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Tetrapyrrole Metabolism in *Arabidopsis thaliana*

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This manuscript is dedicated to Prof. Mamoru Mimuro of Kyoto University who passed away in Februay 2011.

Higher plants produce four classes of tetrapyrroles, namely, chlorophyll (Chl), heme, siroheme, and phytochromobilin. In plants, tetrapyrroles play essential roles in a wide range of biological activities including photosynthesis, respiration and the assimilation of nitrogen/sulfur. All four classes of tetrapyrroles are derived from a common biosynthetic pathway that resides in the plastid. In this article, we present an overview of tetrapyrrole metabolism in Arabidopsis and other higher plants, and we describe all identified enzymatic steps involved in this metabolism. We also summarize recent findings on Chl biosynthesis and Chl breakdown. Recent advances in this field, in particular those on the genetic and biochemical analyses of novel enzymes, prompted us to redraw the tetrapyrrole metabolic pathways. In addition, we also summarize our current understanding on the regulatory mechanisms governing tetrapyrrole metabolism. The interactions of tetrapyrrole biosynthesis and other cellular processes including the plastid-to-nucleus signal transduction are discussed.

1. INTRODUCTION

Arabidopsis and all other higher plants produce four classes of tetrapyrroles, namely, chlorophyll, heme, siroheme, and phytochromobilin. Chlorophyll (Chl) is a tetrapyrrole macrocycle containing Mg^{2+}, a phytol chain, and a characteristic fifth ring (Fig. 1). The five rings in Chls are lettered A through E, and the substituent positions on the macrocycle are numbered clockwise, beginning in ring A (Fig. 1). In plants, Chls are the most abundant tetrapyrroles and they function as photosynthetic pigments to harvest light energy and transfer the absorbed energy to the reaction center in which charge separation occurs. Cyanobacteria and the chloroplasts of algae and plants including Arabidopsis, which evolve oxygen as a byproduct of photosynthesis, synthesize Chl *a* (Fig. 1). A group of cyanobacteria (Prochlorophytes), green algae, and plants also contain Chl *b*. The methyl group at the C7 position of Chl *a* is replaced by a formyl group in Chl *b*. Purple and green photosynthetic bacteria, which do not evolve oxygen, synthesize a variety of related tetrapyrroles, termed bacteriochlorophylls (Bchls) (Chew and Bryant 2007). In the order of their discovery, Chls and Bchls are named *a*-*d,* and *a*-*g*, respectively. More recently, a novel Chl *f* has been identified in cyanobacteria isolated from stromatlite (Chen et al. 2010b). Alterations in the ring structure allow photosynthetic organisms to harvest light at different wavelengths, depending on the type of Chls that are synthesized.

Heme is another closed macrocycle that contains iron and it plays a vital role in various biological processes including respiration and photosynthesis (Fig. 2). Siroheme (see Fig. 9) is a prosthetic group of nitrite and sulfite reductase that plays central roles in nitrogen and sulfur assimilation, respectively. Phytochromobilin (see Fig. 8) is a linear tetrapyrrole and a chromophore of phytochromes that perceives light and mediates its signal to the nucleus. The major site of tetrapyrrole biosynthesis in higher plants is plastids.

In virtually all living organisms except some Archae (Storbeck et al. 2010), common steps of heme synthesis are highly conserved and it serves diverse biological functions as a prosthetic group of various hemoproteins. Some exceptions include parasitic organisms which depend on heme biosynthesis originating within host organisms (Fleischmann et al. 1995; Heinemann et al. 2008). In humans, a malfunction of this pathway leads to severe metabolic disorders termed porphyrias (Ajioka et al. 2006; Moore 1993; Straka et al. 1990). Although some enzymes have initially been studied using plants, most enzymes involved in heme biosynthesis were firstly identified in mammals and bacteria. For Chl biosynthesis, molecular genetic analysis of the photosynthesis gene cluster from purple bacteria, *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, provided the first detailed understanding of genes involved in Bchl *a* biosynthesis (Suzuki et al. 1997). In these bacteria, all of the identified loci essential for Bchl *a* biosynthesis are tightly linked to a 45-kb region of the chromosome termed the “photosynthesis gene cluster.” Sequence analysis of the entire photosynthesis gene cluster, coupled with the construction of defined sets of insertion mutations within each of the open reading frames, have provided the first comprehensive molecular understanding of genes involved in specific steps in the biosyn-

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thetic pathway (Suzuki et al. 1997). Subsequently, many of Chl biosynthesis genes have been identified by virtue of their ability to complement Bchl *a* biosynthesis mutants, as well as by sequence homology comparisons. The remainder of the Chl biosynthesis genes has been subsequently identified by genetic analyses of Arabidopsis mutants (see below).

This chapter provides an overview of tetrapyrrole metabolism in Arabidopsis and other higher plants. The outline of tetrapyrrole biosynthesis is shown in Fig. 3. Conceptually, the pathway can be divided into several sections, each leading to a key intermediate or branch point. Here, we will describe each section in the following order: (1) "Biosynthesis of 5-aminoleculinic acid (ALA)", which is a universal precursor for all tetrapyrrole compounds, (2) "Common steps" consisting from ALA to protoporphyrin IX (Proto IX), which is a common precursor for Chl and heme/bilin biosynthesis, (3) "Chl branch" consisting of the insertion of Mg^{2+} into Proto IX for Chl *a* biosynthesis, (4) "Chl cycle" which refers to the interconversion between Chl *a* and

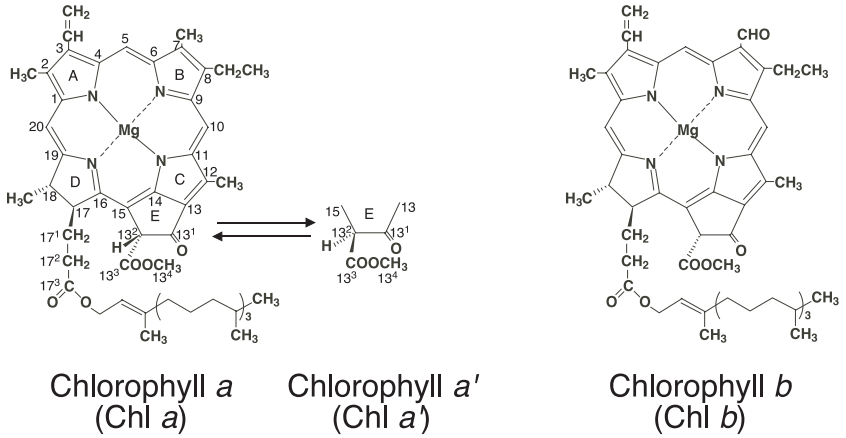


Figure 1. Structures of chlorophyll (Chl) *a* and *b*.

Chl is a tetrapyrrole macrocycle containing Mg^{2+}, a phytol chain, and a characteristic fifth ring. The five rings in Chls are lettered A through E and the substituent positions on the macrocycle are numbered clockwise, beginning in ring A. Chl *a*' is an epimer of Chl *a* at 13^2 position. In Chl *b,* the methyl group at the C7 position of Chl *a* is replaced by a formyl group.

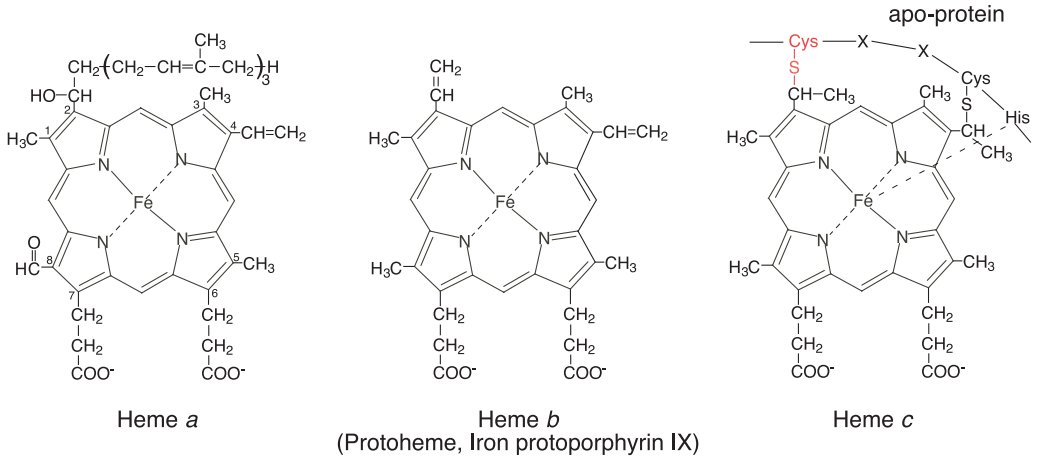


Figure 2. Structures of hemes.

Hemes are classified according to the type of groups attached to the periphery of their tetrapyrrole macrocycle. The *a*-type heme has three methyl groups (C1, C3, and C5), two propionic acids (C6 and C7), a vinyl group (C4), a farnesylated group (C2) and a formyl group (C8). The *b*-type heme has two vinyl groups (C2 and C4), four methyl groups (C1, C3, C5, and C8), and two propionic acids (C6 and C7), and is referred to as protoheme (iron Proto IX) or heme *b*. The general *c*-type heme has two vinyl-thioether groups instead of vinyl groups of heme *b*, while some organisms such as green algae *Euglena gracilis* and marine flagellate *Diplonema papillatum* have only one bound mitochondrial cytochrome *c* heme. In *c*-type heme, the two vinyl thioether side chains are covalently attached to cysteine residues of the hemoprotein, as in cytochrome *c*. The histidine acts as one axial ligand to the heme iron. In single-cysteine cytochrome *c*, the first cysteine (indicated by red) is replaced by other amino acid residues, resulting in formation of one thioether bond. In contrast, the porphyrin periphery of *a*- and *b*-type hemes is not covalently bound to the hemoprotein.

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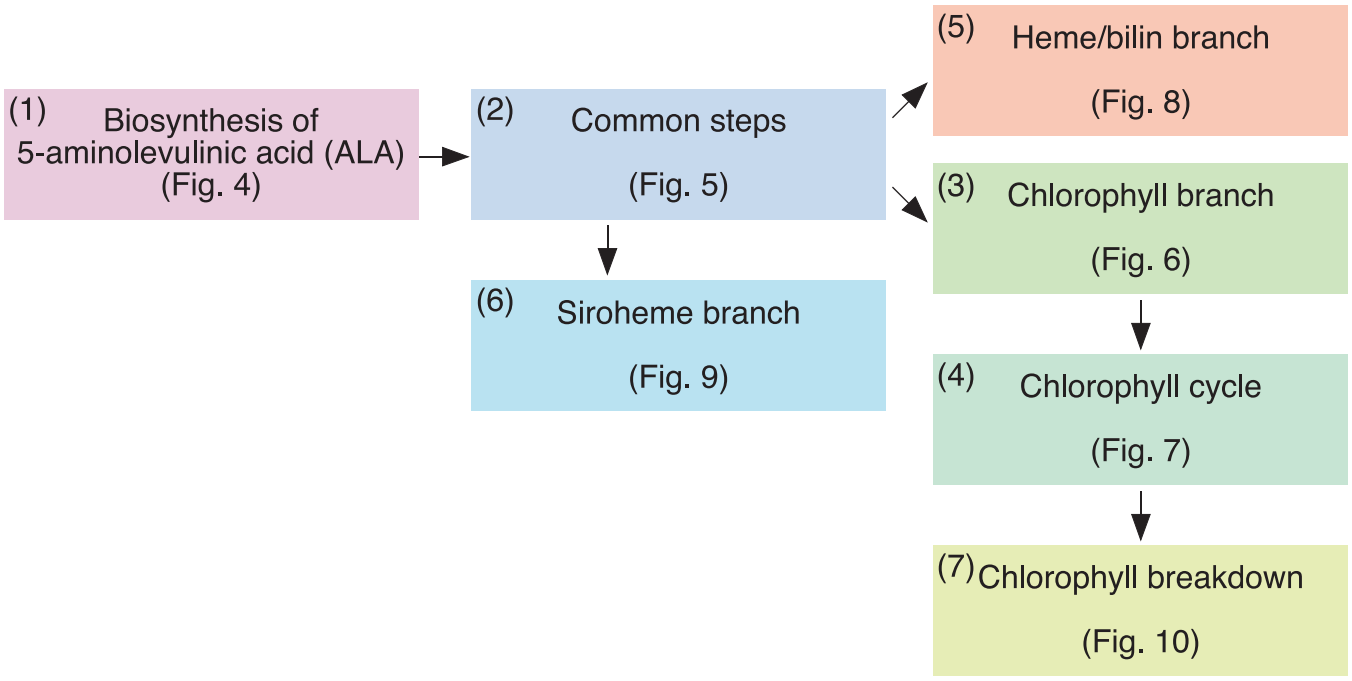


Figure 3. The core pathways of tetrapyrrole metabolism.

The tetrapyrrole metabolic pathways in higher plants can be conceptually divided into several sections: (1) "Biosynthesis of 5-aminoleculinic acid (ALA)"; which is a universal precursor for all tetrapyrrole compounds, (2) "Common steps" consisting from ALA to Proto IX; which is a common precursor for Chl and heme biosynthesis, (3) "Chl branch" consisting of the insertion of Mg^{2+} into Proto IX for Chl *a* biosynthesis, (4) "Chl cycle" which refers to the interconversion between Chl *a* and Chl *b*, (5) "Heme/bilin branch" consisting of biosynthesis and oxidative cleavage of heme to form bilin derivatives, (6) "Siroheme branch" which branched from the common steps to form siroheme, (7) "Chl breakdown" consisting from Chl *a* through the steps to the non-fluorescent Chl catabolites (NCC).

Chl *b*, (5) "Heme/bilin branch" consisting of biosynthesis and oxidative cleavage of heme to form bilin derivatives, (6) "Siroheme branch" which branched from the common steps to form siroheme, (7) "Chl breakdown" consisting from Chl *a* through the steps to the non-fluorescent Chl catabolites (NCC).

All identified enzymatic steps involved in this metabolic pathway are described in this chapter, with gene names and the Arabidopsis Gene Identifier (AGI) codes (Table S1), if available. In addition, the regulatory mechanisms controlling tetrapyrrole metabolism are described with an emphasis placed on recent updates. There are two main reasons why a high degree of regulation is necessary for tetrapyrrole metabolism. Firstly, the control of substrate flow is essential to meet the cellular demands for each product. Secondly, since most of tetrapyrrole intermediates are strong photosensitizers, plants need to prevent excessive accumulation of the intermediate molecules of the metabolic pathway. These molecules can potentially produce reactive oxygen species which result in oxidative damage or cell death under illumination. By absorbing light energy, tetrapyrrole intermediates are excited to a triplet state, and if they interact with ground-state oxygen, they produce singlet oxygen (Krieger-Liszkay et al. 2008; Triantaphylides and Havaux 2009). The regulation of tetrapyrrole metabolism has been extensively studied in higher plants. In many aspects, Arabidopsis mutants and transformants deficient in tetrapyrrole metabolism have contributed significantly to not only the identification of the metabolic enzymes, but also to a better understanding of the regulatory mechanisms of tetrapyrrole metabolism.

This review emphasizes our current knowledge on tetrapyrrole metabolism in Arabidopsis and other higher plants. When necessary, literatures pertaining to other eukaryotes and prokaryotes are included, but are not discussed in detail. For additional information, interested readers are encouraged to refer to comprehensive reviews on this field (Beale 1999; Eckhardt et al. 2004; Grimm et al. 2006; Hörtensteiner and Kräutler 2011; Layer et al. 2010; Masuda 2008; Masuda and Fujita 2008; Masuda and Takamiya 2004; Mochizuki et al. 2010; Moulin and Smith 2005; Tanaka and Tanaka 2006; Tanaka and Tanaka 2007; Terry et al. 2002; Vavilin and Vermaas 2002).

2. BIOSYNTHESIS OF THE UNIVERSAL TETRAPYRROLE PRECURSOR, ALA

The synthesis of tetrapyrroles starts from the first committed precursor, ALA. In non-photosynthetic eukaryotes and α-proteobacteria (Panek and O'Brian 2002), ALA is synthesized by a single step of condensation of succinyl-CoA and glycine in mitochondria via the so-called Shemin pathway. In Arabidopsis and all other higher plants, algae and bacteria (with the exception for

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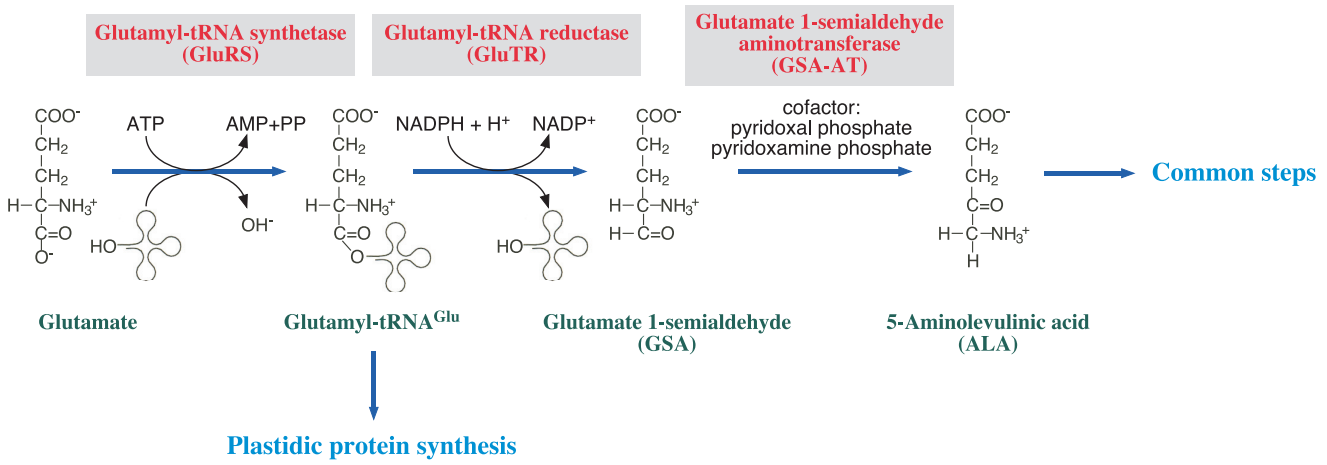


Figure 4. Biosynthetic pathway of ALA.

In Arabidopsis and all other higher plants, algae and bacteria (with the exception for α-proteobacteria), ALA is synthesized from glutamate (Glu) via the so-called C_5 pathway consisting of three enzymatic steps. In the shaded boxes, the corresponding enzyme names are given with abbreviations (in parentheses).

α-proteobacteria) (Panek and O'Brian 2002), ALA is synthesized from glutamate (Glu) via the so-called C_5 pathway consisting of three enzymatic steps as described below (Fig. 4).

2.1. Glu-tRNA synthetase

Glu is first activated by Glu-tRNA synthetase (GluRS: EC 6.1.1.17) to yield Glu-$tRNA^{Glu}$, a reaction which is common to plastidic protein synthesis. Like all other aminoacyl-tRNA synthetases, the enzyme requires the cognate amino acid and tRNA as substrates, and the reaction requires the energy of ATP hydrolysis. GluRS is encoded by two loci in Arabidopsis: At5g26710 and At5g64050. The deduced amino acid sequence of the At5g26710-encoded GluRS (Day et al. 1998) shows close similarity to the "cytoplasmic" one, which is presumed to be involved in translation in cytoplasm ribosomes. In contrast, At5g64050-encoded GluRS is dually targeted into plastid and mitochondria (Duchene et al. 2005). As a T-DNA insertion in the At5g64050 locus in an Arabidopsis mutant (Berg et al. 2005) and a gene silencing in the At5g64050 ortholog (Kim et al. 2005) in tobacco resulted in embryonic lethality and developmental arrest of organelles, respectively, it is hypothesized that this locus is required for translation in both organelles. In addition, it is also likely that At5g64050-encoded GluRS functions in the synthesis of ALA in plastids.

2.2. Glu-tRNA reductase

The second enzyme of ALA biosynthesis is Glu-tRNA reductase (GluTR), which catalyzes the reduction of Glu-$tRNA^{Glu}$ to Glu 1-semialdehyde (GSA) in an NADPH-dependent manner (Fig. 4). This is the rate-limiting step for the synthesis of tetrapyrroles, and is the first step that is unique to the biosynthetic pathway. In angiosperms, GluTR is encoded by the small *HEMA* gene family and all higher plants examined so far contain at least two *HEMA* genes. It should be noted that the gene encoding ALA synthase which catalyzes one-step condensation of glycine and succinyl-CoA in the Shemin pathway is also named *hemA*. However, this type of *hemA* genes is phylogenetically unrelated to the *hemA* genes encoding GluTR. The plant *HEMA* gene was first identified in Arabidopsis by functional complementation of the *E. coli hemA* mutant (Ilag et al. 1994). The deduced amino acid sequence encoded by this gene predicts a protein of 60 kDa (Ilag et al. 1994). Arabidopsis possesses three genes encoding GluTR isoforms and are named: *HEMA1* (At1g58290), *HEMA2* (At1g09940), and *HEMA3* (At2g31250). The expression of *HEMA1* is light–regulated and is predominant in photosynthetic tissues in Arabidopsis (Ilag et al. 1994) and other plants (Bougri and Grimm 1996; Tanaka et al. 1996; Tanaka et al. 1997). Since antisense *HEMA1* Arabidopsis plants showed decreased levels of Chl, noncovalently-bound heme, and ALA; HEMA1 is considered to play the major role in tetrapyrrole biosynthesis (Kumar and Soll 2000). On the other hand, *HEMA2* is preferentially expressed in non-photosynthetic tissues and its expression is not altered by illumination (Kumar et al. 1996). The expression of *HEMA3* is almost undetectable under all experimental conditions tested, therefore it is suggested this gene may have a limited physiological significance (Matsumoto et al. 2004; Ujwal et al. 2002).

Moser et al. (2001) determine the crystal structure of GluTR from the Archaeon *Methanopyrus kandleri* in a complex with the substrate-like inhibitor glutamycin, which shows a V-shaped dimeric structure through its dimerization domain. Modeling suggestes that the large void of the V-shaped structure may be occupied by the subsequent enzyme of this pathway (GSA-AT), thereby facilitating the efficient synthesis of ALA.

