Chapter 15

Chlorophyll Synthesis

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Summary

Chlorophyll is the dominant pigment in a mature plant cell, whether in the leaf of a plant or in the abundant algal species. Chlorophyll is synthesized within the chloroplast from a plentiful precursor, the amino acid glutamate. From glutamate to the tetrapyrrole protoporphyrin IX, at which the pathway branches between chlorophyll and heme, the reactions occur in the plastid stroma and are catalyzed by soluble enzymes. The latter steps to chlorophyll, the first being the insertion of the central magnesium atom, occur with enzyme complexes that are at least partially if not entirely localized on membranes. Magnesium chelatase, the key enzyme in this pathway, is a complex of proteins that includes soluble and membrane-bound subunits. Subsequent reactions occur primarily on membranes and involve modification of structural groups on the periphery of the molecule. The pentultimate precursor of chlorophyll, protochlorophyllide, is reduced by NADPH to chlorophyllide in the only reaction in the pathway that requires light. This reaction, which in angiosperms is catalyzed by light-dependent NADPH:protochlorophyllide oxidoreductase, dramatically changes the property of the molecule and allows the product chlorophyllide, and its esterified product, chlorophyll, to interact with proteins. These chlorophyll-protein complexes become the building blocks of the photosynthetic apparatus. The biosynthetic pathway is tightly regulated, particularly at the key reactions that generate 5-aminolevulinic acid, magnesium-protoporphyrin IX and chlorophyllide. Expression of genes encoding critical enzymes is usually regulated markedly by light, and the activities of the enzymes are also regulated by end-products in typical feedback inhibition.

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I. Introduction: Overview of Chlorophyll Biosynthesis

This chapter emphasizes the reactions in chlorophyll synthesis in the latter part of the pathway. These latter intermediates become important in processes related to chloroplast development, thylakoid biogenesis and regulatory mechanisms of these processes. For a description of the earlier reactions in the pathway, see the forthcoming volume "Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics and Biological Function" (Editors: Bernhard Grimm, Robert J. Porra, Wolfhart Rüdiger and Hugo Scheer) in this series.

Although the plastid has a number of essential functions, photosynthesis occurs only in the chloroplast version of the organelle and requires chlorophyll. The importance of these molecules has attracted a great amount of effort to understand their characteristics and means of synthesis. A robust process must be in place to achieve the synthesis of chlorophyll—one of the most dominant substances in the plant cell—which occurs over the developmental stage of hours to a few days as a leaf matures. Chlorophyll is synthesized from the plentiful amino acid, glutamate. For biosynthetic reactions, including incorporation of glutamate into proteins, glutamate is converted to glutamyl-transfer RNA (tRNA) in a two-step reaction that requires ATP and is catalyzed by glutamyl-tRNA synthetase. The "activated" α-carboxyl group, linked to the tRNA via a reactive ester bond, is then reduced by NADPH to another reactive product, glutamate 1-semialdehyde (GSA), in the reaction catalyzed by glutamyl-tRNA reductase (GTR). GSA is rapidly converted to 5-aminolevulinic acid (ALA), in which the carbon-1 of glutamate becomes carbon-5 in ALA (Fig. 1). This reaction is an example of a rare intramolecular transaminase, in which the amino group, formerly on carbon-2 of glutamate and now carbon-4 of GSA, is transferred to carbon-5 in a reaction that requires the cofactor pyridoxamine-phosphate. GSA, and its more stable isomer, ALA, are the committed precursors for chlorophyll synthesis. Synthesis of ALA, and specifically of GSA, is the rate-controlling step in the pathway, and thus the activities of the enzymes that catalyze these steps are tightly controlled (Vavilin and Vermaas, 2002; Eckhardt *et al.*, 2004). The primary means of feedback control on the activity of glutamyl-tRNA reductase is thought to be mediated by heme. The crystal structures of the key enzymes in ALA synthesis were resolved, glutamyl-tRNA reductase by Schubert *et al.* (2002) and GSA transaminase by Hennig *et al.* (1994, 1997).

Two molecules of ALA are condensed to porphobilinogen by the enzyme porphobilinogen synthase (also called ALA dehydratase). Four molecules of porphobilinogen then are linked to achieve the tetrapyrrole structure. Condensation of the pyrrole rings is catalyzed by porphobilinogen deaminase (also called hydroxymethylbilane synthase). This enzyme has an unusual feature of containing a protein-bound dipyrromethane cofactor. Four porphobilinogen molecules are added sequentially to the cofactor to generate a chain of six pyrrole rings. The outer four are then cyclized, with reversal of the orientation of the last unit added, by uroporphyrinogen III synthase (Jordan, 1994; Beale, 1999). Uroporphyrinogen III is converted in several steps, by trimming of the propionyl side chains and oxidation, to yield protoporphyrin IX.

The early steps in the biosynthetic pathway are catalyzed by soluble proteins in the stroma of the plastid. The latter reactions, which involve increasingly more hydrophobic products, are localized on membranes. Increasing evidence supports localization of the reactions between protoporphyrin IX to protochlorophyllide on the inner membrane of the chloroplast envelope (Joyard *et al.*, 1998; Beale, 1999; Eckhardt *et al.*, 2004). Chlorophyll biosynthesis from glutamyl-tRNA to chlorophyll *b* requires 15 enzymes and 27 genes. With the identification of the gene for 8-vinyl reductase, the enzyme that catalyzes conversion of divinyl-protochlorophyllide or divinyl-chlorophyllide to the monovinyl forms, all the enzymes and genes involved in the biosynthetic pathway in *Arabidopsis thaliana* are known (Nagata *et al.*, 2005).

II. Protoporphyrin IX to Chlorophyll

The steps from protoporphyrin IX onwards are unique to the chlorophyll biosynthetic pathway. Figure 2 shows an overview of the enzymatic steps in the synthesis of chlorophyll from protoporphyrin IX onwards. The insertion of magnesium commits protoporphyrin IX to

Abbreviations: ALA – 5-aminolevulinic acid; CAO – chlorophyll *a* oxygenase; DPOR – dark NADPH:protochlorophyllide oxidoreductase; GSA – glutamate 1-semialdehyde aminotransferase; GTR – glutamyl-tRNA reductase; LHC – light-harvesting complex; Lhcb – apoprotein of light-harvesting complex; PLB – prolamellar body; POR – light-dependent NADPH:protochlorophyllide oxidoreductase; PORA, PORB, PORC – three forms of light-dependent, NADPH:protochlorophyllide oxidoreductase; tRNA – transfer RNA.

