the corresponding barley gene, which may correspond to the *xantha-l* or *viridis-k* gene.

Mutants at the loci *xantha-f* (Figure 2D), *xantha-g*, and *xantha-h* (Figure 2E) accumulate protoporphyrin IX when fed 5-aminolevulinate (von Wettstein et al., 1971, 1974). The products of these three genes are thus required for the insertion of Mg^{2+} into protoporphyrin IX. Disruption of any one of the three open reading frames *bchD*, *bchI*, and *bchH* of *Rhodobacter capsulatus* by interposon mutagenesis also leads to the secretion of protoporphyrin IX (Bollivar et al., 1994b), which suggests that their products are necessary for the Mg-chelatase reaction. When the homologous *bchD*, *bchI*, and *bchH* genes from *R. sphaeroides* are expressed separately in *Escherichia coli*, three proteins of 70, 40, and 140 kD, respectively, are produced. The BchD protein aggregates to form a 650-kD homopolymer. When combined in vitro, the 650-, 40-, and 140-kD proteins catalyze the insertion of Mg^{2+} into protoporphyrin IX in an efficient, ATP-dependent manner, thus confirming their biological function (Gibson et al., 1995).

Nuclear genes corresponding to *bchI* and *bchH* have now been identified in higher plants (Koncz et al., 1990; Hudson et al., 1993; P.F. Jensen, R.D. Willows, B. Larsen-Petersen, C.G. Kannangara, B.M. Stummann, D. von Wettstein, and K.W. Henningsen, manuscript submitted). Thus, four barley *xantha-h* mutants lack the BchI-immunoreactive protein, and cloning of the *xantha-h* gene showed it to encode a protein with ~50% amino acid sequence identity to the *Rhodobacter* 40-kD bchI subunit and 85% to the deduced protein sequence of the *Arabidopsis chlorata* (ch-42) gene. The Arabidopsis and barley genes encoding this Mg-chelatase subunit are located in the nuclear genome, whereas in *Euglena* the gene is found in the chloroplast genome (Orsat et al., 1992). From an evolutionary point of view, it is interesting that the primary structures of the nonidentical domains of this protein are more closely conserved between the higher plant and *Euglena* subunits than between the *Euglena* and *Rhodobacter* subunits.

In two *xantha-f* mutants, no protein was detectable with antibodies directed against the higher plant homolog of the 140-kD *Rhodobacter* BchH protein. By exploiting nucleotide sequence homology to the *bchH* gene from *Rhodobacter*, the barley gene was cloned. It contains three introns and codes for a 1381-amino acid–long protein (153 kD), the N-terminal 50 residues of which are considered to target the precursor of the mature subunit from its site of synthesis in the cytosol into the chloroplast. In these two *xantha-f* mutants, no significant amount of transcripts from this gene were found (P.F. Jensen, R.D. Willows, B. Larsen-Petersen, C.G. Kannangara, B.M. Stummann, D. von Wettstein, and K.W. Henningsen, manuscript submitted), which indicates that the barley *xantha-f* gene is the structural gene for the 140-kD subunit of Mg-chelatase. The barley protein displays 82% amino acid sequence identity to the *olive* protein of snapdragon (Hudson et al., 1993), 66% identity to the cyanobacterial *Synechocystis* protein (Jensen et al., 1995), and 34% identity to the *Rhodobacter* BchH subunit. The snapdragon *olive* mutations were recognized as yellow-green leaf variegations, and the gene was subsequently cloned with the aid of a known transposon insertion that had inactivated the gene. The hunt is on for the gene encoding the third (70 kD) subunit of higher plant Mg-chelatase.

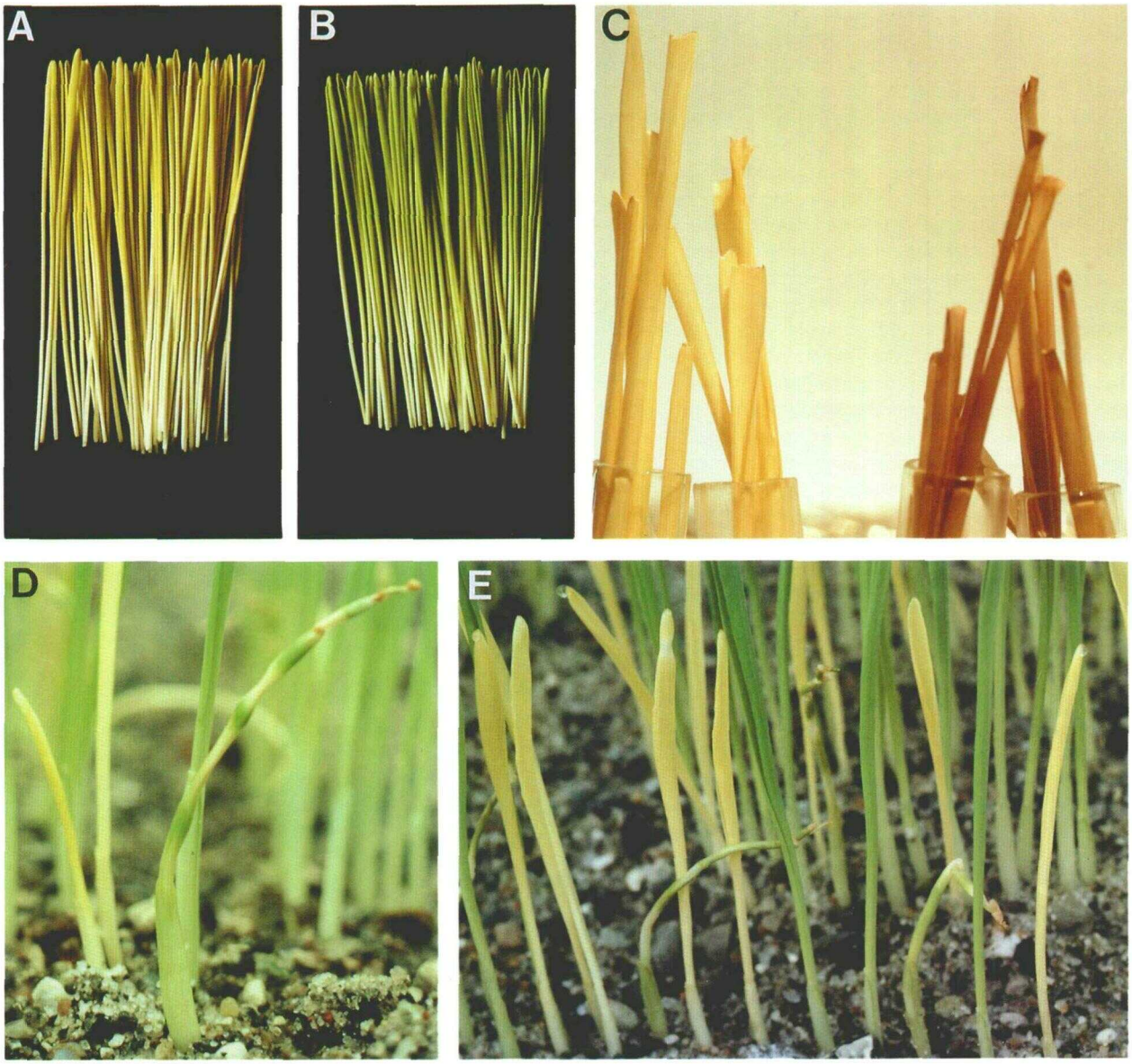

Figure 2. Barley Mutants in Structural and Regulatory Nuclear Genes Involved in the Synthesis of Chlorophyll.

(A) Primary leaves of barley grown for 7 days in darkness at 23°C.
(B) Primary leaves of the mutant *tigrina-d*^{12} grown for 7 days in darkness. The green pigmentation is due to unregulated accumulation of protochlorophyllide. A phenocopy of the mutant can be obtained by feeding wild-type leaves for 24 hr with 5-aminolevulinate.
(C) Primary leaves of the mutant *xantha-l*^{35} fed for 24 hr with water (left) or 0.01 M 5-aminolevulinate (right). These mutants are unable to carry out the cyclase reaction to form ring V of the chlorophyll molecule and thus accumulate red Mg-protoporphyrin IX and its monomethyl ester.
(D) F_2 generation segregating a homozygous *xantha-f*^{10} seedling (yellow) and a homozygous *tigrina-b*^{19} mutant. The double mutant is distinguishable from *xantha-f*^{10} by photometrically detectable overproduction of protoporphyrin IX.
(E) F_2 generation segregating homozygous *xantha-h*^{57} seedlings (yellow) unable to insert Mg^{2+} into protoporphyrin IX and homozygous *tigrina-d*^{12} seedlings (tiger banded). Double mutants are yellow but do not accumulate protoporphyrin IX.

The possibility to express active Mg-chelatase subunits from bacteria and higher plants in heterologous hosts in large quantities makes it possible to study how insertion of Mg^{2+} into the protoporphyrin is carried out by the three subunits, a problem that could not be addressed previously. The mechanism appears to be related to the insertion of other ions into the protoporphyrin ring: cobaltochelatase of *Pseudomonas denitrificans*, which inserts Co to yield cobyrinic acid-a, c-diamide in vitamin B_{12} synthesis, also consists of three polypeptides (Debussche et al., 1992). The porphyrin binding 140-kD *cobN*

gene product shows homology to the large subunit of Mg-chelatase, whereas the other two required polypeptides, CobS and CobT (38 and 80 kD), have a similar size but no sequence homology to BchI and BchD. We surmise that the large subunit in Mg-chelatase also will turn out to carry the porphyrin binding pocket.

In cases in which mutants in steps of chlorophyll synthesis are not obtainable or viable at the seedling stage, information about the function of genes cloned by oligonucleotides derived from amino acid sequences of purified enzymes of the pathway from any organism can be obtained by overexpressing them or by expressing antisense genes using either the cauliflower mosaic virus 35S promoter or a tissue-specific promoter. Figure 3 provides an example of this approach, in which tobacco plants were transformed with an antisense glutamate 1-semialdehyde aminotransferase gene. This enzyme catalyzes the last step in 5-aminolevulinate synthesis (see subsequent discussion), and antisense transformants show a general or tissue-specific reduction in chlorophyll (Höfgen et al., 1994). Partial or complete suppression of the aminotransferase mimics a wide variety of inheritable chlorophyll variegation patterns elicited by nuclear and organelle gene mutations in different higher plants. Such antisense plants provide a sensitive visible marker for activities of chlorophyll synthesis genes in leaves, stems, and flowers. The cell- or tissue-specific action of such antisense genes depends *inter alia* on the position of insertion in the chromosomes of the host, a position we at present cannot control. However, transgenic plants blocked in specific steps in chlorophyll synthesis provide the experimental material to address important questions. For example, translation of chlorophyll binding proteins on chloroplast ribosomes is halted unless chlorophyll is present and can bind to the nascent polypeptide chains. If chlorophyll synthesis is limited by the expression of an antisense gene, does translation of chlorophyll binding proteins on chloroplast ribosomes then take preference over translation of chlorophyll binding proteins on cytosolic ribosomes and their import into the organelle? We would also like to know if angiosperm cells can synthesize haem with a 5-aminolevulinate synthase when they are prevented from producing 5-aminolevulinate from glutamate by complete antisense inhibition of glutamate semialdehyde aminotransferase.

Antisense or sense approaches have led to transgenic tobacco plants with reduced glutamate–tRNA synthase (Andersen, 1992) and reduced glutamyl–tRNA reductase (B. Grimm, personal communication). Like the plants with reduced glutamate 1-semialdehyde aminotransferase (Figure 3), the transgenic plants exhibited reduced chlorophyll content and variegation. Tobacco antisense plants with reduced uroporphyrinogen decarboxylase (Mock et al., 1995) and reduced coproporphyrinogen III oxidase display extensive necrotic lesions on the leaves, presumably because they accumulate photosensitive porphyrin precursors (Kruse et al., 1995a, 1995b).

SURPRISES IN THE BIOSYNTHESIS OF 5-AMINOLEVULINATE

There are two distinct routes for the synthesis of 5-aminolevulinate, one utilizing a condensation reaction of glycine with succinyl-CoA by the enzyme 5-aminolevulinic acid synthase

Figure 3. Tobacco Transformants Expressing a Glutamate 1-Semialdehyde Aminotransferase Antisense Gene.

The transformant at left has pale leaves, whereas in the transformant at center, chlorophyll formation is inhibited along the leaf veins. The plant at right is the untransformed control. From Höfgen et al. (1994) and used with permission of the National Academy of Sciences.

