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REVIEW

Recent overview of the Mg branch of the tetrapyrrole biosynthesis leading to chlorophylls

Tatsuru Masuda

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Abstract In plants, chlorophylls (chlorophyll *a* and chlorophyll *b*) are the most abundant tetrapyrrole molecules and are essential for photosynthesis. The first committed step of chlorophyll biosynthesis is the insertion of Mg^{2+} into protoporphyrin IX, and thus subsequent steps of the biosynthesis are called the Mg branch. As the Mg branch in higher plants is complex, it was not until the last decade—after many years of intensive research—that most of the genes encoding the enzymes for the pathway were identified. Biochemical and molecular genetic analyses have certainly modified the classic metabolic map of tetrapyrrole biosynthesis, and only recently have the molecular mechanisms of regulatory pathways governing chlorophyll metabolism been elucidated. As a result, novel functions of tetrapyrroles and biosynthetic enzymes have been proposed. In this review, I summarize the recent findings on enzymes involved in the Mg branch, mainly in higher plants.

Keywords Chlorophyll · Chloroplast · Mg branch · Tetrapyrrole · Photosynthesis

Abbreviations

AAA	ATPase associated with various cellular activities
ALA	5-aminolevulinic acid
Bchl	Bacteriochlorophyll
CAO	Chlorophyllide *a* oxygenase
Chl	Chlorophyll
DVR	3,8-Divinyl Pchlide *a* 8-vinyl reductase
DPOR	Light-independent protochlorophyllide oxidoreductase
GFP	Green fluorescent protein
gun	Genome uncoupled
LHC	Light-harvesting chlorophyll *a*/*b*-protein complex
LHCII	Light-harvesting chlorophyll *a*/*b*-protein complex of photosystem II
LHPP	Light-harvesting protochlorophyllide *a*/*b*-binding protein complex
Pchlide	Protochlorophyllide
PLB	Prolamellar body
POR	Light-dependent NADPH:protochlorophyllide oxidoreductase
Ptc	Protochlorophyllide translocon complex
SAM	*S*-adenosyl-L-methionine
ZnPP	Zinc-protopheophorbide

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Introduction

Chlorophylls (Chls) and bacteriochlorophylls (Bchls) are essential molecules for photosynthesis. They are responsible for harvesting and transferring solar energy in antenna systems, and for charge separation and electron transport in reaction centers. Chls and Bchls are Mg^{2+}-containing tetrapyrrole compounds. In plants, algae and many bacteria, all of the tetrapyrroles, including Chls and heme, originate from a common biosynthetic pathway. They are synthesized from glutamyl-$tRNA^{glu}$ via 5-aminolevulinic acid (ALA), a linear five-carbon molecule. The formation of ALA is the first committed step of tetrapyrrole biosynthesis, and is therefore assumed to be a regulatory point for the synthesis of total amounts of

tetrapyrroles (Beale 1999). Actually, the ALA synthesizing activity is subjected to feedback regulation by heme and repressed by a regulatory protein, FLU, in the dark (Meskauskiene et al. 2001; Goslings et al. 2004). Two molecules of ALA are then condensed to form a pyrrole molecule. Subsequently, four pyrrole molecules are polymerized to form a linear pyrrole that subsequently undergoes cyclization to yield a macrocyclic tetrapyrrole. The branch point of the Chl and heme biosynthetic pathway is protoporphyrin IX, a closed macrocycle lacking a chelated ion. The first committed step of Chl/Bchl biosynthesis is the insertion of Mg^{2+} into protoporphyrin IX, and thus subsequent steps in this pathway are called the Mg branch (Fig. 1).

The enzymes responsible for the Mg branch have been identified and characterized primarily via molecular genetic analyses of the photosynthetic gene cluster in *Rhodobacter* (*Rba.*) *capsulatus* (Bollivar et al. 1994b) and *Rba. sphaeroides* (Coomber et al. 1990). These gene clusters comprise approximately 45 kbp and contain most of the genes encoding structural and regulatory proteins for assembly of the photosynthetic apparatus as well as enzymes involved in Bchl biosynthesis from protoporphyrin IX. Directed mutagenesis and subsequent heterologous expression in *E. coli* have identified many genes encoding these Bchl biosynthetic enzymes. In addition, molecular genetic studies in plants have identified many genes encoding Chl biosynthetic enzymes, many of which are orthologous to the bacterial genes. Owing to the complexity of this pathway in higher plants, only in the last decade have the genes encoding the enzymes of the Mg branch been identified—and then only after many years of intensive research. Biochemical and molecular genetic analyses have modified the classical metabolic map of tetrapyrrole biosynthesis, but only recently have the molecular mechanisms of regulatory pathways governing Chl metabolism been elucidated. These insights have enabled researchers to propose novel functions for tetrapyrroles and their associated biosynthetic enzymes. Thus, it is an opportune time to review and redraw the metabolic pathway for Chl biosynthesis. The most recently updated biosynthetic pathway is shown in Fig. 1.

In this review, I summarize the recent findings on enzymes involved in the Mg branch, focusing mainly in higher plants. According to the order of function of each enzyme in the metabolic pathway, I review the gene identification, enzyme biochemistry, expression and localization, and regulation. Readers may also refer to recent comprehensive reviews on this field (Beale 1999; Vavilin and Vermaas 2002; Rebiez et al. 2003; Eckhardt et al. 2004; Moulin and Smith 2005; Bollivar 2006; Tanaka and Tanaka 2006, 2007).

Mg-chelatase

Mg-chelatase catalyzes the insertion of Mg^{2+} into protoporphyrin IX. This is the first committed step in Chl biosynthesis with the enzymes prior to this step also being shared with the heme biosynthetic pathway. Although Mg-chelatase and ferrochelatase catalyze virtually the same reaction—for heme, the insertion of Fe^{2+} into protoporphyrin IX to form protoheme—the structures of these enzymes are completely different. Ferrochelatase is a single-subunit enzyme encoded by a single gene and does not require a cofactor for catalysis. By contrast, Mg-chelatase requires ATP for catalysis and is a heterologous complex consisting of 40-, 70-, and 140-kDa subunits, designated I, D and H subunits, respectively. These respective subunits are known as BchI, BchD, and BchH in Bchl-producing organisms and ChlI, ChlD, and ChlH in Chl-producing organisms (Walker and Willows 1997). Mg-chelatase, a key enzyme at the branch point between the heme and Chl biosynthetic pathways, has been extensively studied during the last decade. Structural and functional analyses of individual subunits have provided overall mechanisms for the reaction and enzyme regulation (Walker and Willows 1997; Reid and Hunter 2002; Willows 2003).

Identification and characterization of genes encoding subunits of Mg-chelatase

Genes encoding subunits of Mg-chelatase were first proposed by analysis of directional mutagenesis of the photosynthetic gene cluster of *Rhodobacter*, which accumulated protoporphyrin IX (Coomber et al. 1990; Bollivar et al. 1994b). Reconstitution of Mg-chelatase activity with recombinant proteins demonstrated that the chelatase is composed by three subunits both in Bchl-producing prokaryotes (Gibson et al. 1995; Petersen et al. 1998; Willows and Beale 1998) and Chl-producing cyanobacterium *Synechocystis* PCC6803 (Jensen et al. 1996a). In 1996, natural photosynthesis using Zn-containing Bchl *a* was discovered in the aerobic acidophilic purple photosynthetic bacterium, *Acidiphilium rubrum* (Wakao et al. 1996). This bacterium predominantly produces Zn-containing Bchl *a*, resulting from Zn^{2+} insertion (instead of Mg^{2+}) as the central metal of the tetrapyrrole ring; this Bchl *a* is associated with fully active reaction centers and light-harvesting complexes analogous to those of other purple photosynthetic bacteria (Wakao et al. 1996). Analysis of the photosynthetic gene cluster of *A. rubrum* revealed that this bacterium contains genes encoding three subunits of the functional Mg-chelatase, and thus, substitution with Zn^{2+} may occur after Mg-insertion probably for acclimation to an acidic environment (Masuda et al. 1999). These prokaryotic