2.3. GSA aminotransferase

The third enzyme of the ALA biosynthesis is GSA aminotransferase (GSA-AT) (EC 5.4.3.8), which catalyzes the transamination reaction to form ALA. The GSA-AT enzyme contains a pyridoxal-phosphate or pyridoxamine-phosphate cofactor. Arabidopsis

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possesses two GSA-AT isoforms: GSA1 (At5g63570) and GSA2 (At3g48730). In Arabidopsis, *GSA1* is expressed in all organs and is moderately induced by light (Ilag et al. 1994; Matsumoto et al. 2004). From the determined structure of *Synechocystis* GSA-AT (Hennig et al. 1997), this enzyme is proposed to form a complex with GluTR, which may prevent the release of the highly-reactive aldehyde moiety of GSA by direct channeling of this intermediate from GluTR to GSA-AT (Moser et al. 2001). Physical and kinetic interactions between GluTR and GSA-AT have been demonstrated using recombinant proteins of *Chlamydomonas* (Nogaj and Beale 2005) and *E. coli* (Luer et al. 2005).

3. THE COMMON STEPS

In the common steps (Fig. 5), two molecules of ALA are condensed to form the monopyrrole (porphobilinogen; PBG), four molecules of which are then sequentially polymerized linearly and form the cyclic tetrapyrrole uroporphyrinogen III (Urogen III). The pathway is branched at this step to form siroheme ("siroheme branch"), a cofactor of nitrite and sulfite reductases which function in nitrogen and sulfur assimilation, respectively. Proto IX is formed after further steps including decarboxylations and oxidations.

3.1. ALA dehydratase

ALA dehydratase (ALAD) (EC 4.2.1.24; also known as PBG synthase) catalyzes the asymmetric condensation of two ALA molecules to form PBG, with the release of two molecules of H_2O. The reaction mechanism of ALAD has been extensively studied. Jordan and Seehra (1980) show that mammalian ALAD binds two ALA molecules successively and catalyzes the formation of an aromatic pyrrole ring. ALAD contains two substrate-binding sites, that are termed the A and P sites, respectively. The substrate ALA molecule that is bound to the A site becomes the acetyl-substituted half of PBG, while the propionyl-coordinating half of PBG derives from the P-site bound ALA. The ALAD reaction starts with the binding of ALA to the P site, followed by subsequent binding of the second substrate molecule to the A site. During this process, hydrogen is removed from the enzyme to form the aromatic pyrrole ring (Jordan and Seehra 1980). A plant *ALAD* gene was first isolated from soybean encoding 412 amino acids with a chloroplast transit peptide (Kaczor et al. 1994). Arabidopsis possesses two ALAD isoforms: *ALAD1* (At1g69740) and *ALAD2* (At1g44318). A mutation in the *ALAD1* gene results in pale green leaves with significantly reduced Chl contents and thus demonstrates that this gene is essential in Chl biosynthesis (S. Sawa, personal communication).

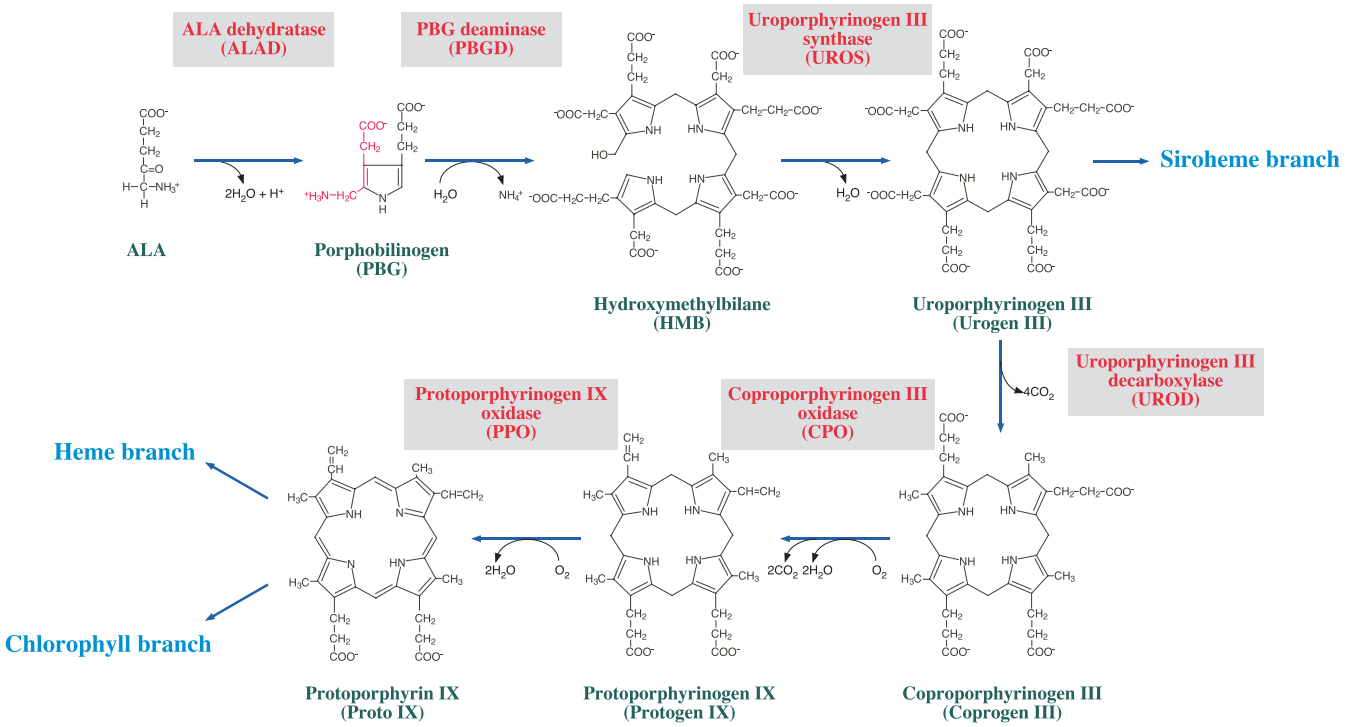


Figure 5. The common steps.

In the common steps, two molecules of ALA are condensed to form the monopyrrole (porphobilinogen; PBG), four molecules of which are then sequentially polymerized linearly and form the cyclic tetrapyrrole (Urogen III). The pathway is branched at this step to form siroheme ("siroheme branch"), a cofactor of nitrite and sulfite reductases which function in nitrogen and sulfur assimilation, respectively. Proto IX is formed after further steps including decarboxylations and oxidation.

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3.2. PBG deaminase

PBG deaminase (PBGD) (EC 2.5.1.61; also known as hydroxymethylbilane (HMB) synthase) condenses four PBG molecules to form the first tetrapyrrole, HMB. This enzyme assembles four PBG units in the order of the A, B, C and D rings, as they appear in Urogen III. Native PBGD is a soluble monomer of ~40 kDa molecular mass. It does not contain a dissociable prosthetic group or require metal ions for activity (Williams et al. 1981). Instead, in bacteria, this enzyme contains a covalently linked dipyrromethane cofactor (Jordan and Warren 1987) which is involved in the binding of the reaction intermediates during the catalysis but is not incorporated into the product (Jordan and Warren 1987). A single *PBGD* gene is present in the Arabidopsis genome (*PBGD*; At5g08280).

3.3. Urogen III synthase

Urogen III synthase (UROS) (EC 4.2.1.75) catalyzes the cyclization of HMB to form the first macrocyclic tetrapyrrole, Urogen III. Free HMB spontaneously cyclizes to form the nonphysiological product Urogen I, in which the D ring is conjugated with the A ring in the reverse orientation (Battersby et al. 1979). Therefore, the presence of UROS is essential for the synthesis of tetrapyrroles. Arabidopsis has a single *UROS* gene (At2g26540) which was identified by functional complementation of the yeast *UROS* mutant (Tan et al. 2008).

3.4. Urogen III decarboxylase

Urogen III decarboxylase (UROD) (EC 4.1.1.37) catalyzes the stepwise decarboxylation of the four acetate residues of Urogen III to form coproporphyrinogen III (Coprogen III). The decarboxylations occur in an ordered fashion, beginning with the residue on ring D and proceed around the molecule in a clockwise direction (Jackson et al. 1976). A plant UROD enzyme was first isolated from tobacco (Mock et al. 1995) and two loci *UROD1* (At2g40490) and *UROD2* (At3g14930) encoding two UROD isoforms were identified in the Arabidopsis genome. In tobacco, *UROD* antisense transgenic lines showed decreased activities of other enzymes involved in tetrapyrrole biosynthesis and led to a necrotic phenotype (Mock and Grimm 1997). Subsequently, the antisense lines were found to induce pathogen defense responses which conferred increased resistance to the tobacco mosaic virus (Mock et al. 1999). In maize, a dominant lesion-mimic mutant (*Les22*) of *UROD* was identified and heterozygotes of this mutant developed light-dependent necrotic lesions that were associated with a 2~3 fold increase in the intermediate Urogen III. On the other hand, homozygotes gave rise to yellow seedlings that quickly died shortly after germination (Hu et al. 1998). The dominant phenotype of *Les22* is analogous to *UROD* mutations in human that cause a specific type of metabolic disorder, porphyria cutanea tarda (Moore 1993; Straka et al. 1990). These data suggest that a full activity of UROD, which is expressed from both chromosomes, is required to sustain tetrapyrrole metabolism.

3.5. Coprogen III oxidase

Coprogen III oxidase (CPO) (EC 1.3.3.3) catalyzes the oxidative decarboxylation of two propionate side chains at ring A and B of Coprogen to vinyl groups. There are two phylogenetically-unrelated CPO enzymes encoded by the *HemF* and *HemN* genes, respectively, among eukaryotes and bacteria. Specifically, HemF protein catalyzes this oxidative decarboxylation reaction using oxygen as the electron acceptor (Phillips et al. 2004), whereas HemN protein catalyzes the same reaction in an oxygen-independent manner (Layer et al. 2003). In higher plants, oxygen-dependent *HemF* genes have been cloned from soybean (Madsen et al. 1993), barley, tobacco (Kruse et al. 1995a), maize (Williams et al. 2006) and Arabidopsis (At1g03475) (Ishikawa et al. 2001). Antisense *hemF* lines of tobacco (Kruse et al. 1995b) and a *hemF* loss-of-function mutant of Arabidopsis (Ishikawa et al. 2001) show a necrotic phenotype and a yellow seedling-lethal phenotype in maize (Williams et al. 2006). Collectively, these data suggest that the HemF-type CPO is the main enzyme responsible for tetrapyrrole metabolism. On the other hand, a role of oxygen-independent HemN in higher plants is still unknown, although this type of CPO is also conserved widely in bacteria and plants including Arabidopsis (At5g63290) (Obornik and Green 2005).

3.6. Protoporphyrinogen IX oxidase

Protoporphyrinogen IX (Protogen IX) oxidase (PPO, EC 1.3.3.4) catalyzes the six-electron oxidation of non-fluorescent Protogen IX to fluorescent Proto IX. Currently three types of PPO have been reported: HemG (Boynton et al. 2009), HemJ (Kato et al. 2010), and HemY (Lermontova et al. 1997; Narita et al. 1996). While HemG and HemJ are both primarily of bacterial origin, HemY is found in both eukaryotes and prokaryotes. Plants use the HemY-type PPO enzyme which requires oxygen as an electron acceptor and FAD as a cofactor (Koch et al. 2004). In land plants, there are two phylogenetically-distinct isoforms of HemY (PPOI; At4g01690 and PPOII; At5g14220) (Obornik and Green 2005). Since knockdown mutations in the PPOI-encoding gene in Arabidopsis (Molina et al. 1999) and reduction of PPOI protein levels by antisense RNA expression in tobacco (Lermontova and Grimm 2006) cause severe growth defects along with necrotic leaf damage, PPOI is considered to be the main isoform for tetrapyrrole synthesis. Thus far, the role of PPOII in land plants remains unknown.

4. THE CHL BRANCH

The first step of the Chl branch (Fig. 6) is the insertion of Mg^{2+} into Proto IX. As Proto IX is also a substrate for heme biosynthesis, the pathway is branched into the synthesis of Chl *a* or heme at this step. In the Chl branch, Mg-protoporphyrin IX (Mg-Proto IX) is sequentially modified by methylation, formation of the fifth isocyclic ring and reduction of a side chain of the tetrapyrrole ring. The D ring of protochlorophyllide (Pchlide) is reduced stereospecifically to form chlorophyllide (Chlide) *a*. The resulting monovinyl Chlide *a* is esterified with a long chain polyisoprenol (geranylgeraniol or phytol) to synthesize Chl *a*. Phytol is provided from geranylgeranyl pyrophosphate, which is produced via isopentenyl pyrophosphate in the non-mevalonate 2-C-methyl-D-erythritol-4-phosphate pathway in plastids.

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4.1. Magnesium chelatase

The first step of the Chl branch is an ATP-dependent insertion of the Mg^{2+} into Proto, a reaction catalyzed by magnesium chelatase (MgCh) that is categolized as class I chelatase (Brindley et al. 2003). This enzyme is composed of three subunits (CHLH, CHLI, and CHLD) which have average molecular weights of 140, 40, and 70 kDa, respectively. In Arabidopsis, MgCh subunits are encoded by *CHLH* (At5g13630), *CHLI1* (At4g18480), *CHLI2* (At5g45930), and *CHLD* (At1g08520). Catalysis by MgCh proceeds with a two-step reaction: an enzyme-activation step, followed by a Mg^{2+} insertion step (see review Masuda 2008; Walker and Willows 1997). The activation step requires ATP, Mg^{2+} and subunits CHLI and CHLD for the formation of a CHLI–CHLD–Mg-ATP complex without ATP hydrolysis. CHLD forms a hexameric ring structure that interacts with another hexameric ring structure made of six CHLI subunits. At the Mg^{2+} insertion step, this complex binds to the Mg–CHLH–Proto IX complex. At this step, Mg chelation occurs with ATP hydrolysis to form Mg-Proto IX. Subsequently, it is assumed that the MgCh complex disassembles for turnover (see review Masuda 2008; Walker and Willows 1997).

In Arabidopsis, at least three of the homozygous *CHLI1* mutants, such as *ch42-1* (Fisherova 1975), *cs* (*ch42-2*) (Koncz et al. 1990), *ch42-3* (Rissler et al. 2002), are recessive and exhibit a pale-green phenotype. The *cs* mutant has a T-DNA insertion, resulting in an extension of the C-terminal end. Meanwhile, a semidominant mutation of *CHLI1* (*aci5*) has been identified as a single amino acid substitution (Soldatova et al. 2005). In wild type, *CHLI1* and *CHLI2* mRNAs accumulate to similar levels, but CHLI2 protein is undetectable in wild type and the *ch42-3* mutant (Rissler et al. 2002). Thus, it was proposed that CHLI2 plays a limited role in the MgCh complex (Apchelimov et al. 2007; Rissler et al. 2002). However, more recent analysis revealed that the *chli2* mutation is semidominant on a homozygous *cs* background. These data demonstrate that although CHLI2 plays a limited role in Chl biosynthesis, this subunit has a functional role that is involved with the assembly of the MgCh complex (Kobayashi et al. 2008). Concerning CHLH, the *cch* (*conditional chlorina*) and *gun5* (*genome uncoupled 5*) loci were found to contain single missense mutations in Arabidopsis (Mochizuki et al. 2001). The *gun5* mutation was found to impart aberrant regulation of chloroplast-to-nucleus signal transduction (see below). A rice *chlorina-1* mutant,

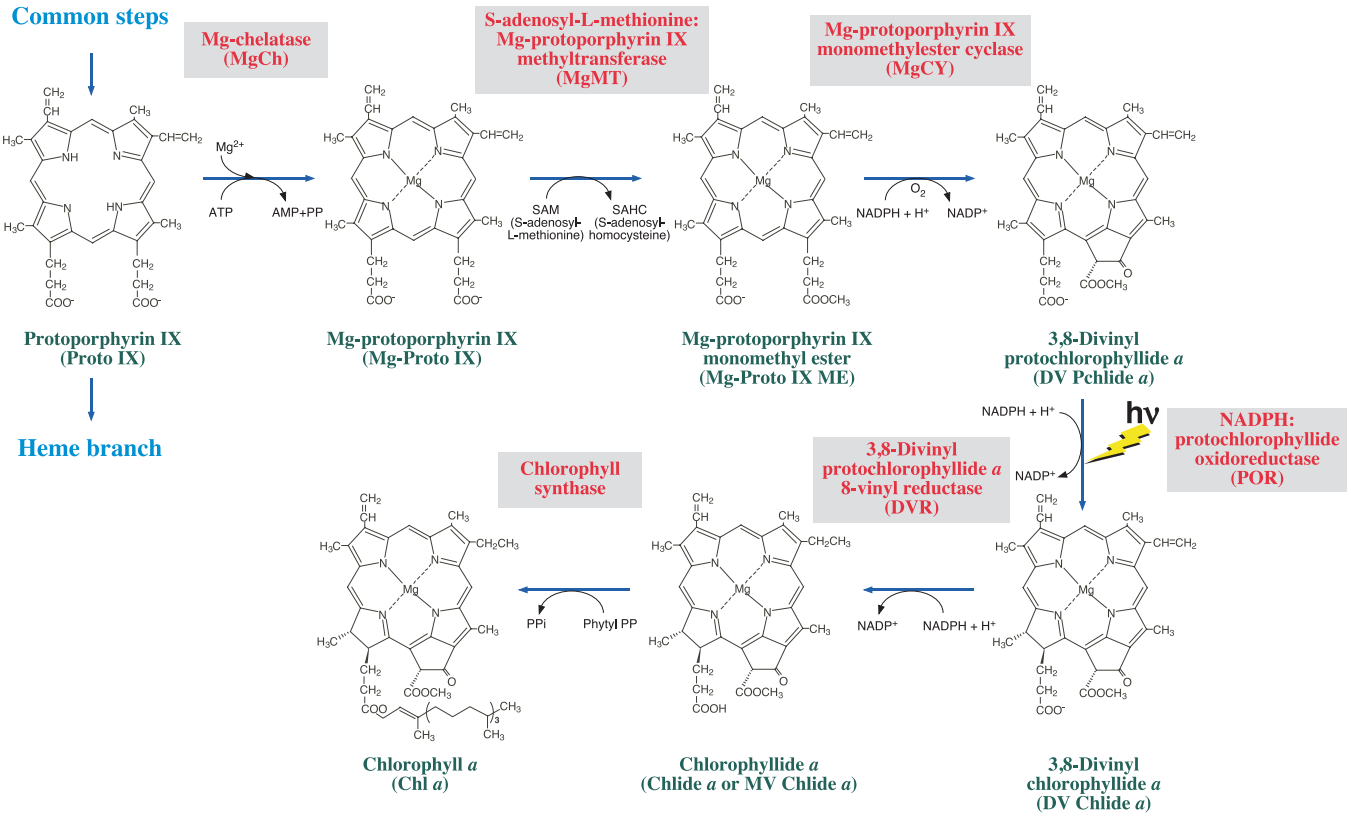


Figure 6. The Chl branch.

The first step of the Chl branch is the insertion of Mg^{2+} into Proto IX. As Proto IX is also a substrate for heme biosynthesis, the pathway is branched into the synthesis of Chl *a* or heme at this step. In the Chl branch, Mg-Proto IX is sequentially modified by methylation, formation of the fifth isocyclic ring and reduction of a side chain of the tetrapyrrole ring. The D ring of protochlorophyllide (Pchlide) is reduced stereospecifically to form chlorophyllide (Chlide) *a*. The resulting movovinyl Chlide *a* is esterified with a long chain polyisoprenol (geranylgeraniol or phytol) to synthesize Chl *a*. Phytol is provided from geranylgeranyl pyrophosphate, which is produced via isopentenyl pyrophosphate in the non-mevalonate 2-C-methyl-D-erythritol-4-phosphate pathway in plastids.

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which has a missense mutation in the *CHLD* gene, had reduced Chl contents in the mutant leaves (Zhang et al. 2006). These results demonstrate that every MgCh subunit is essential in MgCh activity and that this activity is directly linked to the overall activity of Chl synthesis.

MgCh activity is regulated by a porphyrin binding protein, GUN4, which is encoded by the At3g59400 locus. In Arabidopsis, this gene was identified as the defective gene in the *genome uncoupled 4* (*gun4*) mutant, which exhibits aberrant regulation of chloroplast-to-nucleus signal transduction (Larkin et al. 2003). GUN4 binds both Proto IX and Mg-Proto IX and stimulates the activity of MgCh (Davison et al. 2005; Verdecia et al. 2005). Disruption of GUN4 reduces the cellular levels of heme, suggesting that GUN4 is also involved in heme biosynthesis. Although the possibility of indirect effect of heme reduction can not be excluded, it is proposed that GUN4 controls the flow of substrate into the heme or Chl branch (Peter and Grimm 2009). More recently, Davison and Hunter (2011) showed that *Synechocystis* CHLH recombinant proteins introduced *cch*- and *gun5*-type mutations are inactive in MgCh assays, despite being able to bind both substrate and product, and retaining a capacity to form a CHLH–CHLI–CHLD Mg-chelatase complex. The inactivation of MgCh activities in *gun*-type CHLH is reversed upon addition of GUN4. Although the mechanism of MgCh activation by GUN4 is not clear at the present time, GUN4 seems to play an essential role at the branch point of Chl and heme biosynthesis.

4.2. S-adenosyl-L-methionine:Mg-Proto IX methyltransferase

S-adenosyl-L-methionine (SAM):Mg-Proto IX methyltransferase (MgMT) (EC 2.1.1.11) catalyzes the transfer of the methyl group from SAM to the carboxy group of the 13-propionate side chain of Mg-Proto IX. MgMT belongs to the broad family of SAM-dependent methyltransferases (Kagan and Clarke 1994) and a single MgMT gene (*CHLM*; At4g25080) exists in Arabidopsis (Block et al. 2002). Functional analysis of a *CHLM* knockout mutant shows that this gene is essential for the formation of Chl and the subsequent formation of photosystems I and II and cytochrome b_6f complexes in Arabidopsis (Pontier et al. 2007). In antisense- and sense-RNA overexpressing tobacco lines, modification of MgMT activities is linked to changes in ALA-synthesizing and MgCh activities, while ferrochelatase (FeCh) activity showed opposite profiles (Alawady and Grimm 2005). In pea seedlings, MgMT activity is reduced by treatment with a folate biosynthesis inhibitor, methotrexate, via the decrease of methyl-tetrahydrofolate and the subsequent methylation index (SAM/S-adenosylhomocysteine (SAHC) ratio) (Van Wilder et al. 2009). These data suggest that there is a correlation between folate status and the rate of Chl synthesis through the methyl cycle.