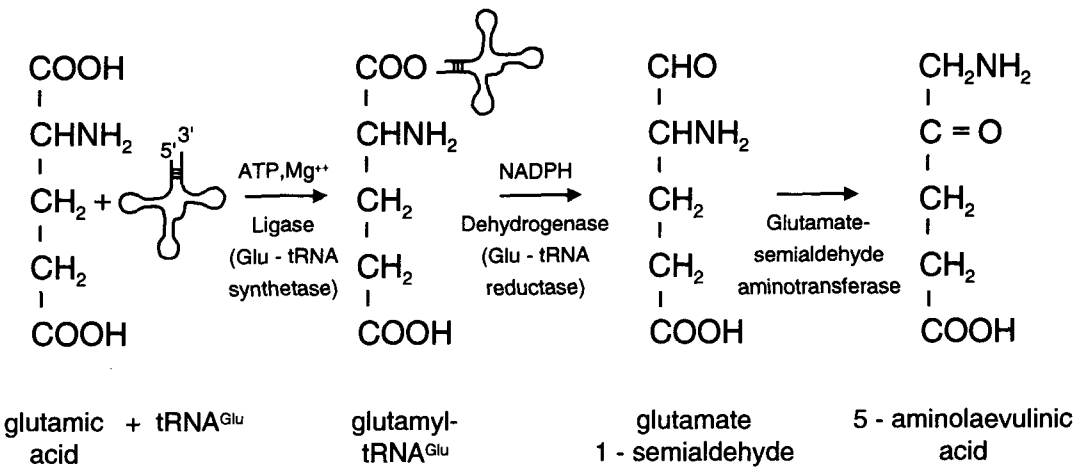

Figure 4. Biosynthesis of 5-Aminolevulinate.

and the other a three-step pathway from glutamate that is called the C_5 pathway (Jordan, 1991). The first route is used by animals, yeast, and a number of bacteria, notably *Rhodobacter*, *Rhodospirillum*, and *Rhizobium*, whereas the C_5 pathway is characteristic of higher plants, bryophytes, cyanobacteria, and many eubacteria. In the phytoflagellate *Euglena gracilis*, the two pathways are used in different compartments. The C_5 pathway operates in chloroplasts and is exclusively responsible for chlorophyll synthesis there, whereas in mitochondria, 5-aminolevulinate synthase is responsible for the synthesis of heme *a* of cytochrome *c* oxidase (Weinstein and Beale, 1983).

When greening plants or algae are treated with levulinate, 5-aminolevulinate accumulates because levulinate inhibits the enzyme 5-aminolevulinate dehydratase (Beale and Castelfranco, 1974a). When ^{14}C-labeled glutamate is fed together with levulinate, the radioactivity is found in the accumulated 5-aminolevulinic acid (Beale and Castelfranco, 1974b). By using glutamic acid labeled with ^{14}C in different positions as precursors and determining the label distribution in the resulting 5-aminolevulinic acid or in the entire chlorophyll molecule, it was shown that the intact five-carbon skeleton of glutamate is incorporated into 5-aminolevulinic acid (Beale et al., 1975; Meller et al., 1975; Porra, 1986).

It was a great surprise when it was discovered that conversion of glutamate to 5-aminolevulinate requires glutamate to be activated at the α-carboxyl by ligation to $tRNA^{Glu}$. It was equally surprising that the *hemA* gene of *E. coli* (Sasarman et al., 1968) encodes not 5-aminolevulinate synthase, as had been thought for 20 years, but glutamyl-tRNA dehydrogenase. Moreover, a second *E. coli* gene, identified as giving rise upon mutation to 5-aminolevulinate auxotrophy (Powell et al., 1973), turned out to be a remarkable enzyme: glutamate–semialdehyde aminotransferase.

As detailed in Figure 4, glutamic acid is first activated by ligation to $tRNA^{Glu}$ with an aminoacyl–tRNA synthetase in the presence of ATP and Mg^{2+}. The activated glutamate is then reduced to glutamate 1-semialdehyde in an NADPH-dependent reaction catalyzed by Glu-tRNA reductase. Glutamate 1-semialdehyde-2,1-aminomutase (EC 5.4.3.8) then carries out the conversion into 5-aminolevulinate. Because we have recently provided a detailed account of our present knowledge of this pathway with relevant references (Kannangara et al., 1994), in this article we highlight additional interesting elements and novel observations of importance for further work.

In higher plants, the gene encoding the $tRNA^{Glu}$ is encoded in chloroplast DNA, whereas the three enzymes involved in 5-aminolevulinate formation—the aminoacyl–tRNA synthetase, the reductase, and the aminotransferase—are encoded by nuclear DNA and are imported into the chloroplast stroma after synthesis on cytoplasmic ribosomes. This single chloroplast $tRNA^{Glu}$ has to serve for both chlorophyll synthesis and protein synthesis on chloroplast ribosomes. In the presence of ATP and glutamate, purified Glu–tRNA synthetase, $tRNA^{Glu}$, and Glu–tRNA reductase can form a complex; this implies that different domains of the $tRNA^{Glu}$ molecule recognize and bind to the two enzymes. This conclusion has recently been verified by studies of a *E. gracilis* mutant in which a cytosine of the T-loop of $tRNA^{Glu}$ was converted to a uracil (Stange-Thomann et al., 1994). This tRNA can still be charged with glutamate by the aminoacyl–tRNA synthetase, as judged by the capacity of the mutant to synthesize ribulose-bisphosphate carboxylase and ATP synthase on chloroplast ribosomes, but it is unable to function with the reductase in the synthesis of 5-aminolevulinate.

The ligation and reduction reactions require both the 3′ CCA sequence and the UUC anticodon with its hypermodified 5-methylaminomethyl-2-thiouridine of the $tRNA^{Glu}$. Although the ligase does not discriminate between $tRNA^{Glu}$ and $tRNA^{Gln}$ and loads glutamate onto both tRNA species, the reductase recognizes glutamyl-$tRNA^{Glu}$ exclusively (glutamyl-$tRNA^{Gln}$ is converted to glutaminyl-$tRNA^{Gln}$ by a specific amidotransferase). Chloroplast glutamyl-$tRNA^{Glu}$ from barley,

wheat, tobacco, spinach, cucumber, *Synechocystis*, and *Chlamydomonas* are equally efficient as substrates for the barley glutamyl–tRNA reductase, but *E. coli* $tRNA^{Glu}$ is a much poorer substrate, and yeast $tRNA^{Glu}$ is only slightly better (Willows et al., 1995). A comparison of the bases conserved among $tRNA^{Glu}$ and $tRNA^{Gln}$ from different species and cell compartments reveals that five nucleotides are probably required for recognition by the glutamyl–tRNA synthetase. An analogous comparison of sequences that can and cannot be used by the reductase indicates that seven nucleotides are involved in recognition by the barley chloroplast glutamyl–$tRNA^{Glu}$ reductase.

Two approaches have been taken to characterize the latter unique enzyme, which converts glutamyl-$tRNA^{Glu}$ to glutamate 1-semialdehyde (reviewed in Pontoppidan and Kannangara, 1994). One approach was to purify this unstable enzyme of low abundance, whereas the other centered around mutations in the *hemA* gene of the *E. coli* K12 strain which leads to auxotrophy for 5-aminolevulinate. Recently, unequivocal evidence was obtained that *hemA* encodes the glutamyl–$tRNA^{Glu}$ reductase and that no other protein is required for activity (Pontoppidan and Kannangara, 1994). The purified barley enzyme, with a molecular mass of ~270 kD, is made up of four to six identical subunits with a molecular weight of 54 kD and has an activity for synthesis of glutamate semialdehyde of 250 pmol min/μg. The sequence of the 18 N-terminal amino acids of the mature subunits of the active enzyme is identical to the N-terminal amino acid sequence deduced from a barley cDNA clone that rescues the *E. coli hemA* mutant. Complementation of the *E. coli* mutant has yielded over the years *hemA* genes from *Salmonella typhinurium*, *Bacillus subtilis*, *Synechocystis*, Arabidopsis, *Clostridium josni*, barley, and other species. Uncertainty has reigned about the function of the HemA gene product for two reasons. (1) The mutant can also be rescued by the genes encoding the monomeric 5-aminolevulinate synthase from *Rhodobacter* and *Rhizobium*, the enzyme using the ubiquitous metabolites succinyl-CoA and glycine as substrates. When the *E. coli hemA* gene was cloned and sequenced, its deduced amino acid sequence showed no homology to that of 5-aminolevulinate synthase from *Rhodobacter* and mouse. At the same time, identification of the *E. coli hemL* gene as the structural gene for glutamate 1-semialdehyde aminotransferase and demonstration that this enzyme as well as glutamyl–tRNA synthetase are active in the *E. coli hemA* mutant established that *E. coli* uses the C_5 pathway. This is a good illustration that complementation of a mutant with expression of a known enzyme does not necessarily identify the enzymatic function of the cognate wild-type gene. (2) Although *hemA* genes from several bacteria and higher plants can complement the *E. coli hemA* mutant and cause *E. coli* cells to overproduce uroporphyrinogen, HemA protein purified from *E. coli* overexpressing the *hemA* gene is not enzymatically active in vitro.

The deduced amino acid sequences of eight glutamyl–$tRNA^{Glu}$ reductases from a range of organisms contain several highly conserved sequence domains, including one of 27 residues that is 70% identical in all eight proteins. Analysis of the function of this domain and elucidation of the mechanism of the reduction of glutamyl-$tRNA^{Glu}$ requires establishing an expression system yielding high amounts of active enzyme.

Barley contains two *hemA* genes, which have different expression patterns. One gene is not transcribed in roots but gives a high transient transcript level during greening; a second is expressed moderately in roots and leaves (O. Bougri and B. Grimm, personal communication). Light-stimulated transcription of the Arabidopsis genes for glutamyl–$tRNA^{Glu}$ reductase and glutamate semialdehyde aminotransferase has been reported by Ilag et al. (1994). Further experiments are necessary to determine to what extent the regulation of 5-aminolevulinate synthesis takes place at the transcriptional and/or translational level.

Glutamate 1-semialdehyde aminotransferase transfers the amino group from the C4 carbon of glutamate semialdehyde to the C5 carbon of 5-aminolevulinic acid, with pyridoxal 5′ phosphate as a cofactor and 4,5-diaminovalerate as an intermediate (Kannangara et al., 1994). Overexpression and purification of recombinant enzyme from *Synechococcus* and barley and site-directed mutagenesis have permitted a detailed investigation of the ping-pong bi-bi mechanism by which the transamination is effected. The 46-kD enzyme of *Synechococcus* can function as a monomer, whereas the barley enzyme operates as homodimeric protein. X-ray crystallography is presently being employed to study the interaction between the two subunits (Hennig et al., 1994).

THREE REMARKABLE ENZYMES GENERATE UROPORPHYRINOGEN III

The biosynthetic pathway of chlorophyll from 5-aminolevulinate to uroporphyrinogen III and beyond to protoporphyrin IX is, according to our present knowledge, essentially the same as that of heme in man, mouse, plants, and bacteria. Much more molecular information for plants is desirable, and the present discussion endeavors to emphasize molecular results in mammals and bacteria that might be confirmed for plants.

The carbon–carbon and carbon–nitrogen bonds in the pyrrole ring of porphobilinogen are formed by the enzyme 5-aminolevulinic acid dehydratase (porphobilinogen synthase), which catalyzes the asymmetric condensation of two molecules of 5-aminolevulinic acid, as shown in Figure 5 (Jordan, 1991; Spencer and Jordan, 1994). Isotopically labeled substrates reveal that the initially bound 5-aminolevulinic acid forms a Schiff base with its keto group and the ε-amino group of a lysine at position 247 of the enzyme (*E. coli* numbering) that, along with an adjacent proline, is conserved in all 16 dehydratases whose sequence is known. These enzymes include representatives from mammals, bacteria, yeast, and plants, including spinach (Schaumburg et al., 1992), pea (Boese et al., 1991), tomato, and soybean. This first bound molecule gives rise to the propionic acid side chain of

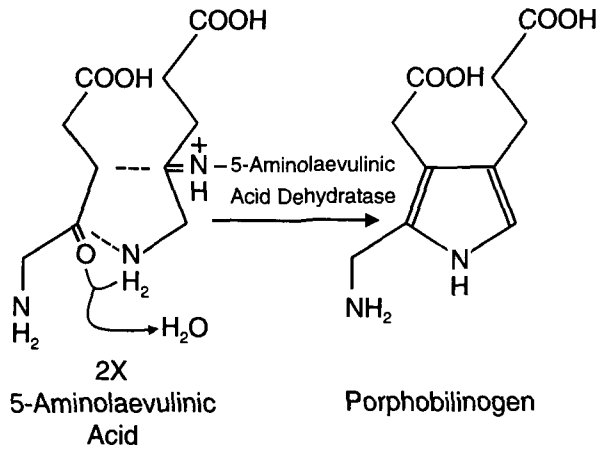

Figure 5. Synthesis of the Pyrrole Ring.

porphobilinogen, whereas the second molecule of 5-aminolevulinate (which gives rise to the acetic acid side chain) is placed in a separate substrate binding site that allows the aldol condensation between the C3 of this second molecule and the C4 of the first molecule to proceed. Condensation is accompanied by detachment from the enzyme's lysine nitrogen. A Schiff base between the keto group of the second 5-aminolevulinate and the amino group of the first one furnishes the pyrrole ring; the enzyme also catalyzes the tautomerization of the molecule with a stereospecific removal of a hydrogen atom at the C2 of the pyrrole ring.