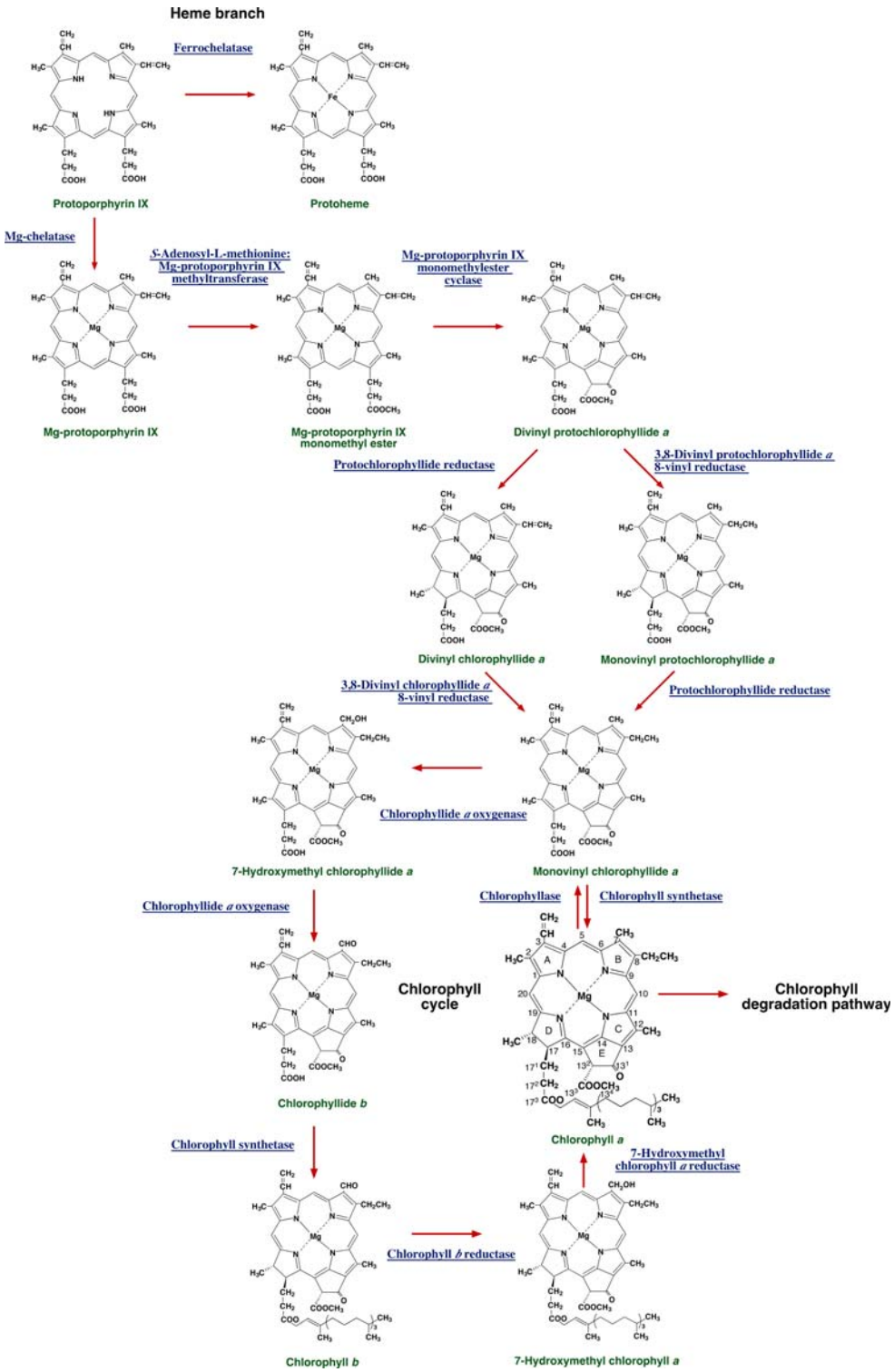

Fig. 1 Recent overview of the Mg branch of the tetrapyrrole biosynthesis leading to chlorophylls. The International Union of Pure and Applied Chemistry (IUPAC) numbering scheme for C atoms is shown on the structure of chlorophyll *a*

genes encoding subunits of Mg-chelatase have been used to analyze the early evolution of photosynthesis. Phylogenetic analysis of these genes suggested that purple bacteria are the earliest photosynthetic lineage to have emerged (Xiong et al. 2000).

In photosynthetic eukaryotes, *cs* and *ch-42* mutants were first identified in *Arabidopsis thaliana* as a novel chloroplast protein (Koncz et al. 1990), and subsequently those mutants were found to be defective in the *ChlI* gene encoding subunit I. These mutants could still make small

amounts of Chl using a second gene, *ChlI2* (Rissler et al. 2002). Owing to a Chl-deficient yellow phenotype and ALA-dependent accumulation of protoporphyrin IX, barley mutants at three genetic loci, termed *xantha-f*, *xantha-g*, and *xantha-h*, were characterized as Mg-chelatase mutants corresponding to the gene locus encoding the H, D, and I subunits, respectively (Jensen et al. 1996b; Petersen et al. 1999b). Three semidominant alleles of the *xantha-h* locus, originally isolated as pale green *chlorina* mutants, each have single missense mutations (Hansson et al. 1999). *ChlI* was identified from soybean as a stromal protein of chloroplasts (Nakayama et al. 1995). In maize, the gene *Oil Yellow1* (*Oy1*) was identified as subunit I (Sawers et al. 2006). In the *Oy1* mutant, amino acid substitutions resulted in a semidominant phenotype probably via competitive inhibition of wild type I subunit. In rice, map-based cloning identified *Chlorina-1* and *Chlorina-9* as genes encoding subunits ChlD and ChlI, respectively (Zhang et al. 2006). These genes occur in the nuclear genome. *ChlI* genes have also been identified from plastid genomes of green (Orsat et al. 1992), red, and cryptomonad algae (Douglas and Reith 1993).

Mutants of *ChlH* have been described in *Antirrhinum majus* (Hudson et al. 1993). *ChlH* was identified from soybean (Nakayama et al. 1998). *ChlD* was identified from tobacco by homology, and recombinant ChlD along with ChlH and ChlI subunits reconstituted active Mg-chelatase (Papenbrock et al. 1997). In *Chlamydomonas reinhardtii*, *ChlH* was identified and defective mutants were characterized (Chekounova et al. 2001). *Arabidopsis* mutants, *cch*, and *gun5* (*g*enome *un*coupled 5), were identified as containing single missense mutations in the H subunit, *ChlH* (Mochizuki et al. 2001). The mutant *gun5* was found to impart aberrant regulation of chloroplast-to-nucleus signal transduction, suggesting that Mg-porphyrins are involved in intracellular signaling (see below).

On the basis of functional and genetic studies and results from sequencing projects, chelatases have been categorized into three main classes: (I) ATP-dependent heterotrimeric chelatases, (II) ATP-independent chelatases, and (III) multifunctional homodimeric chelatases (Brindley et al. 2003; Al-Karadaghi et al. 2006). Co-chelatase inserts Co^{2+} into hydrogenobyrinic acid *a,c*-diamide, a tetrapyrrole precursor of vitamin B_{12}. Both Co-chelatase and Mg-chelatase are type I chelatases, whereas ferrochelatase is a type II chelatase. Co-chelatase consists of a 140-kDa subunit (CobN) that binds tetrapyrrole, and a 450-kDa tetramer of the subunits CobS and CobT (38 and 80 kDa, respectively) that binds ATP (Debussche et al. 1992). As such, Co-chelatase is somewhat similar to Mg-chelatase; there is a significant sequence similarity between CobN and subunit H, although no similarity between other subunits have been identified apart from their respective molecular masses and a consensus ATP-binding site in CobS and subunit I. More recently, a metal ion-dependent adhesion site (MIDAS motif), which is involved in binding of subunits (see below), was found in both CobT and subunit D, suggesting important similarity between these two subunits as well as between the Mg- and Co-chelatases (Fodje et al. 2001).

Surprisingly, recent work proposed that ChlH is a receptor for abscisic acid, which regulates stomata opening and seed development (Shen et al. 2006). It was shown that *A. thaliana* ChlH specifically binds abscisic acid and mediates its signaling as a positive regulator of seed germination, post-germination growth, and stomatal movement.

Characterization of Mg-chelatase activity

A model of the catalytic cycle of Mg-chelatase is shown in Fig. 2. Earlier work on chloroplast extracts suggested that Mg chelation proceeded by a two-step reaction: an enzyme-activation step, followed by a Mg^{2+} insertion step (Walker and Weinstein 1994). Subsequent work using recombinant subunits encoded by genes from photosynthetic bacteria showed that the activation step requires ATP, Mg^{2+} and subunits I and D for the formation of a ternary I–D–Mg–ATP complex (Willows et al. 1996; Gibson et al. 1999). This step does not require the hydrolysis of ATP, as ADP or nonhydrolyzable ATP analogs such as 5′-[γ-thio]triphosphate (ATPγS) can substitute for ATP (Jensen et al. 1999a; Lake et al. 2004). The Mg-insertion step also requires ATP, although at a lower concentration than needed for activation, and this cannot be replaced by ATPγS. Thus, ATP hydrolysis is required for the Mg insertion step (Jensen et al. 1999a). The I–D–Mg–ATP complex uses Mg^{2+} and the porphyrin-bound H subunit as the substrate for the Mg insertion step, with ATP hydrolysis driving the reaction.

The I subunits are classified as members of the AAA^{+}-proteins (ATPase associated with various cellular activities), by sequence (Neuwald et al. 1999), by conservation of a characteristic fold (Lecerof et al. 2000), and by structure analysis (Fodje et al. 2001). The I subunit is an ATPase (Gibson et al. 1999; Jensen et al. 1999a). The ATPase activity of subunit I is repressed when this subunit forms a complex with subunit D (Gibson et al. 1999; Jensen et al. 1999a), although the opposite observation also has been made (Hansson and Kannangara 1997). The N-terminal halves of subunits D and I share high sequence similarity, whereas the C-terminal half of subunit D includes a metal ion coordination motif (MIDAS) characteristic of the integrin I domain (Fodje et al. 2001). The similarity between the D and I subunit N termini suggests that the D subunit is also an

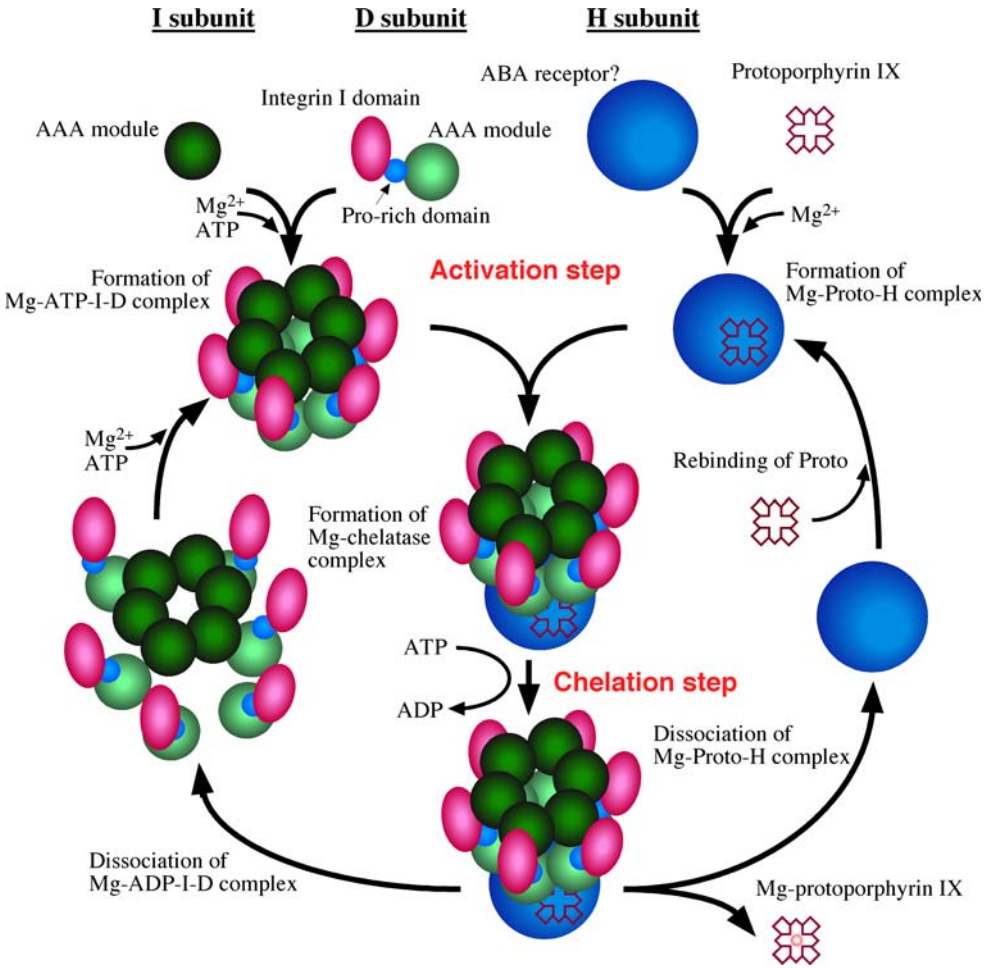

Fig. 2 Model of the catalytic cycle of Mg-chelatase. Activation step: In a Mg^{2+} and ATP-dependent process, six I subunits are assembled into a hexameric ring structure, while six D subunits form a hexameric ring in an ATP-independent manner. The two-tiered hexameric ring forms the Mg–ATP–I–D complex. It is suggested that the ATPase activity of subunit I is inhibited in this complex due to binding of the integrin I domain of subunit D to the integrin I binding domain of subunit I. Meanwhile, subunit H binds to protoporphyrin IX and most likely also the Mg^{2+} substrate—as this subunit is considered the catalytic subunit—to form the Mg-H-protoporphyrin IX complex. Chelation step: ATP hydrolysis is triggered upon switching the binding of the integrin I domain of subunit D to the integrin I binding domain of subunit H. After the formation of Mg-protoporphyrin IX, the complex disassembles. Here, subunit H is shown docking to the D subunits, although it remains unknown if subunit H docks to the I or D side of the complex