4.3. Mg-Proto IX monomethyl ester cyclase

Mg-Proto IX monomethyl ester (ME) cyclase (MgCY) catalyzes the incorporation of atomic oxygen into the Mg-Proto IX ME to form 3,8-divinyl protochlorophyllide (Pchlide or DV-Pchlide). This oxidative cyclization reaction creates the fifth ring of Chl. Two types of MgCY have been identified in photosynthetic organisms. The first type of MgCY is an anaerobic hydratase-type cyclase which incorporates atomic oxygen from water (Porra et al. 1995). The second type of MgCY is an aerobic oxygenase-type cyclase which incorporates atomic oxygen from molecular oxygen (Pinta et al. 2002). Molecular genetic analysis has revealed that *bchE* from *Rhodobacter capsulatus* (Bollivar et al. 1994) encodes the hydratase-type MgCY, while *chlA* from *Synechocystis* sp. PCC 6803 (Minamizaki et al. 2008), *acsF106* from *Rubrivivax gelatinosus* (Pinta et al. 2002), *CRD1* and *CTH1* from *Chlamydomons reinhardtii* (Moseley et al. 2000; Moseley et al. 2002) encode oxygenase-type MgCY. In *Chlamydomonas*, two paralogous proteins (CRD1 and CTH1) are differentially accumulated based on copper nutrition and oxygen supply (Allen et al. 2008b; Moseley et al. 2002).

The aerobic MgCY reaction is proposed to occur in three sequential two-electron oxidations (Porra et al. 1996). The first step requires molecular oxygen as a substrate for hydroxylation. This reaction is analogous to the methane monooxygenase reaction. The activated methylene group reacts with the γ-meso carbon of the porphyrin nucleus in an oxidative reaction involving removal of two hydrogens to yield ring E. There is also an oxygen requirement for the third reaction that converts the keto intermediate to DV-Pchlide.

Aerobic photosynthetic organisms, including higher plants, contain the aerobic MgCY form and all of the anaerobes examined to date only possess the anaerobic cyclase. In some photosynthetic bacteria such as *Rubrivivax gelatinosus*, aerobic and anaerobic cyclases coexist in a single organism. The *bchE* gene that encodes the aerobic cyclase is expressed under high-oxygen conditions, while the anaerobic cyclase functions only under low oxygen-conditions (Ouchane et al. 2004). The aerobic MgCY is an iron-dependent enzyme which is assciated with membranes. It is speculated that this enzyme consists of multiple subunits, based on the observation that multiple mutant loci in barley led to accumulation of Mg-Proto IX ME in ALA-fed plants (von Wettstein et al. 1995). Both soluble and membrane components must be recombined to restore cyclase activity (Walker et al. 1991; Wong and Castelfranco 1984), which further supports the involvement of multiple gene products in the MgCY reaction. Arabidopsis possesses one MgCY gene (*CHL27*; At3g56940) which encodes the membrane-bound catalytic subunit of MgCY (Tottey et al. 2003). In barley, *Xantha-L* is found to encode this subunit (Rzeznicka et al. 2005). Reconstitution systems of MgCY with purified proteins are not yet available. Understanding of the MgCY enzyme properties has remained a great challenge in Chl biosynthesis.

4.4. Pchlide oxidoreductase

Pchlide oxidoreductase catalyzes the reduction of the C17-C18 double bond of the D pyrrole ring of the tetrapyrrole macrocycle. Two types of Pchlide oxidoreductase have been identified in photosynthetic organisms: light-dependent NADPH:Pchlide oxidoreductase (POR: EC 1.3.1.33 or EC 1.6.99.1) (reviewed by Aronsson et al. 2003; Heyes and Hunter 2005; Masuda and Takamiya 2004; Schoefs and Franck 2003) and light-independent Pchlide oxidoreductase. The light-independent Pchlide oxidoreductase enzyme accepts electrons from ferredoxin (Fujita and Bauer 2000; Muraki et al. 2010) and is referred to as “dark” operative POR (DPOR), although it functions both under light and

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dark conditions (reviewed by Armstrong 1998; Fujita and Bauer 2003). POR is a single-subunit enzyme belonging to the short-chain dehydrogenase family that absolutely requires light for catalysis. POR is present in all Chl-synthesizing organisms but is not found in Bchl-synthesizing organisms. In contrast, DPOR is absent in angiosperms but is present in most of the other Chl- and Bchl-synthesizing organisms. Consequently, Arabidopsis is unable to synthesize Chl in darkness. Arabidopsis contains three POR isoforms that are encoded by *PORA* (At5g54190), *PORB* (At4g27440), and *PORC* (At1g03630). *PORA* and *PORB* were first identified in Arabidopsis (Armstrong et al. 1995) and barley (Holtorf et al. 1995). The third POR gene (*PORC*) was subsequently identified in Arabidopsis (Oosawa et al. 2000; Su et al. 2001). The amino acid sequences of PORA, PORB and PORC are nearly identical with the exception of their N-terminal regions which are chloroplast transit peptides. Although the primary structures of these isoforms are similar to each other, their gene expression profiles are strikingly different. Both *PORA* and *PORB* mRNAs are formed in etiolated seedlings, but only *PORB* mRNA continues to accumulate in light-grown plants, whereas *PORA* mRNA rapidly disappears after illumination (Armstrong et al. 1995; Holtorf et al. 1995). 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. The third POR gene (*PORC*) is induced by light (Oosawa et al. 2000).

Studies on the photoreduction of Pchlide were performed on dark-grown angiosperms, which require light to synthesize chlorophyll. In these etiolated seedlings, POR and Pchlide accumulate in large aggregates to form highly organized structures, known as 'prolamellar bodies', in the etioplast membranes (Franck et al. 2000; Sundqvist and Dahlin 1997). Several spectral forms of Pchlide have been identified in these membranes and have been attributed to interactions of the pigment with the membranes and with POR, as well as to pigment aggregation and structural arrangements (Boddi and Franck 1997). On illumination of the plants, the pigment is rapidly photoreduced to chlorophyllide (Chlide), which leads to concomitant breakdown of prolamellar bodies. Thus, the reduction of Pchlide by POR is the first step in the overall greening processes in angiosperms.

The import mechanism for the POR precursor and its assembly into the prolamellar body in plastids has garnered recent attention from researchers and remains highly controversial (Armstrong et al. 2000; Aronsson et al. 2003; Reinbothe et al. 2010). Using *in vitro* reconstitution experiments with the two barley POR isoforms and synthetic zinc analogs of Pchlide *b* and Pchlide *a*, a novel light-harvesting Pchlide *a*/*b*-binding protein complex (LHPP) is proposed with distinct functions for PORA and PORB (Reinbothe et al. 1999). The LHPP complex is predicted 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. 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 proposed to provide a mechanism for photoprotection during the early stage of seedling greening. Meanwhile, it is proposed that the PORA precursor is imported into plastids with a specific translocon which is designated as the Pchlide translocon complex. The import of the PORA precursor occurs in an envelope-bound Pchlide *b*–dependent manner to form a LHPP, whereas PORB is imported with a general translocon.

However, several features of these processes still remain to be confirmed such as the presence of Pchlide *b in vivo*, Pchlide *b*–dependent import of PORA precursor, and the resultant formation of PORA-Pchlide *b* in the LHPP complex (Masuda and Takamiya 2004; Reinbothe et al. 2010). The abundant accumulation of Pchlide *b in vivo* (Reinbothe et al. 2003) has not been reproduced by other researchers (Armstrong et al. 2000; Kollosov and Rebeiz 2003; Scheumann et al. 1999). For discrepancy of the Pchlide-dependent import of PORA, instability of the protein import receptor and the involvement of a high concentration urea in the *in vitro* reaction mixtures have been considered (Aronsson et al. 2000). The import pathway of PORA precursor has been analyzed by using Arabidopsis mutants deficient in Chlide *a* oxygenase (CAO) or one of the Pchlide translocon complex (OEP16), both of which have been implied to be key factors for the import of PORA (Philippar et al. 2007). These mutants possess a normal prolamellar body structure containing PORA. Surprisingly, using the identical mutant of OEP16, the Reinbothe group reported that this mutant showed a conditional lethal phenotype related to defects in import and assembly of PORA (Pollmann et al. 2007). To explain the reason for completely different phenotypes of the same *oep16* line, it is reported that the original seed stock contains two subclasses of T-DNA mutants: one subclass lacking PORA and another subclass with wild-type PORA protein levels (Samol et al. 2011). Further analyses are apparently necessary to conclude the distribution and functional significance of LHPP.

4.5. 3,8-divinyl Pchlide *a* 8-vinyl reductase

Subsequent to the reduction of the D pyrrole ring by POR, the 8-vinyl group on the B ring is reduced by an NADPH-dependent enzyme 3,8-divinyl Chlide 8-vinyl reductase (DVR). Arabidopsis possesses a single *DVR* gene (At5g18660). This gene was identified in the *dvr* mutant that accumulated 3,8-divinyl Chl *a* (often referred to as divinyl Chl or DV-Chl) instead of Chl *a* where an ethyl group is placed at the C8 carbon (Nagata et al. 2005; Nakanishi et al. 2005). Chl *a* is sometimes referred to as monovinyl Chl or MV-Chl in order to distinguish it from DV-Chl. It is important to note that DVR is the only enzyme in plants that is capable of reducing the 8-vinyl group. This conclusion is supported from the analysis of the Arabidopsis *dvr* mutant which lacks functional DVR and consequently results in a complete loss of the MV form of Chls (Nakanishi et al. 2005).

It has been long believed that the reduction of the 8-vinyl group takes place before the reduction of the D ring by POR. This assumption is mainly based on the observation that MV-Pchlide accumulates in etiolated seedlings (see Nagata et al. 2007). Nagata and coworkers (2007) reexamined these steps in etiolated Arabidopsis seedlings and demonstrated that the POR reaction is followed by the DVR reduction in the major route of Chl biosynthesis. Similar to many other plant species, when Arabidopsis seedlings are kept in darkness for several days after germination, they accumulate both DV- and MV-Pchlide (see Nagata et al. 2007). When the authors illuminate these seedlings just for one

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minute, both forms of Pchlide molecules are immediately converted to MV-Chlide without accumulation of a detectable amount of DV-Chlide. These data indicate that once Pchlide is converted to Chlide, the reduction of the DV group to the MV group occurs very quickly. In contrast, when the illuminated seedlings are returned to darkness, DV-Pchlide predominantly accumulates until the MV form dominates the DV form after 120 hours of dark incubation. These results show that the conversion of DV-Pchlide to MV-Pchlide is very slow compared to the quick conversion of DV-Chlide to MV-Chlide.

These physiological data are supported by *in-vitro* experiments. Nagata et al. (2007) examined the substrate specificity of the DVR enzyme using the recombinant DVR protein produced in *E. coli*. Using this system, they showed that DVR is able to reduce the 8-vinyl group of DV-Chlide *a*, but it cannot react with 3,8-DV-Pchlide or DV-Chlide *b* at observable rates *in vitro*. By using an *in vivo* method with isolated cucumber etioplast membranes, Parham and Rebeiz (1995) also report that DV-Chlide *a* can be readily converted to MV-Chlide. The rate of this conversion is estimated to be 50- to 300-fold higher than that of the activity to convert DV-Pchlide to MV-Pchlide in isolated barley etioplasts (Tripathy and Rebeiz 1988). Although the activity within cucumber and barley etioplasts in these experiments cannot be directly compared, these results are consistent with the hypothesis that DVR prefers DV-Chlide to DV-Pchlide. Wang et al. (2010) also report that the recombinant rice DVR protein shows preference of DV-Chlide to DV-Pchlide. Taken together, it is now believed that in the major route for Chl biosynthesis, the POR reaction is followed by the DVR reaction.

4.6. Chl synthase

In the last step of the Chl branch, 17-propionate on the D ring of MV-Chlide *a* is esterified with phytol pyrophosphate by Chl synthase forming Chl *a*. Chl synthase can use either geranylgeranyl pyrophosphate or phytyl pyrophosphate as substrates, but it prefers phytyl pyrophosphate (Oster et al. 1997; Soll et al. 1983). Chl synthase can use both Chlide *a* and Chlide *b* as the substrate, but not bacteriochlorophyllide (Oster et al. 1997; Oster and Rüdiger 1997). Chl synthase cannot use metal-free Chlide *a* derivative or Chlide *a*' (Helfrich et al. 1994).

In Arabidopsis, the *CHLG* (At3g51820) locus encodes Chl synthase (Oster and Rüdiger 1997). In *Avena sativa*, the *CHLG* gene is constitutively expressed in dark-grown and light-grown seedlings (Schmid et al. 2001), although the function of this enzyme in dark-grown seedlings has not yet been identified. From the analysis of esterification kinetics of Chlide, it is suggested that in barley and oat, POR and Chl synthase form a complex having a molar ratio of 7:1 (Domanskii et al. 2003).

4.7. Formation of pheophytin *a* and Chl *a*'

Pheophytin *a* (Omata et al. 1984) and epimeric Chl *a*' (Maeda et al. 1992) are esssential components of photosynthetic reaction center proteins. These molecules are believed to be synthesized (Fig. 1) from Chl *a* molecules. Pheophyrin *a* is a metal-free Chl *a* in which two hydrogen ions replace the central Mg^{2+}. Pheophytin *a* functions as an electron acceptor in photosystem II. Chl *a*' differs from Chl a only in the stereochemistry at $C13^2$ position (Fig. 1). It is found in small but significant amounts in photosystem I, where one molecule forms half of the P700 special pair of Chl that is a primary electron donor. So far, the biosynthetic pathways of these pigments have not been identified.

5. THE CHL CYCLE

5.1. Biosynthesis of Chl *b*

Land plants produce two different species of Chl (Chl *a* and Chl *b*) (Fig. 1) and both function to harvest light energy and subsequently transfer it to other photosynthetic pigments, including other Chl molecules and carotenoids. In addition, Chl *a* is able to carry out photosynthetic charge separation. In accordance with the specific roles of Chl *a*, this pigment is bound to both the core antenna complexes and the peripheral antenna complexes, while Chl *b* is specifically bound to the peripheral antenna complexes.

It is not fully understood why land plants retain Chl *b* in addition to Chl *a*. Researchers have initially thought that the major advantage of the additional presence of Chl *b* is to broaden the ability of plants to absorb different wavelengths of light, mainly in the blue region. However, considering that the blue region of light can also be efficiently absorbed by carotenoids, this advantage may not be as important as initially hypothesized. The major role of Chl *b* is suggested to stabilize the peripheral antenna complexes which are comprised of light-harvesting Chl-binding proteins (LHC proteins) (see Hoober et al. 2007; Tanaka and Tanaka 2010 for detailed discussion on this topic). Supporting this hypothesis, genetically-enhanced biosynthesis of Chl *b* has been reported to result in increased accumulation of LHC (Hirashima et al. 2006; Nagata et al. 2005; Tanaka et al. 2001) in transgenic Arabidopsis plants.

The proposed biosynthetic route of Chl *b* includes two reactions: the conversion of Chlide *a* to Chlide *b* and a subsequent phytylation step (Fig. 7) (Oster et al. 2000). The first reaction is catalyzed by a Rieske-type monooxygenase, Chlide *a* oxygenase (CAO) (Espineda et al. 1999; Oster et al. 2000; Tanaka et al. 1998), and the second reaction is catalyzed by Chl synthase (Oster and Rüdiger 1997). Using a recombinant Arabidopsis CAO enzyme produced in *E. coli*, Oster et al. (2000) show that CAO catalyzes the incorporation of an oxygen atom into the 7-methyl group of Chlide *a*. The authors propose that this enzyme actually catalyzes successive incorporation of two oxygen atoms into the $C7^1$ position to yield an aldehyde hydrate, which then spontaneously loses water to form Chlide *b*. In addition to the aforementioned route, it is also hypothesized that CAO is able to directly convert Chl *a* to Chl *b* *in vivo* (Fig. 7; dotted line) (Tanaka and Tanaka 2010). This hypothesis is based on the observation that chlorophyll *a*-to-*b* conversion takes place in angiosperms in darkness (Tanaka et al. 1995; Tanaka and Tsuji 1981, 1982; Tanaka et al. 1992). This observation indicates two possibilities: Chl *a* is directly converted to Chl *b* (Fig. 7; dotted line), or Chl *a* is first dephytylated to form Chlide *a* by the action of an enzyme called chlorophyllase (see Section 8), and subsequently Chlide *a* may be converted to Chlide *b* by CAO and finally conjugated with phytol to form Chl *b* (Fig. 7, solid line). Now that evidence suggests localization of chlorophyllase outside the chloroplast (see Section

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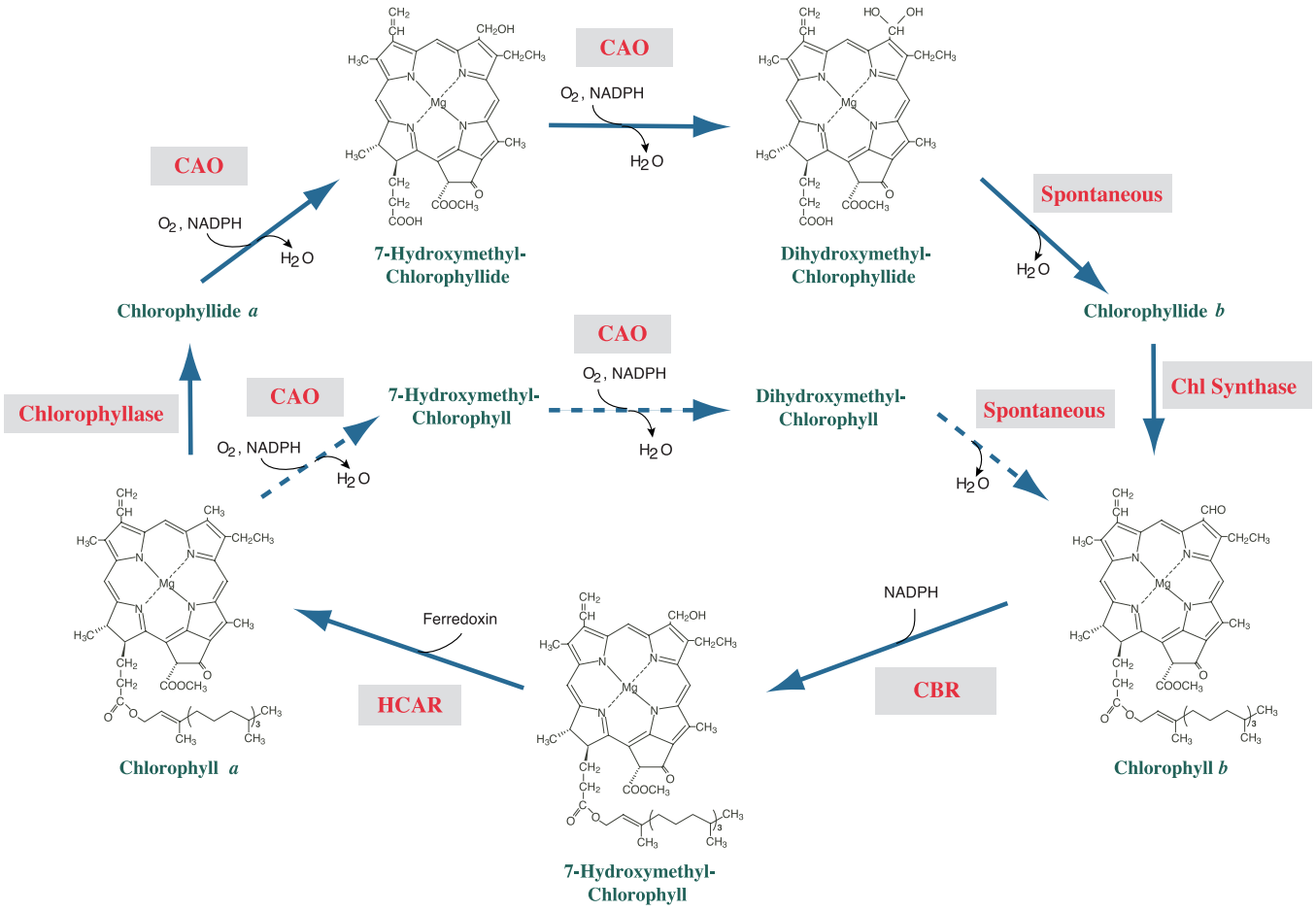


Figure 7. The Chl cycle.

In the Chl cycle, an interconversion between Chl *a* and Chl *b* occurs. The proposed biosynthetic route of Chl *b* includes two reactions: the conversion of Chlide *a* to Chlide *b* and a subsequent phytylation step (solid line). It is also hypothesized that a direct conversion of Chl *a* to Chl *b* occurs *in vivo* (dotted line). The conversion of Chl *b* to Chl *a* consists of two reactions which are catalyzed by two separate enzymes. The first reaction is the reduction of the C7 formyl group of Chl *b* into a hydroxyl group, which yields 7-hydroxymethyl Chl *a*. The second reaction is the reduction of 7-hydroxymethyl Chl *a* to Chl *a*, although the enzyme (7-hydroxymethyl Chl *a* reductase: HCAR) involved in this step has not been identified yet.

8.1 for detailed discussion on this topic), the route for the conversion of Chl *a* to Chl *b* through Chlide forms (Fig. 7; solid line) may not be available in darkness. Taken together, unless chlorophyllase activity is detected within the chloroplast, direct conversion of Chl *a* to Chl *b* is most likely to occur (at least) in darkness.