The plant enzymes and that of *Bradyrhizobium* (Chauhan and O'Brian, 1993) lack the three cysteine residues implicated in the binding of two Zn^{2+} atoms that are required for activity of the mammalian and bacterial enzymes (Jaffe et al., 1994; Spencer and Jordan, 1994); these have been replaced with alanine and aspartic acid residues, which may signal a Mg^{2+} binding site, consistent with the fact that the plant enzymes are activated by Mg^{2+} but not by Zn^{2+}. A second Mg^{2+} binding site has been tentatively located to conserved asparagine and glutamate residues in the spinach and bacterial enzymes. Mg^{2+} can substitute for Zn^{2+} in one of the two Zn^{2+} binding sites of the *E. coli* enzyme (Mitchell and Jaffe, 1993; Spencer and Jordan, 1994). It will be interesting to see whether the plant enzymes have two Mg^{2+} binding sites.

Alternative splicing of transcripts is used in different human cells to provide, on the one hand, mRNA for the prolific production of 5-aminolevulinate dehydratase in erythrocytes and, on the other hand, a continuous housekeeping amount of the enzyme for heme production in other cells (Kaya et al., 1994). It would be interesting to know whether such alternative 5-aminolevulinate dehydratase mRNA splicing is used to satisfy demands for rapid chlorophyll synthesis during greening of leaves and continuous heme synthesis in all organs.

As shown in Figure 6, the enzyme porphobilinogen deaminase (or 1-hydroxymethylbilane synthase), a monomeric protein with a molecular weight of 34 to 44 kD, synthesizes its own cofactor (a dimer of porphobilinogen) and then adds four molecules of porphobilinogen by stepwise deamination to yield the tetrapyrrole 1-hydroxymethylbilane, which is also called preuroporphyrinogen (cf. Jordan, 1994). This enzyme also carries out the hydrolysis that releases the tetrapyrrole from the cofactor. Key features in understanding this enzyme have been obtained from the *E. coli* enzyme, whose three-dimensional crystal structure (Figure 7) has been solved at 1.9Å resolution (Louie et al., 1992).

Although porphobilinigen deaminases from man, mouse, rat, yeast, bacteria, and higher plants have only 15 to 20% overall amino acid sequence similarity, 33 individual residues involved in the catalytic and substrate binding sites are identical in all 15 enzymes analyzed, including those of *Euglena* (Sharif et al., 1989), pea (Witty et al., 1993), and Arabidopsis (Jones and Jordan, 1994; EMBL data base accession number X73418). The dipyrromethane cofactor is covalently bound to Cys-242 and projects into the catalytic cleft from a loop on domain 3 (Figure 7). The side chains of the conserved residues Arg-132, Arg-155, and Lys-83 and of the partially conserved residues Arg-131, Ser-129, and Ser-127 form salt bridges and hydrogen bonds with the acetate and propionate side chains of the cofactor. The substrate binding site is probably formed by the arginines at positions 11, 149, and 155, which may interact with the propionate and acetate side chains of the substrate. Asp-84 is positioned such that its carboxyl side chain can interact with the amino groups of two pyrrole rings and donate or receive protons for deamination and carbon–carbon bond formation (Louie et al., 1992). A site-directed mutant, D84E, in which this residue is changed to glutamic acid, retains <1% activity but can form highly stable dimer and trimer intermediate complexes (Woodcock and Jordan, 1994). Mutants D84A and D84N are inactive in preuroporphyrinogen formation but can assemble the cofactor. By contrast, mutants R131H and R132H prevent dipyrromethane assembly. This indicates that cofactor assembly and tetramerization are carried out by different reactants and provides a hint about why the cofactor remains firmly attached during repeated tetramerizations.

In the next step, uroporphyrinogen (co)synthase carries out the ring closure with isomerization of ring IV, which is thus

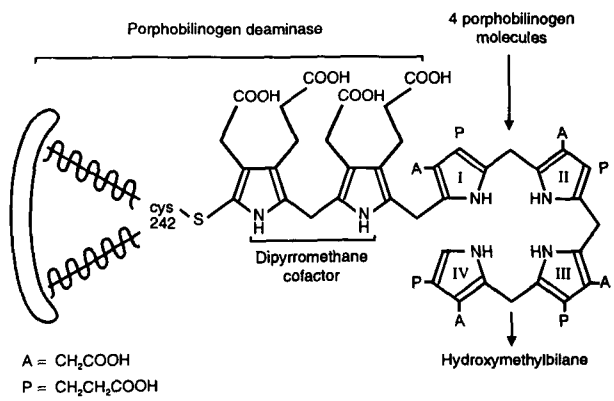

Figure 6. Synthesis of the Tetrapyrrole Chain.

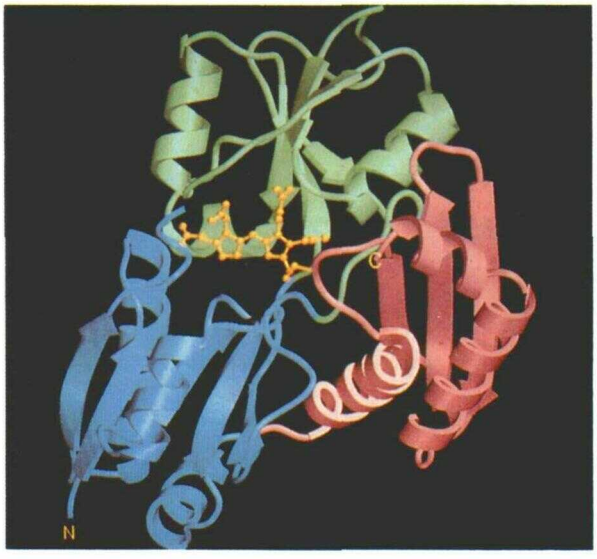

Figure 7. Crystal Structure of *E. coli* Porphobilinogen Deaminase.

The dipyrromethane cofactor is located in a cleft between domain 1 (blue) and domain 2 (green) and is covalently bound to Cys-242 of domain 3 (violet). From Lambert et al. (1994).

turned around (Figure 8; Battersby et al., 1977). This can be seen in the structure of uroporphyrinogen III and the finished chlorophyll molecule because the methyl groups of ring IV and I are located in neighboring positions within the porphyrin ring. The enzymes of human, mouse, *E. coli*, *B. subtilis* (Hansson et al., 1991), and *Synechococcus* (Jones et al., 1994) have a molecular weight of ~30 kD and are highly diverse in primary structure. Only five residues are conserved among the nine enzymes for which the sequence is known.

TRIMMING OF THE SIDE CHAINS AND RED COLOR FORMATION

Figure 9 presents the following steps of chlorophyll formation: the acetic acid side chains are shortened to methyl groups by uroporphyrinogen III decarboxylase, coproporphyrinogen III oxidase trims two of the propionic acid side chains into vinyl groups, and then the molecule is oxidized by protoporphyrinogen IX oxidase to establish the conjugated double-bond system of the red-colored protoporphyrin IX (cf. Akhtar, 1994).

Uroporphyrinogen III decarboxylases from mammalian erythrocytes, and liver, yeast, *Rhodobacter sphaeroides* (Jones and Jordan, 1993), *B. subtilis* (Hansson and Hederstedt, 1992), and the stroma of tobacco and barley plastids (Mock et al., 1995) are monomeric proteins with a molecular weight of ~40 kD. At low-substrate concentrations, the erythrocyte and *Rhodobacter* enzymes catalyze stepwise decarboxylations, beginning at ring IV, proceeding clockwise through rings I and II, and ending at ring III. At high-substrate concentrations, decarboxylation can occur in any order. The 10 primary amino acid sequences show that the eukaryotic enzymes have an overall similarity of 33 to 50%. The tobacco and barley sequences are 73% similar to the sequence of the cyanobacterial (*Synechococcus*) enzyme (Kiel et al., 1992). The sequence comparisons indicate that there are three invariant domains. Their functions, and those of individual invariant amino acids, remain to be determined.

Structural genes for oxygen-dependent coproporphyrinogen III oxidases from three higher plants, including soybean (Madsen et al., 1993), barley, and tobacco (Kruse et al., 1995), have been cloned. Coproporphyrinogen III oxidase is synthesized as a precursor protein on cytoplasmic ribosomes and is imported into the stroma of pea chloroplasts, where it is processed into a catalytically active enzyme with a molecular weight of 39 kD. The three higher plant enzymes are 70% identical at the amino acid sequence level, and an overall comparison from bacteria to human of the eight known primary structures shows 50% identity, which is especially prominent in the C-terminal part of the oxidase. Levels of transcripts for the plant coproporphyrinogen III oxidase are the same in dark-grown and greening leaves of barley; they are also constant during leaf development in tobacco but are strongly reduced in mature leaves. In anaerobic bacteria synthesizing bacteriochlorophyll, the oxygen-requiring coproporphyrinogen III oxidase must be substituted by an oxygen-independent enzyme. A mutant in *R. sphaeroides* excreting coproporphyrin III has defined a gene encoding a putative anaerobic coproporphyrinogen oxidase (Coomber et al., 1992) that is also present in *E. coli*, *Salmonella*, *Pseudomonas*, *Rhizobium*, and *B. subtilis* (cf. Hansson and Hederstedt, 1994).

Protoporphyrinogen IX oxidase has been purified to homogeneity from bovine and mouse liver (cf. Akhtar, 1994) and barley etioplasts (Jacobs and Jacobs, 1987). The mammalian enzymes require molecular oxygen, use a flavin cofactor, and have a molecular mass of 65 kD, whereas the barley enzyme is 36 kD. Expression of the *hemY* gene of the *B. subtilis hemEHY* cluster in *E. coli* results in the production of a soluble 53-kD protein that can oxidize both coproporphyrinogen III and protoporphyrinogen IX (Dailey et al., 1994; Hansson and Hederstedt, 1994). Cloning of the gene for the chloroplast

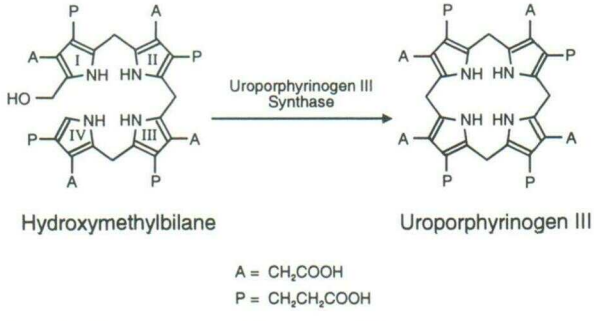

Figure 8. Formation of the Tetrapyrrole Ring.

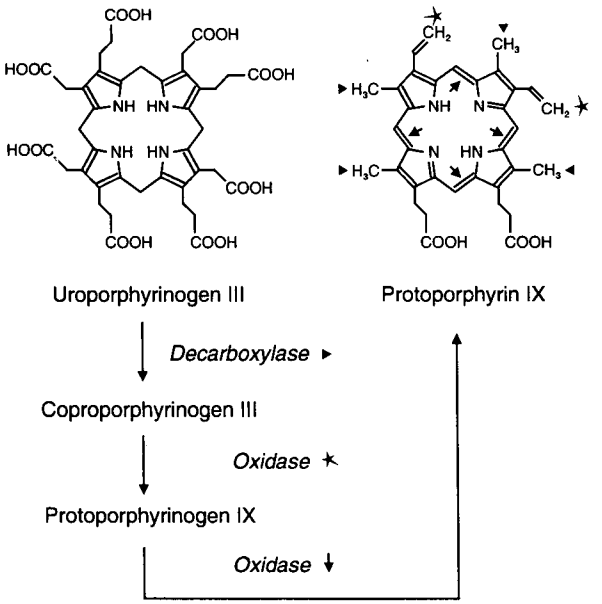

Figure 9. Trimming of the Acetate and Propionate Side Chains and Establishment of the Conjugated Double Bond System.

protoporphyrinogen IX oxidase and its heterologous expression will help verify the catalytic mechanism, which consists of three consecutive dehydrogenations and a subsequent tautomerization yielding the porphyrin through the stereospecific loss of the fourth *meso* hydrogen as a proton (Akhtar, 1994). In the three desaturations, the flavin prosthetic group acts as an acceptor of the hydride ion; the reduced flavin is then reoxidized by O^2 for regeneration of active enzyme.