AAA^+-protein, although no ATPase activity has been detected from this subunit (Hansson and Kannangara 1997; Jensen et al. 1999a; Petersen et al. 1999a). An acidic, proline-rich region linking the N- and C-terminal domains of the D subunit may contribute to the association of subunit D with subunit I by binding to the nucleotide-binding domain of subunit I (Fodje et al. 2001). In tobacco, a special motif (EK-X18-R) has been identified on both sides of the linker domain of subunit D. A region of approximately 110 amino acid residues spanning these special motifs and the linker domain enables interaction of the D subunit with the I subunit and maintenance of the Mg-chelatase activity (Gräfe et al. 1999). Meanwhile, the integrin I domain that is located on the C terminus of subunit D is proposed to bind to the integrin I domain-binding sequence motifs of subunits I and H (Fodje et al. 2001). The binding of subunit D to subunit I may block the ATPase activity of subunit I. It has been proposed that a conformational transition of subunit D is induced upon I–D complex formation and subsequent binding to subunit H may trigger porphyrin metallation, and also relieve the block of the ATP-binding site of subunit I by subunit D, thereby allowing ATP hydrolysis (Fodje et al. 2001). AAA^+-proteins generally form oligomeric ring structures, and subunit I forms high-molecular-mass aggregates in the presence of ATP and $MgCl_2$ (Jensen et al. 1998; Gibson et al. 1999). Negative staining electron microscopy showed that I subunits from *Rba. capsulatus* form a hexameric ring (Willows et al. 2004) and that I subunits from *Synechocystis* PCC6803 form a heptameric ring (Reid et al. 2003).

In *Arabidopsis*, most of the homozygous *CHLI1* mutants of *Arabidopsis thaliana*, such as *ch42-1* (Fisherova 1975), *cs* (*ch42-2*) (Koncz et al. 1990), *ch42-3* (Rissler et al. 2002), are recessive and have a pale-green phenotype. The *cs* mutant has a T-DNA insertion in the 3′-terminal end of *CHLI*, resulting in CHLI malfunction because the C-terminal end is extended (Koncz et al. 1990). These mutants could still make small amounts of Chl probably using a second gene *ChlI2* (Rissler et al. 2002). In wild-type plants, *CHLI1* and *CHLI2* mRNAs accumulate to similar levels, but CHLI2 protein is undetectable in wild type and the *ch42-3* mutant. It has been proposed that unusual post-translational protein instability

limits the function of CHLI2 in the Mg-chelatase complex (Rissler et al. 2002). Meanwhile, a semidominant mutant of *CHLI1* (*aci5*) has been identified as a point mutant with a single amino acid substitution (D240N) (Soldatova et al. 2005). A heterozygous *cs*/*aci5* hybrid plant is completely *albino*, although the plant expresses the mutant genes encoded by both *cs* and *aci5* alleles (Apchelimov et al. 2007). Thus, *cs* allele-induced C-terminal extension of CHLI may perturb the assembly of the CHLI ring structure, whereas displacement by *aci5*-encoded CHLI causes inactivation of the Mg-chelatase complex because an essential residue in the catalytic site of CHLI1 has been substituted (Apchelimov et al. 2007). Since *CHLI2* cannot compensate for the *albino* phenotype in this hybrid plant, it was proposed that CHLI2 is not functional in the Mg-chelatase complex. It was suggested that CHLI2 lost the ability to form effective ring-like structures because of mutations that changed amino acids at the C-terminal end (Apchelimov et al. 2007). Very recently, the physiological contribution of CHLI2 was examined using a *chli2* T-DNA knockout line (Kobayashi et al. submitted). This article shows that the *chli2* mutation is semidominant on a homozygous *cs* background, revealing that although *CHLI2* plays a limited role in chlorophyll biosynthesis, this subunit certainly contributes to the assembly of the Mg-chelatase complex.

Semidominant and recessive mutants deficient in subunit I have been identified in barley *xantha-h* (Hansson et al. 1999; Hansson et al. 2002) and maize (Sawers et al. 2006). The semidominant mutations were all point mutations resulting in changes in single amino acid residues, whereas the recessive mutations were deficient in transcription. In vitro analysis of semidominant mutants of subunit I in expression systems for *Rba. capsulatus* BchI and *Synechocystis* ChlI showed reduced or no ATPase activity. The defective protein could not contribute to the Mg-chelatase activity and rather inhibited when mixed with wild-type I subunits, explaining the dominant effect of the mutated protein (Hansson et al. 2002; Sawers et al. 2006). Thus, dominant mutations in the I subunits function in a cooperative manner within the hexameric complex (Hansson et al. 2002). Subunit D could not be detected in any barley *xantha-h* mutants, suggesting that a functional I subunit is required to maintain the D subunit (Lake et al. 2004). Subunit D also forms ATP-independent oligomeric structures and is classified as an AAA^+ protein (Axelsson et al. 2006). All barley *xantha-g* mutants defective in D subunits that have been identified were recessive. An in-vitro study of mutant proteins of subunit D that can be cooperatively incorporated into the oligomeric structure of *Rba. capsulatus* BchD protein resulted in lower Mg-chelatase activity (Axelsson et al. 2006). This cooperative behavior suggests that the D oligomer takes an active part in the conformational dynamics among the subunits of the enzyme (Axelsson et al. 2006).

The H subunit is regarded as the catalytic subunit since Mg chelation takes place on this subunit (Reid and Hunter 2002). Porphyrin binding analysis has demonstrated that subunit H is the porphyrin binding subunit (Willows et al. 1996; Jensen et al. 1998; Willows and Beale 1998). The binding of deuteroporphyrin IX, a more water-soluble analog of the biological substrate, to subunit H caused quenching of Trp fluorescence, allowing the binding constant to be determined (4.0 μM for *Synechocystis* ChlH and 0.75 μM for *Rba. sphaeroides* BchH) (Karger et al. 2001). Compared to free deuteroporphyrin IX, the red shifts and peak broadening of the spectra of the H subunit-bound deuteroporphyrin IX implied a slightly deformed porphyrin configuration (Karger et al. 2001) that may permit ready chelation (Reid and Hunter 2002). ATPase activities for the various H subunits have been reported for *Rba. sphaeroides* (Hansson and Kannangara 1997), *Chlorobium vibrioforme* (Petersen et al. 1999a) and *Synechocystis* PCC6803 (Jensen et al. 1999a), but a recent study suggests that the H subunit ATPase activity is an artifact of a contaminating *E. coli* protein (Sirijovski et al. 2006).

In *Synechocystis*, the Mg-chelatase-catalyzed reaction requires hydrolysis of ~15 Mg-ATP, and therefore the chelation reaction is energetically unfavorable (Reid and Hunter 2004). The ATPase activity of isolated I subunit is substantially lower than that of the holo-chelatase. Mg^{2+} activates the Mg-chelatase by increasing both the velocity of its reaction and specificity for the substrates, Mg-ATP and porphyrin, possibly through a conformational change (Reid and Hunter 2004).

Regulation of Mg-chelatase

Mg-chelatase is subjected to several forms of regulation. The major ones are transcriptional regulation of the H subunit and post-translational modifications of the subunit that affect the overall activity.

For the expression of each subunit of Mg-chelatase, upregulation of *ChlI* and *ChlH* expression during greening of etiolated tissues has been reported in barley (Jensen et al. 1996b) and soybean (Nakayama et al. 1998). *ChlI* is constitutively expressed in mature green tissues of barley (Jensen et al. 1996b) and *A. thaliana* (Gibson et al. 1996), and the levels of ChlI mRNA and protein also remain constant in soybean (Nakayama et al. 1998). *ChlH* expression follows a circadian rhythm in barley (Jensen et al. 1996b) and in soybean with concomitant dramatic changes of ChlH protein, which peaks during the light phase (Nakayama et al. 1998). Transcriptome analysis that covers the majority of

tetrapyrrole biosynthetic genes of *Arabidopsis* shows that *ChlH* is one of the key regulatory genes, which is coordinately regulated by light and circadian rhythm with other key regulatory genes, such as *HEMA1* (encoding glutamyl-tRNA reductase), *CRD1* (*CHL27*) (encoding a subunit of Mg-protoporphyrin IX monomethyl ester cyclase), and *CAO* (encoding chlorophyllide *a* oxygenase) (Matsumoto et al. 2004). From the gene expression profiles, *CHLI1*, *CHLI2* and *CHLD* were involved in the same group, which are gradually upregulated by light during greening and obeyed diurnal rhythm but not circadian oscillation in matured leaves (Matsumoto et al. 2004). Co-expression of these genes are confirmed by recently developed databases of *Arabidopsis* microarray data, such as the AtGenExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp) and the ATTED-II (*A. thaliana trans*-factor and *cis*-element prediction database; http://www.atted.bio.titech.ac.jp) (Obayashi et al. 2007). Therefore, in *Arabidopsis*, it is assumed that the transcriptional control *CHLH* is a key regulatory point of Mg-chelatase activity, and a primary determinant of the level of active Mg-chelatase holo-complex, as well as orchestrating activities with other regulatory enzymes of tetrapyrrole biosynthesis. Meanwhile, the expression of *CHLI1*, *CHLI2*, and *CHLD* is regulated in a similar manner: they are induced by light during chloroplast development and are then under the control of a diurnal rhythm to maintain constant protein levels in chloroplasts. Recently, Shen et al. (2006) showed that *Arabidopsis* ChlH is ubiquitously expressed in both green and non-green tissues to perceive the abscisic acid signal at the whole-plant level, an expression profile that is quite different from previously published results. Future work must clarify whether the CHLH protein exists alone in non-photosynthetic plastids and functions as a receptor of abscisic acid, separate from its role in tetrapyrrole biosynthesis. In tobacco, *ChlH* and *ChlI* follow a similar circadian profile of expression, but *ChlD* has an inverse expression profile with maximal mRNA levels in the dark phase (Papenbrock et al. 1999). The difference of gene expression profiles are attributable to distinct plant species or developmental conditions.