5.2. Conversion of Chl *b* to Chl *a*

Plants have the ability to reconvert Chl *b* into Chl *a*. This function is considered to be important for two biological processes, that is, light acclimation and Chl breakdown. During the acclimation of low-light grown plants to high-light conditions or other stressful conditions, Chl *a*-to-*b* ratios are known to increase (e.g. Murchie and Horton 1997, 1998) and this adaptive response of plants is probably due to increased activity of the Chl *b*-to-*a* conversion. As described above, Chl *b* levels are correlated with the construction of LHC, therefore, the conversion of Chl *b*-to-*a* likely functions to reduce the amount of LHC per photosynthetic reaction center. This adaptive response functions to compromise the excessive excitation of the photosystems under stressful conditions.

The conversion of Chl *b*-to-*a* is also important during the process of Chl breakdown. Several lines of evidence indicate that plants are capable of degrading only Chl *a*, but not Chl *b*. This specificity in the degradation of Chl is most evident in the observation that Chl breakdown products almost exclusively have a methyl group at the C7 position (see Hörtensteiner and Kräutler 2011). It is also known that at least one enzyme of Chl breakdown is specific for an intermediate of a Chl *a* catabolite (pheophorbide *a*) but does not accept a similar intermediate of Chl *b* catabolite (pheophorbide *b*) (Hörtensteiner et al. 1995). The Chl *b*-to-*a* conversion is necessary for the degradation of LHC during leaf senescence. Specifically, Arabidopsis and rice mutants lacking

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Chl *b*-to-*a* converting activity retain LHC together with the photosynthetic pigments bound to LHC for a substantially longer time period relative to wild type plants during leaf senescence (Horie et al. 2009; Kusaba et al. 2007).

It would be reasonable to assume that when one half of the cycle is active, the other half of cycle is inactive. Otherwise, this would just be an energy consuming futile cycle. At the present time, the mechanisms that balance the activity of both halves of the Chl cycle are not completely understood. Pulse-chase labeling experiments with $^{14}CO_2$ indicate that the turnover of Chl *b* is very low compared to that of Chl *a* in mature Arabidopsis leaves (Beisel et al. 2010). It is likely that Chl cycle activities are maintained at low levels unless the conversion between Chl *a* and Chl *b* is needed under specific conditions or at specific developmental stages. For example, expression of the *NYC1* gene encoding an enzyme involved in the conversion of Chl *b* to Chl *a* (see below) is specifically induced during leaf senescence (Kusaba et al. 2007).

The conversion of Chl *b* to Chl *a* consists of two different reactions which are catalyzed by two separate enzymes. The first reaction is the reduction of the C7 formyl group of Chl *b* into a hydroxyl group, which yields 7-hydroxymethyl Chl *a*. This reaction is performed by Chl *b* reductase (CBR) which functions as an NADPH-dependent short-chain dehydrogenase (Kusaba et al. 2007). Higher plants have two isoforms of this enzyme which are encoded by the *NYC1* and *NOL* genes, respectively (Kusaba et al. 2007). The *NYC1* gene encodes a ~50 kDa protein which contains three putative membrane-spanning regions. In contrast, the *NOL* gene encodes a ~30 kDa protein which lacks any putative membrane-spanning regions. In both Arabidopsis and rice, deficiency of the NYC1 isoform results in a significant delay in Chl *b* breakdown during prolonged dark incubation. These data indicate that NYC1 plays a major role in Chl *b* degradation under dark conditions (Horie et al. 2009; Kusaba et al. 2007). Similarly, a rice mutant lacking the NOL isoform also show a significant delay in the breakdown of Chl *b* (Sato et al. 2009). These results prompt the authors to propose that the NYC1 and NOL isoforms form a functional heterodimer *in vivo* to catalyze the reduction of Chl *b* in rice (Sato et al. 2009). In contrast, the Arabidopsis *nol* mutant shows a slight delay in Chl *b* breakdown under the same conditions (Horie et al. 2009). These results indicate that Arabidopsis NYC1 is able to catalyze the reduction of Chl *b* independent from the NOL isoform. It should be noted that the both of the NOL isoforms from rice and Arabidopsis are able to catalyze Chl *b* reduction *in vitro* (Horie et al. 2009; Kusaba et al. 2007). Taken together, it is likely that both NYC1 and NOL possess CBR activity and that they function as a hetero- and homo-oligomers under different conditions.

The second reaction of the Chl *b* to Chl *a* conversion is the reduction of 7-hydroxymethyl Chl *a* to Chl *a* (Fig. 7), which is catalyzed by 7-hydroxymethyl Chl *a* reductase (HCAR). This enzyme has not been identified yet. The activity of this enzyme was first demonstrated with isolated barley etioplasts, in which exogenously added 7-hydroxymethyl Chlide *a* and phytylpyrophosphate is converted to Chl *a* by the combined action of HCAR and Chl synthase (Ito et al. 1996). HCAR requires reduced ferredoxin for its reaction (Scheumann et al. 1998). Although the activity of HCAR was detected in barley leaves that are not senescing, it increases nearly five folds after the senescence of the leaves are induced in continuous darkness (Scheumann et al. 1999). These results support the notion that the conversion of Chl *b* to Chl *a* via 7-hydroxylmethyl Chl *a* is essential in Chl breakdown (see Section 8).

6. THE HEME/BILIN BRANCH

Hemes are essential molecules that are responsible for various biological activities including oxygen metabolism and transfer, electron transfer, and secondary metabolism. Hemes are classified according to the type of functional groups that are attached to the periphery of their tetrapyrrole macrocycle (Fig. 2). Specifically, the *c*-type heme has four methyl groups, two propionic acids, and two vinyl-thioether groups. The *b*-type heme has two vinyl groups and is referred to as protoheme (iron Proto IX) or heme *b*. The *a*-type heme has a formyl group instead of the methyl group and also has a farnesylated group instead of a vinyl group. In most organisms two vinyl thioether linkages are formed between cysteine side chains and both heme *b* vinyl groups in *c*-type cytochromes, while some organisms such as a unicellular protist *Euglena gracilis* and a marine flagellate *Diplonema papillatum* have only one bound mitochondrial cytochrome *c* heme (Allen et al. 2008a). In contrast, the porphyrin periphery of *a*- and *b*-type hemes is not covalently bound to the hemoprotein. In the heme/bilin branch (Fig. 8), protoheme is the first product formed by the insertion of Fe^{2+} into Proto IX. Protoheme is further used as substrate for heme *a* and heme *c* biosynthesis, as well as bilin biosynthesis, by oxidative cleavage. Although the precise subcellular localization of heme *a* biosynthesis has not been confirmed in plants, it is hypothesized to occur in mitochondria (Schneegurt and Beale 1986).

6.1. Ferrochelatase

Proto IX ferrochelatase, or ferrochelatase (FeCh), inserts Fe^{2+} into Proto IX to form protoheme. Although FeCh and MgCh catalyze similar reactions, the structure of FeCh is completely different from MgCh. FeCh is a single-subunit enzyme encoded by a single gene and does not require a cofactor or external energy source for catalysis that is categolized as class II chelatase (Brindley et al. 2003). cDNA clones of Arabidopsis (Smith et al. 1994) and cucumber (Miyamoto et al. 1994) FeCh are first isolated by functional complementation of *hemH* mutants of yeast and *E. coli*, respectively. Subsequently, Arabidopsis was found to possess two FeCh isoforms: *FC1* (At5g26030) and *FC2* (At2g30390) (Chow et al. 1998). A characteristic feature of FC2 is the hydrophobic C-terminal extension with a putative Chl-binding motif (LHC motif) which is conserved in the FeCh sequences from cyanobacteria and higher plants (Suzuki et al. 2002a). In *Synechocystis*, Sobotka et al. (2008) first showed that the LHC motif is necessary for the activity of cyanobacterial FeCh, and subsequently they proposed that the LHC motif plays a regulatory role and a spacer region between catalytic and LHC motifs designated “region II” is essential for catalysis (Sobotka et al. 2011). In Arabidopsis, *FC2* is only expressed in photosynthetic tissue (Singh et al. 2002) and is therefore likely involved with heme production for photosynthetic cytochromes and hemoproteins. The *FC1* gene, which does not encode the LHC motif, is ubiquitously expressed throughout

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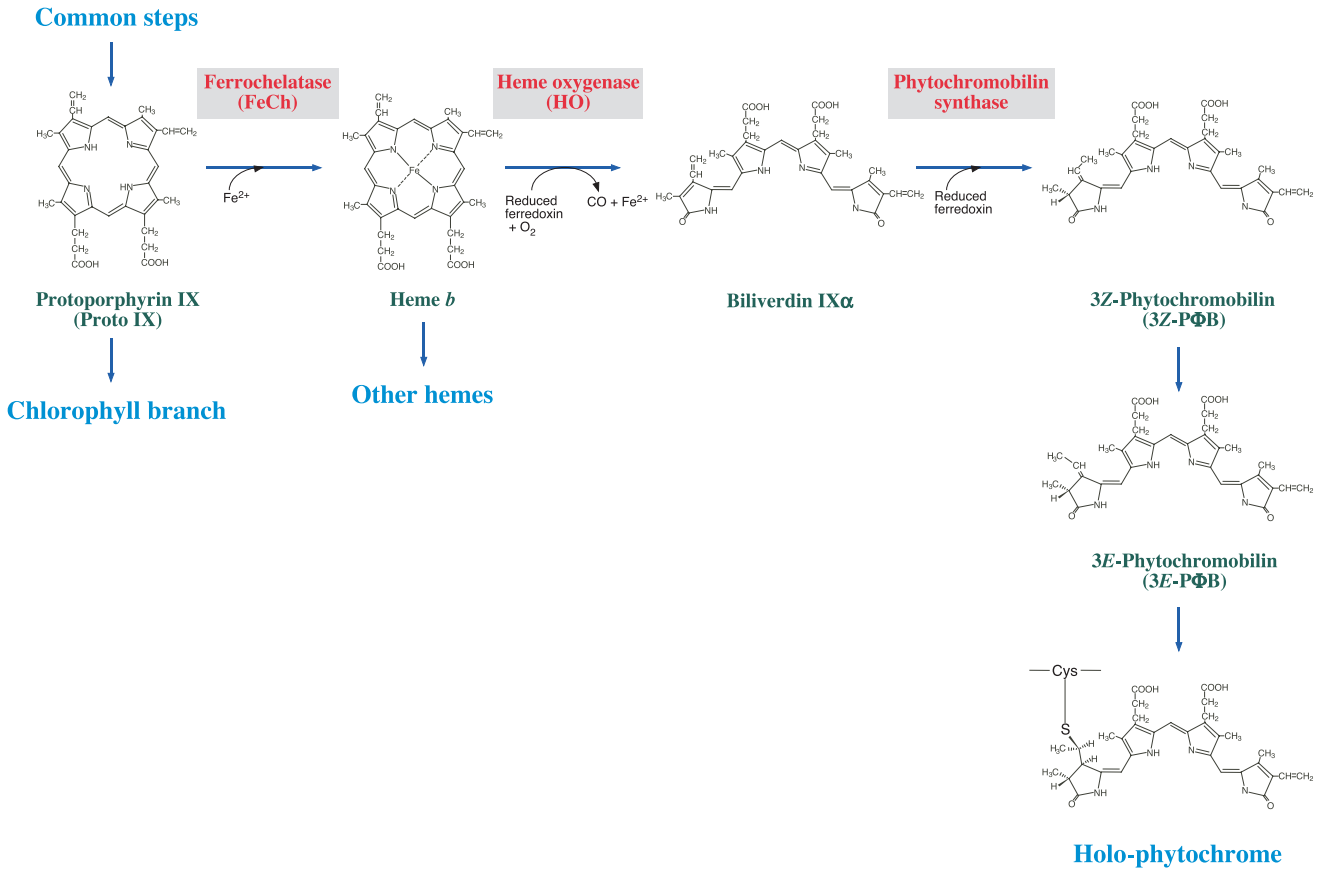


Figure 8. The heme/bilin branch.

In the heme/bilin branch, heme *b* (protoheme) is synthesized by the insertion of Fe^{2+}. Plants synthesize other hemes, such as heme *a* and heme *c*, however the biosynthetic steps responsible for their biosynthesis have not yet been identified in higher plants. Heme is subsequently oxidized and its ring structure is opened and then reduced to form 3*Z*-phytochromobilin (PΦB), the chromophore of the phytochrome family of photoreceptors. Enzymatic or spontaneous isomerization of 3*Z*-PΦB into the 3*E* isomer occurs before the chromophore is bound to the phytochrome apoprotein.

plant tissues (Smith et al. 1994). Expression of *FC1* in leaves is markedly increased in response to environmental stresses such as wounding or viral infection (Singh et al. 2002). Expression of the *FC1* gene may be regulated in response to the cellular demands for heme in the respiratory cytochromes and hemoproteins, which are involved in the defense response (Nagai et al. 2007; Singh et al. 2002).

6.2. Heme oxygenase

Heme oxygenase (HO) catalyzes the oxidation and ring opening of protoheme and yields biliverdin IXα, CO, and Fe^{2+} in a reaction requiring molecular oxygen and electrons from ferredoxin (Gisk et al. 2010; Terry et al. 2002). In plants, HOs are required for the synthesis of the chromophore of the phytochrome family of photoreceptors. Arabidopsis possesses four HO isoforms: HO1 (At2g26670), HO2 (At2g26550), HO3 (At1g69720), and HO4 (At1g58300). The HO isoforms fall into two distinct subfamilies: the HO1 subfamily (HO1, HO3 and HO4) and the HO2 subfamily (HO2), which are widely present in higher plants (Davis et al. 2001; Terry et al. 2002). In Arabidopsis, *HO1* shows the highest expression level followed by *HO2* and expression of *HO3* and *HO4* is essentially very low in any type of tissue (Emborg et al. 2006; Matsumoto et al. 2004). The *hy1* mutant is deficient in HO1 activity and exhibits a characteristic phytochrome-deficient long hypocotyl phenotype, showing that HO1 plays a major role in biliverdin IXα synthesis (Davis et al. 1999; Muramoto et al. 1999). In addition to HO1, both HO3 and HO4 are also capable of converting heme to biliverdin IXα in *E. coli,* which coexpresses the phytochrome apoprotein (BphP) from *Deinococcus radiodurans* and naturally employs biliverdin IXα as its chromophore (Emborg et al. 2006). Furthermore, these isoforms show HO activity as monomeric enzymes *in vitro*, demonstrating that all HO1 subfamily members are *bona fide* HOs (Gisk et al. 2010). In accordance with these results, it is shown that disruption of HO3 and HO4 in the *hy1* background result in further reduction in the responsiveness to red and far-red light, reflecting a supportive role of these less abundant isoforms (Emborg et al. 2006). Since kinetic parameters are comparable among the three HO1 subfamily members (Gisk et al. 2010), transcriptional activities of these isoforms may differentiate their importance *in vivo*. Compared with the HO1 subfamily, the HO2 subfamily contains an inserted ~35 amino acid spacer

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sequence which is rich in Glu, Asp and Gly residues. Moreover, all HO2 subfamily members lack the conserved His residue in the active site, which is considered to be required for heme-iron binding (Davis et al. 2001). Indeed, *in vitro* studies using the recombinant Arabidopsis HO2 proteins have failed to detect HO activity, implying that HO2 is not a functional HO (Emborg et al. 2006; Gisk et al. 2010). Nevertheless, a T-DNA insertion mutant of Arabidopsis HO2 showed small decreases in holo-phytochrome and light responses, suggesting that this isoform also has a minor role in phytochromobilin (PΦB) synthesis (Davis et al. 2001). Intriguingly, although this protein is unable to bind heme, it is capable of binding strongly to Proto IX (Gisk et al. 2010). It is suggested that the HO2 subfamily has a role in the regulation of the tetrapyrrole flux in the plastid through the binding of tetrapyrrole intermediates (Gisk et al. 2010).

6.3. PΦB synthase

In plants, biliverdin IXα is reduced to PΦB, which is the chromophore of the phytochrome family of photoreceptors, by PΦB synthase. In Arabidopsis, a single isoform of PΦB synthase (HY2) is encoded by the At3g09150 locus (Kohchi et al. 2001). The primary product of PΦB synthase is actually the 3*Z*-isomer of PΦB, while 3*E*-PΦB is believed to be the immediate precursor of the bound chromophore (Terry et al. 2002) (Fig. 8). In addition, both Beale and Cornejo (1991) and Frankenberg et al. (2001) observe the glutathione and heat stimulated 3*Z* to 3*E* spontaneous isomerization of phycobilins *in vitro*. Thus, isomerization of 3*Z*-PΦB into the 3*E* isomer occurs before the chromophore is bound to the phytochrome apoprotein. At the present time, it is not known whether this isomerization step is catalyzed by an enzyme or if it proceeds spontaneously (Terry et al. 2002). HY2 is a member of the ferredoxin-dependent bilin reductase family which includes phycocyanobilin:ferredoxin oxidoreductase (PcyA; EC 1.3.7.5) from cyanobacteria. Using Arabidopsis as a model, Chiu et al. (2010) analyze the interaction of HY2 and six isoforms of ferredoxin and concluded that the AtFd2 isoform of ferredoxin is the main electron donor of HY2. It is hypothesized that ferredoxin tentatively binds the biliverdin-bound form of HY2 and directly transfers an electron to the biliverdin molecule. Subsequent to protonation, ferredoxin may dissociate from HY2 because of conformational changes in ferredoxin and HY2 (Chiu et al. 2010).

6.4. Heme *c* biosynthesis (CCM machinery)

Regarding the biosynthesis of heme *c*, the mechanism of mitochondrial cytochrome *c* biogenesis involved in the respiratory chain has been extensively studied (reviewed by Giege et al. 2008; Kranz et al. 2009). In general, two cysteines in a heme binding motif, "CXXCH ", of the protein are linked by two thioether bonds to the two vinyl groups of heme (Fig. 2). Three assembling systems for heme *c* have been identified. Prokaryotes use the pathways called systems I and II. In general, α-, γ-, and some other proteobacteria and Archae use system I (alternatively referred to as the CCM system) comprising eight proteins (CcmABCDEFGH), while the other bacteria use system II which minimally consists of two cytochrome *c* synthase (CCS) proteins, CcsB and CcsA. Fungal and animal use system III, which is dependent on the cytochrome *c* heme lyase (CCHL) (Kranz et al. 2009). Higher plants have specific proteins that are similar to system I bacterial counterparts which are required for the assembly of cytochrome *c* (CCM proteins; *C*ytochrome *C M*aturation) (Giege et al. 2008). In Arabidopsis, genes encoding these proteins are present within both the nuclear and mitochondrial genomes and encode an ABC transporter (CcmA and CcmB), a heme delivery pathway (CcmC), a heme chaperone (CcmE), a putative heme lyase (CcmF), and a redox protein (CcmG). In Arabidopsis, 5 *ccm* genes (*ccmB*, *ccmC*, $ccmF_{N1}$, $ccmF_{N2}$ and $ccmF_{C}$) are identified in the mitochondrial genome, while *CCMA*, *CCME* (At3g51790) (Spielewoy et al. 2001) and *CCMH* (At1g15220) (Meyer et al. 2005) are found in the nucleus.

Chloroplasts contain two *c*-type cytochromes, membrane-anchored cytochrome *f* and soluble cytochrome c_6 (Mathews 1985). Components involved in the photosynthetic cytochrome *c* assembly have been identified by genetic screening of *C. renihardtii* (Howe and Merchant 1992; Howe et al. 1995; Xie et al. 1998). It is proposed that the chloroplast cytochrome *c* is assembled by the system II (CCS). The *ccsA* gene and four nuclear *CCS1–CCS4* genes are required for the heme attachment step during assembly of both holocytochrome *f* and holocytochrome c_6 (Xie et al. 1998). Xie et al. (1998) propose the multisubunit "holocytochrome *c* assembly complex" in which CCS1 is associated with CcsA, probably together with other CCS subunits.

Determination of crystal structures of the cytochrome b_6f complex have identified three prosthetic groups, a Chl *a*, a β-carotene, and a unique heme *x* molecule, which are not found in the cytochrome bc_1 complex from the mitochondrial respiratory chain (Kurisu et al. 2003; Stroebel et al. 2003). Heme *x,* in which "x" nomenclature is used because the coordination of the heme in the protein is unprecedented, is covalently bound by a single thioester link to cytochrome b_6f. Heme *x* seems to be electronically coupled to heme b_n and occupy the position correspoinding to the ubiquinone-binding site in the bc_1 complex, suggesting its involvement in the Q cycle reaction mechanism and probably in PS I-mediated cyclic electron transport (Cramer et al. 2005). For function of the Chl *a* molecule whose phytol tail is bound to the quinone/quinol transfer domain of the subunit IV of the cytochrome b_6f, it is suggested that this molecule participates in the activation of the LHC kinase and photochemical generation of its triplet state may be quenched by the β-carotene (Cramer et al. 2005).