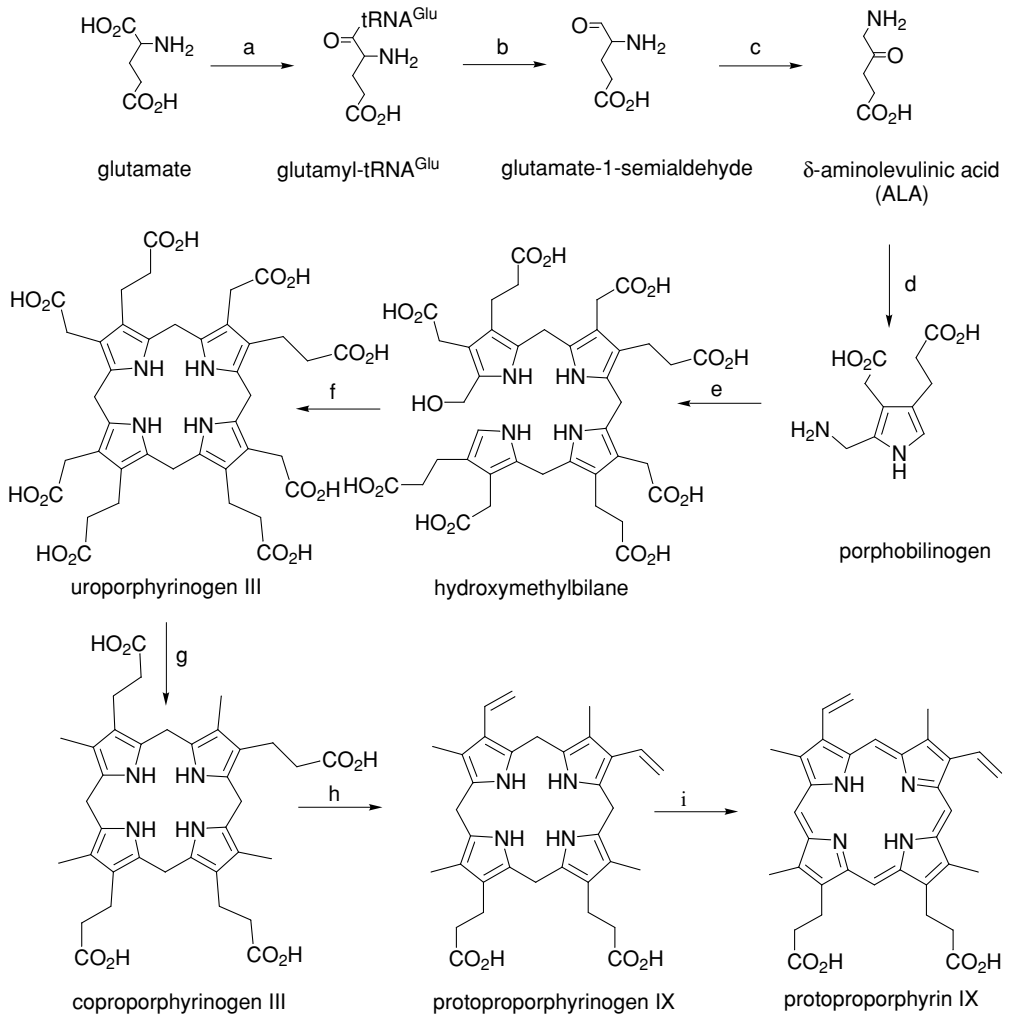

Fig. 1. Scheme showing the pathway for the synthesis of protoporphyrin IX from glutamate. (a) glutamyl tRNA synthetase; (b) glutamyl tRNA reductase (GTR); (c) glutamyl tRNA aminotransferase (GSAT); (d) ALA dehyratase (ALAD) or porphobilinogen synthase (PBGS); (e) porphobilinogen deaminase (PBGD) or hydroxymethylbilane synthase; (f) uroporphyrinogen IIII synthase; (g) uroporphyrinogen III decarboxylase (UROD); (h) coproporphyrinogen oxidase (COPOX); (i) protoporphyrinogen oxidase (PROTOX).

chlorophyll synthesis rather than heme synthesis. Thus there is a need for regulating the flux of intermediates between these two pathways. This chapter will deal with the overall regulatory mechanisms of chlorophyll biosynthesis in section III. This section will be confined to a discussion to the enzymes and the transcriptional regulation of the genes encoding these enzymes.

A. Magnesium Chelatase

Magnesium chelatase is a complex enzyme consisting of three distinct types of subunits. It is the first committed step in chlorophyll biosynthesis, as the preceding steps in the pathway are shared by the heme biosynthetic pathway (Willows, 2003; Willows and Hansson, 2003). The porphyrin substrate for both magnesium chelatase and ferrochelatase is protoporphyrin IX and, as both enzymes are found in the chloroplast, there is a requirement for regulation of these activities so that the demand for the end products of each pathway is satisfied. These two enzymes are quite different in structure, cofactor requirement and mechanism of metal ion insertion, partly because it is more difficult to insert Mg^{2+} than Fe^{2+} into the tetrapyrrole macrocycle. Magnesium chelatase requires Mg^{2+} ions and the hydrolysis of ATP for metal ion insertion into protoporphyrin IX, as shown in Fig. 2. Compared to the complex mechanism for magnesium chelatase, ferrochelatase is