Mg-chelatase activity is regulated by GUN4, a recently identified porphyrin-binding protein (Larkin et al. 2003). *GUN4* was identified as the defective gene in the *gun4* mutant, which exhibits aberrant regulation of chloroplast-to-nucleus signal transduction like *gun5*. GUN4 is regarded to be involved in intracellular signaling (see below), and it binds both protoporphyrin IX and Mg-protoporphyrin IX and stimulates the activity of Mg-chelatase in *Synechocystis* (Verdecia et al. 2005) and *Thermosynechococcus elongatus* (Davison et al. 2005). Disruption of *GUN4* reduces the cellular levels of heme, suggesting that GUN4 is also involved in heme biosynthesis and thus may control the flow of substrate into the heme or Chl branch (Wilde et al. 2004).

In addition to regulation via rhythmic gene expression, Mg-chelatase activity is also regulated by diurnal cycling, which is attributed to fluctuations in the stromal concentration of free Mg^{2+} and the [ATP]:[ADP][Pi] ratio. Mg^{2+} triggers a conformational change in Mg-chelatase, resulting in a more active form that promotes cooperative binding of the other substrates, ATP and porphyrin (Reid and Hunter 2004). The free Mg^{2+} concentration in stroma increases from 0.5 to 2.0 mM upon shift from darkness to light in spinach (Ishijima et al. 2003). In the presence of GUN4, the Mg^{2+} concentration required for full activation of Mg-chelatase is lowered from 6 to 2 mM, suggesting that GUN4 controls Mg-chelatase activity at a physiologically relevant Mg^{2+} concentration (Davison et al. 2005). Stromal Mg^{2+} concentration may also affect subcellular localization of ChlH. ChlH associates with the envelope membrane when chloroplasts are disrupted in the presence of 5 mM Mg^{2+}, whereas it is detected in the soluble stromal fraction in the presence of 1 mM Mg^{2+} (Gibson et al. 1996; Nakayama et al. 1998). This suggests that translocation of ChlH from stroma to the envelope membrane is required for formation of active Mg-chelatase complex as well as to bind the substrate, protoporphyrin IX, such that the reaction may proceed.

In chloroplasts, the regulation of the activity of a number of enzymes involved in the photosynthetic reactions is coupled to photosynthetic electron transport via the thioredoxin system in which a light-induced change in enzyme activity is linked to the redox state of a disulfide bond located within each enzyme (Buchanan and Balmer 2005). Using a thioredoxin-affinity chromatography technique, ChlI was identified as a potential thioredoxin-target protein (Balmer et al. 2003). In fact, Mg-chelatase activity is sensitive to thiol-modifying reagents (Fuesler et al. 1984a, b; Walker and Weinstein 1991a, b), suggesting that thiol groups in the molecule are essential for proper function and optimal activity of this enzyme. The thiol-modifying reagent *N*-ethylmaleimide specifically binds to ChlI and inhibits its ATPase activity (Jensen et al. 2000). The ATPase activity of *A. thaliana* ChlI1 is fully inactivated by oxidation but is easily recovered by thioredoxin-assisted reduction, suggesting that ChlI1 is a target of regulation by thioredoxin (Ikegami et al. 2007). Moreover, the in vivo redox state of ChlI is light dependent (Ikegami et al. 2007).

Thus, taken together, the current data indicate that Mg-chelatase activity is tightly regulated by a diurnal cycle and photosynthetic electron transport in order to coordinate Chl biosynthesis with the photosynthetic activities in chloroplasts.

S-Adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase

S-Adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase (EC 2.1.1.11) catalyzes the transfer of the methyl group from *S*-adenosyl-L-methionine (SAM) to the carboxyl group of the 13-propionate side chain of Mg-protoporphyrin IX. Initially, the gene *BchH* was mistakenly assigned to code for SAM:Mg-protoporphyrin IX methyltransferase in *Rhodobacter* because Mg chelation is obligatorily coupled to the methyltransferase reaction in purple bacteria (Gorchein et al. 1993). Later, heterologous expression of the *BchM* genes of *Rba. capsulatus* and *Rba. sphaeroides* in *E. coli* demonstrated that the gene products had the SAM:Mg-protoporphyrin IX methyltransferase activity in vitro (Bollivar and Bauer 1992; Bollivar et al. 1994a). The enzyme belongs to the broad family of SAM-dependent methyltransferases (Kagan and Clarke 1994). The *Rba. capsulatus* SAM:Mg-protoporphyrin IX methyltransferase activity is stimulated by the BchH subunit of Mg-chelatase (Hinchigeri et al. 1997), suggesting that Mg-protoporphyrin IX may be channeled directly to this enzyme from Mg-chelatase through the BchH subunit. Assembly of functional Mg-chelatase and SAM:Mg-protoporphyrin IX methyltransferase in *E. coli* by heterologous expression of *Rba. sphaeroides BchM*, together with *BchI, BchD*, and *BchH*, resulted in the introduction of a new branch point in the tetrapyrrole biosynthetic pathway of *E. coli*, causing the accumulation of Mg-protoporphyrin IX monomethyl ester (Jensen et al. 1999b).

In Chl-producing organisms, the gene *chlM* was cloned from *Synechocystis* PCC6803 by functional complementation of the *bchM* mutant of *Rba. capsulatus* (Smith et al. 1996). The reaction mechanism of SAM:Mg-protoporphyrin IX methyltransferase was examined using a recombinant protein of *Synechocystis* ChlM. Transient kinetic analysis showed that a rapid porphyrin binding step to ChlM is preceded by a slower enzyme conformational change (isomerization step). For the subsequent partial reaction, a lag phase attributable to the formation of stable intermediate has been identified (Shepherd and Hunter 2004).

The SAM:Mg-protoporphyrin IX methyltransferase gene has also been cloned from *A. thaliana* (Block et al. 2002) and tobacco (Alawady et al. 2005). In *Arabidopsis*, the enzyme was found to localize to both the thylakoid and envelope membranes within chloroplasts (Block et al. 2002). In antisense transgenic tobacco, reduction of SAM:Mg-protoporphyrin IX methyltransferase activity was directly linked to a reduction of Mg-chelatase activity and ALA synthesis; ferrochelatase activity, however, was elevated. Moreover, overexpression of the enzyme in sense plants showed the opposite phenotypic profile. Due to changes in ALA synthesis and Mg-chelatase activity correlate with transcript levels of *HemA* (encoding glutamyl-tRNA reductase), *Gsa* (encoding glutamte-1-semialdehyde aminotransferase), and *ChlH*, it has been proposed that changes in methyltransferase activity are communicated to the cytoplasm to coordinate transcriptional activities of these regulatory enzymes (Alawady and Grimm 2005).

Involvement of Mg-porphyrins in intracellular signaling

The photosynthetic apparatus is composed of proteins that are encoded in the nuclear and chloroplast genomes. The mechanism that evolved to coordinate nuclear and organellar gene expression includes communication between the nucleus and chloroplasts. It is believed that chloroplasts send signals to the nucleus in various ways—so called retrograde signaling. Mg-protoporphyrin IX was identified as one of the plastid-derived retrograde signals involved in intracellular communication between chloroplasts and the nucleus (Rodermel 2001; Rodermel and Park 2003; Strand 2004; Beck 2005; Nott et al. 2006). In *Chlamydomonas reinhardtii*, administration of Mg-protoporphyrin IX and Mg-protoporphyrin IX monomethyl ester induces the expression of the light-responsive nuclear gene *HSP70*, which encodes a heat shock protein (Kropat et al. 1997, 2000). Analysis of *cis* elements in the promoter region of *HSP70A* identified two enhancer regions that are involved in Mg-protoporphyrin IX and light inducibility (von Gromoff et al. 2006).

In *A. thaliana*, mutants termed *gun* (genome uncoupled) were identified in which intracellular signaling was disrupted (Susek et al. 1993). These mutants express nuclear-encoded photosynthesis genes even when chloroplast function is disrupted by treatment with norflurazon, an inhibitor of carotenoid biosynthesis. Among five *gun* mutants (*gun1–5*), four of them (*gun2–5*) have mutations in tetrapyrrole biosynthetic enzymes. The *gun2* and *gun3* mutants are alleles of *hy1* and *hy2*, which encode heme oxygenase and phytochromobilin synthase, respectively. The gene *GUN5* encodes the H subunit of Mg-chelatase (Mochizuki et al. 2001), and *GUN4* encodes a chloroplast porphyrin-binding protein that activates Mg-chelatase (Larkin et al. 2003). Wild type plants accumulate high amounts of Mg-protoporphyrin IX when grown on norflurazon, whereas this accumulation occurs only partially or is absent in *gun2* and *gun5* mutants (Strand et al. 2003). Analysis of nuclear gene expression in an *A. thaliana* knockout mutant of SAM:Mg-protoporphyrin IX methyltransferase also suggested that Mg-protoporphyrin IX is a negative effector of nuclear photosynthetic gene expression (Pontier et al. 2007). Based on this observation, Mg-

protoporphyrin IX has been proposed to accumulate under stress conditions and act as a negative regulator of photosynthetic gene regulation.