7. SIROHEME BRANCH

Higher plant sulfite and nitrite reductases contain siroheme as a prosthetic group. Siroheme is synthesized from the common tetrapyrrole precursor Urogen III in three steps involving methylation, oxidation, and ferrochelation reactions (Fig. 9). In *E. coli*, a single enzyme encoded by *cysG* catalyzes all three reactions (Stroupe et al. 2003), while in yeast, this transformation requires two enzymes: Met1p (a Urogen III methyltransferase) and Met8p (a bifunctional dehydrogenase and sirohydrochlorin FeCh) (Schubert et al. 2002). In bacteria, SAM-dependent Urogen III methyltransferase is identified from eubacteria (Blanche et al. 1989) and Archae (Blanche et al. 1991). In some bacteria including *Bacilus megaterium*, the transformation of dihydrosirohydrochlorin (precorrin-2) into siroheme is catalyzed by two separate enzymes called SirC (dihydrosirohydrochlorin dehydro-

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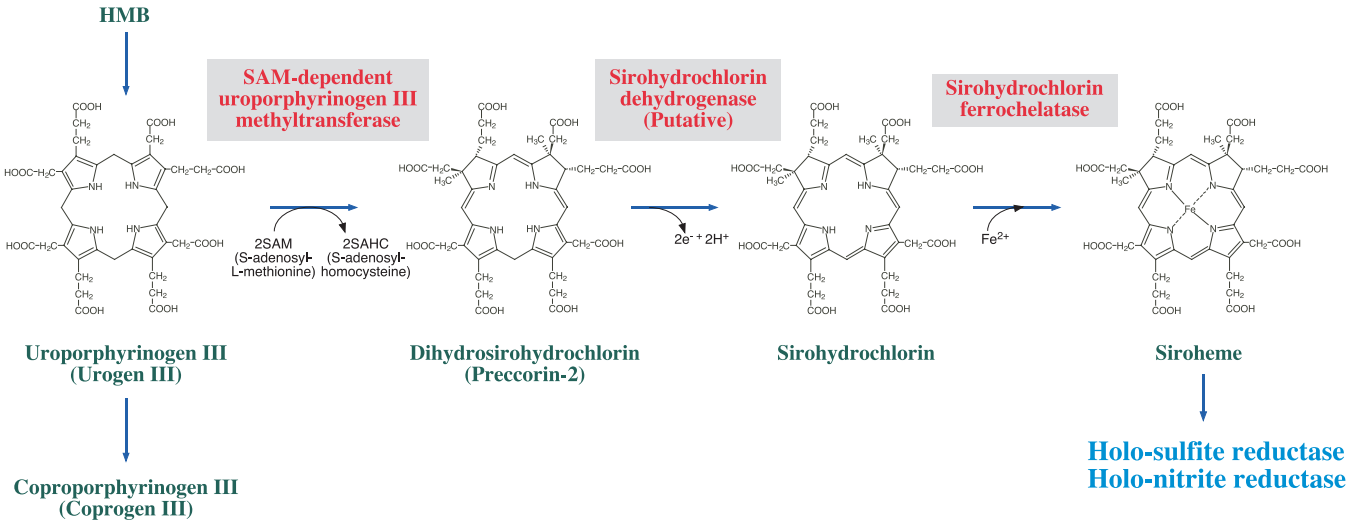


Figure 9. The siroheme branch.

Higher plant sulfite and nitrite reductases contain siroheme as a prosthetic group that play central roles in nitrogen and sulfur assimilation, respectively. Siroheme is synthesized from the Urogen III in three steps involving methylation, oxidation, and ferrochelation reactions. Although a single enzyme is known to catalyze all three reactions in *E. coli*, it is presumed that these activities are separated into three distinct enzymes in higher plants in which the first and third enzymes have been identified so far.

genase) and SirB (sirohydrochlorin ferrochelatase) (Johansson and Hederstedt 1999; Raux et al. 2003). However, in the plastids of higher plants, these activities seem to be separated into three distinct enzymes.

7.1. SAM-dependent Urogen III methyltransferase

The first step of the siroheme branch is the methylation of Urogen III to form precorrin-2. In Arabidopsis, this reaction is catalyzed by an SAM-dependent Urogen III methyltransferase encoded by *UPM1* (At5g40850) (Leustek et al. 1997). *UPM1* encodes a 39.9-kDa protein containing two regions that are identical to consensus sequences found in bacterial Urogen III and precorrin methyltransferases encoded by *SirA*.

7.2. Precorrin-2 dehydrogenase

Subsequently, precorrin-2 is oxidized by an unidentified oxidase to form sirohydrochlorin. In *Bacillus megaterium* bacterium, precorrin-2 is converted to sirohydrochlorin by an NAD-dependent dehydrogenase encoded by the *SirC* gene (Raux et al. 2003). Intriguingly, *SirC* homologues appear to be absent from the Arabidopsis genome, suggesting the possibility that Arabidopsis is capable of synthesizing siroheme without precorrin-2 dehydrogenase. In *E. coli,* the presence of SAM-dependent Urogen III methyltransferase and sirohydrochlorin FeCh is sufficient for siroheme biosynthesis (Warren et al. 1994). In this organism, it is hypothesized that dihydrosirohydrochlorin is spontaneously oxidized to sirohydrochlorin with a dinucleotide (NAD^+ or $NADP^+$) dependent manner (Warren et al. 1994).

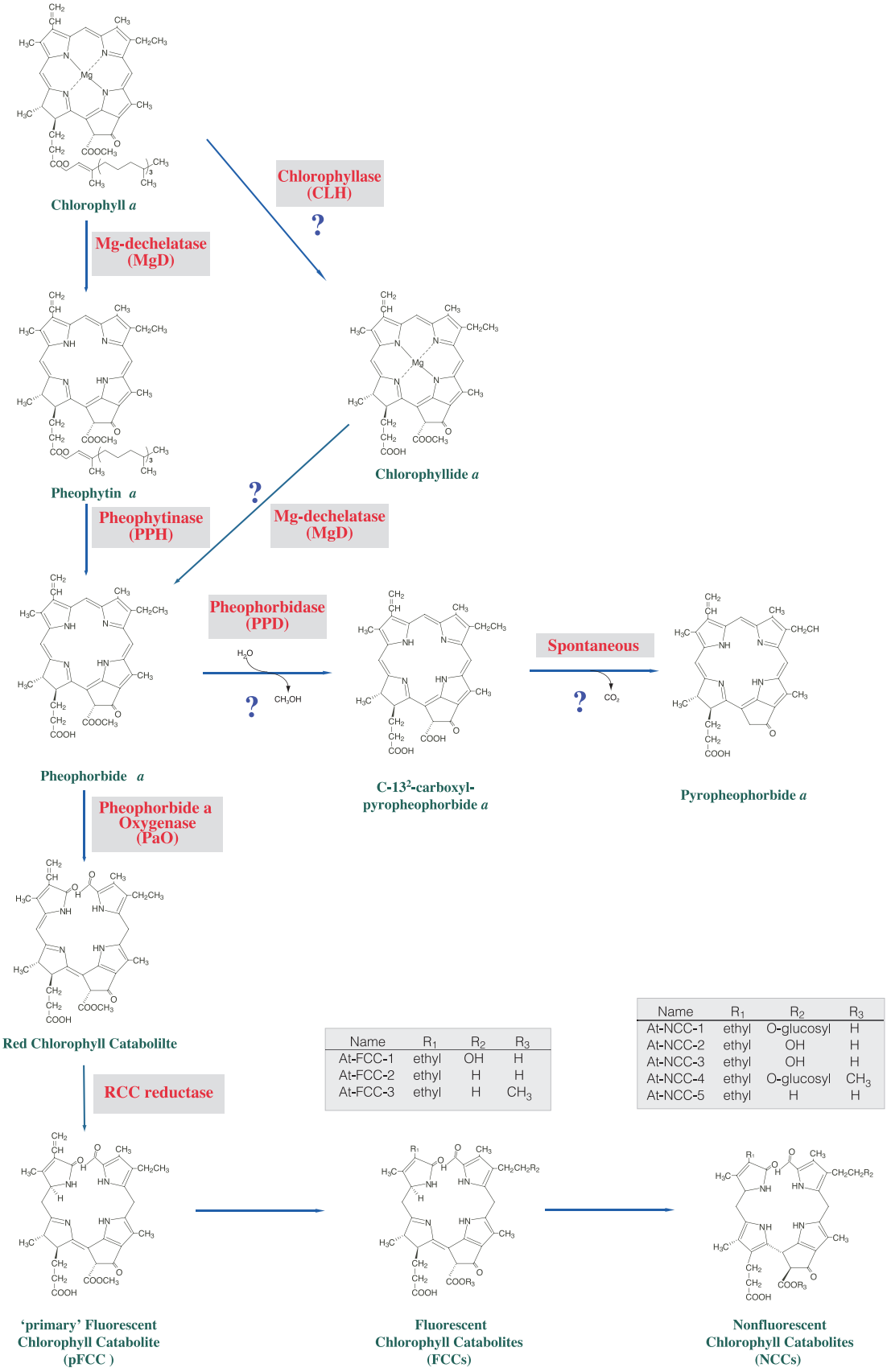
7.3. Sirohydrochlorin FeCh

In Arabidopsis, the *SirB* gene (At1g50170) encodes sirohydrochlorin FeCh which functions to insert Fe^{2+} into sirohydrochlorin for the formation of siroheme (Raux-Deery et al. 2005). The Arabidopsis SirB protein is synthesized as a precursor consisting of 225 amino acids, including a putative 79 amino acid N-terminal transit peptide. Sirohydrochlorin FeCh activity of SirB is demonstrated with both *in vitro* and *in vivo* methods. Transformation of the *E. coli* mutant lacking siroheme-synthesizing ability with the Arabidopsis *SirB* gene and the *Pseudomonas denitrificans cobA* gene encoding SAM-dependent Urogen III methyltransferase complement the deficiency of siroheme (Raux-Deery et al. 2005). The mature form of SirB is suggested to contain a 2Fe-2S center (Raux-Deery et al. 2005). The structure of this enzyme resembles that of Proto IX FeCh in the heme/bilin branch (Al-Karadaghi et al. 1997; Raux-Deery et al. 2005). The possibility that SirB is also involved in the precorrin-2 dehydrogenase reaction cannot be ruled out.

8. CHL BREAKDOWN

Chl breakdown is an important biological process for two main reasons. Firstly, this process enables the dissociation of Chl from Chl-binding proteins, which is an essential step for the degradation of these proteins and the remobilization of nitrogen to sink organs. In addition, the removal of Chl and its breakdown intermediates is crucial as these molecules readily generate singlet oxygen and are potentially toxic to cells. Within plastids, a series of enzymatic reactions convert Chl into linear colorless tetrapyr-

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Name	R_1	R_2	R_3
At-FCC-1	ethyl	OH	H
At-FCC-2	ethyl	H	H
At-FCC-3	ethyl	H	CH_3

Name	R_1	R_2	R_3
At-NCC-1	ethyl	O-glucosyl	H
At-NCC-2	ethyl	OH	H
At-NCC-3	ethyl	OH	H
At-NCC-4	ethyl	O-glucosyl	CH_3
At-NCC-5	ethyl	H	H

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role derivatives which are referred to as primary fluorescent Chl catabolites (pFCC, see Fig. 12). pFCC molecules are then exported to the vacuole and are finally broken down to monopyrrole molecules (reviewed by Hörtensteiner 2006; Hörtensteiner and Kräutler 2011). It is possible that the monopyrrole molecules are further metabolized to smaller molecules, although the exact fate of the monopyrroles is not known at the present time.

In the Chl breakdown pathway, it has been long hypothesized that dephytylation is the first step in the degradation of Chl *a* that is catalyzed by a hydrolysis enzyme (chlorophyllase) (Fig. 10). Supporting this model, Harpaz-Saad et al. (2007) demonstrate that overexpression of chlorophyllase is sufficient to induce Chl breakdown. However, Hörtensteiner and his coworkers present evidence that Chl breakdown begins with dechelation during leaf senescence (Schelbert et al. 2009). Although their data are convincing, it still seems plausible that two Chl breakdown pathways coexist. In this section, the "old" and "new" pathways are both described with the respective supporting evidence for either of the pathways (Fig. 10).

8.1. The "old" pathway

As described above, removal of the phytol tail from the tetrapyrrole ring of Chl has been long described as the first step of Chl *a* breakdown (Fig. 10). This step is catalyzed by the very active and stable chlorophyllase enzyme which shows enzymatic activity even in the presence of 20% acetone or after precipitation with acetone and resolubilization (Tsuchiya et al. 1999; Tsuchiya et al. 2003). It is possible that the stability of chlorophyllase may be the reason why it is one of the earliest enzymes whose activity were characterized by researchers (Willstätter and Stoll 1913). The activity of chlorophyllase is so pronounced in plant tissues that researchers never hypothesized that it may not be involved in Chl breakdown until Hörtensteiner's group questioned its role in Chl breakdown (Schelbert et al. 2009; Schenk et al. 2007).

Genes encoding chlorophyllase were first isolated from *Chenopodium album* (Tsuchiya et al. 1999) and *Citrus sinensis* (Jacob-Wilk et al. 1999) and two homologous genes are found in Arabidopsis (*CLH1*; At1g19670 and *CLH2*; At5g43860) (Tsuchiya et al. 1999). The Arabidopsis *CLH1* gene encodes a chlorophyllase isoform with a putative N-terminal endoplasmic reticulum signal sequence, however, the precise intracellular localization of CLH1 protein is under debate. Schenk et al. (2007) use a CLH1-GFP fusion protein in a protoplast based system and report that the Arabidopsis CLH1 protein is localized outside the chloroplast. In contrast, Shemer et al. (2008) detect citrus CLH1 in the plastid by *in-situ* immunofluorescence labeling. Further experiments may be necessary to solve this discrepancy. Expression of the *CLH1* gene is induced by methyl-jasmonate in leaves (Tsuchiya et al. 1999), suggesting that the *CLH1*-encoded chlorophyllase is involved in the breakdown of Chl which is induced by certain types of biotic and abiotic stresses, including wounding and microbial attack. This suggestion is consistent with the report of Kariola et al. (2005) in which they describe that down-regulation of the *CLH1* gene results in increased susceptibility to the necrotrophic fungus *Alternaria brassicicola*. The authors speculate that CLH1 may induce accumulation of Chlide, which may lead to the production of reactive oxygen species (ROS). As ROS plays an essential role in the protective responses of plants against pathogens, reduction in the ROS production may lead to alter the susceptibility of plants to pathogens. This hypothesis is intriguing as it explains the link between the Chl breakdown pathway and pathogen responses. However, the same report also describes an oppsite effect of *CHL1* down-regulation, in which the RNAi interferance silencing of *CLH1* results in resistance to a pathogenic bacterium (*Erwinia carotovora*) in transgenic Arabidopsis (Kariola et al. 2005). In the future, it will be important to elucidate the mechanism which enables CLH1 to modify plant responses to pathogen attack.

The *CLH2* gene encodes the second form of chlorophyllase in Arabidopsis. In contrast to *CLH1*, it encodes the putative transit peptide for the import into plastids. Therefore, it would seem plausible that the *CLH2*-encoded chlorophyllase functions within the chloroplast. However, to the best of our knowledge, there are no experimental data reported for the presence of CLH2 in chloroplasts. Many reports describe chlorophyllase activity associated with the chloroplast (summarized in Shemer et al. (2008)), but these activities could be attributed to contamination of membranes from other organelles. Schenk et al. (2007) also report that a CLH2-GFP fusion protein is localized outside the chloroplast. Taken together with other experimental evidence provided by Hörtensteiner's group, the hypothesis that pheophytinase, rather than chlorophyllase, plays a more significant role in Chl breakdown appears to be the dominant hypothesis at the present time.

In the "old" hypothesis of the Chl breakdown pathway, removal of the Mg^{2+} ion from the tetrapyrrole ring follows the dephytylation step. Although there have been significant efforts from many researchers, the Mg-dechelatase enzyme which is responsible for

Figure 10. Chl breakdown.

There are two separate pathways for Chl breakdown that have been proposed to date. The first is the "old" pathway in which Chl *a* is first dephytylated by a hydrolysis enzyme, chlorophyllase, and subsequently dechelated by putative Mg-dechelatase. The second is the recently identified "new" pathway in which Chl breakdown begins with dechelation, which is followed by dephytylation by pheophytinase. Genetic analysis suggests the "new" pathway is much more physiologically significant than the "old" one during leaf senescence. In either pathway, the final step is the ring opening of the pheophorbide *a* which is a crucial step for red Chl catabolite (RCC) formation. Pheophorbidase, which is distributed among the Brassicaceae and a few other species, catalyzes the demethylation of pheophorbide *a* to form C-13(2)-carboxylpyropheophorbide *a*. This compound is spontaneously converted to pyropheophorbide *a*. At the present time, it is not clear how pyropheophorbide is further metabolized. RCC is further degraded into primary fluorescent Chl catabolite by RCC reductase. Subsequently, pFCC is modified at the C3, $C8^2$ and $C13^2$ positions to form modified FCCs. The enzymes that are responsible for the modification of pFCC remain to be identified. Modified FCCs are imported into vacuoles and nonenzymatically tautomerized to NCCs (non-fluorescent Chl catabolites) under the acidic conditions in vacuoles.

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this step has not yet been identified. Shioi and coworkers (Shioi et al. 1996; Suzuki et al. 2002b) propose that low-molecular weight compounds (less than 400 Da) are responsible for this reaction. Costa et al. (2002) also report low-molecular weight compounds of 2180 Da with Mg-dechelatase activity from strawberries (Fragaria x ananassa).

8.2. The "new" pathway

Schenk et al. (2007) report that Chl degradation in the Arabidopsis *clh1*/*clh2* double mutant occurrs as fast as it does in wild type plants. These results implicate the existence of other Chl-degrading activities within the chloroplast. Schelbert et al. (2009) hypothesize that additional lipases/hydrolases besides chlorophyllase may be involved in Chl breakdown and they search the Arabidopsis genome for a corresponding gene encoding a protein with a hydrolase/lipase motif. As a result of their inquiry, 462 proteins containing hydrolase/lipase motifs are identified and the candidates are narrowed by the following two criteria: 1) genes with induced expression during leaf senescence and 2) the presence of putative transit peptide sequences for plastids. Only three genes fulfill these criteria and the At5g13800 locus is finally identified and found to encode an enzyme (pheophytinase) that cleaves a phytol chain from pheophytin. It is also shown that insertion of a tag into the At5g13800 locus results in impairment of Chl breakdown during leaf senescence. Taken together, these data enable the researchers to propose that Chl breakdown begins with dechelation, which is followed by dephytylation by the pheophytinase enzyme (Fig. 10). This hypothesis is later supported by results from a rice mutant in which the lack of rice pheophytinase results in a stay-green phenotype (Morita et al. 2009).

8.3. Pheophorbidase

The complexity of the Chl breakdown pathway is illustrated in the distribution of pheophorbidase. This enzyme catalyzes the demethylation of pheophorbide *a* and forms C-13(2)-carboxyl-pyropheophorbide *a*, which is spontaneously converted to pyropheophorbide *a* (Fig. 10). At the present time, it is not clear how pyropheophorbide is further metabolized. The pheophorbidase enzyme is identified with radish (*Raphanus sativus*) (Suzuki et al. 2006) and homologues are found in the Arabidopsis genome (At4g16690) and other plant species including *Capsella rubella* and two *Brassica* species. The distribution of homologues in a limited number of species is consistent with the findings that pheophorbidase activity is detected with the Brassicaceae and a few other species (Suzuki et al. 2002b). Therefore, it is likely that the formation of pyropheophorbide is an auxiliary pathway of Chl breakdown.

8.4. Pheophorbide *a* oxygenase

Regardless of which pathway of Chl breakdown is the dominant process, the opening of the tetrapyrrole ring appears to be a crucial step which is catalyzed by pheophorbide *a* oxygenase (PaO) (Fig. 10). Enzyme activity of PaO has been extensively investigated with senescent thylakoid membranes from *Brassica napus* and PaO is found to function as a non-heme-iron type monooxygenase (Vicentini et al. 1995). This enzyme was subsequently identified by two independent laboratories who utilized bioinformatics approaches to identify candidate genes for PaO. Tanaka et al. (2003) hypothesized that some Rieske-type oxygenases might be involved in this reaction, and they identified three genes from the Arabidopsis genome which encode chloroplast-localized Rieske oxygenases: Tic55 (At2g24820), ACD1 (At3g44880) and an ACD1-like gene (At4g25650). To understand the function of these genes, the authors down-regulated their expression with corresponding antisense-RNA species in Arabidopsis. As a result of these studies, they determined that only suppression of the *ACD1* gene expression led to accumulation of pheophorbide *a* during leaf senescence. Therefore, they conclude that *ACD1* is necessary for the oxygenation of pheophorbide *a*. Likewise, Pruzinska et al. (2003) narrowed down the candidates to the *ACD1* and *ACD1-like* genes among nine genes encoding putative Rieske-type oxygenases and 4200 genes encoding di-iron-oxo-motif-containing proteins in Arabidopsis. They later tested the *in vitro* activity of PaO using recombinant proteins expressed in *E. coli* and found that only the *ACD1* product exhibited PaO activity.

Prior to the identification of the *ACD1* gene encoding PaO, the mutants defective in the *ACD1* gene, or its maize ortholog *LLS1*, were extensively analyzed, because it was hypothesized that the *ACD1* gene is involved in the control of programmed cell death (Gray et al. 2002; Greenberg and Ausubel 1993). In general, it is assumed that plants restrict the propagation of pathogens by inducing programmed cell death around the site of pathogen infection. Since pathogen-induced cell death is capable of spreading into the whole area of the *acd1* mutant leaves, researchers speculate that ACD1 functions to suppress the programmed cell death in the neighboring cells of the infection site. Since researchers confirmed that the *ACD1* gene encodes PaO, a defect in the *ACD1* gene is now hypothesized to promote cell death through the accumulation of pheophorbide *a*.

It is noteworthy that pheophorbide *a* induces cell death in a unique manner that differs from other tetrapyrrole molecules. Many tetrapyrrole molecules, including Proto IX, Mg-Proto IX and Pchlide are activated by light and transfer their energy to oxygen, resulting in the generation of singlet oxygen. Pheophorbide *a* also induces the generation of singlet oxygen upon illumination. However Hirashima et al. (2009) found that excessive accumulation of pheophorbide *a* induces cell death under darkness. Although the mechanism for inducing cell death is not known at the present time, the authors hypothesized that pheophorbide *a* might emit an intracellular signal which induces programmed cell death. Since the Arabidopsis *acd1* or the maize *lls1* mutants exhibit the stay-green phenotype, Pruzinska and colleagues (2003) hypothesized a feedback mechanism of Chl breakdown in which pheophorbide *a* inhibits the initial step of Chl degradation. However, as in the Arabidopsis mutants with reduced PaO activity, Chl breakdown in the dark proceeded until 30% of total Chl levels is reached with concomitant accumulation of a large amount of pheoporbide *a*, a simple feedback hypothesis is unlikely. The possibility that pheoporbide *a* disturbs certain cellular functions which may result in cell death in the dark can be considered. This hypothesis should be carefully examined in the future.

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8.5. Red Chl catabolite reductase

Red Chl catabolite reductase (RCCR) catalyzes the ferredoxin-dependent conversion of red Chl catabolite (RCC) to primary fluorescent Chl catabolites (pFCC) (Fig. 10). It is suggested that RCCR forms a protein complex with PaO and that the formation of this complex may prevent the release of RCC into the stroma (Rodoni et al. 1997; Wuthrich et al. 2000). Interestingly, the Arabidopsis *acd2* mutant lacking RCCR protein accumulates several different derivatives of FCC and the nonfluorescent Chl catabolite (NCC) (Pruzinska et al. 2007). This accumulation indicates a possible non-enzymatic conversion of RCC derivatives to FCC or NCC. Nevertheless, evidence suggests that enzymatic conversion of RCC to FCC is essential in Chl breakdown. In the *acd2* mutant, an excessive amount of RCC accumulates during leaf senescence, which leads to lesion formation in a light-dependent manner (Pruzinska et al. 2007). Rapid conversion of RCC to pFCC is necessary to protect cells from excessive accumulation of RCC and from subsequent induction of cell death.