FROM BRIGHT RED TO GREEN

The chlorophyll- and bacteriochlorophyll-specific pathway from protoporphyrin IX to chlorophyll (Figure 10) begins with the insertion of the Mg^{2+} ion (Bauer et al., 1993; Bollivar et al., 1994b; Castelfranco et al., 1994). Recent advances in the characterization of this reaction were described earlier, in the discussion of the use of mutants. After insertion of the Mg^{2+} ion, the magnesium–protoporphyrin methyltransferase esterifies the propionic side chain of ring III in preparation for the cyclization reaction that produces ring V. The *S*-adenosyl-L-methionine that donates the methyl group must be imported into the chloroplast, which also contains a methyl esterase and an oxidase to degrade unused Mg-protoporphyrin IX monomethyl ester (Castelfranco et al., 1994). Expression of the *bchM* gene of *R. capsulatus* and *R. sphaeroides* in *E. coli* have identified this gene's product as the methyltransferase for Mg-protoporphyrin IX (Bollivar et al., 1994a; Gibson and Hunter, 1994). $^{18}O_2$ labeling indicates that the oxygen atom in ring V derives from atmospheric O_2 and that β-hydroxypropionate

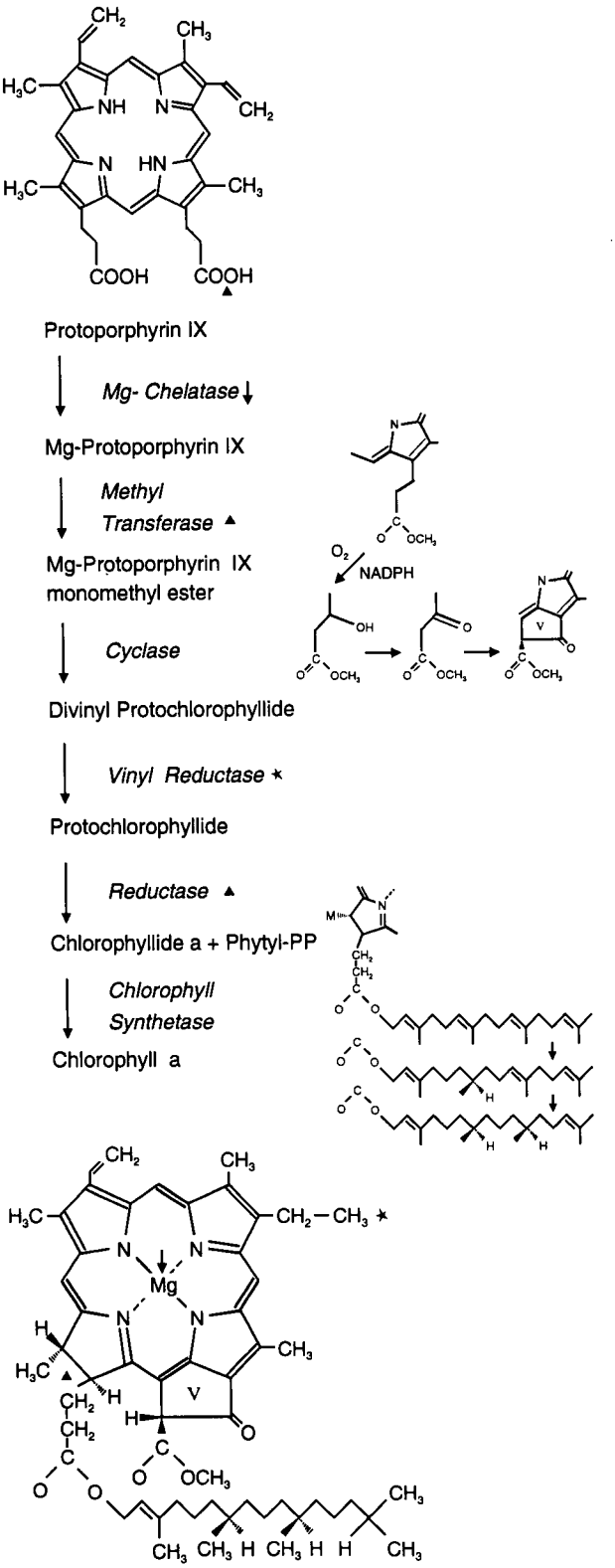

Figure 10. Pathway from Protoporphyrin IX to Chlorophyll *a*.

and β-oxopropionate are intermediates in the cyclization process (Castelfranco et al., 1994).

Reduction of divinyl protochlorophyllide to monovinyl-protochlorophyllide has been inferred from product characterization (Whyte and Griffiths, 1993). Because disruption of the *bchJ* gene in *R. capsulatus* leads to accumulation of divinyl protochlorophyllide, this gene is thus a candidate for a structural gene for this enzyme (Bollivar et al., 1994b).

Protochlorophyllide reduction in plants and bacteria has been studied extensively and is the topic of the next section. Briefly, the transfer of a hydrogen from NADPH to the carbon atom carrying the propionic side chain of ring IV and a hydrogen from water or protein to the carbon atom carrying the methyl group by protochlorophyllide reductase is carried out in angiosperms by a photochemical reaction. In cotyledons of gymnosperms and in mosses, liverworts, algae, and photosynthetic bacteria, the reduction can be catalyzed by a very different enzyme in the dark.

The last step in the synthesis of chlorophyll *a* is catalyzed by chlorophyll synthetase. This enzyme esterifies the propionic acid side chain of ring IV with either phytyl pyrophosphate, as is preferred in chloroplasts, or geranylgeranyl pyrophosphate, with subsequent reduction of the double bonds at positions 6, 10, and 14, as is preferred in etioplasts (Rüdiger et al., 1980). Nuclear magnetic resonance analysis of bacteriochlorophyll synthesized by *R. sphaeroides* from 5-aminolevulinic acid labeled at the carboxyl group with ^{13}C and ^{18}O revealed retention of both ^{18}Os in the formation of the ester bond and thus a carboxyl–alkyl transfer mode (cf. Akhtar, 1994). In *Rhodobacter*, the products of two genes (*bchG* and *bchP*) are implicated in the phytolation of bacteriochlorophyll *a*.

After many years of debate, there is now strong evidence that the formyl group in chlorophyll *b* that replaces the methyl group in position 3 (cf. Figure 1) of chlorophyll *a* is inserted by an oxygenase enzyme. Mass spectrometry of chlorophyll *b* synthesized in dark-grown maize leaves in the presence of either $H_2{}^{18}O$ or $^{18}O_2$ has documented that the oxygen in the formyl group of chlorophyll *b* is incorporated exclusively from atmospheric O_2 (Porra et al., 1994), as is the case in *Chlorella* (Schneegurt and Beale, 1992). Oxygenation of chloropyll *a* by a single oxygenase is supported by the observation that 10 independently isolated chlorophyll *b*–deficient mutants in barley and 54 *b*-less mutants in *Chlamydomonas* belong to a single complementation group (Simpson et al., 1985; Chunayev et al., 1991).

BACTERIA-INVENTED PROTOCHLOROPHYLLIDE REDUCTION WITH AND WITHOUT LIGHT

Rhodobacter reduces protochlorophyllide to chlorophyllide in the dark, using an enzyme consisting of three polypeptides, encoded by *bchL*, *bchN*, and *bchB* (Burke et al., 1993a). Elimination of the double bond in ring IV in the course of protochlorophyllide reduction is similar to elimination of the double bond in ring II by chlorin reductase, which converts chlorophyllide *a* into 2-desacetyl-2 vinyl bacteriochlorophyllide *a*. Chlorin reductase comprises the products of the *bchX*, *bchY*, and *bchZ* genes. BchX and BchL share 34% amino acid sequence identity with each other, as well as 30 to 37% identity with the Fe protein (NifH) of the *Acetobacter vinelandii* nitrogenase (Burke et al., 1993b). The nucleotide binding site and the 4Fe-4S cysteine binding ligands are especially conserved in all known BchL subunits (Koonin, 1993). In addition, *bchN* is homologous to *nifK*, the gene encoding the β-subunit of the MoFe protein of nitrogenase, with its 4Fe-4S cluster pair. The three cysteine ligands of NifK are conserved in the BchN sequences. The *bchB* product is homologous to the Vd-Fe alternative α subunit of nitrogenase (*nifD*). However, the FeMo binding ligands Cys-275 and His-442 are replaced by tyrosines, indicating that another cofactor reduces protochlorophyllide.

Genes encoding the three polypeptides that are implicated in light-independent protochlorophyllide reduction have been identified in the cyanobacteria *Plectonema boryanum*, *Synechocystis*, and *Synechococcus* (Li et al., 1993; Suzuki and Bauer, 1995). Genes encoding these polypeptides have also been identified in the chloroplast genomes of the liverwort *Marchantia polymorpha*, *Chlamydomonas reinhardtii*, *C. moewusii*, the red alga *Porphyra purpurea*, ferns, and gymnosperms (Li et al., 1993; Suzuki and Bauer, 1995), all of which are known to synthesize chlorophyll in the dark. Disruption of the *Chlamydomonas bchL* homolog by particle gun transformation produces a "yellow in the dark" phenotype; that is, protochlorophyllide reduction in the dark is blocked (Suzuki and Bauer, 1992). The presence of these genes has been documented in the chloroplast DNA of the ferns *Cystopteris fragilis* and *Athyrium filix femina*; the horsetail *Equisetum arvense*; and the coniferous trees *Gingko biloba* (Richard et al., 1994), *Pseudotsuga menziesii*, *Taxus*, *Juniperus*, *Araucaria*, *Pinus contorta*, and *Picea abies*. The genes are, however, absent in the chloroplast genome of the angiosperms tobacco, rice, maize, Arabidopsis, and *Bougainvillea glabra*, all of which are incapable of synthesizing chlorophyll in the dark.

In contrast to lower plants and gymnosperms, angiosperms use exclusively the nuclear-encoded light- and NADPH-dependent protochlorophyllide oxidoreductase to synthesize chlorophyllide. The barley and Arabidopsis enzymes have a molecular weight of 35 kD and are synthesized in the cytosol with transit peptides for import into etioplasts and chloroplasts. After expression of the plant cDNA genes in *E. coli*, the extracted enzyme was able to reduce the substrate protochlorophyllide only in the presence of light and NADPH (Schulz et al., 1989; Benli et al., 1991). Monovinyl and divinyl protochlorophyllide are equally acceptable as substrates (Knaust et al., 1993). Light-dependent protochlorophyllide reductases from barley, oats, wheat, pea, and Arabidopsis are 80 to 95% identical at the amino acid sequence level (Suzuki and Bauer, 1995) but bear no homology to the three peptides of the light-independent protochlorophyllide reductase.

Barley contains two genes encoding light-dependent protochlorophyllide oxidoreductase isoenzymes that are 75% identical

at the amino acid sequence level (Holtorf et al., 1995). One isoenzyme is abundantly synthesized in the dark, but its synthesis declines rapidly upon illumination and greening of dark-grown seedlings. Transcription of the gene for this isoenzyme is turned off upon illumination. The gene for the other isoenzyme is constitutively transcribed in the dark and in the light, and the transcripts are translated continuously into active enzyme, which is responsible for chlorophyll synthesis during greening. This explains why transcripts for the light-dependent protochlorophyllide reductase as well as the protein itself were found to be abundant in dark-grown seedlings of bean, pea, tomato, sunflower, mustard, Arabidopsis, and maize, but both mRNA and enzyme levels declined when the seedlings were illuminated and rapid chlorophyll synthesis ensued (Forreiter et al., 1990; Benli et al., 1991). These experiments, as it turns out, focused on the "wrong" isoenzyme.

In gymnosperms such as pine and spruce, the chloroplast genome encodes the light-independent protochlorophyllide reductase and the nuclear genome two light-dependent protochlorophyllide reductases. The light-independent enzyme is expressed only during development of the cotyledons in the dark but not during development of the primary and secondary needles, in which chlorophyll synthesis relies on the light-dependent protochlorophyllide reductase. As in barley, there are two light-dependent isoenzymes in *Pinus mugo*, *P. strobus*, and *P. taeda*; one is synthesized specifically in the dark and the other constitutively in the dark as well as during chloroplast development in the light (Forreiter and Apel, 1993). Both of them are synthesized in the cotyledons together with the light-independent enzyme.

It was originally thought that the light-dependent protochlorophyllide oxidoreductase was an evolutionary invention of angiosperms. However, in a seminal experiment that illustrates the power of using *Rhodobacter* mutants in the analysis of chlorophyll biosynthesis genes and enzymes from cyanobacteria and higher plants, Suzuki and Bauer (1995) demonstrate that cyanobacteria had probably already invented the light-dependent enzyme before the advent of angiosperms. An *R. capsulatus* mutant that is unable to reduce protochlorophyllide as a consequence of the disruption of the *bchL* gene was mated with an *E. coli* strain library containing cosmids of 19 genome equivalents of *Synechocystis* (strain PCC6803) under a strong *Rhodobacter* promoter. Some exconjugants selected under anaerobic light conditions permitted the synthesis of bacteriochlorophyll and photosynthesis. The *Synechocystis* gene that conferred the ability for light-dependent protochlorophyllide reduction encodes a protein that is 53% identical and 73% similar to the light-dependent protochlorophyllide reductase of Arabidopsis.

This experimental approach was developed further by Wilks and Timko (1995) into an assay for identifying substrate binding and catalytic sites in the protochlorophyllide reductase from pea. The pea enzyme is able to restore bacteriochlorophyll synthesis to the *bchN*, *bchB*, or *bchL* mutants of *Rhodobacter*. By site-directed mutagenesis of a tyrosine or a lysine residue in the pea enzyme, complementation was abolished, even though inactive protein was synthesized. It was suggested that in the reduction of ring IV, the proton transferred to the carbon atom carrying the methyl group is donated by the tyrosine. Such complementation and expression assays in *Rhodobacter* permit the identification and molecular analysis of other chlorophyll biosynthetic enzymes from higher plants.