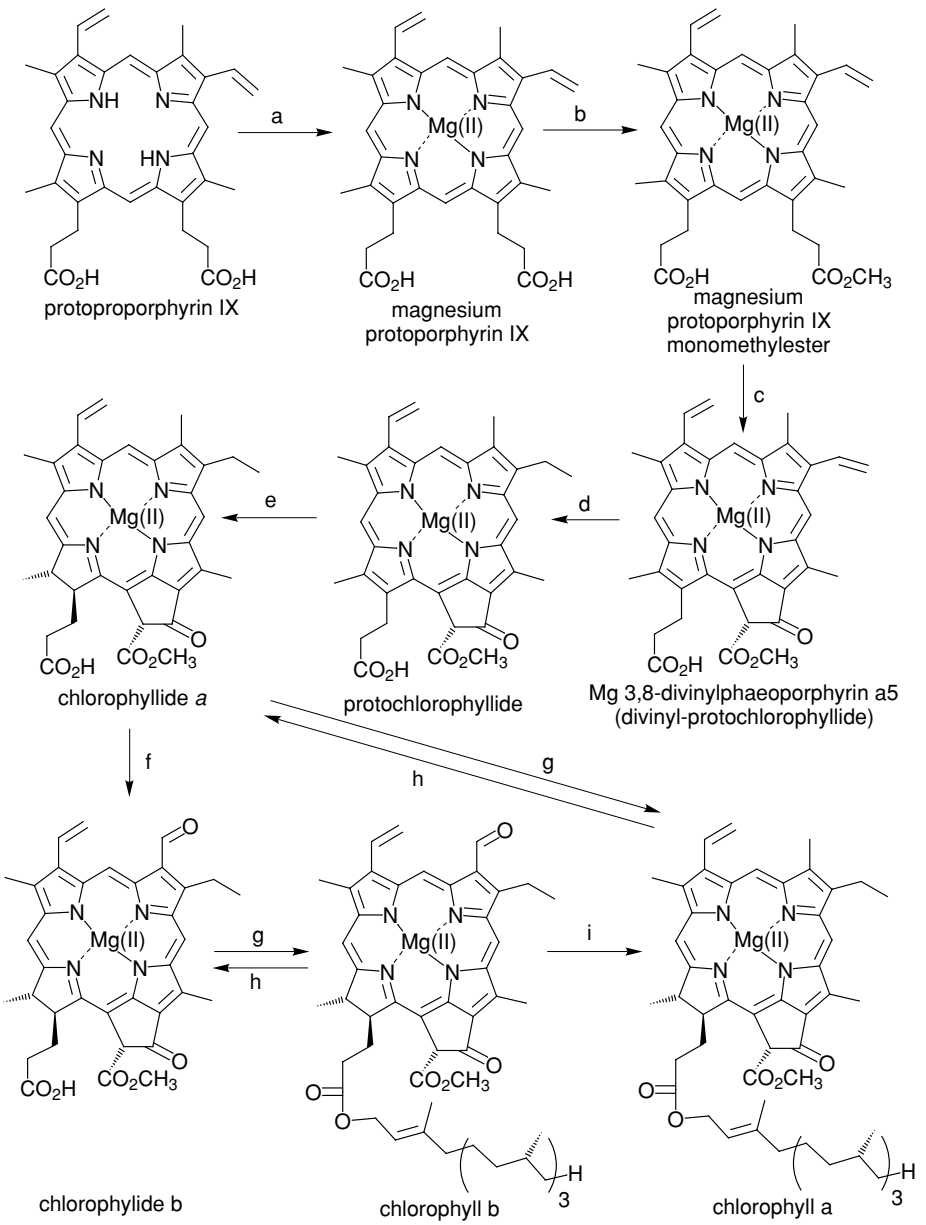

Fig. 2. Scheme showing the pathway for the synthesis of chlorophyll *a* and *b* from protoporphyrin IX. (a) magnesium chelatase; (b) S-adenosylmethionine:magnesium protoporphyrin IX O-methyltransferase; (c) magnesium protoporphyrin IX monomethylester oxidative cyclase; (d) 8-vinyl reductase; (e) protochlorophyllide oxidoreductase; (f) chlorophyll *a* oxidase; (g) chlorophyll synthase; (h) chlorophyllase; (i) chlorophyll *b* reductase.

a single subunit enzyme of about 40-kDa that catalyses Fe^{2+} insertion into protoporphyrin IX without the involvement of any additional cofactors (Ferreira, 1999).

The magnesium chelatase subunits consist of a small ~40-kDa subunit, an intermediate sized subunit protein of ~70-kDa, and a large subunit protein of ~140-kDa. These subunits will be referred to as **I**, **D** and **H**, respectively, when referring to the proteins, and ***I***, ***D*** and ***H***, respectively, when referring to the genes encoding these proteins. The naming of the different subunits of the magnesium chelatase in the literature may be rather confusing for many people as various plant mutants have different names. The corresponding genes in these plants share the mutant name and the suffixes to the names are often

Table 1. Magnesium chelatase mutants

Species	Subunit	Mutant, allele, and/or gene name/s	References
Antirhinnum majus	**H**	*olive-605*	Hudson *et al.*, 1993
A. thaliana	**I**	*cs*, *ch-42*	Koncz *et al.*, 1990 Rissler *et al.*, 2002
	H	*gun5*, *cch*	Larkin *et al.*, 2003 Mochizuki *et al.*, 2001 Strand *et al.*, 2003
	D	*chlD*	Strand *et al.*, 2003
	Unknown	*xan2*, *xan3*	Runge *et al.*, 1995
Chlamydomonas reinhardtii	**H**	*chl1*, *brs-1*	Chekounova *et al.*, 2001
	Unknown	*brs-2, brc-1, brc-2*	Wang *et al.*, 1974
Chlorella vulgaris	Unknown	W_5-brown	Granick, 1948
Hordeum vulgare	**I**	*xantha h*	Jensen *et al.*, 1996b
	D	*xantha g*	Petersen *et al.*, 1999
	H	*xantha f*	Jensen *et al.*, 1996b
Nicotiana tabacum	**I**	*sulfur*, *chlI*	Nguyen, 1995 Papenbrock *et al.*, 2000b
	H	*chlH*	Papenbrock *et al.*, 2000a
Rhodobacter species	**H**	*bchH*	Bollivar *et al.*, 1994; Coomber *et al.*, 1990 Gorchein *et al.*, 1993 Taylor *et al.*, 1983 Zsebo and Hearst, 1984
	D	*bchD*	Bollivar *et al.*, 1994 Coomber *et al.*, 1990 Gorchein *et al.*, 1993 Taylor *et al.*, 1983 Zsebo and Hearst, 1984
	I	*bchI*	Bollivar *et al.*, 1994 Coomber *et al.*, 1990 Gorchein *et al.*, 1993 Taylor *et al.*, 1983 Zsebo and Hearst, 1984
Triticum species	Unknown	Driscoll's chlorina, *chlorina-1, CD3, chlorina-214*	Falbel and Staehelin, 1994 Freeman *et al.*, 1987 Pettigrew *et al.*, 1969 Sears and Sears, 1968
Zea mays	Unknown	*l*-Blandy4, l*-Blandy3drk, l*-EMS1, oy-1039, oy-1040, l13-Neuffer2, l13-1050*	Mascia, 1978

contradictory, as detailed in Table 1. The molecular changes resulting from mutations in magnesium chelatase genes are known for mutants of barley, *Arabidopsis thaliana*, *Antirhinnum majus*, *Nicotiana tabacum*, *Chlamydomonas reinhardtii*, and the purple bacteria *Rhodobacter capsulatus*, and *Rhodobacter sphaeroides.* Putative magnesium chelatase mutants have also been identified in *Zea mays*, *Triticum* sp. and *Chlorella vulgaris*. A complete list of these magnesium chelatase mutants identifying the affected subunits was recently published (Willows and Hansson, 2003). All of the plant mutants have a chlorophyll-deficient, pale-green to yellow phenotype and accumulate protoporphyrin IX when fed ALA. Most of the mutants are recessive with the exception of three semi-dominant barley mutants that have single missense mutations in the **I** subunit (Hansson *et al.*, 1999). The algal mutants in contrast have a pale-brown phenotype due to the accumulation of protoporphyrin IX. This suggests that there is a difference in the regulation of chlorophyll biosynthesis in algae compared to plants, or at least angiosperms.