An additional role of RCCR may be to determine the stereospecificity of pFCC. It is known that the *Brassicaceae* and a few *Gramineae* species produce pFCC-1, while all other species examined produce pFCC-2 the C1 stereoisomer of pFCC-1. Pruzinska et al. (2007) found that the RCCR sequences from the *Brassicaceae* contain phenylalanine-218, while RCCR sequences from many other plants, including tomato, have valine in this position. In their studies, phenylalanine and valine residues were exchanged between Arabidopsis and tomato RCCR sequences and these exchanges were found to be sufficient for conferring the species-specific stereospecificity to pFCC. In fact, Arabidopsis RCCR with a phenylalanine-218-valine substitution produces pFCC-2, while tomato RCCR with a valine to phenylalanine substitution produces pFCC-1. Crystallographic analysis of the RCCR structure revealed that phenylalanine-218 locates at the substrate-binding pocket (Sugishima et al. 2009). Although the physiological significance of the presence of the stereospecific isomers is not clear at the present time, these results unequivocally demonstrate the *in vivo* participation of RCCR in the process of Chl breakdown.

Surprisingly, it is found that the *ACD2* gene encoding RCCR is expressed in roots (Wüthrich et al. 2000), suggesting the possibility that RCCR is a bifunctional enzyme. Yao and Greenberg (2006) found that RCCR is localized in both chloroplasts and the mitochondria and that overexpression of *ACD2* suppresses programmed cell death in protoplasts from leaves and roots. As a result, they speculate that RCCR functions in controlling programmed cell death which is independent and not related to Chl breakdown.

8.6. Formation of NCCs

pFCC is further modified at C3, $C8^2$ and $C13^2$ positions to form modified FCCs. These modifications are specific to plant species (see review Hörtensteiner 2006) and the enzymes responsible for the modification of pFCC remain to be identified. In Arabidopsis, the C3 position appears to be unmodified during Chl breakdown (Hortensteiner 2006). Modified FCCs are imported into vacuoles and nonenzymatically tautomerized to NCCs (non-fluorescent Chl catabolites) under the acidic conditions in vacuoles (Oberhuber et al. 2003). In Arabidopsis, five NCCs have been identified (Pruzinska et al. 2005) (see Fig. 10). The amount of NCCs accumulated in senescent leaves is almost equivalent to the number of Chl molecules that are catabolized during leaf senescence (Pruzinska et al. 2005). Thus, it is most likely that the NCCs are the final products of Chl breakdown in Arabidopsis under normal growth conditions. Nevertheless, it is possible that NCCs are further metabolized to monopyrrolic compounds in certain plant species or in certain growth stages, since such compounds are detected in several plant species during leaf senescence. A dipyrrolic compound, rollipyrrole, which is most likely derived from the A and B pyrrole rings of chlorophyll molecules, is isolated from *Rollinia muscosa* leaves (Kuo et al. 2001). This result also indicates the existence of chlorophyll degradation activity in nature which does not involve the oxygenation of the methine bridge between the A and B rings. Suzuki and Shioi suggested that NCCs are further metabolized to monopyrrolic compounds in barley (Suzuki and Shioi 1999).

9. INTRACELLULAR LOCALIZATION OF ENZYMES

Because of the hydrophilic nature of early intermediates of tetrapyrroles, the initial steps in tetrapyrrole biosynthesis from ALA to Protogen IX occur in the stroma of plastids, whereas the enzymes involved in the subsequent steps that catalyze hydrophobic intermediates are membrane-bound (Masuda and Fujita 2008; Mochizuki et al. 2010; Tanaka and Tanaka 2007). It should be noted that certain pairs of enzymes involved in tetrapyrrole synthesis form protein complexes to enable the efficient channeling of substrates. As in the case of GluTR-anchoring protein (see below), it is possible that some enzymes catalyzing early steps of tetrapyrrole biosynthesis are interacting with other proteins and are anchored to the plastid membrane. Meanwhile, it has been suggested in higher plants that several enzymes have a dual localization in both plastids and mitochondria. In Arabidopsis, the sub-cellular compartmentation of plastid proteins, including the enzymes involved in tetrapyrrole biosynthesis, has been comprehensively examined by proteomic analysis (Joyard et al. 2009). Current understanding of the localization of tetrapyrrole biosynthetic enzymes and regulatory proteins is summarized in Fig. 11.

ALA-synthesizing activity has been shown to be localized in the stroma in barley and other plant species (Gough and Kannangara 1976). Proteomic analyses in Arabidopsis fail to identify any of the proteins involved in the first two steps of ALA biosynthesis (GluRS and GluTR). Subsequent enzymes from GSA-AT to CPO are detected in the stroma by the same analyses (Joyard et al. 2009). In Arabidopsis, CPO was only detected in the stroma of plastids (Joyard et al. 2009; Santana et al. 2002), while one of the two isoforms in maize CPO is reported to be localized in mitochondria (Williams et al. 2006). It is interesting to note that FLU, a negative regulator of ALA biosynthesis that binds to HEMA1, is detected in both thylakoid and envelope membranes. It has been suggested that GluTR is anchored to thylakoid membranes via a GluTR-binding protein (Grimm et al. unpublished). Thus, it is possible that the negative feedback regulation of GluTR activity via FLU and heme occurs in plastid membranes.

For the reactions occurring later than PPO, the topography of the pathway is different. Proteomic analysis identified both

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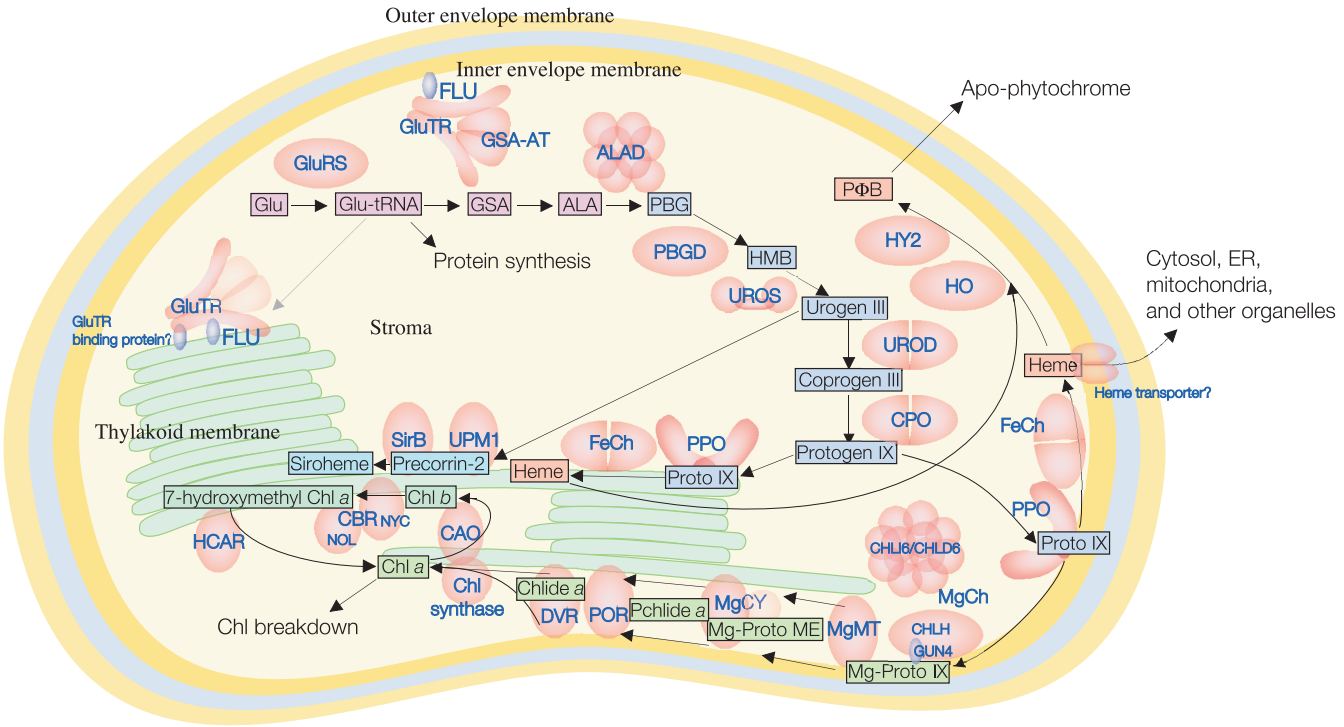


Figure 11. Localization of tetrapyrrole biosynthetic enzymes in plastids.

Localization of tetrapyrrole biosynthetic enzymes (indicated by red spheres) and regulatory proteins (indicated by blue spheres) are shown with enzyme names (indicated by blue letters). When subunit composition or crystal structure is available, we showed the complex composition and simplified structures. Interested readers may refer an excellent review of Layer et al. (2010) about the biochemistry and structure of enzymes involved in ALA biosynthesis, the common steps, and the heme/bilin branch. Biosynthetic intermediates are showen with colored boxes with each color representing categorization of the biosynthetic pathways (see Section 3). Localization of proteins are mainly based on the proteomic analyses done by Joyard and colleagues (Joyard et al. 2009) and are partly based on other data sets that are described in the text. For UPM, SIRB and CAO, there is no experimental data available to indicate sub-organellar localization, and therefore, we predicted locations of these enzymes based on the predicted solubility of these enzymes and the substrates. The location of enzymes represents their major sub-organellar localizations (stroma, envelope or thylakoids), but differences between grana- or stroma-thylakoids are not intended to be indicated. The subunits of Mg-chelatase show complex patterns of localization responding to cellular conditions, therefor, only a representative pattern of localization is dipicted for these subunits. One of the proposed multiple routes is shown for the Chl cycle in order to simplify the illustration. This cycle is described in more detail in Fig. 7. Dual localizations of enzymes in the thylakoid and envelope membranes are reported in the Chl branch and FeCh. Thus, these enzymes are placed inbetween these two membrane systems.

PPO isoforms in plastids membranes (Joyard et al. 2009); PPO1 (At4g01690) is associated with both envelope and thylakoid membranes, whereas PPO2 (At5g14220) is restricted to the envelope membrane. On the other hand, dual localization of PPO in both plastids and mitochondria is proposed in tobacco (Lermontova et al. 1997) and spinach (Watanabe et al. 2001). In spinach, the PPO2 isoform has translational variants and the larger (57 kDa) and smaller (55 kDa) forms are targeted into plastids and mitochondria, respectively (Watanabe et al. 2001). These data indicate that PPO isoforms have dual localizations in both plastids and mitochondria in higher plants. However, only a single gene encoding *PPO* is present in a green alga (*Chlamydomonas reinhardtii*), the product of which is exclusively found in plastids (van Lis et al. 2005). In the future, it will be important to clarify the physiological function of the PPO2 subfamily whose distribution is restricted in land plants.

Enzymes involved in the Chl branch have been exclusively found in plastids. Specifically, the CHLI subunit of MgCh is detected in stroma (Nakayama et al. 1995), while the catalytic CHLH subunit binds to envelope membranes in Mg^{2+}- and porphyrin-dependent manners (Adhikari et al. 2009; Gibson et al. 1996; Nakayama et al. 1998). Since the CHLD subunit binds CHLI (Walker and Willows 1997), it is presumably localized to stroma as well. Proteomic analysis showed that all MgCh subunits (CHLI1, CHLI2, CHLD and CHLH) were detected in the stroma, but CHLH and CHLD were also present in envelope membranes and thylakoids, respectively (Joyard et al. 2009). The reason why CHLD is found in thylakoids remains to be clarified even though CHLI is absent in this location. Since CHLD tends to form large molecular weight aggregates, it is possible that such an aggregated form of CHLD co-migrates with thylakoid membranes during the biochemical separation process. It is likely that the behavior of GUN4 is similar to CHLH showing localization in the stroma, envelope, and thylakoids (Larkin et al. 2003) and binds to the membrane in Mg^{2+}- and porphyrin-dependent manners (Adhikari et al. 2009). Following the MgCh reaction, it is originally hypothesized

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that Chl biosynthesis mainly occurs in envelope membranes. However, recent analyses show that many of the enzymes are localized in both thylakoid and inner envelope membranes. Such a dual localization is observed in the cases of MgMT (Block et al. 2002), MgCY (Tottey et al. 2003), and POR (Masuda and Block, unpublished). In fact, proteomic analysis confirmed the dual localization of these enzymes (Joyard et al. 2009). The reason why there are two spatially separated pathways in chloroplasts is not well understood, although several possibilities can be considered (Masuda and Fujita 2008; Tanaka and Tanaka 2007). Considering the large volume of the thylakoid membranes relative to the envelope, it would be reasonable to assume that the thylakoid membranes are the major site for Chl biosynthesis. In this regard, one should be careful in looking at proteomic profiles, as the "probability" of protein localization suggested by proteomic analysis should not be confused with the "amounts" of proteins in specific localizations. This caution is particularly important when we consider enzymes that are localized in more than two sites. If an enzyme is estimated to localize in both envelope and thylakoid membranes with 60% and 30% probabilities, respectively, it does not mean that two thirds of the enzyme population reside in envelope and the rest is in thylakoids. If we assume that the volume of thylakoid membranes is typically 100 times larger than envelope membranes in leaf chloroplasts, it is reasonable to estimate that the majority of this enzyme is localized in thylakoid membranes.

For enzymes involved in the Chl cycle, Chl synthase activity is demonstrated in the thylakoid (Soll et al. 1983) and this enzyme has been actually detected in the thylakoid proteome (Joyard et al. 2009). CAO is reported to be localized in both inner envelope and thylakoid membranes (Reinbothe et al. 2006) and proteomic analysis failed to detect this protein (Joyard et al. 2009) probably because CAO protein levels are tightly restricted by proteolytic regulation (see below). CBR comprising NOL and NYC1 (Kusaba et al. 2007) are proposed to be co-localized in thylakoids (Sato et al. 2009). On the other hand, while NYC1 is undetectable, proteomic analysis detects NOL only in the envelope suggesting distinct roles of this protein in the envelope and thylakoid membranes (Joyard et al. 2009).

For the heme/bilin branch, it has been widely believed that plant FeCh exists in both plastids and mitochondria, because yeast and animal FeChs localize in mitochondria. The presence of mitochondrial heme synthesizing activity has been reported in pea (Cornah et al. 2002) and tobacco (Papenbrock et al. 2001). It has been proposed that FC1 is dual-targeted into both plastids and mitochondria (Chow et al. 1997), however, the mitochondrial targeting of FC1 is disputed since pea mitochondria appeared to accept a variety of chloroplast proteins in this assay (Lister et al. 2001). Later an in-vitro study using cucumber subfractions showed both FeCH isoforms are present solely in plastids (Masuda et al. 2003). FC2 is only detected in Arabidopsis (Chow et al. 1997) and cucumber (Suzuki et al. 2002a). Although FC2 is detected both in thylakoid and envelope membranes in cucumber (Suzuki et al. 2002a), proteomic analysis detected FC2 only in thylakoid membrane. FC1 is predicted to be hydrophobically attached to membranes. However, this protein is undetectable in any chloroplast sub-fraction (Joyard et al. 2009). More recently, Woodson et al. (2011) showed that in Arabidopsis FC1 and FC2 are co-localized within plastids. In *C. reinhardtii*, only a single gene encoding FeCh is present, the product of which is exclusively found in plastids (van Lis et al. 2005). Obornik and Green (2005) performed the phylogenetic analysis of genes involved in heme biosynthesis in eukaryotes. They found that red algal FeCh sequences from *Cyanidioschyzon merolae*, *Porphyra yezoensis*, and *Galdieria sulfuraria*, do not cluster either with the other plastid sequences or with cyanobacterial sequences and appear to have a proteobacterial origin like that of the apicomplexan parasites *Plasmodium* and *Toxoplasma*. Since *Plasmodium* FeCh localizes in mitochondria (Sato and Wilson 2003), it is presumed that these red algal FeChs are also localized in mitocondria.

All of the identified steps for PΦB synthesis also localize within plastids (Kohchi et al. 2001), although attachment of the chromophore to the apo-phytochrome by a thioether bond occurs in cytosol (Terry et al. 2002). HO1 was detected in stroma by the proteomic analysis (Joyard et al. 2009). PΦB synthase and the enzymes involved in sirohme synthesis have not been detected by proteomic analysis (Joyard et al. 2009).

10. OVERVIEW OF REGULATION OF TETRAPYRROLE METABOLISM

Since the requirements for tetrapyrroles varies dramatically within different cell types at different developmental stages, various amounts of tetrapyrroles originating from the common steps of biosynthesis need to be supplied appropriately. In de-etiolating seedlings, Chl levels increase dramatically whereas heme levels remain constant (Castelfranco and Jones 1975). It is likely that Chl and heme levels do not always follow the same accumulation profiles, though both Chl and heme are essential components of the photosynthetic electron transport chain. With respect to tetrapyrrole metabolism, these patterns suggest that there is a greater allocation of intermediates to the Chl branch at this stage. To meet the variable demands for tetrapyrroles within the cell, plants adopt several regulatory mechanisms which govern tetrapyrrole metabolism. In particular, the enzymes of the common steps, as well as those of the branch points, are under multiple levels of regulation. Moreover, the regulation of each enzyme is well coordinated as a means to avoid the accumulation of tetrapyrrole intermediates which could result in the formation of singlet oxygen and toxic radicals upon illumination.

The first level of the regulation of tetrapyrrole metabolism consists of transcriptional activation and repression of genes that are involved in this metabolism. Light is probably the most important stimulus for the regulation. Blue light receptors (cryptochromes), phytochromes and the circadian clock machinery mediate the light signaling which modulates the expression of genes that are involved in tetrapyrrole metabolism. Furthermore, recent findings suggest that phytohormones and environmental (stress) signals also regulate the expression of genes that are related to tetrapyrrole metabolism. The transcriptional control of tetrapyrrole metabolism also shows specificity with respect to tissues (organs) and stages of development. A finer level of regulation for tetrapyrrole metabolism is accomplished at the translational level and also through the control of subsequent import of enzymes into plastids. In addition, several regulatory proteins are also known to control enzymatic activity, which affects the redox status of the chloroplast and the level of biosynthesized products. The final

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level of regulation is completed through the proteolysis of the enzymes. Taken together, we can clearly see that tetrapyrrole metabolism is a true showcase and a marvel of multiple regulatory mechanisms occurring within the plastid.

11. TRANSCRIPTIONAL REGULATION OF TETRAPYRROLE METABOLISM

11.1. Coordinated transcriptional regulation of Chl biosynthesis

Coordinated gene expression is the central mechanism for synchronizing the synthesis of Chl and cognate proteins. In tobacco, coordinated expression of *HEMA* and *CHLH* with a *LHC* gene occurs in diurnal and circadian rhythms, which appears to influence ALA synthesis and tetrapyrrole metabolisms (Papenbrock et al. 1999). A miniarray analysis for genes involved in Chl biosynthesis reveals that genes encoding key enzymes of tetrapyrrole biosynthesis, which include *HEMA1*, *CHLH*, *CHL27* and *CAO*, exhibited a strong and coordinated response to light and circadian rhythms (Matsumoto et al. 2004). Analysis for gene expression networks using the ATTED-II database (*Arabidopsis thaliana* trans-factor and *cis*-element prediction database; http://atted.jp/) reveals that these genes form a gene cluster of highly correlated expression profiles. This gene cluster also includes *GUN4*, *CHLP* (geranylgeranyl pyrophosphate reductase; At1g74470), and *CLA1* (1-deoxy-D-xyluose-5-phosphate synthase; At4g15560) the latter two of which are required for the synthesis of the phytol tail of Chl (Masuda and Fujita 2008). In addition, many genes encoding Chl-binding subunits of the photosynthetic apparatus are described as being co-expressed with the tetrapyrrole biosynthesis-related genes mentioned above, suggesting that these genes share a large transcriptional regulatory system. It is likely that this regulatory co-expression plays a central role in the assembly of the photosynthetic apparatus.

In contrast to these aforementioned light-inducible genes, the *POR* genes show unique expression patterns. As it was described in Section 4.4, *PORA* and *PORB* transcripts accumulate in etiolated seedlings and decrease rapidly after illumination (Armstrong et al. 1995; Matsumoto et al. 2004), which is the unique feature of angiosperm *POR* genes. In gymnosperms and mosses, the expression profiles of POR (*PORA* and *PORB*) are light inducible, which is well correlated with other photosynthesis-related genes (Skinner and Timko 1999; Takio et al. 1998). This is probably because these organisms do not accumulate a high level of Pchlide in darkness, and accordingly, they do not need a large amount of POR in darkness. The expression patterns of the genes encoding the DPOR subunits (*ChlL*, *ChlN*, and *ChlB*) in response to light conditions are varied among plant species (Demko et al. 2009; Skinner and Timko 1999; Suzuki et al. 2001). Meanwhile, in adult Arabidopsis plants, *PORA* and *PORB* show circadian rhythmic expression patterns that are similar to the light-inducible Chl synthesis genes, although the oscillation peak is somewhat delayed (Matsumoto et al. 2004). Consistent with the reports that *PORA* mRNA is almost undetectable in light-adapted mature plants (Armstrong et al. 1995; Oosawa et al. 2000; Su et al. 2001), *PORA* transcript levels are much lower than that of *PORB* during the light-dark cycle (Matsumoto et al. 2004). On the other hand, the expression pattern of *PORC* is largely different from that of the other two *POR* genes. Specifically, *PORC* transcript is not detectable in the dark but it accumulates after illumination with a light intensity dependent manner (Oosawa et al. 2000; Su et al. 2001). Unlike the genes encoding key enzymes of tetrapyrrole biosynthesis, *PORC* expression is not regulated by the circadian clock (Matsumoto et al. 2004). These characteristic expression profiles of the *POR* genes are most likely reflecting the unique role of POR compared to other enzymes participating in tetrapyrrole metabolism. POR is not only essential in Chl synthesis in the light, but also plays an important role in maintaining Pchlide under dark conditions. Such a mixture of demands for POR may explain why many plants have multiple *POR* genes in their genomes.