In barley, mutants defective in all three subunits of Mg-chelatase displayed the *gun* phenotype, whereas a mutant defective in Mg-protoporphyrin IX monomethyl ester cyclase (see below) did not, although this mutant accumulated Mg-protoporphyrin IX monomethyl ester (Gadjieva et al. 2005). For this reason, it was suggested that Chl intermediates might only function as signal molecules early in chloroplast development (Gadjieva et al. 2005). Taking advantage of the fact that tetrapyrroles have intrinsic fluorescence, the accumulation of Mg-protoporphyrin IX was visualized in vivo using confocal laser scanning microscopy (Ankele et al. 2007). Under stress conditions, Mg-protoporphyrin IX accumulated both in the chloroplast and in the cytosol upon feeding with ALA, suggesting that this intermediate is exported from chloroplasts to the cytosol. Since the accumulation of Mg-protoporphyrin IX was only observed in ALA-treated samples that might be involved in artificial effects, the physiological significance of this phenomenon should be carefully examined. The stress-induced accumulation of Mg-protoporphyrin IX coordinates nuclear- and plastid-encoded photosynthesis genes by repressing the expression of nuclear-encoded sigma factors, which are essential components of plastid-encoded RNA polymerase (Ankele et al. 2007).

However, contradictory observations have been made; *cs* and *ch-42* mutants with a defect in *ChlI* did not display the *gun* phenotype, although the Mg-chelatase activity was severely impaired (Mochizuki et al. 2001). Reduction of the endogenous level of Mg-protoporphyrin IX by over-expressing SAM:Mg-protoporphyrin IX methyltransferase did not alter the expression of a nuclear-encoded photosynthesis gene (Alawady and Grimm 2005).

Very recently, the protein GUN1, the mutation of which caused *gun* phenotype but was independent of tetrapyrrole biosynthesis, was identified as a chloroplast-localized pentatricopeptide-repeat protein (Koussevitzky et al. 2007). Surprisingly, GUN1 and ABI4, an Apetala 2 (AP2)-type transcription factor, are common to retrograde signaling pathways that mediate GUN5-dependent modulation of Mg-protoporphyrin IX levels, as well as the reduction/oxidation (redox) state of the photosynthetic electron transfer chain. It was thus proposed that multiple indicators of aberrant plastid function in *Arabidopsis* are integrated upstream of GUN1 within plastids, which leads to ABI4-mediated repression of nuclear-encoded genes (Koussevitzky et al. 2007).

Moreover, it has been recently proposed that the *gun* phenotype is not directly (or not proportionally) correlated with the levels of Mg-protoporphyrin IX in mutants of tetrapyrrole biosynthesis. Several groups independently show the contradict results of the accumulation of Mg-protoporphyrin IX under stress conditions. Previously, Strand et al. (2003) showed that norflurazon-treated *Arabidopsis* seedlings accumulate more than 6 nmol g^{-1} FW of Mg-protoporphyrin IX, which is more than 15-fold higher than untreated wild type seedlings. This level is unusually high without taking into account of up-regulation or activation of upsteam enzymes of tetrapyrrole biosynthesis including ALA biosynthesis. However, gene expression analyses of upstream tetrapyrrole biosynthetic genes showed that the treatment of norflurazon to *Arabidopsis* seedlings substantially repressed the transcripts levels of almost all genes (Masuda et al. unpublished data; M.J. Terry, personal communication). Furthermore, Mochizuki et al. (manuscript in preparation) found that Mg-protoporphyrin IX and its methyl ester do not accumulate in norflurazon-treated mutants defective in genes involved in Mg-chelatase, SAM:Mg-protoporphyrin IX methyltransferase, or Mg-protoporphyrin IX monomethyl ester cyclase (see below), and the endogenous levels of these intermediates in wild type and these mutants (including *gun*) are not correlated with the *LHC* expression. Independently, using a sensitive LC/MS method, Moulin and Smith (2008) also showed that no accumulation of Mg-protoporphyrin IX under conditions in which the *LHC* genes are repressed. These observations suggest that the levels of Mg protoporphyrins do not simply account for the regulation of *LHC* transcript level. Thus, future work must clarify how defect of tetrapyrrole biosynthesis in *gun* mutants correlates to the control of expression of nuclear-encoded photosynthetic genes.

Mg-protoporphyrin IX monomethyl ester cyclase

Mg-protoporphyrin IX monomethyl ester cyclase catalyzes the incorporation of atomic oxygen into Mg-protoporphyrin IX monomethyl ester to form 3,8-divinyl protochlorophyllide (Pchlide). This oxidative cyclization reaction creates the fifth ring of Chl. Two types of Mg-protoporphyrin IX monomethyl ester oxidative cyclase have been identified in photosynthetic organisms. One is the anaerobic cyclase, which incorporates atomic oxygen from water; the activity of this enzyme depends on a functional *bchE* gene. Directed mutagenesis of *Rba. capsulatus* revealed that Mg-protoporphyrin IX monomethyl ester accumulated in a *bchE* mutant, suggesting that the *bchE* gene product is required for Mg-protoporphyrin IX monomethyl ester oxidative cyclase activity (Bollivar et al. 1994b). Similarities of the deduced sequence of *bchE* to that of the methylcobalamin-requiring P-methylase from *Streptomyces hygroscopicus* (Kuzuyama et al. 1995) indicated that the *bchE* gene product uses a cobalamin cofactor, and indeed two vitamin

B_{12}–requiring *Rba. capsulatus* mutants (*bluE* and *bluB*) accumulated Mg-protoporphyrin IX monomethyl ester (Gough et al. 2000). Supplementation with adenosylcobalamin or methylcobalamin restored the cyclase activity in permeabilized *bluE* and *bluB* cells. Another type of the cyclase is the an aerobic cyclase, which incorporates atomic oxygen from O_2. The activity of this enzyme depends on the gene *acsF*, homologs of which are found only in photosynthetic organisms. The *acsF* gene was first identified from *Rubrivivax gelatinosus* as a protein containing a putative binuclear iron cluster (Pinta et al. 2002).

The strict aerobic photosynthetic organisms, including higher plants, seem to contain the aerobic Mg-protoporphyrin IX monomethylester cyclase, whereas the strict anaerobes examined so far contain only the anaerobic cyclase. In facultative aerobic photosynthetic bacteria, aerobic and anaerobic cyclases coexist in the same organism (Porra et al. 1998; Pinta et al. 2002), apparently providing the competitive advantage of producing Chls under both aerobic and anaerobic conditions (Ouchane et al. 2004). Although *C. reinhardtii* can grow and produce photosystems under oxygen-limited conditions, this alga lacks a *bchE* gene. In *C. reinhardtii*, two homologs of *acsF*, *CRD1* and *CTH1*, were identified. The *CRD1* was shown to be induced under low oxygen tension and copper deficiency, and a *crd1* mutant is chlorotic under such conditions. In contrast, *CTH1* was expressed primarily during copper sufficiency and hence could not cover the loss of *CRD1* function in *crd1* mutants (Moseley et al. 2000; Eriksson et al. 2004). Therefore, it is probable that Chl biosynthesis is supplied by *CRD1* under low oxygen tension in *Chlamydomonas*. It is possible that lack of *bchE* in *Chlamydomonas* is related to oxygen-evolving photosynthesis in this organism. In purple bacteria, *puc* and *puf* operons, encoding proteins of the photosynthetic apparatus, are regulated by oxygen level. Ouchane et al. (2004) suggested that the existence of two types of Mg-protoporphyrin IX monomethyl ester cyclase encoded by *bchE* and *acsF* in purple bacteria may be advantageous to rapidly build up the photosystem under aerobic or anaerobic conditions, enabling an immediate start of photosynthetic growth following exposure to light.

In higher plants, an *acsF* homolog, *CHL27*, has been found in the *Arabidopsis* genome. Antisense *Arabidopsis* plants with reduced amounts of CHL27 accumulate Mg-protoporphyrin IX monomethyl ester upon ALA feeding, and the plants have chlorotic leaves with reduced levels of all Chl-binding proteins (Tottey et al. 2003). An in vitro assay for the aerobic cyclase reaction required membrane-bound and soluble components from the chloroplasts (Walker et al. 1988). It was proposed that CHL27 is membrane-bound catalytic subunit of the aerobic cyclase (Tottey et al. 2003). In barley, the gene *Xantha-I*, a homolog of *acsF* and *CHL27*, also was identified as a membrane-bound subunit. In addition to the *Xantha-I* gene product, it was proposed that the aerobic cyclase requires at least one soluble and two membrane-bound components (Rzeznicka et al. 2005). Biochemical characterization showed that the aerobic cyclase requires the cofactors NADPH, Fe^{2+}, and O_2 (Walker et al. 1989).

3,8-divinyl Pchlide *a* 8-vinyl reductase (DVR)

According to the number of vinyl side chains, chlorophylls of oxygenic photosynthetic organisms are classified into two groups: 3,8-divinyl chlorophyll (divinyl chlorophyll) and 3-vinyl chlorophyll (monovinyl chlorophyll). DVR catalyzes reduction of the 8-vinyl group on the tetrapyrrole to an ethyl group (Parham and Rebeiz 1995) using NADPH as the reductant. Reduction of the 8-vinyl group can probably occur at various steps of Chl biosynthesis before and after the reduction of the D pyrrole ring. Therefore, it is likely that the monovinyl and divinyl Chl biosynthesis reactions may occur in parallel rather than by separate linear pathways (Parham and Rebeiz 1995).

Almost all of the oxygenic photosynthetic organisms contain monovinyl chlorophylls, regardless of the variation in their indigenous environments (Porra 1997). The exceptions are species of *Prochlorococcus marinus*, marine picophytoplanktons that contain divinyl chlorophylls as their photosynthetic pigments (Chisholm et al. 1992; Coleman and Chisholm 2007). The advantage for *Prochlorococcus* sp. to have divinyl Chls is that the absorbance maximum of the pigments in the Soret region red shifts by $\sim$10 nm compared with monovinyl Chls, enabling more efficient absorption of green light that is enriched in deep water layers.

Two independent groups recently isolated the *DVR* gene by characterizing *A. thaliana* mutants that exhibit a predominant replacement of monovinyl Chl with divinyl Chl and another mutant that is pale green and has a reduced level of Chl *b* (Nagata et al. 2005; Nakanishi et al. 2005). Interestingly, a *DVR* homolog was identified in marine *Synechococcus* but not in *Prochlorococcus* (Nagata et al. 2005). It has been proposed that the common ancestor of *Prochlorococcus* lost *DVR* and acquired divinyl Chls, which allow photosynthetic growth in deep-sea waters by absorbing green light, which predominates in this niche.

Arabidopsis DVR can reduce 3,8-divinyl Pchlide as well as 3,8-divinyl chlorophyllide, but the efficiency of the former substrate is substantially lower than that of the latter in vitro and in vivo (Nagata et al. unpublished results). Thus, it is possible that the DVR reaction occurs after the NADPH:Pchlide oxidoreductase (POR) reaction, and thus

should be designated as 3,8-divinyl chlorophyllide *a* 8-vinyl reductase.