Mutants of the ***D*** and ***H*** genes of *A. thaliana* have a "genomes uncoupled" (*gun*) phenotype and

are defective in chloroplast to nucleus communication (Mochizuki *et al.*, 2001; Larkin *et al.*, 2003; Strand *et al.*, 2003; also, see Chapter 9). The *gun* mutants were selected for their ability to express the chlorophyll *a*/*b* binding protein of photosystem II, Lhcb1, under conditions where it is normally not expressed. The *gun5* and *cch* alleles were found to have missense mutations resulting in an alanine to valine substitution in *gun5* and a proline to leucine substitution in *cch* (Mochizuki *et al.*, 2001). Subsequent work showed that magnesium protoporphyrin IX or its monomethyl ester is part of the chloroplast-nuclear signalling process and is possibly the primary signalling molecule (Strand *et al.*, 2003). A protein involved in the downstream signalling process called GUN4 was also shown to interact with the **H** subunit of magnesium chelatase (Larkin *et al.*, 2003). Thus the magnesium chelatase may have a dual role in chloroplast to nuclear signalling; it produces the nuclear signal *and* one of the subunits interacts with a downstream signalling component.

Transgenic tobacco plants that express the **I** and **H** gene in the antisense orientation were produced. These plants have a uniformly pale-green phenotype typical of some of the barley mutants in these two genes. Perhaps unsurprisingly, protoporphyrin IX did not accumulate to high levels in these mutants, which tends to support the theory that feedback inhibition of ALA biosynthesis by heme is a major controlling factor in the pathway. However, the transcript levels of the *Gtr* and *Alad* genes, encoding glutamyl-tRNA reductase and ALA dehydratase, respectively, were also reduced in these lines, suggesting that expression may be synchronised with magnesium chelatase transcripts in some way (Papenbrock *et al.*, 2000a,b). It is possible that this regulation of transcript levels is via magnesium protoporphyrin signalling.

In plants, all of the magnesium chelatase genes are nuclear encoded. In algae and cyanelles, with the exception of *C. reinhardtii*, the ***I*** gene of magnesium chelatase is located on the chloroplast genome while the ***H*** and ***D*** are located on the nuclear genome. The pattern of nuclear gene expression for magnesium chelatase genes is not consistent across plant and algal species. The only common features are that magnesium chelatase genes are regulated by a circadian clock when grown under normal day/night cycles and etiolated or dark adapted plants show light-induced expression of ***H*** and ***I*** (Koncz *et al.*, 1990; Jensen *et al.*, 1996b). The pattern of expression of ***H*** varies across species with *A. majus* having a maximum in the dark phase and a minimum in the light phase, with low to non-existent transcript levels at medium light intensity (Hudson *et al.*, 1993). The opposite pattern is observed for ***H*** of tobacco, soybean, barley and *A. thaliana,* with all species having maximum expression in the light phase (Gibson *et al.*, 1996; Jensen *et al.*, 1996b; Nakayama *et al.*, 1998; Papenbrock *et al.*, 1999). Expression of the ***I*** gene in tobacco, barley and *A. thaliana* follows a similar pattern to the ***H*** transcript (Gibson *et al.*, 1996; Jensen *et al.*, 1996b; Papenbrock *et al.*, 1999). The ***D*** and the ferrochelatase genes of tobacco have an inverse expression to ***H*** (Papenbrock *et al.*, 1999). In *C. reinhardtii* there are maxima in both the light and dark phases for all three genes (Lake and Willows, 2003). It was suggested that the differences in the pattern of expression of magnesium chelatase may be related to both light intensity and to the ability of plants or algae to adapt to various light conditions (Lake and Willows, 2003).

Most of the details of the mechanism for magnesium chelatase have been gleaned from studies of the *Rhodobacter* and *Synechocystis* enzymes. Although the enzymes from different sources have slightly different properties, the overall mechanism is likely to be similar and much of the information on the structure and catalytic mechanism discussed in this section is from studies with the cyanobacterial or purple bacterial enzymes. The magnesium chelatase reaction has been dissected into two phases. The first phase involves formation of an activation complex between subunits **I** and **D,** which is dependent on protein concentration and ATP (Walker and Weinstein, 1991; Jensen *et al.*, 1996a; Willows and Beale, 1998; Willows *et al.*, 1996; Guo *et al.*, 1998). This activation complex catalyses magnesium insertion into protoporphyrin only when combined with the **H** protein, Mg-ATP, protoporphyrin IX and Mg^{2+}. The **H** protein behaves as a substrate in the magnesium chelatase reaction and has a K_m in the low micromolar range (Jensen *et al.*, 1998; Willows and Beale, 1998; Gibson *et al.*, 1999). The structure of the **I** protein from *R. capsulatus* was determined by X-ray crystallography (Fodje *et al.*, 2001). The **I** protein forms an ATP-dependent hexameric ring (Willows *et al.*, 2004), which is proposed to interact with a similar **D** hexameric ring to form a double-ring complex (Fodje *et al.*, 2001; Willows and Hansson, 2003). The **I** protein and a domain of the **D** protein belong to the extended class of triple-A proteins (AAA+) which are one of the largest and most diverse classes of proteins known and generally form ring-like structures (Confalonieri and Duguet, 1995; Vale, 2000). AAA+ proteins have also been called mechanoenzymes due to the mechanical nature of the large conformational changes that occur on ATP hydrolysis (Vale, 2000). The **I:D** double ring

structure presumably catalyses an ATP-dependent conformation change in **H** to effect magnesium insertion into a protoporphyrin IX bound to **H** (Hansson *et al.*, 2002; Willows and Hansson, 2003).

Studies that reported on compounds that specifically inhibit magnesium chelatase have been somewhat confusing, as inhibition of activity has been examined in a variety of ways ranging from *in vivo* studies, *in organello* studies, to true *in vitro* inhibition experiments. The *in vivo* and *in organello* studies suffer from problems of access of the inhibitor to the enzyme, which was highlighted in a recent review (Willows and Hansson, 2003). The inhibitors can be catalogued based on the mechanism of inhibition, being protein modifying agents, ATPase inhibitors, tetrapyrrole analogues and other inhibitors of undefined mechanism (Willows and Hansson, 2003). Light has also been shown to inhibit the magnesium chelatase of barley (Pöpperl *et al.*, 1997) and *Rhodobacter* (Willows and Beale, 1998), and this mode of inhibition probably occurs via photooxidative damage of the **H** subunit (Willows and Beale, 1998; Willows *et al.*, 2003). The inhibition by light of barley magnesium chelatase was also demonstrated with isolated chloroplasts. This contrasts with the situation *in planta* where isolated chloroplasts of barley from etiolated barley seedlings exposed to 4 h of light have considerably higher activity than chloroplasts from plants not exposed to light (Jensen *et al.*, 1996b). This increase in activity *in planta* can be attributed to the increased synthesis of the **I** and **H** subunits (Jensen *et al.*, 1996b) and is supported by data showing that magnesium protoporphyrin and magnesium protoporphyrin monomethyl ester levels increase dramatically in leaves from barley or tobacco when transferred from dark to light (Pöpperl *et al.*, 1997).