CHLOROPHYLL SYNTHESIS AND CHLOROPLAST DEVELOPMENT

Chloroplasts of higher plants develop from proplastids that consist of envelope and internal vesicular membranes in addition to invaginations from the inner envelope membrane (von Wettstein, 1958; Henningsen et al., 1993). In light-exposed tissue, the proplastids grow in size and differentiate a membrane system composed of unpaired stroma thylakoids (disclike sacs) that are continuous with paired or appressed thylakoids in the grana (Figures 11D and 11E). The membranes of both types of thylakoid contain chlorophyll bound to individual proteins. If seedlings, especially those of the family Poaceae (for example, barley, maize, and wheat), are germinated in the dark, the plastids develop into etioplasts. These contain perforated primary lamellar layers and develop crystalline prolamellar bodies (Figure 11A). The major protein constituent of the membranes in the prolamellar body is a ternary protochlorophyllide:NADPH-oxidoreductase complex (Henningsen et al., 1993). Upon illumination, the etioplasts synthesize chlorophyll, develop a lamellar system, and become photosynthetically active chloroplasts.

The protochlorophyllide in the prolamellar bodies is reduced to chlorophyllide, and the membrane tubes lose their ordered arrangement (Figure 11B) and flow out into fenestrated primary lamellar layers (Figure 11C). The dispersal of the prolamellar body is preceded or accompanied within minutes by a spectral shift of protochlorophyllide absorption from 684 to 672 nm and esterification of chlorophyllide to chlorophyll *a* (Henningsen et al., 1993). Chlorophyll synthetase seems to be present in the crystalline prolamellar bodies but does not become active, at least in wheat, until the protochlorophyllide has been reduced by light and the tubes have lost their regular configuration (Lindsten et al., 1993). Over the next 24 hr, chlorophyll is synthesized and the complexes of photosystem I and II and cytochrome b_6/f (Figure 12) are assembled. Incipient grana are found as overlapping, paired primary lamellar layers, and respectable grana are present after 24 hr (Figure 11D).

Assembly of the crystalline configuration of the prolamellar body requires the presence of protochlorophyllide and NADPH. The *xantha-f*^{60} mutant, which is unable to synthesize Mg-protoporphyrin, and other mutants stringently blocked in protochlorophyllide synthesis contain only unfenestrated thylakoids (Figure 11F) and lack prolamellar bodies, even though the 36-kD protochlorophyllide reductase protein is still synthesized (Dehesh et al., 1986). Arabidopsis mutants with lesions in the nuclear transcription factor DET1 also form expanded leaves in the dark and etioplasts lacking prolamellar bodies.

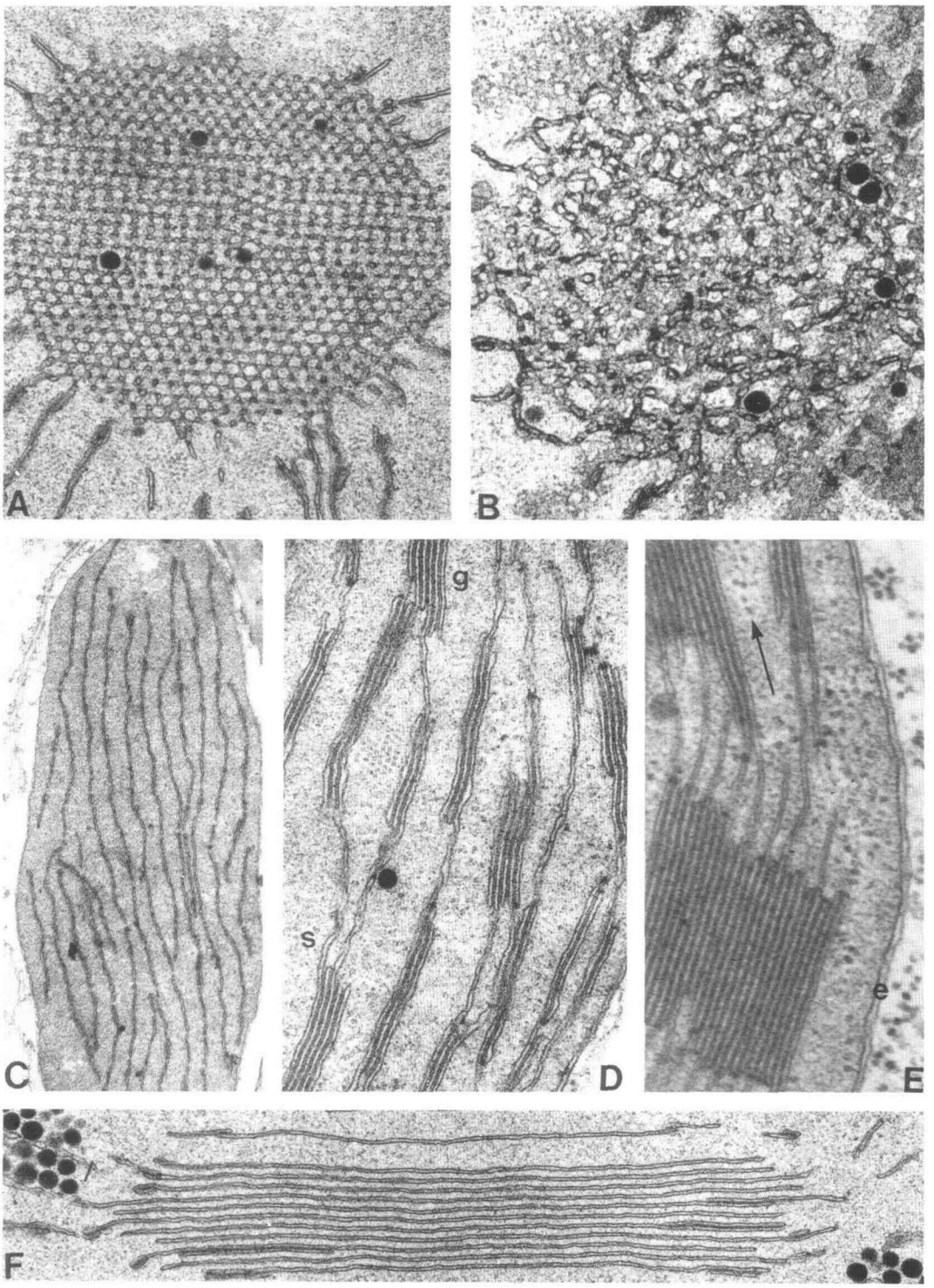

Figure 11. Membrane Structures in Etioplasts, Developing Plastids, and Chloroplasts of Barley Seedlings.

(A) Etioplasts of 7-day-old dark-grown primary leaves contain crystalline prolamellar bodies. Protochlorophyllide oxidoreductase is the major protein in the prolamellar bodies.

(B) Upon transfer into light and reduction of protochlorophyllide to chlorophyllide, the membrane tubes of the prolamellar body lose their ordered arrangement and flow out into primary lamellar layers.

DET1 is required to initiate chloroplast development upon illumination (Chory et al., 1989; Pepper et al., 1994), and in *det1* mutants, the synthesis of the protochlorophyllide reductase necessary for prolamellar body formation is probably prevented.

Reorganization and dispersal of the prolamellar body require protochlorophyllide and the capacity for phytylation. Thus, in senescing etioplasts, in which crystalline prolamellar bodies are depleted of protochlorophyllide, transformation into the unordered tubular configuration upon illumination is severely impeded. Several mutants in barley can be exploited to analyze the relationship between chlorophyll synthesis and development of membrane structures. In *xantha-j* mutants, protochlorophyllide oxidoreductase accumulates in normal amounts, crystalline prolamellar bodies are formed (Figure 11A), and reduction to chlorophyllide-684 and tube transformation are instantaneous upon illumination. However, dispersal of the prolamellar body, shift of the chlorophyllide absorption peak to 672 nm, and esterification by chlorophyll synthetase are blocked, and no further chloroplast development is possible (Henningsen et al., 1993). On the other hand, if the mutants are grown at low temperature in the light, ~10% of normal levels of chlorophyll and some grana can develop. Taking note of the recent discovery of two light-dependent protochlorophyllide reductases in barley, we suggest that the limited amount of chlorophyll formed in the light in the *xantha-j* mutants uses the constitutively expressed enzyme, whereas the dark-specific enzyme, with its bound photoreceptor protochlorophyllide, is located in the prolamellar bodies with the specific function to initiate the reorganization of this membrane storage body upon illumination. It is this reorganization that is defective in the *xantha-j* mutants.

Stacking of thylakoids into grana requires the presence of chlorophyll, and it has variously been suggested that thylakoid stacking is dependent on the presence of chlorophyll *b*, the light-harvesting complex LHCII, or the PSII complex (Simpson et al., 1989). However, even a double mutant in barley (*chlorina-f2/viridis-115*) that lacks LHCII because of a block in the conversion of chlorophyll *a* to chlorophyll *b* and that is unable to assemble a functional inner core of photosystem II can still form grana (Simpson et al., 1989). The molecular basis for thylakoid stacking therefore remains unresolved.

When etiolated seedlings are returned to the dark after conversion of protochlorophyllide with a short illumination, protochlorophyllide is resynthesized and crystalline prolamellar bodies reassemble. Chloroplasts with both crystalline prolamellar bodies containing protochlorophyllide as well as many large, chlorophyll-containing grana develop in dark-grown barley seedlings illuminated for 24 hr with only 1 $\mu E\ m^{-2}\ sec^{-1}$ of light. Upon illumination with higher intensity light, the protochlorophyllide is reduced and the prolamellar bodies disperse (Henningsen et al., 1993). The discovery of the two differentially regulated light-dependent protochlorophyllide reductases may explain the finding that the chlorophyll in the grana of the "low-light" chloroplasts is likely to be synthesized by the protochlorophyllide reductase isozyme that is constitutively expressed in the dark and during greening, whereas the "prolamellar body–specific reductase" maintains the prolamellar bodies under very low light intensity.

Crystalline prolamellar bodies and extensive grana are also found in chloroplasts of cotyledons of Norway spruce and other conifers developed in the dark (von Wettstein, 1958; Selstam and Widell, 1986). The light-independent protochlorophyllide reductase and the two light-dependent protochlorophyllide reductases expressed in the dark in developing conifer cotyledons make this differentiation of plastid structures understandable. The light-independent enzyme permits the synthesis of chlorophyll and the assembly of active photosystems and light-harvesting complexes in the grana (Lewandowska and Öquist, 1980), whereas a light-dependent protochlorophyllide reductase is responsible for the assembly of the prolamellar bodies. From an evolutionary point of view, cyanobacteria and algae (which neither store protochlorophyllide in the dark nor form prolamellar bodies) "invented" the light-independent and light-dependent protochlorophyllide reductases. By duplicating the gene for the latter enzyme, the seed-producing spermatophytes have evolved the prolamellar body–specific reductase.

CHLOROPHYLL BINDING PROTEINS

All chlorophyll molecules in the chloroplast are bound noncovalently to proteins in the photosynthetic membrane. Our present knowledge of the structure of these chlorophyll binding proteins has recently been compiled and reviewed by Jansson (1994) and Simpson and Knoetzel (1995).

In the reaction center of photosystem II (Figure 12), the D1 and D2 transmembrane polypeptides hold the "special pair" chlorophyll molecules in place by coordination with two

Figure 11. (continued).

(C) Formation of primary lamellar layers completed.

(D) Concomitant with intensive synthesis of chlorophyll, the grana (g) and stroma thylakoids (s) are assembled.

(E) Chlorophyll biosynthetic enzymes are encoded in the nucleus, translated as precursors on cytoplasmic ribosomes, and imported through the chloroplast envelope (e) into the stroma of the chloroplast. Six chlorophyll *a* binding proteins are encoded in chloroplast DNA and translated on chloroplast ribosomes (arrow). Ten chlorophyll *a*/*b* binding proteins are encoded in the nucleus, translated as precursors on cytoplasmic ribosomes, imported into the chloroplast, and inserted into the thylakoids.

(F) The mutant *xantha-f*^{60}, which is blocked in the insertion of Mg^{2+} into protoporphyrin IX, forms no prolamellar bodies in the dark (and no grana in the light; data not shown). Instead, it forms loose stacks of large unperforated thylakoids.

histidine residues. A few additional scavenger chlorophyll molecules are also located in this protein dimer. These two polypeptides are encoded in the chloroplast genome, as are the chlorophyll *a* binding proteins CP47 and CP43, which comprise the inner antenna of the reaction center. These proteins each bind ~45 chlorophyll molecules and approximately six β-carotene molecules. In photosystem I, the dimer-forming PSIA and PSIB membrane-spanning polypeptides are encoded in the chloroplast DNA and coordinate the special pair chlorophyll P700 between them. They also bind together ~90 antenna chlorophyll *a* molecules and nine to 18 β-carotene molecules.