DVR has been identified in higher plants and green algae, but no homologs have been found in the genome of the unicellular red alga *Cyanidioscyzon merolae*, a eukaryotic photosynthetic organism, although this organisms synthesizes monovinyl Chl *a*. Among cyanobacteria, five *Synechococcus* species were found to contain a *DVR* homolog, but no other species, including eleven *Synechococcus* species, *Synechocystis* sp PCC6803, *Nostoc* sp. PCC7120, and *Gloeobacter violaceus* PCC7421, contain a *DVR* homolog although all of these organisms synthesize monovinyl Chl *a*. The gene *bchJ* has been also reported to be essential in the reduction of the 8-vinyl group in *Rba. capsulatus* (Bollivar et al. 1994b), but neither *bchJ* nor the *DVR* homolog has been found in the majority of the cyanobacterial genomes. Interestingly, recent analysis of the green sulfur bacterium *Chlorobium tepidum* led to the proposal that BchJ may play an important role in substrate channeling and/or regulation of Chl biosynthesis but showed that it is not a vinyl reductase (Chew and Bryant 2007). These observations imply the existence of a third type of DVR that is not related to *A. thaliana* DVR or to *Rba. capsulatus* BchJ (Nagata et al. 2005). Very recently a novel cyanobacterial *DVR* was identified by bioinformatic survey of whole-genome comparisons (personal communication from Dr. Ayumi Tanaka).

Pchlide oxidoreductase

Pchlide oxidoreductase catalyzes the reduction of the C_{17}–C_{18} double bond of the D pyrrole ring of the tetrapyrrole macrocycle. Two types of Pchlide oxidoreductase have been identified in photosynthetic organisms. One is light-dependent NADPH:Pchlide oxidoreductase (POR: EC 1.3.1.33 or EC 1.6.99.1), which has been the subject of a number of recent reviews (Aronsson et al. 2003b; Schoefs and Franck 2003; Masuda and Takamiya 2004; Heyes and Hunter 2005). Thus, here I discuss only the most recent findings on POR. POR is a single-subunit enzyme that requires light for catalysis and is present in all Chl-synthesizing organisms but is not found in Bchl-synthesizing organisms. Another type is light-independent Pchlide oxidoreductase (DPOR) (Armstrong 1998; Fujita and Bauer 2003). DPOR is a multisubunit enzyme consisting of three subunits related to nitrogenase. DPOR is not found in angiosperms but is present in most other Chl- and Bchl-synthesizing organisms; this allows these organisms to synthesize Chl or Bchl in darkness. When angiosperms are germinated in darkness, they accumulate small amounts of Pchlide in a ternary complex with NADPH and POR, and they are unable to synthesize Chl until this bound Pchlide is converted to chlorophyllide upon exposure to light.

One aspect of POR on which recent research has focused is the import mechanism for the POR precursor (pPOR) and its assembly into the prolamellar body (PLB) in plastids. Two types of POR, PORA and PORB, have been identified in *A. thaliana* (Armstrong et al. 1995) and barley (Holtorf et al. 1995). PORA and PORB are highly similar except in the N-terminal region that contains structural features such as the chloroplast transit peptide, but their gene expression profiles are substantially different. Both *POR* mRNAs are formed in etiolated seedlings, but only *PORB* mRNA continues to accumulate in light-grown plants, whereas *PORA* mRNA rapidly disappears after illumination. These results suggest that PORB operates throughout the greening process and in light-adapted mature plants, whereas PORA is active only in etiolated seedlings at the beginning of illumination. Thereafter, a third POR, PORC, the expression of which is induced by light, was identified in *A. thaliana* (Oosawa et al. 2000). The in vitro POR activity of PORC is recently confirmed (Pattanayak and Tripathy 2002).

Reinbothe et al. (1995a, b) originally suggested that the import of the precursor of barley pPORA into chloroplasts was totally dependent on envelope-bound Pchlide, the enzyme's substrate. It was suggested that the pPORA transit peptide directly interacts with Pchlide in the plastid envelope for translocation (Reinbothe et al. 1996, 1997). Moreover, Reinbothe et al. (1999) performed in vitro reconstitution experiments with the two barley POR enzymes and synthetic zinc analogs of Pchlide *b* and Pchlide *a*, zinc-protopheophorbide *b* (ZnPP*b*) and zinc-protopheophorbide *a* (ZnPP*a*). These experiments led to the hypothesis of a novel light-harvesting Pchlide *a*/*b*-binding protein complex, named LHPP, and a new proposal for distinct functions of PORA and PORB in vivo. The LHPP complex is thought to consist of a 5:1 ratio of the dark-stable ternary complexes of PORA and PORB, which specifically bind to Pchlide *b* and *a*, respectively, and are embedded in the lipid bilayers of PLB of etioplasts. Only the PORB-bound Pchlide *a* in the LHPP complex appears to be reduced immediately upon illumination, whereas the PORA-bound Pchlide *b* is proposed to function initially as a light-harvesting pigment. Energy transfer from Pchlide *b* to Pchlide *a* is speculated to provide a mechanism for photoprotection during the early stage of seedling greening. Subsequently, several papers from the same group have been published (Reinbothe et al. 2000, 2004a, b, 2005; Pollmann et al. 2007; Schemenewitz et al. 2007). The basic concept of their proposal is that pPORA is imported into plastids with a specific translocon, designated Pchlide translocon complex (Ptc), in an envelope-bound Pchlide *b*-dependent manner to form a PORA-Pchlide *b* complex

with LHPP, whereas PORB is imported with the general translocon. However, several papers dispute this concept. Thus, the following sections first review a series of recent studies—mainly from Reinbothe's group—that support the LHPP concept, followed by a discussion of criticisms of this concept.

Reinbothe et al. (2003c) reported that Pchlide *b* is abundant in barley etioplasts but metabolically unstable because it is rapidly converted to Pchlide *a* by the activity of 7-formyl reductase, an enzyme involved in the Chl *b*-to-Chl *a* reaction. They proposed that 7-formyl reductase finely tunes the levels of Pchlide *b* and Pchlide *a*, and thereby may regulate the steady-state level of LHPP (Reinbothe et al. 2003c). Reconstitution of LHPP complexes in vitro from barley PORA and PORB, chemically synthesized Pchlide *a*, Pchlide *b*, and lipids showed pigment binding characteristics of PORA and PORB specific to Pchlide *b* and Pchlide *a*, respectively (Reinbothe et al. 2003a). The reconstituted ~480-kDa complex displayed the same characteristics as photoactive Pchlide. Upon illumination, only the PORB-bound Pchlide *a* was photoactive and it was converted to Chlide *a*, whereas Pchlide *b* bound to PORA remained photoinactive. Fractionation of isolated barley etioplasts identified POR-pigment complexes that are similar in size, stoichiometry, and photochemical properties to the reconstituted LHPP (Reinbothe et al. 2003a). The authors further analyzed deletion mutants of barley POR and found that the central region of the polypeptide, the so-called "extra-loop" that distinguishes POR from the structurally related short-chain alcohol dehydrogenase, is dispensable for pigment binding but required for the assembly of LHPP (Reinbothe et al. 2003b).

Pchlide-dependent import of the transit peptide of barley pPORA has been shown in chloroplasts of tobacco, *A. thaliana*, and five other monocotyledonous and dicotyledonous plant species (Reinbothe et al. 2000). A competition assay with the precursors of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (pSSU) and ferredoxin as well as an antibody-blocking experiment with anti-TOC86 and anti-TOC75 indicated that the import site is specific for pPORA (Reinbothe et al. 2000). By chemical crosslinking using a reagent that interacts with the transit peptide of pPORA, the outer plastid envelope protein Oep16 was identified as the translocase for pPORA (Reinbothe et al. 2004b). Furthermore, analysis of components of the Ptc complex identified a putative Pchlide *a* oxygenase (Ptc52), a tyrosine aminotransferase, GTP-binding proteins Toc33/34, and Oep16 (Reinbothe et al. 2004a). As Pchlide *b* was detected as part of the Ptc complex, it was proposed that the putative Pchlide *a* oxygenase, which is homologous to Lls1, Toc55, and chlorophyllide *a* oxygenase (CAO), provides Pchlide *b* as import substrate for pPORA to form the major component, PORA-Pchlide *b*, of the LHPP complex (Reinbothe et al. 2004a). Oep16 forms a larger complex with TOC33 but not TOC34 in *Arabidopsis* chloroplasts (Reinbothe et al. 2005). Plastids of the *ppi1* mutant of *Arabidopsis* lacking Toc33 were unable to import pPORA but imported pSSU, pPORB, and a precursor of ferredoxin (Reinbothe et al. 2005). A loss-of-function mutation in *Arabidopsis* Oep16 led to defects in import and assembly of PORA, which caused excess accumulation of Pchlide in the dark. Then, the pigment operated as a photosensitizer and provoked cell death during greening (Pollmann et al. 2007). In the Oep16 mutant, the PLB was completely lacking as compared with wild-type etioplasts. Schemenewitz et al. (2007) reported that pPORA can use two different plastid import pathways that differ in their requirements for cytosolic 14:3:3 proteins and Hsp70. pPORA synthesized in vitro in wheat germ lysate segregated into two different fractions by size exclusion chromatography, one of which bound with 14:3:3 proteins and Hsp70, and pPORA in this fraction was imported into Pchlide-free chloroplasts by the standard translocon, whereas other free and Hsp70-bound pPORA were imported in Pchlide-dependent manner. They also showed that Ptc52, encoding the putative Pchlide *b* oxygenase, but not CAO, is indispensable for Pchlide *b*–dependent transport (Schemenewitz et al. 2007).

Their hypothesis is supported by several reports by other research groups. Smith et al. (2004) reported that the main receptor of transit peptides that are imported into chloroplasts, Toc159, interacts with precursors to photosynthetic proteins; however, this protein had little activity in binding assays with nonphotosynthetic precursors, including pPORA. Using stable *Arabidopsis* transformants expressing pPOR-green fluorescent protein (GFP) fusion constructs, Kim and Apel (2004) showed that substrate-dependent import of pPORA into plastids of cotyledons occurred in the *flu* mutant, which over-accumulates Pchlide in the dark, but not in the *xantha2* mutant, which is devoid of Pchlide. In true leaves, PORA uptake does not require the presence of Pchlide. However, Kim et al. (2005) showed that in *ppi3*, *ppi1* and *ppi2* mutants, which are deficient in atToc34, atToc33 and atToc159, respectively, none of these translocon constituents is required for substrate-dependent and organ-specific import of PORA, whereas atToc33 is necessary for the import of PORB.