B. S-Adenosyl-L-Methionine:Magnesium Protoporphyrin IX-O-Methyltransferase

S-Adenosylmethionine:magnesium protoporphyrin IX-O-methyltransferase catalyses the S-adenosylmethionine-dependent methylation of the carboxyl group of the 13-propionate on magnesium protoporphyrin IX. This enzyme is membrane associated and the activity has been characterised for a number of plant species (reviewed in Bollivar, 2003). The gene for S-adenosylmethionine:magnesium protoporphyrin IX-O-methyltransferase was cloned and sequenced from tobacco and *A. thaliana* (Block *et al.*, 2002), and antisense transgenic tobacco plants were produced and are the subject of a patent (Reindl *et al.*, 2001). Two barley mutants, *xantha-n* and *albina-e,* have no detectable S-adenosylmethionine:magnesium protoporphyrin IX-O-methyltransferase activity. These mutants also have defective membrane structure and the reduction in activity may be a pleiotrophic effect of the defective membrane structure (Moller *et al.*, 1997).

The enzymes from *Euglena gracilis* (Richards *et al.*, 1981; Hinchigeri and Richards, 1982), wheat (Hinchigeri *et al.*, 1981) and more recently *Synechocystis* (Shepherd *et al.*, 2003) have been kinetically characterised. The *Euglena* and *Synechocystis* enzymes operate via a random ternary mechanism where the porphyrin and substrate may bind in any order. However, the wheat enzyme appears to operate via a ping-pong mechanism with S-adenosylmethionine binding first and presumably methylating the enzyme. If this is confirmed, it would represent a novel mechanism for a methyltransferase.

C. Magnesium-Protoporphyrin IX Monomethylester Oxidative Cyclase

An oxidative cyclization is required to create the fifth ring of chlorophyll, a reaction that is catalysed by magnesium protoporphyrin IX monomethyl ester oxidative cyclase. The origin of the oxygen atom in the fifth ring was studied by ^{18}O labelling using $^{18}O_2$ and/or $H_2^{18}O$. The oxo group in the fifth ring of chlorophyll is derived from molecular oxygen in cucumber (Walker *et al.*, 1989), while the oxo group in the fifth ring of bacteriochlorophyll of anaerobic photosynthetic bacteria is derived from water (Porra *et al.*, 1995, 1996; Porra and Scheer, 2001). This implies a completely different mechanism and subsequently a different type of enzyme that is required for formation of the fifth ring in these organisms.

There are only a limited number of reports demonstrating oxidative cyclase activity in oxygenic organisms. These reports include activity from chloroplasts of *C. reinhardtii* (Bollivar and Beale, 1996), developing chloroplasts from cucumber cotyledons (Vijayan *et al.*, 1992), lysed cucumber and *C. reinhardtii* chloroplasts (Walker *et al.*, 1991b; Whyte *et al.*, 1992; Whyte and Castelfranco, 1993; Bollivar and Beale, 1996) and cell-free extracts from cyanobacteria (Bollivar and Beale, 1996). The cucumber enzyme was resolved into membrane and soluble components, and inhibition studies suggested that the enzyme was probably not a member of the cytochrome P-450 family (Whyte and Castelfranco, 1993). In contrast to the cyclases from cucumber, *C. reinhardtii* cyclase activity did not require a soluble component and activity was found associated with

membranes. This enzyme is also not a member of the P-450 family based on inhibitor studies (Bollivar and Beale, 1995). The herbicide 2,2'-dipyridyl, which is a Fe^{2+} chelator, inhibits most oxidative cyclases and reduced chlorophyll synthesis (Mostowska *et al.*, 1996). Other iron chelating inhibitors of this enzyme include 8-hydroxyquinoline, desferal mesylate (Walker *et al.*, 1991a) and β-thujaplicin (Oster *et al.*, 1996). Thus the one common feature of all known cyclases is that they are inhibited by chelators of Fe^{2+}, suggesting that non-heme iron or an iron sulphur cluster is involved in the reaction. As only hydrophobic Fe^{2+} chelators appear to be effective inhibitors, it was suggested that the Fe^{2+} requirement is associated with the cyclase membrane fraction (Bollivar and Beale, 1996).

Although no plant genes have been positively identified, hints at the identity of the plant oxidative cyclase genes come from *Chlamydomonas* mutants and the purple bacterium *Rubrivivax gelatinosus*. Unlike many purple bacteria, *Rx. gelatinosus* is able to synthesize bacteriochlorophyll *a* under both aerobic and anaerobic conditions. Disruption of the *AcsF* gene of *Rx. gelatinosus* prevents bacteriochlorophyll *a* synthesis and causes accumulation of magnesium protoporphyrin IX monomethyl ester under aerobic conditions but not under conditions of low aeration. The designation *acsF* stands for aerobic cyclization system Fe-containing subunit, as AcsF and its homologs have a conserved putative binuclear-iron-cluster motif (Pinta *et al.*, 2002). The AcsF protein is homologous to previously identified gene products in *C. reinhardtii* called Crd1 (Moseley *et al.*, 2000) and Cth1 (Moseley *et al.*, 2002) and homologs of AcsF were also identified in *A. thaliana* and *Synechocystis* (Pinta *et al.*, 2002).

Crd1 and *Cth1* expression in *C. reinhardtii* is reciprocal and is regulated by copper and/or oxygenation conditions. *Crd1* is expressed under low aeration and/or low copper conditions and *Cth1* is expressed under oxygenated and copper sufficient conditions. Mutation of either of these genes and growth under conditions where the alternative protein is not expressed results in a chlorotic phenotype with reduced photosystem I and light-harvesting complex 1 accumulation (Moseley *et al.*, 2000, 2002). These results suggest that the Crd1 and Cth1 proteins probably encode two isoforms of the oxidative cyclase. Two mutant loci in barley called *xantha-l*^{35} and *viridis-k*^{23} also have defective cyclase activity (Walker *et al.*, 1997). Extracts of either *xantha-l* or *viridis-k* showed no activity in an *in vitro* assay nor did components in one extract complement the other when mixed. Fractionation studies showed that the *xantha-l* and *vividis-k* components are membrane-bound subunits and that cyclase activity also required a soluble, stromal component. The barley *Xantha-l* gene is homologous to the *Arabidopsis Crd1*, the ortholog of *AcsF* (Rzeznicka *et al.*, 2005). These putative cyclase encoding genes are nuclear encoded, but an ortholog of *AcsF* is found in the chloroplast genome of the red algae *Porphyra purpurea* (Reith and Munholland, 1995).