Both reaction center complexes are surrounded by chlorophyll *a* and *b* binding proteins (shown in light green in Figure 12). These proteins are encoded in the nucleus, synthesized on cytoplasmic ribosomes, imported into the chloroplast, and inserted into the photosynthetic membrane. Six polypeptides encoded by the *Lhcb* genes are associated with photosystem II, and four proteins encoded by the *Lhca* genes are attached to photosystem I. Most of the genes are present in one or two copies per nuclear genome, but the most abundant protein in the light-harvesting complex II of photosystem II, Lhcb1, is encoded by a multigene family with three to 16 members in different plants. Their molecular weights range from 22 to 28 kD, and they contain three highly conserved helix domains and two conserved loops (cf. Jansson, 1994). Electron crystallography at 3.4 Å resolution reveals three LHCII particles as a trimeric complex in the unit cell. The individual light-harvesting polypeptides form two tilted membrane-spanning α-helices (1 and 3) of unusual length (31 to 33 amino acids) and a perpendicular membrane-spanning helix of ~20 amino

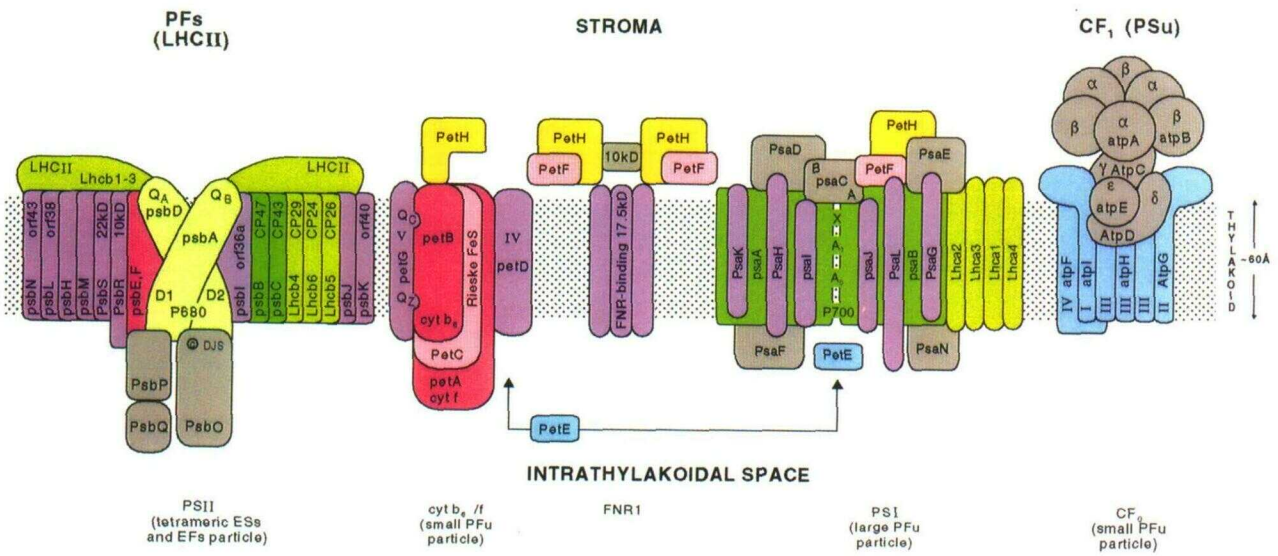

Figure 12. Model of the Thylakoid Membrane Showing the Known Polypeptides in the Major Complexes.

The polypeptides are identified by gene and/or protein name and are color coded according to their electron transport cofactors. Light-harvesting proteins binding only chlorophyll *a* are shown in dark green, whereas those binding both chlorophyll *a* and *b* are shown in light green (Lhca1-4 and Lhcb1-6). The "special pair" chlorophyll in photosystem II (P680) is coordinated between the D1 and D2 polypeptides (lightest green). That of photosystem I (P700) is ligated between the *psaA*- and *psaB*-encoded polypeptides, which also bind 90 light-harvesting antenna chlorophyll *a* molecules.

PSII, photosystem II. Q_A, tightly bound quinone; Q_B, exchangeable quinone; psbE,F, cytochrome b_{559}; psbO,P,Q, extrinsic oxygen-evolving complex; psbH to psbS, polypeptides of PSII and water-splitting enzyme. Freeze fracture membrane particles: PFs, protoplasmic fracture face of stacked membranes; LHCII, light-harvesting complex of photosystem II; EFs, ectoplasmic fracture face of stacked membranes; ESs, ectoplasmic surface face of stacked membranes.

Cyt b_6/f, cytochrome b_6 (red)–cytochrome *f* (red) complex participating in the photosynthetic electron transport between photosystem II and I and pumping protons across the membrane. pet, photosynthetic electron transport protein (chloroplast encoded); Pet (nucleus encoded); PetH, ferredoxin nucleotide reductase (FNR); PetG, polypeptide binding quinone that accepts electrons plus protons and donates electrons to the next carrier together with a release of protons as part of the proton pump across the membrane; PetE, copper-containing plastocyanin (blue); Pfu, protoplasmic fracture face of unstacked membrane.

FNR1, ferredoxin nucleotide reductase complex for cyclic photophosphorylation. PetF, ferredoxin.

PSI, photosystem I. A_0, chlorophyll molecule; A_1, phylloquinone; X, 4Fe-4S cluster; psaC, polypeptide carrying two 4Fe-4S clusters, A and B; PsaD, PsaE, PsaF, PsaG, PsaH, PsaK, PsaL, and PsaN, nucleus-encoded subunits of PSI; psaI and psaJ, chloroplast-encoded subunits of PSI.

CF_1, extrinsic part and CF_0, membrane-embedded part of coupling factor (ATP synthase). The CF_0 component forms a proton channel, whereas the CF_1 component protrudes into the stroma and couples ATP synthesis to proton translocation. The α, β, and ε subunits are encoded in chloroplast DNA, as are the channel subunits I, III, and IV. Subunits γ and δ and II are encoded in the nucleus. PSu, protoplasmic surface face of unstacked membranes.

Updated from Simpson and von Wettstein (1989).

acids. Helices 1 and 3 bind all eight chlorophyll *a* and six chlorophyll *b* molecules, which are held in close proximity by the helices inside the photosynthetic membrane to ensure energy transfer. The helices also bind the xanthophyll lutein, and some of the polypeptides bind violaxanthin, β-carotene, and neoxanthin. These carotenoids serve as accessory pigments for absorption of blue light (see Bartley and Scolnik, 1995, this issue).

Assembly of the imported light-harvesting polypeptides and their insertion into the thylakoid membrane seem to require the presence of chlorophyll and lutein, as indicated by the following experiments. The major chlorophyll *a/b* binding protein can be expressed in *E. coli*, isolated, and folded in vitro, but only in the presence of chlorophyll *a*, chlorophyll *b*, and lutein (Paulsen et al., 1993). In barley mutants that are blocked in chlorophyll *a* synthesis, transcription of the structural genes for light-harvesting polypeptides proceeds at normal rates (Batschauer et al., 1986). However, the polypeptides do not accumulate in the thylakoids, probably because they are not stabilized with chlorophyll and thus are proteolytically degraded. The viable barley chlorophyll *b*–less mutant *chlorina-f2* is deficient in LHCII, although normal levels of LHCII mRNA are present in the mutant (Batschauer et al., 1986). A limited amount of the translated and imported polypeptide binds chlorophyll *a* and is inserted into the thylakoids as an intrinsically unstable chlorophyll protein (Bassi et al., 1985).

It will be interesting to explore to what extent attachment of chlorophyll to the chlorophyll *a/b* binding proteins is required for their transport from the chloroplast envelope through the stroma into the thylakoid membrane (Nielsen et al., 1994; Robinson and Klösgen, 1994). It would also be helpful to identify mutants that are disturbed in the transport pathway. The temperature-sensitive viable mutant *chlorina-104* fails to incorporate the Lhca2 and Lhcb1 light-harvesting proteins of photosystem I into the photosynthetic membrane at the restrictive temperature, but it does so when moved to the permissive temperature (Knoetzel and Simpson, 1991). Isolation of mutant plastids developed at restrictive or permissive temperature and import experiments with in vitro–transcribed and translated Lhca2 and Lhcb1 precursor proteins will show whether an import or transport component is affected in the mutant.

Insight is also being gained into chlorophyll's importance for translating mRNA for the proteins that bind chlorophyll *a* exclusively. These proteins are encoded in chloroplast DNA and are translated on polysomes attached to the thylakoids. In wild-type etioplasts and in plastids of mutants blocked in the synthesis of porphyrins and chlorophyll, transcription of the genes encoding D1, CP43, and CP47 of photosystem II and PSIA and PSIB of photosystem I proceeds, and the transcripts form polysomes. Translation is interrupted because the ribosomes pause at specific sites of the mRNA until chlorophyll and other electron carriers become available (Klein et al., 1988; Kim et al., 1991). For the D1 polypeptide, the sites of ribosome pausing were determined and found to coincide with the transmembrane helices that bind the special pair chlorophyll, quinone, and pheophytin (Kim et al., 1991). It was suggested that chlorophyll has to bind to the transmembrane helix before it can be incorporated cotranslationally into the thylakoid membrane.

In an interesting experiment, lysed barley etioplasts containing chlorophyll synthetase were supplied with chlorophyllide *a* and phytyl pyrophosphate and used in translation assays in the dark: chlorophyll *a* was synthesized, ribosome pausing was released, and the PSIA, PSIB, CP47, CP43, and D1 polypeptides accumulated. The barley nuclear gene mutant *viridis-115* lacks the reaction center core peptides of photosystem II, including polypeptide D1. Investigation of the translation of the mRNA-encoding reaction center polypeptide D1 revealed ribosome pausing as in the wild type, but the mRNA intermediates characteristic for pausing polysomes were missing (Kim et al., 1994). It was concluded that the *viridis-115* protein stabilizes the mRNA intermediates such that in its absence, incorporation of the polypeptides of the photosystem II core into the membrane cannot take place. In barley, the nuclear gene mutant *viridis-zb*^{63} lacks the reaction center complex of photosystem I but incorporates its chlorophyll *a/b* binding proteins and assembles a complete photosystem II (Hiller et al., 1980). This mutant provides the opportunity to analyze the assembly of photosystem I by in vitro expression of all 13 cloned subunit genes and import of the subunit precursors into the mutant plastids.

PERSPECTIVES

The identification, physical mapping, and nucleotide sequencing of the 45-kb photosynthesis gene cluster in *Rhodobacter* and closely related organisms have probably provided us with the primary structure of all genes necessary for the synthesis of chlorophyll and a basic frame of the photosynthetic apparatus. Fortunately, the primary structure of these genes is also conserved considerably in the corresponding genes of cyanobacteria, which in turn reveal sequence homology to the chlorophyll biosynthesis genes in algae and higher plants. This provides access to sufficient amounts of the enzymes and regulatory molecules required for the synthesis of the chlorophyll molecule by their expression in vitro and in heterologous hosts. Biochemical and structural analysis by spectroscopy, x-ray crystallography, and nuclear magnetic resonance spectroscopy will remain the cornerstones for elucidating the mechanisms of the individual enzymatic steps. Complementation of mutants in *Rhodobacter* defective in individual biosynthetic steps with proteins from higher plants and algae provides a powerful tool to identify the corresponding enzymes and their catalytic domains. The biggest challenge is to understand the regulation of chlorophyll synthesis and the assembly of the membrane proteins that hold the chlorophyll molecules in place and in close proximity for their tasks in harvesting sunlight and converting the light energy into chemical energy. Studies with isolated chloroplasts, isolated photosynthetic membranes, and isolated protein complexes will continue to provide exciting insights into the molecular mechanisms of photosynthesis.

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REFERENCES

Akhtar, M. (1994). The modification of acetate and propionate side chains during the biosynthesis of haem and chlorophylls: Mechanistic and stereochemical studies. In The Biosynthesis of the Tetrapyrrole Pigments, Ciba Foundation Symposium 180, D.J. Chadwick and K. Ackrill, eds (Chichester, England: John Wiley and Sons), pp. 131–151.

Andersen, R.V. (1992). Characterization of a barley tRNA synthetase involved in chlorophyll biosynthesis. In Research in Photosynthesis, Vol. 3, N. Murata, ed (The Hague: Kluwer), pp. 27–30.

Bartley, G.E., and Scolnik, P.A. (1995). Plant carotenoids: Pigments for photoprotection, visual attraction, and human health. Plant Cell **7**, 1027–1038.

Bassi, R., Hinz, U., and Barbato, R. (1985). The role of the light harvesting complex and photosystem II in thylakoid stacking in the *chlorina-f2* barley mutant. Carlsberg Res. Commun. **50**, 347–367.

Batschauer, A., Mösinger, E., Kreuz, K., Dörr, I., and Apel, K. (1986). The implication of a plastid-derived factor in the transcriptional control of nuclear genes encoding the light-harvesting chlorophyll a/b protein. Eur. J. Biochem. **154**, 625–634.

Battersby, A.R., McDonald, E., Williams, D.C., and Wurziger, H.K.W. (1977). Biosynthesis of the natural (type-III) porphyrins: Proof that rearrangement occurs after head-to-tail bilane formation. J. Chem. Soc. Commun. 113–117.