On the contrary, other studies have reported results that conflict with the Pchlide-dependent import and the LHPP model (Armstrong et al. 2000). The import into chloroplasts in a homologous system from *Arabidopsis* (Jarvis et al. 1998; Jarvis and Soll 2001), pea (Dahlin et al. 1995; Aronsson et al. 2000, 2001, 2003a) and wheat (Teakle and Griffiths 1993) was not dependent on Pchlide. The absence or presence of Pchlide did not significantly affect the

capacity to import pPORA and pPORB in barley (Dahlin et al. 1995; Aronsson et al. 2000). A competition assay with pSSU and crosslinking analysis indicated that pPORA uses the general import pathway into chloroplasts (Aronsson et al. 2000). Re-examination of pigment extraction from etiolated barley failed to detect Pchlide *b* irrespective of the extraction protocol, and disputed the existence of LHPP complexes in barley etioplasts (Kollosov and Rebeiz 2003). Phillipar et al. (2007) recently analyzed homozygous loss-of-function mutant lines of Oep16 and showed that etioplasts from *oep16* mutants contained PORA protein, as assessed by mass spectrometry. Strikingly, even though they analyzed the same T-DNA insertional SALK line of Oep16 that was analyzed by Pollmann et al. (2007), they showed that mutants had a normally structured PLB that contained the protochlorophyllide holochrome.

There is ongoing disagreement/discussion regarding the presence of Pchlide *b* in vivo, Pchlide *b*–dependent import of pPORA, and the resultant formation of PORA-Pchlide *b* in the LHPP complex. For the presence of Pchlide *b* in vivo, although Pchlide *b* was reported in several green plants (Shedbalkar et al. 1991), the abundant accumulation of Pchlide *b* in etioplasts observed by Reinbothe et al. (2003c) was not reproduced by the same extraction protocol that inhibits putative 7-formyl reductase (Kollosov and Rebeiz 2003). Furthermore, molecular nature of this 7-formyl reductase, which may have unusually high activity to convert Pchlide *b* to Pchlide *a* is still unidentified. For production of Pchlide *b*, Ptc52 is proposed as the putative Pchlide *a* oxygenase (Reinbothe et al. 2004a), however in vitro activity of this protein has not been demonstrated. In addition, the Pchlide *b* added to etioplast membrane was photoreduced to Chlide *b* by POR (Scheumann et al. 1999). Scheumann et al. (1999) also detected a substantial reduction of exogenously added ZnPP*b* to zinc-71-hydroxy-protopheoporbide *a* in darkness, which is catalyzed by Chl *b* reductase (see below).

For discrepancy of the Pchlide-dependent import of pPORA, instability of the protein import receptor and the involvement of a high concentration of urea in the reaction mixtures have been considered (Aronsson et al. 2000; Philippar et al. 2007). A major protein import receptor, TOC160, is extremely sensitive to proteolysis upon cell lysis, and degrades with a half-life of a few minutes if strict precautions are not taken (Botler et al. 1998). The degradation of TOC160 lowers the import efficiency of pSSU, although the degraded 86-kDa form still supports the import reaction. High concentrations of urea used in Reinbothe's experiments seem to influence chloroplast integrity and import competence. Aronsson et al. (2000, 2003b) proposed that differential functional integrity of the TOC complex might influence the requirement for import of pPOR into plastid in vitro. Very recently, Schemenewitz et al. (2007) proposed that cytosolic targeting factors, 14:3:3 proteins, present in wheat germ lysates, which were used to in vitro translation in other research groups, may target pPORA to an import site other than the Ptc complex. They proposed the level of the 14:3:3 proteins may physiologically and developmentally regulate the import of PORA. Further analysis is necessary to identify the effects of urea and the involvement of such cytosolic targeting factor on the substrate-dependent import of pPORA. Moreover, why the same T-DNA knockout line of Oep16, a putative component of Ptc complex, showed very distinct phenotypes should be clarified (Phillipar et al. 2007; Pollmann et al. 2007).

For the formation of the LHPP, it is still difficult to generalize as the central structural determinant of the PLBs in etioplasts of angiosperms. The given 5:1 stoichiometry of PORA to PORB is not consistent with the levels of PORA and PORB mRNA in etiolated seedlings of *Arabidopsis* (Armstrong et al. 1995). Furthermore, the total quantities of either PORA or PORB mRNA in *Arabidopsis* can be specifically manipulated by their constitutive overexpression or knockout by DNA tagging (Sperling et al. 1997, 1998; Franck et al. 2000; Frick et al. 2003; Masuda et al. 2003a). Such studies provide no evidence that alterations in the PORA to PORB ratio, independent of the total quantity of POR, dramatically influence the extent of PLB formation and the photoactive Pchlide. More strikingly, overexpression of cyanobacterial *POR* from *Synechocystis* PCC6803 or *Gloeobacter violaceus* in *Arabidopsis PORA* knockdown mutant showed significant complementation of Chl deficiency, with concomitant formation of PLB in etioplasts (Shinji Masuda et al. unpublished data). These cyanobacteria, producing only Chl *a* but not Chl *b*, contain single *POR* gene but do not form PLB in the dark, since they also contain DPOR. Thus, it is likely that the PLB forming capacity is instinct nature of POR proteins, irrespective of species of photosynthetic organisms. Moreover, cucumber (Fusada et al. 2000) and pea (Spano et al. 1992; Sundqvist and Dahlin 1997) provide an example of angiosperms that apparently contain only one POR gene. Therefore, the in vivo data from *Arabidopsis*, cucumber and pea are not consistent with specific functions for PORA and PORB within an LHPP complex of the type postulated especially for barley. One possibility is that the LHPP complex is specific to monocotyledonous plants, since Reinbothe et al. (2003a) found a similar solubilization of PORA during the isolation of etioplast inner membranes from oat and wheat etioplasts. However, the predominance of non-photoactive Pchlide, which is one essential prediction of the LHPP model, is not fulfilled in these plants, since most of the Pchlide was phototransformed by flash illumination of wheat etioplast (Ryberg and Sundqvist 1988), etiolated wheat seedlings

(Franck et al. 1999), and oat seedlings (Domanski and Rüdiger 2001). Thus, although the proposed LHPP model is intriguing, it is generally untenable in view of many opposing arguments.

DPOR

Many gymnosperms, ferns, mosses, cyanobacteria, and photosynthetic bacteria have the ability to synthesize Chl in the dark, indicating the existence of a light-independent mechanism for Pchlide reduction. Analysis of mutants of *Rhodobacter* species identified three genetic loci, known as *BchL*, *BchB*, and *BchN*, that are required for light-independent Pchlide reduction in a photosynthetic gene cluster in photosynthetic bacteria (Zsebo and Hearst 1984; Coomber et al. 1990; Yang and Bauer 1990; Burke et al. 1993; Bollivar et al. 1994b). Fujita and Bauer (2000) subsequently reconstituted the activity of light-independent Pchlide reduction in vitro with recombinant BchL, BchN, and BchB from *Rba. capsulatus*.

DPOR is an oxygen-sensitive nitrogenase-like enzyme consisting of the L protein (BchL/ChlL-dimer) as the reductant component and the NB proteins (BchN/ChlN–BchB/ChlB-heterotetramer) as the catalytic component (Fujita and Bauer 2003). In the cyanobacteria, *Synechocystis* sp PCC 6803 (Wu and Vermaas 1995) and *Leptolyngbya boryana* (formerly *Plectonema boryanum*) (Fujita et al. 1998), analysis of *ChlL*-disrupted mutants revealed that DPOR contributes to Chl synthesis in cells growing in light, suggesting that DPOR is tolerant of oxygen to some extent or has a mechanism of protection from the oxygen evolved in oxygenic phototrophs. The extent of the contribution by DPOR decreases with increasing light intensity, suggesting inefficient catalysis when oxygen evolution is maximal (Fujita et al. 1998). Yamazaki et al. (2006) showed that anaerobic conditions are required for DPOR to compensate for the loss of POR in a mutant of *L. boryana.* In the POR-lacking mutant, DPOR activity was stimulated by an increase in levels of the DPOR subunits, especially ChlL and ChlN, suggesting that this increase stimulates DPOR activity that is protected efficiently from oxygen by anaerobic environments, resulting in complementation of the loss of POR. The metallocenter of BchL was identified as an oxygen-sensitive [4Fe–4S] cluster similar to the nitrogenase Fe protein (Nomata et al. 2006). Considering the oxygen sensitivity of DPOR, the evolution of light-dependent POR is considered to have been critical for the survival of ancestral cyanobacteria that were growing in oxidative environments exposed to high light (Yamazaki et al. 2006). Furthermore, dual Pchlide reductases are assumed to operate differentially in oxygenic photosynthetic cells grown under natural environments where oxygen levels change dynamically.

The Chl cycle

It is now considered that the chlorophyllide *a* produced by Pchlide reductase is subsequently converted to Chl *a* by a Chl synthase-catalyzed phytol chain attachment. A portion of chlorophyllide *a* is converted to chlorophyllide *b* by a Reiske-type monooxygenase, CAO (Oster et al. 2000) and subsequently is converted to Chl *b*—one of the major light-harvesting pigments essential for optimal light harvesting. The phytol chain of Chl *a* can be removed by chlorophyllase, and the resultant chlorophyllide *a* can be used as a substrate of CAO. Chl *b* that is produced by the action of CAO can be reversibly converted to Chl *a* through the intermediate 7-hydroxymethyl Chl *a* (Ito et al. 1993, 1994) via the action of Chl *b* reductase and 7-hydroxymethyl Chl *a* reductase (Scheumann et al. 1998; Kusaba et al. 2007). Thus, Chl *a* and Chl *b* are interconvertible, with the entire interconversion system being designated as the Chl cycle (Ito et al. 1996; Rüdiger 2002). The Chl cycle is considered to be important for altering the size of light-harvesting antennae to allow plant acclimation to various light intensities (Tanaka et al. 2001; Tanaka and Tanaka 2005). In addition, the conversion of Chl *b* to Chl *a* is crucial because this is the first step in Chl degradation (Kusaba et al. 2007).