D. 8-Vinyl Reduction

Virtually all photosynthetic organisms require reduction of the 8-vinyl group of chlorophyll to an ethyl group. 8-Ethyl and 8-vinyl derivatives of intermediates from protochlorophyllide to chlorophyllide *a* have been detected in a number of studies by low temperature fluorescence spectroscopy (Rebeiz *et al.*, 1994; JS Kim and Rebeiz, 1995; Parham and Rebeiz, 1995; JS Kim *et al.*, 1997). The relative amounts of 8-ethyl and 8-vinyl intermediates and the stage at which reduction occurs is complex and depends on numerous factors such as species, developmental stage, time in the dark or light, the age of the tissue, and light intensity (Rebeiz *et al.*, 1994). Separation of 8-vinyl-protochlorophyllide and 8-ethyl-protochlorophyllide using a solid phase polyethylene column was used to analyse the biosynthesis of these intermediates in wheat and cucumber cotyledons. The activity in wheat was higher than in cucumber and it was suggested that the reaction is reversible (Whyte and Griffiths, 1993). An 8-vinyl reductase activity was detected in plastid membranes from cucumber that converts 8-vinyl-chlorophyllide *a* to chlorophyllide *a* but was unable to convert 8-vinyl-protochlorophyllide to 8-ethyl-protochlorophyllide (Parham and Rebeiz, 1992, 1995). To explain the diversity of other 8-ethyl intermediates it was suggested that a soluble component may mediate the substrate specificity of the 8-vinyl reductase allowing other 8-vinyl intermediates to be converted to 8-ethyl forms (JS Kim *et al.*, 1997). A gene that encodes a 3,8-divinyl-protochlorophyllide *a* 8-vinyl reductase was recently identified and cloned. When expressed in *Escherichia coli*, the gene product reduced divinyl chlorophyllide *a* to monovinyl-chlorophyllide *a* (Nagata *et al.*, 2005).

E. Protochlorophyllide Oxidoreductases

Two types of enzymes have been identified that reduce the D pyrrole ring of protochlorophyllide to form chlorophyllide. Of these two enzymes the light-requiring or light-dependent NADPH- protochlorophyllide oxidoreductase (EC 1.3.1.33 or EC 1.6.99.1,

abbreviated POR) has been the subject of a large number of reviews (Fujita, 1996; Reinbothe and Reinbothe, 1996; S Reinbothe *et al.*, 1996; Adamson *et al.*, 1997; Lebedev and Timko, 1998; Schoefs, 2001a,b; Rüdiger, 2003). POR is a single subunit enzyme that requires light as a substrate and it appears to be present in all organisms that synthesize chlorophyll. It has not been found in bacteriochlorophyll-synthesizing organisms. In the dark this enzyme forms a ternary complex with protochlorophyllide and NADPH and the bound protochlorophyllide is only reduced to chlorophyllide upon exposure to light.

The second type of enzyme, known as the light-independent protochlorophyllide oxidoreductase or DPOR, consists of three subunits (Armstrong, 1998; Fujita and Bauer, 2003). The multi-subunit DPOR has not been found in flowering plants (angiosperms) but appears to be present in most other chlorophyll and bacteriochlorophyll synthesizing organisms and allows these organisms to make chlorophyll in the dark. In contrast, flowering plants (angiosperms) are unable to synthesize chlorophyll in the dark, at least during the early stages of development, and thus do not appear to have a DPOR-type of enzyme. However, there are numerous reports that mature green leaves of some angiosperms can synthesize chlorophyll in the dark (reviewed in Adamson *et al.*, 1997). Thus DPOR may be present in mature leaves of some angiosperms or another as yet uncharacterised mechanism exists in these plants to allow chlorophyll synthesis in the dark.

1. Light-Dependent Protochlorophyllide Oxidoreductase (POR)

The barley (*Hordeum vulgare*) *Por* gene encoding POR was the first to be sequenced (Schulz *et al.*, 1989). Since then many *Por* genes have been cloned and sequenced from a variety of sources and some plants have been found to contain multiple *Por* genes encoding different isoforms. *A. thaliana* has three *Por* genes encoding proteins termed PORA, PORB and PORC (Armstrong *et al.*, 1995; Oosawa *et al.*, 2000; Su *et al.*, 2001), while *Pinus taeda* (Skinner and Timko, 1998),*Pinus mugo* (Forreiter and Apel, 1993), barley (Holtorf *et al.*, 1995) and tobacco (Masuda *et al.*, 2002) have at least two genes encoding different POR isoforms. *Por* genes have also been identified in *Triticum aestivum* (Teakle and Griffiths, 1993), cucumber (Fusada *et al.*, 2000), *Pisum sativum* (Spano *et al.*, 1992), *C. reinhardtii* (Li and Timko, 1996) as well as from the cyanobacteria *Synechocystis* PCC6803 (Suzuki and Bauer, 1995). In barley and *A. thaliana* the isoforms are differentially expressed and the isoform called PORA is negatively regulated by light and appears to have a role only in the de-etiolation process (Armstrong *et al.*, 1995; Holtorf *et al.*, 1995; Holtorf and Apel, 1996). However, this type of differential regulation of isoforms does not appear to be universal as the two tobacco *Por* genes are regulated in a similar way and are not negatively regulated by light (Masuda *et al.*, 2002). Some plants such as cucumber have only a single *Por* gene indicating that multiple isoforms are not essential for plant growth and development (Fusada *et al.*, 2000).

POR is responsible for large crystalline-like membrane structures that form within chloroplasts known as prolamellar bodies (PLBs). These structures are visible by electron microscopy and are found in developing angiosperm chloroplasts that have not been exposed to light. These membrane-associated complexes consist of the protochlorophyllide:NADPH:POR ternary complex aggregated within a lipid matrix in the etioplast, which are poised, waiting for the final substrate, light, to allow photoconversion of the protochlorophyllide to chlorophyllide. The main spectral form of protochlorophyllide observed *in vivo* is due to these ternary complexes that make up the PLBs (Wiktorsson *et al.*, 1992, 1993, 1996b). Pigment binding to POR is reported to be essential for the formation of PLBs, as mutants that are unable to make protochlorophyllide do not make PLBs (Henningsen *et al.*, 1993) and PLB formation can also be inhibited by treatment of plants with gabaculine, which inhibits protochlorophyllide formation (Younis *et al.*, 1995). Import of PORA into chloroplasts has been reported to require the presence of protochlorophyllide within the chloroplast (S Reinbothe *et al.*, 1995a, 1995b, 2000). It has been suggested that this finding is an artifact (Aronsson *et al.*, 2000, 2003; Dahlin *et al.*, 2000). A recent paper reconciling these findings indicates protochlorophyllide is indeed required for the import of PORA but this only occurs in etioplasts within developing cotyledons (C Kim and Apel, 2004). A complicating factor in all of these analyses is that a light-induced protease that breaks down PORA is also present within developing chloroplasts (C Reinbothe *et al.*, 1995). Lipids are also required for the formation of PLBs (Klement *et al.*, 2000), and flavins (Belyaeva *et al.*, 2000), violaxanthin and zeaxanthin (Chahdi *et al.*, 1998) have been detected in PLBs and may be involved in their formation. PLBs have been detected in mutants of organisms that are normally able to synthesize chlorophyll in the dark such as the *yellow-in-the-dark* mutants of *C. reinhardtii*. This suggests that most PORs are capable of forming PLBs, and the demonstration that both PORA and PORB of