Bauer, C.E., Bollivar, D.W., and Suzuki, J.Y. (1993). Genetic analyses of photopigment biosynthesis in Eubacteria: A guiding light for algae and plants. J. Bacteriol. **175**, 3919–3925.

Beale, S.I., and Castelfranco, P.A. (1974a). The biosynthesis of δ-aminolaevulinic acid in higher plants. 1. Accumulation of δ-aminolevulinic acid in greening plant tissue. Plant Physiol. **53**, 291–296.

Beale, S.I., and Castelfranco, P.A. (1974b). The biosynthesis of δ-aminolevulinic acid from labeled precursors in greening plant tissues. Plant Physiol. **53**, 297–303.

Beale, S.I., Gough, S.P., and Granick, S. (1975). Biosynthesis of δ-aminolevulinic acid from the intact carbon skeleton of glutamic acid in greening barley. Proc. Natl. Acad. Sci. USA **72**, 2719–2723.

Benli, M., Schulz, R., and Apel, K. (1991). Effect of light on the NADPH-protochlorophyllide oxidoreductase of *Arabidopsis thaliana*. Plant Mol. Biol. **16**, 615–625.

Boese, Q.F., Spano, A.J., Li, J., and Timko, M.P. (1991). Aminolaevulinic acid dehydratase in pea (*Pisum sativum* L.): Identification of an unusual metal-binding domain in the plant enzyme. J. Biol. Chem. **266**, 17060–17066.

Bollivar, D.W., Jiang, Z.-Y., Bauer, C.E., and Beale, S.I. (1994a). Heterologous expression of the *bchM* gene product from *Rhodobacter capsulatus* and demonstration that it encodes *S*-adenosyl-L-methionine: Mg-protoporphyrin IX methyltransferase. J. Bacteriol. **176**, 5290–5296.

Bollivar, D.W., Suzuki, J.Y., Beatty, J.T., Dobrowolski, J.M., and Bauer, C.E. (1994b). Directed mutational analysis of bacteriochlorophyll *a* biosynthesis in *Rhodobacter capsulatus*. J. Mol. Biol. **237**, 622–640.

Burke, D.H., Alberti, M., and Hearst, J.E. (1993a). *bchFNBH* bacteriochlorophyll synthesis genes of *Rhodobacter capsulatus* and identification of the third subunit of light-independent protochlorophyllide reductase in bacteria and plants. J. Bacteriol. **175**, 2414–2422.

Burke, D.H., Alberti, M., and Hearst, J.E. (1993b). The *Rhodobacter capsulatus* chlorin reductase-encoding locus, *bchA*, consists of three genes, *bchX*, *bchY*, and *bchZ*. J. Bacteriol. **175**, 2407–2413.

Castelfranco, P., Walker, C.J., and Weinstein, J. (1994). Biosynthetic studies on chlorophylls: From protoporphyrin IX to protochlorophyllide. In The Biosynthesis of the Tetrapyrrole Pigments, Ciba Foundation Symposium 180, D.J. Chadwick and K. Ackrill, eds (Chichester, England: John Wiley and Sons), pp. 194–204.

Chauhan, S., and O'Brian, M.R. (1993). *Bradyrhizobium japonicum* δ-aminolevulinic acid dehydratase is essential for symbiosis with soybean and contains a novel metal-binding domain. J. Bacteriol. **175**, 7222–7227.

Chory, J., Peto, C., Feinbaum, R., Pratt, L., and Ausubel, F. (1989). Arabidopsis thaliana mutant that develops as a light-grown plant in absence of light. Cell **88**, 991–999.

Chunayev, A.S., Mirnaya, O.N., Maslov, V.G., and Boschetti, A. (1991). Chlorophyll *b* and chloroxanthin-deficient mutants of *Chlamydomonas reinhardtii*. Photosynthetica **25**, 291–301.

Coomber, S.A., Jones, R.M., Jordan, P.M., and Hunter, C.N. (1992). A putative anaerobic coproporphyrinogen III oxidase in *Rhodobacter sphaeroides*. I. Molecular cloning, transposon mutagenesis and sequence analysis of the gene. Mol. Microbiol. **6**, 3159–3169.

Dailey, T.A., Meissner, P., and Dailey, H.A. (1994). Expression of a cloned protoporphyrinogen oxidase. J. Biol. Chem. **269**, 813–815.

Debussche, L., Conder, M., Thibaut, D., Cameron, B., Crouzet, J., and Blanche, F. (1992). Assay, purification, and characterization of cobaltochelatase, a unique complex enzyme catalyzing cobalt insertion in hydrogenobyrinic acid a,c-diamide during coenzyme B_{12} biosynthesis in *Pseudomonas denitrificans*. J. Bacteriol. **174**, 7445–7451.

Dehesh, K., van Cleve, B., Ryberg, M., and Apel, K. (1986). Light-induced changes in the distribution of the 36000-M_r polypeptide of NADPH-protochlorophyllide oxidoreductase within different cellular compartments of barley (*Hordeum vulgare* L.) II. Localization by immunogold labeling in ultrathin sections. Planta **169**, 172–183.

Forreiter, C., and Apel, K. (1993). Light independent and light dependent protochlorophyllide-reducing activities and two distinct NADPH-protochlorophyllide oxidoreductase polypeptides in mountain pine (*Pinus mugo*). Planta **190**, 536–545.

Forreiter, C., van Cleve, B., Schmidt, A., and Apel, K. (1990). Evidence for a general light-dependent negative control of NADPH-protochlorophyllide oxidoreductase in angiosperms. Planta **183**, 126–132.

Gibson, L.D.C., and Hunter, C.N. (1994). The bacteriochlorophyll biosynthesis gene, *bchM*, of Rhodobacter sphaeroides encodes *S*-adenosyl-L-methionine: Mg-protoporphyrin IX methyltransferase. FEBS Lett. **352**, 127–130.

Gibson, L.C., Willows, R.D., Kannangara, C.G., von Wettstein, D., and Hunter, C.N. (1995). Magnesium-protoporphyrin chelatase of *Rhodobacter sphaeroides*: Reconstitution of activity by combining the products of the *bchH*, *I* and *D* genes expressed in *E. coli*. Proc. Natl. Acad. Sci. USA **92**, 1941–1944.

Hansson, M., and Hederstedt, L. (1992). Cloning and characterization of the *Bacillus subtilis hemEHY* gene cluster, which encodes protoheme IX biosynthetic enzymes. J. Bacteriol. **174**, 8081–8093.

Hansson, M., and Hederstedt, L. (1994). *Bacillus subtilis* HemY is a peripheral membrane protein essential for protoheme IX synthesis and which can oxidise coproporphyrinogen III and protoporphyrinogen IX. J. Bacteriol. **176**, 5962–5970.

Hansson, M., Rutberg, L., Schröder, I., and Hederstedt, L. (1991). The *Bacillus subtilis hemAXCDBL* gene cluster, which encodes enzymes of the biosynthetic pathway from glutamate to uroporphyrinogen III. J. Bacteriol. **173**, 2590–2599.

Hennig, M., Grimm, B., Jenny, M., Müller, R., and Jansonius, J.N. (1994). Crystallization and preliminary x-ray analysis of wild type and K272A mutant glutamate 1-semialdehyde aminotransferase from *Synechococcus*. J. Mol. Biol. **242**, 591–594.

Henningsen, K.W., Boynton, J.E., and von Wettstein, D. (1993). Mutants at *xantha* and *albina* loci in relation to chloroplast biogenesis in barley (*Hordeum vulgare* L.). Royal Dan. Acad. Sci. Lett. Biol. Skrifter **42**, 1–349.

Hiller, R.G., Möller, B.L., and Høyer-Hansen, G. (1980). Characterization of six putative photosystem I mutants in barley. Carlsberg Res. Commun. **45**, 315–328.

Höfgen, R., Axelsen, K.B., Kannangara, C.G., Schüttke, J., Pohlenz, H.-D., Willmitzer, L., Grimm, B., and von Wettstein, D. (1994). A visible marker for antisense mRNA expression in plants: Inhibition of chlorophyll synthesis with a glutamate-1-semialdehyde aminotransferase antisense gene. Proc. Natl. Acad. Sci. USA **91**, 1726–1730.

Holtorf, H., Reinbothe, S., Reinbothe, C., Bereza, B., and Apel, K. (1995). Two distinct NADPH-protochlorophyllide oxidoreductases are differentially expressed during the light-induced greening of dark-grown barley (*Hordeum vulgare* L.) seedlings. Proc. Natl. Acad. Sci. USA, in press.

Hudson, A., Carpenter, R., Doyle, S., and Coen, E.S. (1993). *Olive*: A key gene required for chlorophyll biosynthesis in *Antirrhinum majus*. EMBO J. **12**, 3711–3719.

Ilag, L.L., Kumar, A.M., and Söll, D. (1994). Light regulation of chlorophyll biosynthesis at the level of 5-aminolevulinate formation in Arabidopsis. Plant Cell **6**, 265–275.

Jacobs, J.M., and Jacobs, N.J. (1987). Oxidation of protoporphyrinogen to protoporphyrin, a step in chlorophyll and haem biosynthesis. Biochem. J. **244**, 219–224.

Jaffe, E.K., Volin, M., Myers, C., and Abrams, W.R. (1994). 5-Chloro [1,4-^{13}C] levulinic acid modification of mammalian and bacterial porphobilinogen synthase suggests an active site containing two Zn (II). Biochemistry **33**, 11554–11562.

Jansson, S. (1994). The light-harvesting chlorophyll *a/b* binding proteins. Biochim. Biophys. Acta **1184**, 1–19.

Jones, M.C., Jenkins, J.M., Smith, A.G., and Howe, C.J. (1994). Cloning and characterization of genes for tetrapyrrole biosynthesis from the cyanobacterium *Anacystis nidulans* R2. Plant Mol. Biol. **24**, 435–448.

Jones, R.M., and Jordan, P.M. (1993). Purification and properties of the uroporphyrinogen decarboxylase from *Rhodobacter sphaeroides*. Biochem. J. **293**, 703–712.

Jones, R.M., and Jordan, P.M. (1994). Purification and properties of porphobilinogen deaminase from *Arabidopsis thaliana*. Biochem. J. **299**, 895-902.

Jordan, P.M. (1991). The biosynthesis of 5-aminolaevulinic acid and its transformation into uroporphyrinogen III. In Biosynthesis of Tetrapyrroles, New Comprehensive Biochemistry Vol. 19, P.M. Jordan, ed (Amsterdam: Elsevier), pp. 1–66.

Jordan, P.M. (1994). The biosynthesis of uroporphyrinogen III: Mechanism of action of porphobilinogen deaminase. In The Biosynthesis of the Tetrapyrrole Pigments, Ciba Foundation Symposium 180, D.J. Chadwick and K. Ackrill, eds (Chichester, England: John Wiley and Sons), pp. 50–64.

Kahn, A., Avivi-Bleiser, N., and von Wettstein, D. (1976). Genetic regulation of chlorophyll synthesis analyzed with double mutants in barley. In Genetics and Biogenesis of Chloroplasts and Mitochondria. T. Bücher, W. Neupert, W. Sebald, and S. Werner, eds (Amsterdam: North Holland), pp. 119–131.

Kannangara, C.G., Andersen, R.V., Pontoppidan, B., Willows, R., and von Wettstein, D. (1994). Enzymic and mechanistic studies on the conversion of glutamate to 5-aminolaevulinate. In The Biosynthesis of the Tetrapyrrole Pigments, Ciba Foundation Symposium 180, D.J. Chadwick and K. Ackrill, eds (Chichester, England: John Wiley and Sons), pp. 3–20.

Kaya, A.H., Plewinska, M., Wong, D.M., Desnick, R.J., and Wetmur, J.G. (1994). Human δ-aminolevulinate dehydratase (ALAD) gene: Structure and alternative splicing of the erythroid and housekeeping mRNAs. Genomics **19**, 242–248.

Kiel, J.A.K.W., Ten Berge, A.M., and Venema, G. (1992). Nucleotide sequence of the Synechococcus sp. PCC7942 *hemE* gene encoding the homologue of mammalian uroporphyrinogen decarboxylase. DNA Seq. **2**, 415–418.

Kim, J., Gamble-Klein, P., and Mullet, J.E. (1991). Ribosomes pause at specific sites during synthesis of membrane-bound chloroplast reaction center protein D1. J. Biol. Chem. **266**, 14931–14938.

Kim, J., Gamble-Klein, P., and Mullet, J.E. (1994). Vir-115 gene product is required to stabilize D1 translation intermediates in chloroplasts. Plant Mol. Biol. **25**, 459–467.

Klein, R.R., Mason, H.S., and Mullet, J.E. (1988). Light regulated translation of chloroplast proteins. I. Transcripts of psaA-psaB, psbA, and rbcL are associated with polysomes in dark-grown and illuminated barley seedlings. J. Cell Biol. **106**, 289–301.