Chl synthase

Chl synthetase, encoded by *ChlG*, catalyzes the esterification of chlorophyllide *a* with phytol. For the substrates of Chl synthetase, early evidence indicated that during greening alcohol moiety is provided by geranylgeranyl diphosphate but not phytol (Shioi and Sasa 1983; Maloney et al. 1989). It has been suggested that chlorophyll *a* phytol is formed in two steps: the esterification of chlorophyllide *a* and geranylgeraniol and followed by three successive hydrogenations of the alcohol moiety. The corresponding enzyme in photosynthetic bacteria catalyzes the esterification of bacteriochlorophyllide *a* to Bchl *a*. Chl synthase activity was originally detected in etioplast membranes of *Avena sativa L.* (Rüdiger et al. 1980). The activity and substrate specificity of Chl synthase was demonstrated with the recombinant enzymes from *Rba. capsulatus*, *Synechocystis* sp. PCC6803 (Oster et al. 1997), *A. thaliana* (Oster and Rüdiger 1997) and *Avena sativa* (Schmid et al. 2001). *ChlG* is constitutively expressed as the same transcript level is found in dark-grown and light-grown seedlings (Schmid et al. 2001). Analysis of esterification kinetics of

chlorophyllide suggested that POR and Chl synthase form a complex having the ratio 7:1 (Domanskii et al. 2003). Experiments with chemically modified substrates suggested that tetrapyrroles bind with the same orientation on both enzymes; this excludes simultaneous binding of substrate to both enzymes (Rüdiger et al. 2005).

Chlorophyllide *a* oxygenase (CAO)

CAO catalyzes the conversion of chlorophyllide *a* to chlorophyllide *b*. The gene *CAO* was first identified from a Chl *b*-less insertional mutant of *C. reinhardtii* (Tanaka et al. 1998). The *CAO* gene encodes an oxygenase with a Riesk-type [2Fe–2S] cluster and a mononuclear iron-binding site. *CAO* genes have since been cloned from *A. thaliana* (Espineda et al. 1999; Tomitani et al. 1999), *Oryza sativa*, *Marchantia polymorpha*, *Dunaliella salina*, and Chl *b*-producing prokaryotes *Prochlorothrix hollandica* (Nagata et al. 2004) and *Prochloron didemini* (Tomitani et al. 1999). *CAO* has not been found in the genomes of *Prochlorococcus* species, although these organisms also produce Chl *b*. Using bioinformatics and molecular genetic techniques, Satoh and Tanaka (2006) identified the Chl *b* synthesis gene from *Prochlorococcus marinus* MIT9313, and this gene is distantly related to *CAO* genes. *CAO* is a single nuclear gene in *Chlamydomonas* (Tanaka et al. 1998) and *Arabidopsis* (Espineda et al. 1999), and mutants lacking *CAO*, such as *chlorina1*, fail to accumulate Chl *b* (Espineda et al. 1999; Oster et al. 2000). The recombinant *Arabidopsis* CAO protein requires oxygen and reduced ferredoxin for its activity, and it catalyzes a two-step oxygenation reaction of chlorophyllide *a* to chlorophyllide *b* via 7-hydroxymethyl chlorophyllide (Oster et al. 2000; Reinbothe et al. 2006). The enzyme can also use Zn-chlorophyllide *a* as a substrate but not pheophorbide *a* or Chl *a*. Heterologous expression of *Arabidopsis* CAO in *Synechocystis* PCC6803 caused the accumulation of Chl *b* (Satoh et al. 2001).

In the antenna systems of higher plants and green algae, Chl *b* binds to peripheral light-harvesting complexes (LHC). The antenna size can vary in response to growth at different light intensities, with concomitant changes in the Chl *a/b* ratio. Overexpression of *CAO* in transgenic *Arabidopsis* (Tanaka et al. 2001) and tobacco (Pattanayak et al. 2005) decreased the Chl *a/b* ratio, implying an enlargement of antenna size. When low-light-grown *Dunaliella salina* was transferred to high-light conditions, the *CAO* transcript level decreased in concert with an increased Chl *a/b* ratio (Masuda et al. 2002, 2003b). Such light intensity–dependent changes of *CAO* mRNA levels have been observed in tobacco (Pattanayak et al. 2005) and *Arabidopsis* (Harper et al. 2004; Tanaka and Tanaka 2005). These results suggested that Chl *b* synthesis is regulated in part at the *CAO* mRNA level.

The level of the CAO protein is also regulated by protein turnover. CAO is almost undetectable in wild type plants, even though *CAO* mRNA can readily be detected. CAO is comprised of three domains, designated A, B, and C (Nagata et al. 2004). The catalytic domain C can catalyze the reaction independently of domains A and B. Domain A senses the presence of Chl *b* and controls the overall level of the CAO protein because this domain is recognized by protease. When domain C alone was expressed in *Arabidopsis*, the levels of CAO dramatically increased, resulting in extremely low Chl *a/b* ratios (Yamasato et al. 2005). It was proposed that such a feedback mechanism at the protein level effectively controls the level of Chl *b* in response to cellular demand for this protein (Tanaka and Tanaka 2006). An *Arabidopsis* line overexpressing a CAO-GFP fusion was used to identify mutants that had defects in the control of CAO accumulation (Nakagawara et al. 2007). This analysis revealed that mutation of the gene *ClpC1*, encoding the chloroplast protease ClpC1, yielded stronger CAO-GFP fluorescence with decreased Chl *a/b* ratio, suggesting that this protease is involved in regulating Chl *b* synthesis through the destabilization of CAO in response to the accumulation of Chl *b*.

CAO was found on the chloroplast envelope and thylakoid membranes in chloroplasts (Eggink et al. 2004; Reinbothe et al. 2006). In dark-grown *Chlamydomonas* cells, CAO was detected only on the envelope inner membrane (Eggink et al. 2004). CAO was identified as a part of a tranlocon involved in the regulated import and stabilization of LHC proteins Lhcb1 (LHCII) and Lhcb4 (CP29) (Reinbothe et al. 2006).

Chl *b* reductase

Chl *b* is converted to Chl *a* by Chl *b* reductase and 7-hydroxymethyl Chl *a* reductase. The rice *non-yellow coloring 1* (*nyc1*) mutant, which displays the "stay-green" phenotype, retained Chl *b* and LHCII in the light as well as in the dark during leaf senescence. Map-based cloning showed that NYC1 encodes a chloroplast-localized short-chain dehydrogenase (Kusaba et al. 2007). The NYC1-like protein (NOL) showed Chl *b* reductase activity, suggesting that *NYC1* and *NOL* encode Chl *b* reductases having divergent functions and physiological roles (Kusaba et al. 2007). It was suggested that 7-hydroxymethyl Chl *a* reductase is a ferredoxin-dependent enzyme (Scheumann et al. 1998), but the gene encoding this enzyme has not been identified.

Chlorophyllase

Chlorophyllase catalyzes the hydrolysis of Chl *a* to yield chlorophyllide *a* and phytol (Takamiya et al. 2000). Chlorophyllase is not only the first enzyme in the Chl degradation pathway but also the enzyme involved in the Chl cycle because CAO uses chlorophyllide *a* as substrate (Oster et al. 2000). The gene encoding chlorophyllase was originally identified in *Chenopodium album* and *Arabidopsis* (Tsuchiya et al. 1999), and in ethylene-treated citrus fruit (Jacob-Wilk et al. 1999). Cloning of this gene from *Chenopodium album* revealed that this enzyme contained a lipase motif (Tsuchiya et al. 1999). Site-directed mutagenesis of recombinant chlorophyllase of *C. album* revealed that the catalytic mechanism is analogous to that of serine hydrolase, which has the catalytic triad composed of Ser, His, and Asp in its active site (Tsuchiya et al. 2003).

One of the two isoforms of *Arabidopsis* chlorophyllase (AtCLH1) was originally isolated as the CORI1 (coronatine-induced) protein from the mutant *coi1*. AtCLH1 is rapidly induced by methyl jasmonate, which promotes senescence and Chl degradation in plants (Tsuchiya et al. 1999). The in vivo function of AtCLH1 was shown in wild type and *coi1* plants overexpressing *AtCLH1*, and these plants had increased levels of Chlide without a substantial change in the total amount of extractable Chl, resulting in high Chlide to Chl ratios in leaves (Benedetti and Arruda 2002). Furthermore, Kariola et al. (2005) reported that AtCLH1 is involved in plant damage control and can modulate the balance among defense pathways. In an *AtCLH1* RNAi-silenced line, failure to degrade free Chl upon damage caused hydrogen peroxide to accumulate, with concomitant induction of defense genes, under both pathogen inoculation and high-light conditions. Downregulation of *AtCLH1* resulted in resistance to the bacterial necrotroph, *Erwinia carotovora*, but increased susceptibility to the necrotrophic fungus, *Alternaria brassicicola*, resistance to which requires jasmonate signaling (Kariola et al. 2005).

Meanwhile, the expression of citrus chlorophyllase in tobacco protoplast or squash leaves suggested post-translational regulation of this enzyme through cleavage of its N-terminal peptide, which targets the enzyme for degradation (Harpaz-Saad et al. 2007). Heterologous expression of chlorophyllase lacking N-terminal 21 residues resulted in a chlorotic phenotype, whereas no visible phenotype was observed in plants expressing full-length chlorophyllase. As processing of the N-terminal peptide appeared to occur in tobacco, it was proposed that chlorophyllase functions as a rate-limiting enzyme in Chl catabolism controlled via post-translational regulation (Harpaz-Saad et al. 2007).

Concluding remarks

During the last decade, except for 7-hydroxymethyl Chl *a* reductase and unidentified subunits of Mg-protoporphyrin IX monomethyl ester cyclase, all enzymes involved in the Mg branch of Chl biosynthetic pathway have been identified. Analyses of enzymes in vitro and in vivo have modified the classical biosynthetic pathway as redrawn in Fig. 1. Based on the identified enzymes and pathway, novel molecular mechanisms for regulatory pathways governing Chl metabolism have been proposed such as those involving newly identified regulatory proteins like FLU and GUN4, a new protein import and assembly mechanism for POR, and the degradation mechanism of CAO that is triggered by pigments. Further refinement of these mechanisms will provide insight as to how Chl biosynthesis is controlled to meet the exact cellular demands for this essential but potentially toxic molecule in plants. Moreover, recent data indicate that Chl intermediates and degradation products have various functions in diverse physiological processes such as retrograde signaling and programmed cell death (Hörtensteiner 2004; Pruzinska et al. 2005). The complete elucidation of the network comprising these processes linked to Chl biosynthesis will enhance our basic understanding of the photosynthetic system as well as other cellular processes.

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