A. thaliana are able to form PLBs supports this suggestion (Sperling *et al.*, 1998; Franck *et al.*, 2000).

On exposure to light, protochlorophyllide bound to POR is converted to chlorophyllide and then rapidly to chlorophyll. The PLBs then disperse or disaggregate as the photosystems are assembled. Protein phosphorylation appears to be involved in both this disaggregation process and in the formation of the PLBs (Wiktorsson *et al.*, 1996a; Kovacheva *et al.*, 2000). Details of the fine structure of the PLBs and what occurs during the photoconversion process have been the source of much contention. It was proposed, based on *in vitro* experiments with zinc analogues of protochlorophyllide *a* and *b*, that in barley a ternary complex of NADPH:PORA:protochlorophyllide *b* and a ternary complex of NADPH:PORB:protochlorophyllide *a* form a 5:1 complex, respectively, within the PLBs. In this complex the NADPH:PORA:protochlorophyllide *b* acts as a light-harvesting complex transferring light to the NADPH:PORB:protochlorophyllide *a,* allowing photoconversion of protochlorophyllide *a* to chlorophyllide *a* (C Reinbothe *et al.*, 1999). Two of the problems with this model are that this was based on *in vitro* experiments with artificial substrates and that protochlorophyllide *b* had not been detected in the quantities required within developing chloroplasts of barley by other investigators (Willows, 1999; Armstrong *et al.*, 2000). Reinbothe *et al.* recently followed up these criticisms with two papers, one showing that protochlorophyllide *b* does in fact occur in barley and that it is rapidly converted to protochlorophyllide *a* by a reductase (S Reinbothe *et al.*, 2003) and the second showing that the *in vitro* produced 5:1 PORA:PORB complex can also be made using authentic protochlorophyllides *a* and *b* (C Reinbothe *et al.*, 2003). However, the controversy continues as Kolossov and Rebeiz (2003), using the methods of Reinbothe, found protochlorophyllide *b* only in mature green barley leaves and not in etiolated barley and thus proposed that the protochlorophyllide *b* complex does not occur *in vivo*.

2. Light-Independent (Dark) Protochlorophyllide Oxidoreductase (DPOR)

Cyanobacteria, green algae and most non-flowering plants have both POR and DPOR. DPOR was reviewed by Armstrong (1998). Green algae and most nonflowering land plants are able to make chlorophyll in the dark with protein products of the chloroplast-encoded genes *ChlL*, *ChlN* and *ChlB.* Mutation or deletion of these chloroplast genes in the green algae *C. reinhardtii* prevented chlorophyll synthesis in the dark (Roitgrund and Mets, 1990; Suzuki and Bauer, 1992; Li *et al.*, 1993; Liu *et al.*, 1993). Seven *C. reinhardtii* nuclear mutants have a similar lack of chlorophyll in the dark and in all cases these mutations prevent the translation of mRNA from the chloroplast-encoded *ChlL* gene (Cahoon and Timko, 2000). The *ChlL* gene of *C. reinhardtii* hybridises to DNA from bacteria and nonflowering land plants, which can clearly synthesize chlorophyll in the dark, but no bands are evident when hybridised to DNA from the representative angiosperms, *Z. mays*, *A. thaliana*, *N. tabacum* and *Bougainvillea glabra* (Suzuki and Bauer, 1992). Unlike most other chlorophyll biosynthetic genes, when the *ChlL*, *ChlN* and *ChlB* genes are present, they are invariably found in the chloroplast genomes (Lidholm and Gustafsson, 1991; Burke *et al.*, 1993; Suzuki *et al.*, 1997; Armstrong, 1998).

F. Chlorophyll a Synthase

Chlorophyll *a* synthase catalyses the final step in the synthesis of chlorophyll *a* with the esterification of a phytol group to the 17-propionate. Chlorophyll synthase genes, *ChlG*, have been cloned and the enzymes heterologously expressed in *Escherichia coli* from both oat (*Avena sativa*) and *A. thaliana.* The C*hlG* is nuclear encoded and encodes a chloroplast transit sequence for translocation of the enzyme into the chloroplast. Phytyl-pyrophosphate and geranylgeranyl-pyrophosphate are both substrates for chlorophyll synthases. *A. thaliana* chlorophyll synthase preferred geranylgeranyl-pyrophosphate as the substrate (Oster *et al.*, 1997; Oster and Rüdiger, 1997; Schmid *et al.*, 2001).

The *ChlP* gene product is required for the reduction of geranylgeraniol to phytol and it appears that reduction can occur either before or after esterification to chlorophyllide *a*. The *ChlP* genes in *N. tabacum* and *A. thaliana* are located in the nuclear genome and encode a putative 52-kDa precursor protein. Transgenic tobacco plants expressing antisense *ChlP* RNA have both reduced tocopherol and chlorophyll synthesis, indicating that this enzyme provides phytol and/or phytyl-pyrophosphate for both of these pathways (Tanaka *et al.*, 1999). Two types of reductase may be present in chloroplasts, as there is a reductase activity in the chloroplast envelope that converts geranylgeranyl-pyrophosphate to phytyl-pyrophosphate and a second in the thylakoids converts geranylgeraniol esterified to chlorophyllide *a* into chlorophyll *a* (Soll *et al.*, 1983).

G. Chlorophyll a*-Chlorophyll* b *Cycle*

Chlorophyll *a* oxygenase (CAO) is the enzyme that catalyses the conversion of chlorophyll *a* to chlorophyll *b. CAO* genes have been identified in *C. reinhardtii*, *A. thaliana, Oryyza sativa, Marchantia polymorpha, Dunaliella salina, Prochlorothrix hollandica* and *Prochloron didemni* (Tanaka *et al.*, 1998; Espineda *et al.*, 1999; Tomitani *et al.*, 1999). The *A. thaliana* CAO was heterologously expressed in *E. coli* and required oxygen and reduced ferredoxin to convert chlorophyllide *a* to chlorophyllide *b*. Traces of a 7^1-hydroxy intermediate were detected, and the enzyme could also use Zn-chlorophyllide *a* as a substrate but not pheophorbide or chlorophyll *a* (Oster *et al.*, 2000), indicating that the enzyme is a chlorophyllide *a* oxidase rather than a chlorophyll *a* oxidase.

Rüdiger (2002) reviewed the synthesis of chlorophyll *b* and suggested that the interconversion of chlorophyll *a* and *b* operates as a cycle. Chlorophyll *b* can be converted to chlorophyll *a* by a chloroplast localised reductase activity. The gene encoding this reductase has yet to be identified. This reductase activity is probably required for both alteration of the chlorophyll *a* to *b* ratio and in the degradation of chlorophyll. Both chlorophyll *a* and *b* can also be converted to their corresponding chlorophyllides by chlorophyllase. Thus the cycle exists from chlorophyllide *a* to *b,* followed by esterification to chlorophyll *b,* reduction to chlorophyll *a,* and deesterification back to chlorophyllide *a* (Rüdiger, 2002).