Knaust, R., Seyfried, B., Schmidt, L., Schulz, R., and Senger, H. (1993). Phototransformation of monovinyl and divinyl protochlorophyllide by NADPH:protochlorophyllide oxidoreductase of barley expressed in *Escherichia coli*. J. Photochem. Photobiol. Biol. **20**, 161–166.

Knoetzel, J., and Simpson, D. (1991). Expression and organization of antenna proteins in the light- and temperature-sensitive mutant *chlorina*-^{104}. Planta **185**, 111–123.

Koncz, C., Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Reiss, B., Redei, G.P., and Schell, J. (1990). Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in *Arabidopsis thaliana*. EMBO J. **9**, 1137–1146.

Koonin, E.V.A. (1993). A superfamily of ATPases with diverse functions containing either classical or deviant ATP-binding motifs. J. Mol. Biol. **229**, 1165–1174.

Kruse, E., Mock, H.-P., and Grimm, B. (1995a). Coproporphyrinogen III oxidase from barley and tobacco—Sequence analysis and initial expression studies. Planta, in press.

Kruse, E., Mock, H.-P., and Grimm, B. (1995b). Reduction of coproporphyrinogen oxidase level by antisense RNA synthesis leads to deregulated gene expression of plastid proteins and affects the oxidative defense system. EMBO J., in press.

Lambert, R., Brownlie, P.D., Woodcock, S.C., Louie, G.V., Cooper, J.C., Warren, M.J., Jordan, P.M., Blundell, T.L., and Wood, S.P. (1994). Structural studies on porphobilinogen deaminase. In The Biosynthesis of the Tetrapyrrole Pigments, Ciba Foundation Symposium 180, D.J. Chadwick and K. Ackrill, eds (Chichester, England: John Wiley and Sons), pp. 97–104.

Lewandowska, M., and Öquist, G. (1980). Development of photosynthetic electron transport in *Pinus silvestris*. Physiol. Plant. **48**, 134–138.

Li, J., Goldschmidt-Clermont, M., and Timko, M.P. (1993). Chloroplast-encoded *chlB* is required for light-independent protochlorophyllide reductase activity in *Chlamydomonas reinhardtii*. Plant Cell **5**, 1817–1829.

Lindsten, A., Wiktorsson, B., Ryberg, M., and Sundquist, C. (1993). Chlorophyll synthetase activity is relocated from transforming prolamellar bodies to developing thylakoids during irradiation of dark-grown wheat. Physiol. Plant. **88**, 29–36.

Louie, G.V., Brownlie, P.D., Lambert, R., Cooper, J.B., Blundell, T.L., Wood, S.P., Warren, M.J., Woodcock, S.C., and Jordan, P.M. (1992). Structure of porphobilinogen deaminase reveals a flexible multidomain polymerase with a single catalytic site. Nature **359**, 33–39.

Madsen, O., Sandal, L., Sandal, N.N., and Marcker, K.A. (1993). A soybean coproporphyrinogen oxidase gene is highly expressed in root nodules. Plant Mol. Biol. **23**, 35–43.

Meller, E., Belkin, S., and Harel, E. (1975). The biosynthesis of δ-aminolevulinic acid in greening maize leaves. Phytochemistry **14**, 2399–2402.

Mitchell, L.W., and Jaffe, E. (1993). Porphobilinogen synthase from *Escherichia coli* is a Zn (II) metalloenzyme stimulated by Mg (II). Arch. Biochem. Biophys. **300**, 169–177.

Mock, H.-P., Trainotti, L., Kruse, E., and Grimm, B. (1995). Isolation, sequencing and expression of cDNA sequences encoding uroporphyrinogen decarboxylase from tobacco and barley. Plant Mol. Biol., in press.

Nielsen, O.F. (1974). Macromolecular physiology of plastids. XII. *Tigrina* mutants of barley. Hereditas **76**, 269–304.

Nielsen, O.F., and Gough, S. (1974). Macromolecular physiology of plastids. XI. Carotenes in etiolated *tigrina* and *xantha* mutants of barley. Physiol. Plant. **30**, 246–254.

Nielsen, V.S., Mant, A., Knoetzel, J., Møller, B.L., and Robinson, C. (1994). Import of barley photosystem I subunit N into the thylakoid lumen is mediated by a bipartite presequence lacking an intermediate processing site. J. Biol. Chem. **269**, 3762–3766.

Orsat, B., Monfort, A., Chatellard, P., and Stutz, E. (1992). Mapping and sequencing of an actively transcribed *Euglena gracilis* chloroplast gene (*ccsA*) homologous to the *Arabidopsis thaliana* nuclear gene *cs* (*ch42*). FEBS Lett. **303**, 181–184.

Paulsen, H., Finkenzeller, B., and Kühlein, N. (1993). Pigments induce folding of light-harvesting chlorophyll *a*/*b*-binding protein. Eur. J. Biochem. **215**, 809–816.

Pepper, A., Delaney, T., Washburn, T., Poole, D., and Chory, J. (1994). *DET1*, a negative regulator of light-mediated development and gene expression in Arabidopsis, encodes a novel nuclear-localized protein. Cell **78**, 109–116.

Pontoppidan, B., and Kannangara, C.G. (1994). Purification and partial characterization of barley glutamyl-tRNA^{Glu} reductase, the enzyme that directs glutamate to chlorophyll biosynthesis. Eur. J. Biochem. **225**, 529–537.

Porra, R.J. (1986). Labelling of chlorophylls and precursors by [2-^{14}C] glycine and 2-[1-^{14}C] oxoglutarate in *Rhodopseudomonas spheroides* and *Zea mays*. Eur. J. Biochem. **156**, 111–121.

Porra, R.J., Schäfer, W., Cmiel, E., Katheder, I., and Scheer, H. (1994). The derivation of the formyl-group oxygen of chlorophyll *b* in higher plants from molecular oxygen: Achievement of high enrichment of the 7-formyl-group oxygen from $^{18}O_2$ in greening maize leaves. Eur. J. Biochem. **219**, 671–679.

Powell, K.A., Cox, R., McConville, M., and Charles, H.P. (1973). Mutations affecting porphyrin biosynthesis in *Escherichia coli*. Enzyme **16**, 65–73.

Richard, M., Tremblay, C., and Bellemare, G. (1994). Chloroplastic genomes of *Gingko biloba* and *Chlamydomonas moewusii* contain a *chlB* gene encoding one subunit of light-independent protochlorophyllide reductase. Curr. Genet. **26**, 159–165.

Robinson, C., and Klösgen, R.B. (1994). Targeting of proteins into and across the thylakoid membrane—A multitude of mechanisms. Plant Mol. Biol. **26**, 15–24.

Rüdiger, W., Benz, J., and Guthoff, C. (1980). Detection and partial characterization of activity of chlorophyll synthetase in etioplast membranes. Eur. J. Biochem. **109**, 193–200.

Sasarman, A., Surdeann, M., and Horodniceanu, T. (1968). Locus determining the synthesis of delta-aminolevulinic acid in *Escherichia coli* K-12. J. Bacteriol. **96**, 1882–1884.

Schaumburg, A., Schneider-Poetsch, H.A.W., and Eckerskom, C. (1992). Characterization of plastid 5-aminolevulinate dehydratase (ALAD; EC 4.2.1.24) from spinach (*Spinacea oleracea* L.) by sequencing and comparison with non-plant ALAD enzymes. Z. Naturforsch. **47C**, 77–84.

Schneegurt, M.A., and Beale, S.I. (1992). Origin of the chlorophyll *b* formyl oxygen in *Chlorella vulgaris*. Biochemistry **31**, 11677–11683.

Schulz, R., Steinmüller, K., Klaas, M., Forreiter, C., Rasmussen, S., Hiller, C., and Apel, K. (1989). Nucleotide sequence of a cDNA coding for the NADPH-protochlorophyllide oxidoreductase (PCR) of barley (*Hordeum vulgare* L.) and its expression in *Escherichia coli*. Mol. Gen. Genet. **217**, 355–361.

Selstam, E., and Widell, A. (1986). Characterization of prolamellar bodies from dark-grown seedlings of Scots pine, containing light- and NADPH-dependent protochlorophyllide oxidoreductase. Physiol. Plant. **67**, 345–352.

Sharif, A.L., Smith, A.G., and Abell, C. (1989). Isolation and characterization of a cDNA clone for a chlorophyll synthesis enzyme from *Euglena gracilis*: The chloroplast enzyme hydroxymethylbilane synthase (porphobilinogen deaminase) is synthesized with a very long transit peptide in *Euglena*. Eur. J. Biochem. **184**, 353–359.

Simpson, D.J., and Knoetzel, J. (1995). Light harvesting complexes of plants and algae: Introduction, survey and nomenclature. In Oxygenic Photosynthesis: The Light Reactions, D.R. Ort and C.F. Yocum, eds (Dordrecht: Kluwer), in press.

Simpson, D.J., and von Wettstein, D. (1989). The structure and function of the thylakoid membrane. Carlsberg Res. Commun. **54**, 55–65.

Simpson, D., Machold, O., Høyer-Hansen, G., and von Wettstein, D. (1985). *Chlorina* mutants of barley (*Hordeum vulgare* L.). Carlsberg Res. Commun. **50**, 223–238.

Simpson, D.J., Vallon, O., and von Wettstein, D. (1989). Freeze-fracture studies on barley plastid membranes. VIII. In viridis-115, a mutant completely lacking photosystem II, oxygen evolution enhancer I (OEE1) and α-subunit of cytochrome b-559 accumulate in appressed thylakoids. Biochim. Biophys. Acta **975**, 164–174.

Spencer, P., and Jordan, P.M. (1994). 5-Aminolaevulinic acid dehydratase: Characterization of the α and β metal-binding sites of the *Escherichia coli* enzyme. In The Biosynthesis of Tetrapyrrole Pigments, Ciba Foundation Symposium 180, D.J. Chadwick and K. Ackrill, eds (Chichester, England: John Wiley and Sons), pp. 50–64.

Stange-Thomann, N., Thoman, H.-U., Lloyd, A.J., Lyman, H., and Söll, D. (1994). A point mutation in *Euglena gracilis* chloroplast $tRNA^{Glu}$ uncouples protein and chlorophyll biosynthesis. Proc. Natl. Acad. Sci. USA **91**, 7947–7951.

Suzuki, J.Y., and Bauer, C.E. (1992). Light-independent chlorophyll biosynthesis: Involvement of the chloroplast gene *chlL* (*frxC*). Plant Cell **4**, 929–940.

Suzuki, J.Y., and Bauer, C.E. (1995). A prokaryotic origin of light-dependent chlorophyll biosynthesis of plants. Proc. Natl. Acad. Sci. USA, in press.

von Wettstein, D. (1958). The formation of plastid structure. In The Photochemical Apparatus, Its Structure and Function. Brookhaven Symposium Biology 11 (Upton, NY: Brookhaven National Laboratory), pp. 138–159.

von Wettstein, D., Henningsen, K.W., Boynton, J.E., Kannangara, C.G., and Nielsen, O.F. (1971). The genetic control of chloroplast development in barley. In Autonomy and Biogenesis of Mitochondria and Chloroplasts, N.K. Boardmann, A.W. Linnane, and R.M. Smillie, eds (Amsterdam: North Holland), pp. 205–223.

von Wettstein, D., Kahn, A., Nielsen, O.F., and Gough, S.P. (1974). Genetic regulation of chlorophyll synthesis analyzed with mutants of barley. Science **184**, 800–802.

Weinstein, J.D., and Beale, S.I. (1983). Separate physiological roles and subcellular compartments for two tetrapyrrole biosynthetic pathways in *Euglena gracilis*. J. Biol. Chem. **258**, 6799–6807.

Whyte, B.J., and Griffiths, W.T. (1993). 8 Vinyl reduction and chlorophyll *a* biosynthesis in higher plants. Biochem. J. **291**, 939–944.

Wilks, H.M., and Timko, M.P. (1995). A light-dependent complementation system for analysis of NADPH:protochlorophyllide oxidoreductase: Identification and mutagenesis of two conserved residues that are essential for enzyme activity. Proc. Natl. Acad. Sci. USA **92**, 724–728.

Willows, R., Kannangara, C.G., and Pontoppidan, B. (1995). Nucleotides of $tRNA^{Glu}$ involved in recognition by barley chloroplast glutamyl–tRNA synthetase and glutamyl–tRNA reductase. Biochim. Biophys. Acta, in press.

Witty, M., Wallace-Cook, A.D.M., Albrecht, H., Spano, A.J., Michel, H., Shabanowitz, J., Hunt, D.F., Timko, M.P., and Smith, A.G. (1993). Structure and expression of chloroplast-localized porphobilinogen deaminase from pea (*Pisum sativum* L.) isolated by redundant polymerase chain reaction. Plant Physiol. **103**, 139–147.

Woodcock, S.C., and Jordan, P.M. (1994). Evidence for participation of aspartate-84 as a catalytic group at the active site of porphobilinogen deaminase obtained by site-directed mutagenesis of the *hemC* gene from *Escherichia coli*. Biochemistry **33**, 2688–2695.