11.2. Regulation by light signaling pathways

The importance of light signaling in Chl metabolism is evident. Phytochromes play a central role as observed in a quintuple mutant lacking all Arabidopsis phytochromes (Strasser et al. 2010). Light signaling pathways involved in transcriptional regulation of Chl biosynthesis have been extensively studied during photomorphogenesis. Specifically, *HEMA1* is mainly expressed in photosynthetic tissues and is regulated by blue, red and far-red light through phytochromes and cryptochromes (McCormac et al. 2001). Under far-red and red light conditions, two major isoforms of phytochrome (PHYA and PHYB) regulate this gene, whereas two cryptochrome isoforms (CRY1 and CRY2) are involved in its response to blue light together with PHYA and PHYB. Downstream signaling pathways from these photoreceptors are more complex. *HEMA1* expression is repressed in the mutants for several known regulatory factors of the light signaling pathway (*FIN219, FHY1, FHY3, NDPK2, PAT1* and *HY5*), suggesting that these factors are involved in the acute light induction of *HEMA1* (McCormac and Terry 2002a). Among the components of the phytochrome-mediating signaling pathway, *FHY1* and *FHY3* are required for *HEMA1* expression in response to red and far-red light, whereas *FIN219* and *NDPK2* appear to be involved in the blue light induced expression of *HEMA1*. Similar to *HEMA1*, the *CHLH* and *GUN4* genes are primarily controlled by the signaling pathway through PHYA and PHYB, in which the FHY1 and FHY3 are also involved. In contrast, it is likely that cryptochromes play a minor role in the control of these genes (Stephenson and Terry 2008). However, it is reported that CRY1 suppresses the activity of COP1, a ubiquitin E3 ligase controlling the abundance of several light-signaling components including PHYA and HY5, in a blue-light dependent fashion (Liu et al. 2011). Although interactions between phytochromes and cryptochromes have not been fully understood, these photoreceptors are hypothesized to regulate Chl metabolisms by modulating activities of several light-signaling transcription factors as described below.

One of the pivotal transcription factors which functions downstream of the photoreceptors in light signaling is a basic Leu zipper transcription factor (HY5) which functions as a positive regulator of photomorphogenesis. HY5 is negatively regulated through COP1/DET1-mediated degradation in the dark (Osterlund et al.

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2000). Mutation in the *HY5* gene resulted in a large reduction in responses of *HEMA1* gene expression to blue, red and far-red light. These data clearly suggest that HY5 plays a role in the convergence of these light signaling pathways (McCormac and Terry 2002a). A genome-wide chromatin immunoprecipitation (ChIP)-chip analysis confirms that *GluRS* (At5g64050), *URO2*, *PPO1, CHLH, GUN4, CHL27, DVR, PORC, CAO, CHLP* and *HO1* are the putative targets of HY5 together with many photosynthesis-related nuclear genes. *HEMA1* is not identified as a target of HY5 transcriptional regulation (Lee et al. 2007). An involvement of HY5 in Chl synthesis has been clearly observed in the roots. Although roots are heterotrophic organs in Arabidopsis, they accumulate a high amount of Chl in the presence of light in the *det1* (Chory and Peto 1990) and *cop1* mutants (Deng and Quail 1992). In direct contrast, the accumulation of Chl is absent in the *hy5* mutant (Oyama et al. 1997; Usami et al. 2004). Therefore, Chl accumulation in the root is dependent on HY5 function, but is repressed by the COP1/DET1-mediating signaling pathway via the degradation of HY5. Indeed, our recent analysis in roots reveals a considerable reduction in *CHLH*, *CHL27* and *CHLP* expression in the *hy5* mutant (Kobayashi et al., unpublished). These data are in good accordance with the report that these genes are direct targets of HY5 (Lee et al. 2007). Since the expression of *HEMA1* in roots is not abolished in the *hy5* mutant, it is probable that *HEMA1* is not a direct target of HY5.

Phytochrome-interacting basic helix-loop-helix transcription factors, which are termed PIF or PIL (PIF3-like), are the other important regulators which control chloroplast biogenesis downstream of the regulation of phytochromes. Phytochromes are translocated to the nucleus upon the perception of light and they bind and target PIF and PIL proteins for proteolysis in phytochrome nuclear bodies (Chen et al. 2010a). This translocation subsequently triggers a wide range of light responses including seed germination, the inhibition of hypocotyl elongation, cotyledon opening and greening (Leivar and Quail 2011). Out of six well-characterized PIF proteins (Castillon et al. 2007), PIF1 and PIF3 are known to be involved in the regulation of tetrapyrrole metabolism. The *pif1* mutant accumulates an excessive amount of Pchlide in the dark and even after illumination, resulting in photobleaching (Huq et al. 2004). It is suggested that PIF1 directly induces *PORC* expression in the dark by binding to a G-box element on its promoter region whereas it indirectly upregulates *PORA* expression (Moon et al. 2008). On the other hand, PIF1 and PIF3 are proposed to be negative regulators of *HEMA1*, *GUN4* and *CHLH* since both *pif1* and *pif3* mutants exhibited increased expression of these genes at early time points after dark germination (Stephenson et al. 2009). PIF5 is also suggested to be involved in negative regulation of *CHLH* expression in etiolated Arabidopsis seedlings (Shin et al. 2009). Moreover, a quadruple mutant (*pifQ*) lacking four PIFs (PIF1, 3, 4, and 5) shows global upregulation of genes involved in Chl biosynthesis with many nuclear-encoded photosynthetic genes in the dark (Leivar et al. 2009; Shin et al. 2009), indicating that these PIF members are pivotal transcriptional factors negatively regulating Chl biosynthesis during dark growth. Considering that these multiple *pif* mutants have a very strong photobleaching phenotype (Leivar et al. 2009; Shin et al. 2009; Stephenson et al. 2009), these factors play important roles in fine tuning of tetrapyrrole metabolism during photomorphogenesis.

While HY5 and PIF proteins act downstream of phytochrome signaling, GOLDEN2-LIKE (GLK) 1 and GLK2 transcription factors influence the expression of Chl synthesis genes independently of the PHYB signaling pathway (Waters et al. 2009). A deficiency of both isoforms resulted in the reduced expression of nuclear-encoded photosynthetic genes, especially those associated with Chl biosynthesis and light harvesting (Fitter et al. 2002). Moreover, overexpression of GLK1 and GLK2 induces the expression of Chl synthesis genes. In particular, GLKs strongly upregulate *HEMA1, CHLH, CHL27, PORB* and *CAO* (Waters et al. 2009). With the exception of *PORB*, it is important to note that these tetrapyrrole-biosynthesis genes form a cluster within a gene expression network (Masuda and Fujita 2008), indicating that GLKs are main components responsible for the transcriptional regulation of these co-expressed genes in Arabidopsis. In fact, a ChIP analysis revealed that key Chl synthesis genes (*HEMA1, CHLH, GUN4, CHLM, CHL27, PORA, PORB, PORC* and *CAO*) are direct targets of GLK1 *in vivo* (Waters et al. 2009).

11.3. Regulation by phytohormone signaling pathways

It has been shown that several phytohormones are involved in Chl metabolism. One prominent factor affecting Chl metabolism is ethylene, which upregulates the expression of *PORA* and *PORB* in etiolated seedlings (Zhong et al. 2010; Zhong et al. 2009). The effects of ethylene are mediated by an ethylene-inducible transcription factor (EIN3/EIL1) which binds to the promoter regions of both *PORA* and *PORB* and likely triggers their expression in the dark. The protein accumulation of EIN3/EIL1 is enhanced by COP1 but is decreased by light, suggesting that EIN3/EIL1 is involved in the COP1-mediated repression of excess Pchlide accumulation in dark grown seedlings (Zhong et al. 2010; Zhong et al. 2009). The ethylene-inducing signaling can negatively regulate the Pchlide synthesis pathway in the dark because ethylene treatment and EIN3/EIL1 overexpression largely decreases Pchlide levels in the dark, implying that EIN3/EIL1 suppresses ALA synthesis in dark conditions.

Cytokinin has also been shown to influence photomorphogenesis. Dark-grown Arabidopsis plants exposed to exogenous cytokinin have expanded cotyledons, developed leaves, short hypocotyls and partially developed chloroplasts (Chory et al. 1994). Cytokinin is also known to activate light-regulated promoters in the dark (Chory et al. 1994). In cucumber cotyledons, cytokinin induces the gene expression for GluTR and activates ALA synthesis in both darkness and after illumination (Masuda et al. 1995). In Arabidopsis, cytokinin treatment to etiolated seedlings activates the expression of *HEMA1*, *CHLH* and *CHL27*, whereas double mutations in cytokinin receptors (*ahk2* and *ahk3*) reduce their expression levels in the light (Kobayashi et al., unpublished). Consistent with these reports, Pchlide levels are increased in dark grown seedlings by inactivation of cytokinin breakdown in a quadruple mutant of cytokinin oxidase, while Pchlide is decreased in the *ahk2/ahk3* double mutant (Hedtke et al., unpublished). The positive effect of cytokinin on Chl synthesis is more obvious in roots. Specifically, cytokinin treatment increases Chl accumulation in roots and upregulates the expression of *HEMA1, CHLH, GUN4, CHL27, CHLP* and *GLK2* with other many photosynthesis-related genes (Kobayashi et al., unpublished). In Arabidopsis seedlings,

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Vandenbussche et al. (2007) report that cytokinin can increase the abundance of HY5 protein through a hypothesized reduction in degradation by COP1. Considering the involvement of HY5 in light signaling, this protein may function in Chl synthesis as a point of convergence between light and cytokinin signaling pathways.

In the auxin signaling pathway, degradation of a group of auxin-responsive proteins (Aux/IAA family) is essential. Several mutations stabilizing Aux/IAA proteins have been reported to cause de-etiolation in the dark, supporting an antagonizing role for auxin to the light signal (Tian et al. 2002). One member of this Aux/IAA family, SHY2/IAA3, is initially shown to suppress the phenotype of *hy2* mutants as well as *phyB* mutants by its gain-of-function mutations, suggesting a negative involvement of auxin in photomorphogenesis (Tian and Reed 1999). Actually the SHY2/IAA3-stabilizing *shy2* mutations cause an enhanced accumulation of Pchlide and increase the expression of *HEMA1* and *CHLH* in the dark (Hedtke et al., unpublished). These observations are consistent with the reports that *shy2* mutants accumulate *CAB2* (*Lhcb1.1*) transcripts in the dark (Kim et al. 1998; Tian et al. 2002). Moreover, the impairment of auxin signaling causes upregulation of Chl synthesis genes and excess accumulation of Chl in roots, suggesting that auxin signaling is also involved in the repression mechanism of Chl biosynthesis in roots (Kobayashi et al., unpublished). However, the signaling pathway of auxin is largely unknown in respect to photomorphogenesis and Chl synthesis. Further analyses are required to reveal the interaction between the auxin and light signaling pathways with respect to the regulation of tetrapyrrole metabolism.

Seedling de-etiolation is also subject to regulation by gibberellin and lack of gibberellin signaling de-represses photomorphogenesis in the dark (Alabadi et al. 2008; Alabadi et al. 2004). A family of nuclear proteins, DELLAs, negatively regulates gibberellin signaling and they are also known to play an important role in light-regulated seedling development (de Lucas et al. 2008; Feng et al. 2008; see review Lau and Deng 2010). DELLAs can inhibit the DNA binding activity of PIF3 and PIF4 through a physical interaction with these factors. Gibberellins induce the degradation of DELLAs, resulting in the activation of PIF3 and PIF4 and a consequent repression of photomorphogenesis in the dark. Moreover, Cheminant et al. (2011) demonstrated that gibberellins also modulate DNA binding activity of PIF1 to the promoter regions of photosynthetic genes (*LHCB2.2*, *PSAG* and *PSAE-1*) and Chl synthesis genes (*CHLH*, *PORC* and *CAO*). It is proposed that, in the absence of gibberellins, DELLA upregulates the expression of genes involved in Chl synthesis and photosynthesis in the dark through inactivation of PIFs (PIF1, PIF3, PIF4, and PIF5). Intriguingly, DELLAs can also upregulate *PORA* and *PORB* in the dark in a PIF-independent manner, which leads to the repression of photodamages caused by long dark treatment in de-etiolated seedlings (Cheminant et al. 2011). These observations are consistent with the observation that a transgenic Arabidopsis with reduced gibberellin signaling (*35S::gai-1*) showed more rapid accumulation of Chl during de-etiolation (Alabadi et al. 2008). Gibberellins also affect light-regulated seedling development by reducing the abundance of HY5 through a proposed modulation of COP1 activity; which is in contrast to the effect of cytokinin. However, the effect of HY5 on gibberellin-mediated regulation of photosynthetic gene expression in the dark seems more moderate than that of PIFs (Cheminant et al. 2011).

Strigolactones are first identified as factors involved in regulation of shoot branching and also as communication chemicals between plant roots and fungi and parasitic plants. However, Tsuchiya et al. (2010) report that strigolactones are also involved in light signaling via their regulation of the nuclear localization of COP1, which in part determines the levels of light regulators such as HY5. Exogenous application of a synthetic strigolactone (GR24) downregulates the expression of PORA and PORB. On the other hand, it upregulates light-inducible genes such as *Lhcb1.2* in the dark, suggesting that this hormone can activate the light signaling pathway in the dark and can influence Chl metabolism during photomorphogenesis. Because strigolactones are newly-characterized plant hormones, unraveling their physiological roles will bring new insights into our understanding of the signaling network that regulates Chl metabolism and photosynthesis.

Brassinosteroid (BR) is also well known phytohormones linked to the process of de-etiolation. BR signaling-deficient plants exhibit de-etiolation phenotypes in the dark with elevated expression of many light-induced genes including *LHC* genes. It is assumed that BR signaling represses photomorphogenesis as well as photosynthetic gene expression in the absence of light (Asami et al. 2000; Chory et al. 1991; Szekeres et al. 1996). Examining the public microarray data sets reported with Arabidopsis BR mutants, we found that many genes involved in Chl synthesis are upregulated in dark-grown BR-insensitive *bri1-116* seedlings (Sun et al. 2010), whereas none of these genes are listed as upregulated in dark-grown BR-deficient *det2* seedlings (Song et al. 2009). Luo et al. (2010) identify a key transcriptional factor (GATA2) that mediates the crosstalk between BR and light signaling pathways. They suggest that GATA2 is a strong upregulator of light-inducible genes but is repressed transcriptionally through BR signaling and post-translationally through COP1-mediated degradation in the dark. Although overexpression of GATA2 caused elevated expression of many photosynthetic genes in the absence of light, it does not influence the expression of Chl synthesis genes. Thus, it is unlikely that GATA2 plays a role in transcriptional regulation of Chl synthesis in the dark. The relationship between transcriptional regulation of Chl synthesis genes and BR signaling during photomorphogenesis remains to be clarified.

11.4. Regulation by sugar signaling pathways

Sugars are not only important energy sources and structural components but also physiological signaling molecules involved in various cellular processes. In higher plants, sugars act as a regulatory signal for feedback control of photosynthetic genes (Sheen 1994). While sugar depletion activates photosynthetic gene expression (Krapp et al. 1993; Oswald et al. 2001), glucose feeding and overexpression of invertases results in leaf bleaching and repression of photosynthetic gene expression (Jang et al. 1997; Krapp et al. 1993). In addition, a microarray study shows that some genes involved in Chl synthesis are downregulated together with nuclear-encoded photosynthetic genes by exogenous feeding of sucrose to Arabidopsis seedlings (Osuna et al. 2007), showing an importance of sugar signaling for Chl metabolism. A role of hexokinase (HXK) for glucose sensing has been well studied in Arabidopsis. It is known that exogenous application of glucose to plants represses Chl accumulation and photosynthetic gene expression

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(*LHCB1.3* and *RBCS*). This effect of glucose is further enhanced by overexpression of *HXK1,* and is inhibited by antisense silencing and null mutation of this gene (Jang et al. 1997; Moore et al. 2003). It has been shown that mutant *HXK1* alleles encoding catalytically inactive HXK1 proteins are able to complement an *HXK1*-null mutant (*gin2*). These results demonstrate that HXK1 is a true glucose sensor uncoupled from its enzymatic functions (Moore et al. 2003). Furthermore, it has been shown that HXK1 can localize to the nucleus and bind to the promoter of *LHCB1.1* and *LHCB1.2*, suggesting a direct involvement of this factor in transcriptional regulation of photosynthetic genes (Cho et al. 2006). Considering coordinated regulation between key Chl synthesis genes and *LHC* genes (Masuda and Fujita 2008) and the impact of glucose signaling in Chl metabolism (Jang et al. 1997; Moore et al. 2003), it is likely that glucose sensing through *HXK1* is involved in transcriptional regulation of Chl synthesis genes. Interactions between sugar and various hormone signaling pathways are also suggested (Ramon et al. 2008). Future studies in this respect will elucidate the complex signaling networks involved in tetrapyrrole metabolisms and plant growth.

11.5. Regulation by the endogenous circadian rhythm

Several key genes involved in Chl synthesis are under circadian regulation (Matsumoto et al. 2004; Papenbrock et al. 1999; Stephenson et al. 2009). The core components of the circadian clock machinery in Arabidopsis is mainly composed of the TOC1 (TIMING of CAB EXPRESSION 1) protein, which is related to the bacterial response regulator family and two Myb-related transcription factors, CCA1 (CIRCADIAN CLOCK-ASSOCIATED1) and LHY (LATE ELONGATED HYPOCOTYL). TOC1 is active in the evening and it promotes the transcription of *LHY* and *CCA1* genes. Subsequently, LHY and CCA1 repress the expression of TOC1, thus, these proteins form a negative feedback loop. The interaction of these three proteins is essential for the clock function (Imaizumi 2010). It is suggested that TOC1 binds to the promoter of *CHLH* and controls its circadian expression (Legnaioli et al. 2009). On the other hand, a physical interaction between HY5 and CCA1 has been reported to be important for the circadian expression of *Lhcb1.1* and *Lhcb1.3* (Andronis et al. 2008). Since HY5 is one of the key transcriptional regulators for Chl metabolism, this mechanism may also be involved in the clock regulation of the Chl synthesis genes via HY5. An involvement of PIF1 and PIF3 in the circadian control of tetrapyrrole metabolism is also evident because circadian expression of *HEMA1*, *GUN4* and *CHLH* are largely perturbed in *pif1* and *pif3* (Stephenson et al. 2009). They propose that PIF1 and PIF3 function in the output from the circadian clock to control chloroplast development. In addition, the expression of *GLK2* is also under circadian control (Fitter et al. 2002). Considering that GLK2 functions as a direct activator of the expression of key Chl genes (Waters et al. 2009), it is likely that oscillation of *GLK2* transcript levels affect the circadian rhythms of these key Chl genes.

11.6. Regulation by plastid signaling pathway

It is known that expression of photosynthesis-related nuclear genes is shut off when the function of the plastids is significantly impaired by inhibitor treatments or under stressful conditions. Thus, it is hypothesized that the functional status of the plastids is transmitted via an unidentified signaling pathway to the nucleus (Larkin and Ruckle 2008). This hypothetical signaling pathway is called a retrograde signaling pathway and signaling molecules are tentatively referred to as “plastid signals”. Most of the genes involved in Chl synthesis are strongly downregulated in response to chloroplast dysfunction by the treatment of the norflurazon (NF) herbicide in wild-type Arabidopsis plants (Moulin et al. 2008; Strand et al. 2003). Susek et al. (1993) first identified five Arabidopsis mutants (*genome-uncoupled 1 to 5*; *gun1 –gun5)* in which the retrograde signaling pathway is impaired upon NF treatment. All *gun* mutations were subsequently identified by positional cloning (see section 13). However, the downregulation of these genes by NF is attenuated in the *gun* mutants, showing that Chl synthesis is globally subject to the regulation through plastid signaling (Moulin et al. 2008; Strand et al. 2003). Moreover, the expression of *GLK1* and *GLK2*, which are direct upregulators of key Chl genes, is also under control by plastid signaling (Waters et al. 2009). Indeed, it is suggested that GUN1 represses the expression of photosynthesis-related nuclear genes through a downregulation of *GLK1* expression when plastids are dysfunctional (Kakizaki et al. 2009). Thus, GLK1 and GLK2 are thought to function downstream of the retrograde signaling pathway and control Chl synthesis at the transcriptional level to optimize synthesis of Chl in varying environmental and developmental conditions.

11.7. Regulation by biotic and abiotic stresses

As described in Sections 3–8, many enzymes involved in tetrapyrrole metabolism are encoded by multigene families. Each member of a gene family often shows distinct expression patterns from the other members of the same gene family, such as the good example of the *HEMA* and *FC* gene families. While Arabidopsis *HEMA1* and *FC2* are abundantly expressed in photosynthetic tissues, expression of their counterparts *HEMA2* and *FC1* is low in these tissues. On the other hand, *HEMA2* and *FC1* expression is strongly upregulated in response to wounding, ozone, paraquat and rose bengal (a singlet oxygen generator), suggesting that these genes are responsive to reactive oxygen species (Nagai et al. 2007). Consistent with this notion, heme levels are found to increase in response to ozone stress in wild type but not in the *hema2* and *fc1* mutants. It is likely that upregulation of these genes by reactive oxygen species is required to supply hemes to hemoproteins in response to stresses such as wounding. These data demonstrate that the differential expression of individual members from multigene families contributes to the diverse coordinated responses of plants to biotic and abiotic stresses.

12. POST-TRANSLATIONAL REGULATION

The post-transcriptional regulations of tetrapyrrole metabolism, such as RNA processing, small RNA mediated interference, and translational regulation, are poorly understood. On the other hand, it is known that plants exert multiple levels of the post-translational regulation over tetrapyrrole metabolism (summarized in Fig. 12). These regulatory mechanisms promptly adjust the rate of

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biosynthesis depending on tetrapyrrole demand in response to environment factors and plant growth and development.

12.1. Regulation of ALA biosynthesis

ALA biosynthesis is the first regulatory point and it is subjected to multiple regulatory mechanisms. The original regulatory model for ALA biosynthesis was proposed by Castelfranco and coworkers (Beale 1978; Castelfranco and Beale 1983; Castelfranco and Jones 1975; Chereskin and Castelfranco 1982). In this model, heme is a potent feedback inhibitor that directly suppresses ALA formation. In particular, the regulation seems focused on GluTR among the three steps of ALA biosynthesis. A reason why GluTR is a regulatory point is because this step is actually the first committed step of tetrapyrrole biosynthesis, as the substrate of this enzyme Glu-tRNA^{Glu} is shared with protein synthesis in plastids. As described above, *HEMA1* and *HEMA2* gene expression is transcriptionally regulated by light and other signals, respectively. In addition, GluTR activity is feedback-regulated by the end products of the pathway. Two mechanisms have been reported to regulate GluTR activity. In the first example, heme directly binds GluTR and inhibits its activity (Vothknecht et al. 1996, 1998). GluTR is inhibited by heme at a site distinct from the catalytic center of the enzyme (Pontoppidan and Kannangara 1994; Vothknecht et al. 1996), and thus the control of tetrapyrrole biosynthesis in higher plants is exclusively attributed to heme (Beale 1999). In the Arabidopsis *hy1* mutant and the tomato *aurea* and *yellow-green-2* mutants, impairment in the activity of HO or PΦB synthase led to repression of GluTR activity (Goslings et al. 2004; Terry and Kendrick 1999). It is likely that these mutations cause excessive heme accumulation, which may result in inhibition of GluTR activity. It has been reported that soluble proteins are necessary for heme to exert its inhibitory effects on the activity of recombinant GluTR from *C. reinhardtii* (Srivastava et al. 2005). Meanwhile, recombinant GluTR protein from *Chlorobium vibrioforme* forms a dimer and contains one tightly bound heme per subunit (Srivastava and Beale 2005). Heme does not inhibit the activity of this protein *in vitro*, but when the recombinant GluTR protein is expressed under heme deficient condition in *E. coli*, heme inhibits the GluTR activity. Further characterization of GluTR protein is necessary to understand the mechanism of heme inhibition on GluTR activity.

The second example of regulatory mechanisms of GluTR involves the FLU protein, which represses GluTR activity in the dark. This protein was first identified by analysis of the Arabidopsis conditional fluorescence (*flu*) mutant. In this mutant, ALA synthesis is not repressed in darkness, which results in greater accumulation of Pchlide relative to wild type plants (Meskauskiene et al. 2001). FLU is a ~ 27-kDa protein which is localized in plastid membranes and contains two tetratricopeptide-repeat motifs that are presumed to be involved in mediating protein-protein interactions (Meskauskiene et al. 2001). Using a yeast two-hybrid system, the FLU protein is found to directly interact with GluTR1 encoded by *HEMA1* (Meskauskiene and Apel 2002). Interestingly, FLU does not interact with GluTR2, which is encoded by *HEMA2*. These results indicate that distinct roles exist for GluTR1 and GluTR2 with respect to ALA synthesis.

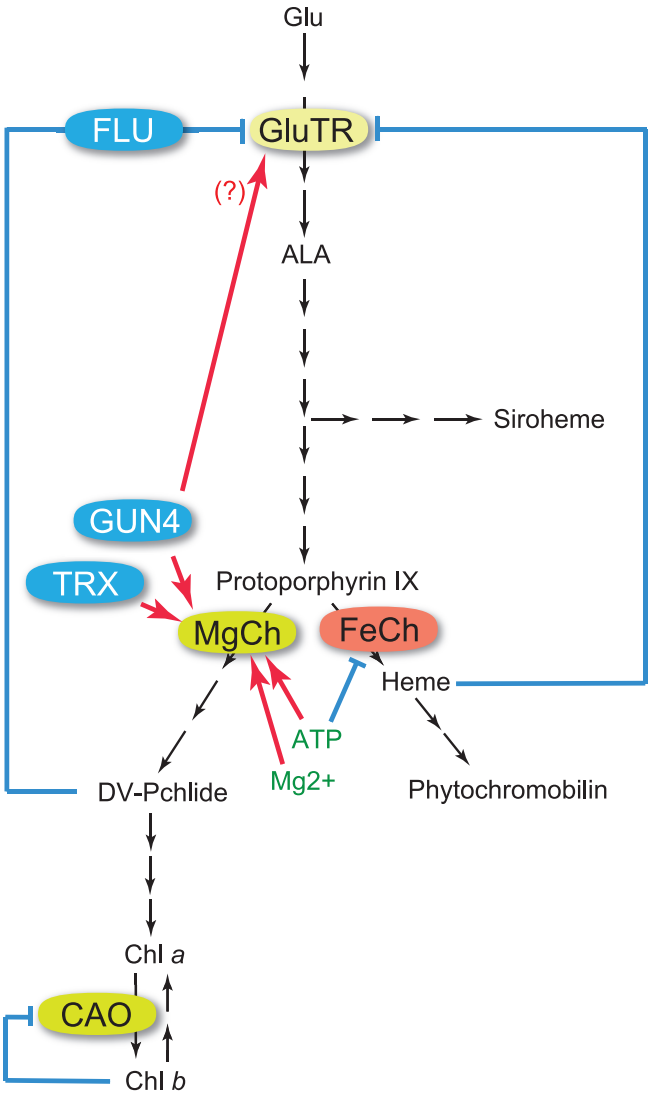


Figure 12. Summary of the proposed regulatory mechanisms for post-translational control of tetrapyrrole biosynthesis.

Red arrows indicate positive regulation, and blue arrows indicate negative regulation. GUN4 is proposed to regulate both MgCh activity and ALA formation. It is not clear which step of ALA formation is controlled by GUN4 at the present time. Therefore, we present a tentative model showing that GUN4 regulates the activity of GluTR which catalyzes the rate-limiting step of ALA formation. TRX, thioredoxin; GluTR, glutamyl-tRNA reductase; FeCh, ferrochelatase; MgCh, Mg-protoporphyrin IX chelatase.

12.2. Regulation at the branch points of the biosynthetic pathway

Although little is known about the regulation at the first branch point of the pathway leading to siroheme biosynthesis, the regulation at the second branch point between the heme/bilin and Chl branches has been extensively investigated. Papenbrock et al (1999) showed that MgCh activity is highest at the beginning of the day, while FeCh activity is highest at the beginning of the

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night. The inversely-related changes in MgCh and FeCh activity are possibly regulated by the fluctuation in the stromal concentrations of ATP and Mg^{2+}. ATP is required for MgCh activity (Gibson et al. 1995; Reid and Hunter 2004), whereas it inhibits FeCh activity (Cornah et al. 2002). In maize chloroplasts, ATP/ADP ratios increase from 1 in the dark to 4 in the light (Usuda 1988). Likewise, Mg^{2+} concentrations increase from 0.5 mM in the night to 2 mM in the day (Ishijima et al. 2003). MgCh activity is also stimulated by an increase in Mg^{2+} concentration (Reid and Hunter 2004). GUN4 is involved in this Mg^{2+}-induced MgCh activation (Davison et al. 2005; Verdecia et al. 2005). The Mg^{2+} concentration required for the full activation of *Synechocystis* MgCh is lowered from approximately 6 mM to 2 mM *in vitro* in the presence of GUN4 (Davison et al. 2005). Mg^{2+} also influence the localization of CHLH subunits within plastids. It is reported (Gibson et al. 1996; Nakayama et al. 1998) that when chloroplasts are disrupted in the presence of 5 mM Mg^{2+}, CHLH is membrane associated, whereas in the presence of 1 mM Mg^{2+}, CHLH is found in the stroma fraction. It is possible that translocation of CHLH to the envelope enables the substrate binding of CHLH. Interestingly, it is found that CHLH and GUN4 are similarly translocated to membrane in a porphyrin-dependent manner (Adhikari et al. 2009). The authors propose that GUN4 enhances MgCh activity not only by interacting with this enzyme, but also by facilitating the interaction of the CHLH subunit to chloroplast envelope membranes. More recently, Adhikari et al. (2011) showed that the associations of ChlH and GUN4 with chloroplast membranes are dependent on porphyrin-binding activity, but they use distinct mechanisms.

In addition, the redox state is proposed to regulate MgCh activity. It has been shown that the presence of dithiothreitol (DTT) is required for MgCh activity *in vitro*, indicating that essential thiol residues are involved in catalytic activity (Jensen et al. 2000). In chloroplasts, the regulation of activity for a number of enzymes involved in photosynthetic reactions is coupled to photosynthetic electron transport. Specifically, this level of regulation is coupled 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). The CHLI subunit of MgCh is identified as a thioredoxin-target protein (Balmer et al. 2003). The ATPase activity of Arabidopsis CHLI1 (Ikegami et al. 2007) and CHLI2 (Kobayashi et al. 2008) is fully inactivated by oxidation but is easily recovered by thioredoxin-assisted reduction, suggesting that CHLI is a target of regulation by thioredoxin. However, Stenbaek and Jensen described in their review (Stenbaek and Jensen 2010) that NADPH-dependent thioredoxin reductase with a C-terminal thioredoxin domain can stimulate the ATPase activity of the CHLI subunit, but is unable to stimulate total MgCh activity. These data suggest that certain cysteines presumably located in CHLH that are required for MgCh activity need reduction but are not targets for thioredoxins.

12.3. Post-translational regulation of CAO

As described in Section 5, the balance between the Chl *a* and Chl *b* levels is essential in light acclimation. During vegetative growth under constant light conditions, Chl *b* levels are primarily determined by the activity of Chl *a*-to-*b* conversion whereas the Chl *b*-to-*a* conversion seems to have a smaller impact at this developmental stage. Increased CAO protein levels in transgenic Arabidopsis plants result in elevated Chl *b* levels (Hirashima et al. 2006; Sakuraba et al. 2007; Tanaka and Tanaka 2005; Yamasato et al. 2005), while defects in CBR activity have little or no effect on Chl *b* levels during vegetative growth of rice and Arabidopsis (Horie et al. 2009; Kusaba et al. 2007; Sato et al. 2009).

Chl *b* synthesizing activity is feedback-regulated through the stability of CAO. The CAO protein sequence can be divided into four parts. The first part is the transit peptide which mediates the import into the chloroplast and is subsequently cleaved off upon import. This domain is followed by so-called “A”, “B” and “C” domains. The A domain is responsible for the regulation of CAO stability, and this domain is only present in the CAO sequences from photosynthetic eukaryotes and is absent from the cyanobacterial CAO sequences (Nagata et al. 2004). The B domain is most likely a linker between the A and C domain (Sakuraba et al. 2007). Compared to the A or C domain, the B domain is less conserved among plant CAO sequences. The C domain has a catalytic function and is conserved among nearly all CAO sequences, an exception to which are the CAO sequences from the Prasinophyceae which are split into two separate genes (Tanaka et al. 2010).

The regulatory role of the A domain is revealed by overexpressing the CAO sequence lacking its A domain in Arabidopsis (Yamasato et al. 2005). Removal of the A-domain coding sequence leads to a significant accumulation of CAO protein without affecting the transcription and translation of the transgene (Yamasato et al. 2005). As a result, the authors conclude that the A domain affects the stability of CAO (Yamasato et al. 2005). It is also demonstrated that the A domain destabilizes other proteins such as green fluorescent protein (GFP) (Yamasato et al. 2005). Interestingly, it is shown that this destabilization effect is dependent on the accumulation of Chl *b* (Yamasato et al. 2005).

The mechanism how the A domain destabilizes itself is not clearly understood. It is suggested that the chloroplast Clp protease is involved in the degradation of the A domain, which is based on the observation that the A domain is more stable in the Arabidopsis *clpC* mutant lacking a subunit of chloroplast protease (Nakagawara et al. 2007). Sakuraba et al. (2009) identified a specific amino acid sequence (QDLLTIMILH) within the A domain which is essential in the destabilization mechanism of CAO. Unlike the entire A domain, the degron sequence is destabilized in the knockout *chlorina1* mutant background, indicating that this degron sequence alone is not sufficient to discriminate the presence/absence of Chl *b.* Taken together, a working hypothesis has been proposed as follows: in the absence of Chl *b,* the A domain may shield the degron sequence from Clp protease. After the synthesis of Chl *b,* this pigment may somehow modify the structure of the A domain so that the degron is exposed to exterior of the protein. The ClpC subunit of Clp protease may recognize the degron and drag the whole CAO protein into its ClpP proteolytic subunits to digest the CAO protein. Such a mechanism may provide a fine and prompt regulation of CAO activity, which may be essential in the coordination of pigment supply and its assembly into the photosynthetic apparatus.

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13. SIGNALING FUNCTION OF TETRAPYRROLES

The function of tetrapyrroles is not limited to their roles as prosthetic groups, they are also capable of serving as signaling molecules. In mammals and yeast, heme has several important signaling roles. It has been identified that heme functions in cellular signal transductions, such as transcription (Guarente and Mason 1983; Shan et al. 2004; Sun et al. 2004; von Gromoff et al. 2008; Zitomer and Lowry 1992), translation (Joshi et al. 1995), post-translational protein modification (Chen et al. 1989), translocation (Lathrop and Timko 1993), and ion-channel function (Tang et al. 2003). Other tetrapyrroles have also been linked with signaling functions. In the red alga *Cyanidioschyzon merolae*, the synchronization of nuclear DNA replication with organellar DNA replication has been shown to be mediated by Mg-Proto IX (Kobayashi et al. 2009). It is shown that Mg-Proto IX binds to an F-box protein, SCF-type E3 ubiquitin ligase (Fbx3), and inhibits cyclin ubiquitylation, which is a part of the regulatory mechanism of nuclear DNA replication (Kobayashi et al. 2011). Similarly, there is evidence for tetrapyrrole regulation of gene expression in *Chlamydomonas reinhardtii* where feeding exogenous Mg-Proto IX has been shown to substitute for light in inducing nuclear *HSP70* expression (Kropat et al. 2000). Using *Chlamydomonas* MgMT mutants in which Mg-Proto IX is accumulated, it is shown that Mg-Proto IX and heme are involved in the transient activation of gene expression by light probably as second messengers (Meinecke et al. 2010). Experiments using MgCh mutants suggest that heme might also be an active signaling molecule in this system (von Gromoff et al. 2008). More recent transcriptome analysis show that in *Chlamydomonas* Mg-Proto IX and heme have global impacts on the expression of genes encoding enzymes of the tricarboxylic acid cycle, heme-binding proteins, stress-response proteins, and protein folding and degradation (Voss et al. 2011). Based on this observation, the authors suggest a signaling role of both tetrapyrroles as secondary messengers for adaptive responses affecting the entire cell and not only organellar proteins.

13.1. Involvement of tetrapyrroles in retrograde signaling

The photosynthetic apparatus is composed of proteins that are encoded by the nuclear and plastid 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. In Arabidopsis, mutants termed *gun* (*genome uncoupled*) are identified in which intracellular signaling was disrupted (Pfannschmidt 2010; Susek et al. 1993). These mutants express nuclear-encoded photosynthesis genes, even when chloroplast function is disrupted by treatment with NF, an inhibitor of carotenoid biosynthesis. Among five *gun* mutants (*gun1–5*), four of them (*gun2–5*) have mutations in tetrapyrrole biosynthetic enzymes. Besides the already mentioned *gun4* and *gun5*, the *gun2* and *gun3* mutants are alleles of *hy1* and *hy2*, which encode HO and PΦB synthase, respectively. On the other hand, *gun1* is a mutant of chloroplast localized pentatricopeptide protein, which appeared to act independently from tetrapyrrole pathway (Koussevitzky et al. 2007). Interestingly, POR-overexpressing Arabidopsis also shows a *gun* phenotype (McCormac and Terry 2002b). Subsequently, it is reported that wild type plants accumulate high amounts of Mg-Proto IX when grown on NF, 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 Arabidopsis knockout mutant lacking MgMT also suggests that Mg-Proto IX is a negative effector of nuclear photosynthetic gene expression (Pontier et al. 2007). Based on these observations, Mg-Proto IX has been proposed to accumulate under stress conditions and act as a negative regulator of photosynthetic gene regulation. The accumulation of Mg-Proto IX is visualized *in vivo* using confocal laser scanning microscopy (Ankele et al. 2007). Under stress conditions, Mg-Proto IX accumulates both in the chloroplast and in the cytosol, suggesting that this intermediate is exported from chloroplasts to the cytosol. However, these results should be interpreted with caution, as the authors only observed Mg-Proto IX accumulation in the cytosol upon feeding of ALA. Under such conditions, excessive accumulation of various intermediates of tetrapyrrole biosynthesis causes photooxidative damage, resulting in potential disruption of membrane integrity and leakage of tetrapyrrole intermediates to the cytosol. To substantiate the Mg-Proto IX signaling hypothesis in plants, it would be important to detect Mg-Proto IX in the cytosol when the cells are intact.

Compelling evidence that refutes the Mg-Proto IX signaling hypothesis in plants has been accumulating through the findings of various studies. The Arabidopsis *cs* and *ch-42* mutants, which have a defect in *CHLI1*, did not display the *gun* phenotype, although the MgCh activity is severely impaired (Mochizuki et al. 2001). Reduction of the endogenous level of Mg-Proto IX by overexpressing MgMT in tobacco does not alter the expression of a nuclear-encoded photosynthesis gene (Alawady and Grimm 2005). The role of Mg-Proto IX in plastid signaling after NF-treatment has been examined in detail. Using different detection techniques, two independent groups present complementary findings indicating that Mg-Proto IX is actually reduced after NF-treatment in Arabidopsis (Mochizuki et al. 2008; Moulin et al. 2008). Transcriptome data show a strong down-regulation of all tetrapyrrole synthesis genes after NF treatment and no correlation between Mg-Proto and *Lhcb1* expression levels are observed. Voigt et al. (2009) confirmed these conclusions and also showed that total heme accumulation is not correlated to the *gun* phenotype. Taken together, direct involvement of Mg-Proto IX as a mobile signal is not substantiated at the present time. More recently, Woodson et al. (2011) report that overexpression of *FC1* shows a *gun* phenotype, while *FC2* overexpressors do not. They propose an interesting model in which a specific heme pool that is produced by FC1 functions as a retrograde signal to coordinate nuclear gene expression. Existence of distinct heme pools that are probably produced by FC1 and FC2, respectively, should be examined in order to substantiate their model. In summary, the mechanism that correlates the impairment of tetrapyrrole biosynthesis and the reduction of nuclear-encoded photosynthesis genes remains challenging for researchers (see review by Mochizuki et al. 2010).

13.2. Involvement in abscisic acid (ABA) signaling

It was surprisingly reported that the CHLH subunit of MgCh functions as a novel receptor for a phytohormone, abscisic acid (ABA), which regulates plant responses to stressful conditions

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and various developmental processes (Shen et al. 2006). However, the function of CHLH as an ABA receptor is highly controversial. The CHLH subunit was shown to bind ABA *in vitro* at a dissociation constant of 32 nM. In Arabidopsis, *CHLH* overexpressors showed hypersensitivity to ABA, whereas *CHLH*-deficient mutants showed ABA-insensitive phenotypes (Shen et al. 2006). Moreover, Wu et al. (2009) proposed that the C-terminal half of CHLH plays a central role in ABA binding and signaling in Arabidopsis. On the other hand, another report showed that the barley *XanF* (a *CHLH* homolog)-deficient mutants did not exhibit any ABA-related phenotypes and the ABA-binding activity of XanF was not detected (Müller and Hansson 2009). However, Shang et al. (2010) reported that CHLH spans the chloroplast envelope membranes two times and that the cytosolic C terminus of CHLH interacts with negative transcriptional regulators of ABA signaling. Although this model is fascinating, their model of CHLH localization contrasts previous reports from several laboratories that are described in Section 9 (Adhikari et al. 2009; Gibson et al. 1996; Joyard et al. 2009; Nakayama et al. 1998). More recently, Tsuzuki et al. (2011) showed that CHLH affects ABA signaling in stomatal guard cells but is not itself an ABA receptor. Moreover, RCAR/PYR1/PYL–PP2C complexes were identified to govern main ABA responses (reviewed by Raghavendra et al. 2010). At the present time, it is not clearly understood whether CHLH is involved in ABA signaling.

14. CONCLUSIONS AND PERSPECTIVES

During the last few decades, almost all of the genes encoding enzymes involved in tetrapyrrole metabolism have been identified in higher plants. Novel insights on tetrapyrrole metabolism are obtained by genetic and biochemical studies with higher plants, which inspires us to update the classical tetrapyrrole metabolic pathways as described in this review. There are still a number of questions on tetrapyrrole metabolism which remain to be answered. We have not yet identified the complete set of regulatory factors that constrain and determine the total Chl and other tetrapyrroles contents. For Chl catabolism, the fate of Chl breakdown products after NCC formation is not yet clarified. Moreover, the exact route of Chl breakdown, which may be via Chlide or pheophytin, is still under consideration.

The interaction of tetrapyrrole metabolism and other metabolic pathways within a cell is another challenging area of research. Chl metabolism not only shares its substrate with other metabolic pathways including plastid protein synthesis and isoprenoid (phytol) metabolism, it also affects the turnover of photosynthetic proteins, degradation of thylakoid membranes, mobilization of nitrogen during senescence and the cellular program of senescence. Reflecting the complexity of the pathways and the chemical nature of tetrapyrrole intermediates that produce ROS by photodynamic reaction necessitated the evolution of a sophisticated system for complex regulatory mechanisms in plants. Understanding multiple levels of regulatory mechanisms on tetrapyrrole metabolism will lead to revealing yet-unidentified systems which function to control plastid metabolism, such as the good example of the ClpC- and Chl *b*-dependent degradation of CAO. The precise involvement of tetrapyrrole metabolism in the retrograde and ABA signaling pathways is still not clearly understood at this time. In order to gain a full understanding of these processes, it will be necessary to combine classical approaches with new technologies such as metabolomics of tetrapyrroles, live imaging, and next generation sequencing, together with biochemical techniques. Large-scale genome sequencing using next generation sequencers will broaden our understanding of gene arrangements and expression patterns in thus far uncharacterized plant and algal species. The outcome of these collective efforts will contribute greatly towards enhancing our understanding of the complex network of cellular processes related to tetrapyrrole metabolism in plants.

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