III. Regulation of Chlorophyll Biosynthesis

The three main regulatory points in chlorophyll biosynthesis appear to be the steps involved in ALA biosynthesis, magnesium chelatase and protochlorophyllide reductase. Fig. 3 shows an overview of the regulatory mechanisms that affect these steps. The regulation of key steps in chlorophyll biosynthesis includes;

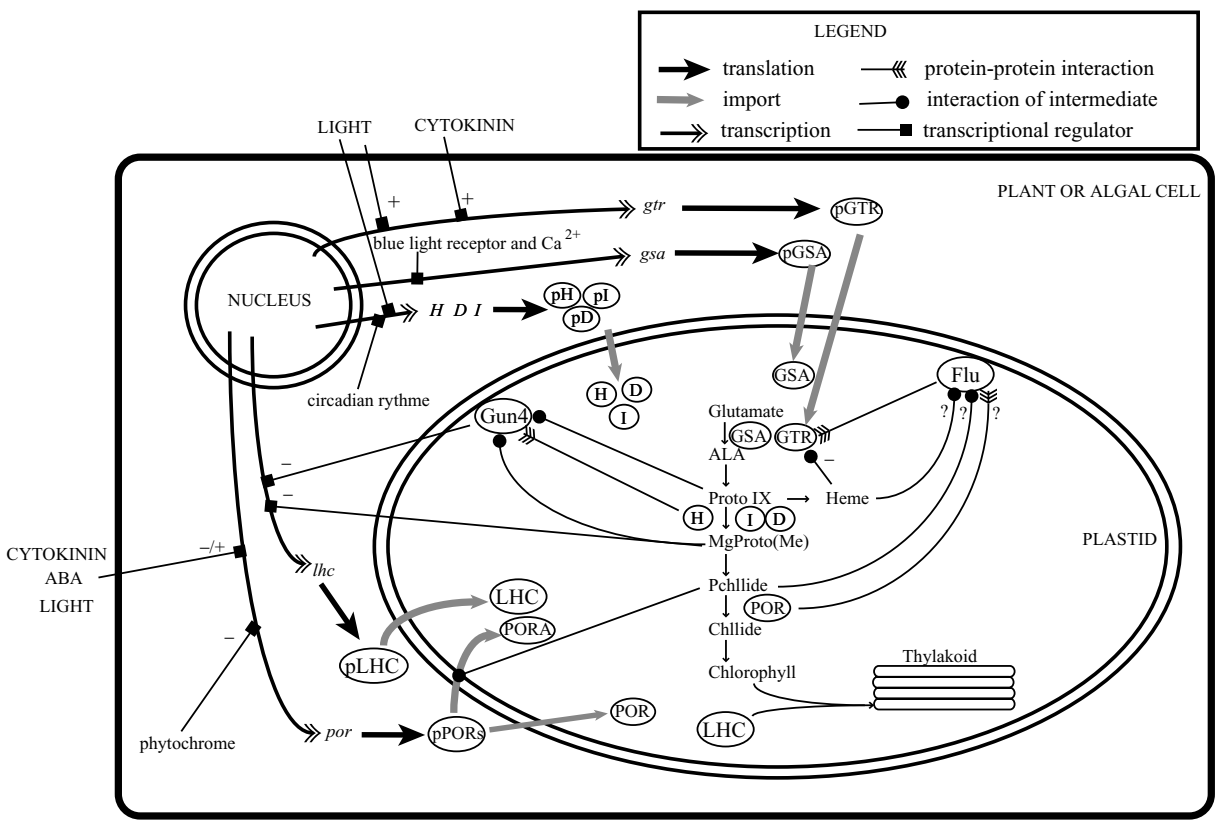

Fig. 3. Regulatory network within plant and algal cells involving the chlorophyll biosynthetic pathway enzymes and intermediates. (−) indicates inhibition, (+) indicates activation, and (?) indicate putative effects. Ellipses represent proteins or preproteins with small "p" prefix. H, D and I are magnesium chelatase subunits. For all other abbreviations see text.

(i) mechanisms to control quantities of individual enzymes using transcriptional or translational controls, and (ii) mechanisms to control activities of various enzymatic steps within the pathway using feedback inhibition or other modifiers of enzymatic activity. An additional feature that may or may not impact on regulation of the pathway but which is involved in plastid development is that both the product of the magnesium chelatase reaction and the magnesium chelatase itself are implicated in control of nuclear gene expression, specifically the control of LHC gene expression.

A. Regulation of ALA Synthesis

The primary regulatory step in chlorophyll biosynthesis is at the level of ALA biosynthesis. This is clear from feeding studies with ALA, which causes the unregulated synthesis of chlorophyll in the light or of protochlorophyllide in the dark. In plants the enzyme glutamyl-tRNA reductase (GTR) is the rate determining step of the entire tetrapyrrole biosynthetic pathway (Grimm, 2003). Feedback inhibition and transcriptional regulation are both used to regulate the activity of this enzyme. Feedback regulation by heme is difficult to demonstrate conclusively because of the detergent like properties of heme and its low solubility. However, recombinant and natural barley GTR have been shown to be inhibited by heme and also appear to have a bound heme (Pontoppidan and Kannangara, 1994; Vothknecht *et al.*, 1998). The inhibition and heme binding were both abolished when the N-terminal end of the barley enzyme was truncated by 30 amino acids, which tends to confirm that the heme inhibition is not an artifact (Vothknecht *et al.*, 1998). Protochlorophyllide is known to limit its own synthesis in dark-grown plants and the feedback regulation occurs at the level of ALA synthesis. This inhibition is likely to be via the FLU protein, as mutations in the *Flu* gene result in deregulation of synthesis of protochlorophyllide, and FLU been shown to interact with GTR (Meskauskiene *et al.*, 2001; Meskauskiene and Apel, 2002).

All plants studied to date have multiple GTR genes, which are differentially expressed in various plant organs and under a variety of conditions, and both light and cytokinin have been shown to induce expression of one *Gtr* gene. Thus a *Gtr* of barley is induced by both light and cytokinin and is also circadian regulated (Bougri and Grimm, 1996). Light induced expression of both the *Gsa* and one of the *Gtr* genes of *A. thaliana* (Ilag *et al.*, 1994). Other regulators of *Gtr* transcription have included temperature, photooxidative stress, and sugar (Ujwal *et al.*, 2002; Grimm, 2003). These transcriptional studies are consistent with *in vivo* studies showing chlorophyll accumulation is enhanced by red-light and cytokinin treatment in developing cucumber cotyledons which involves Ca^{2+} as a second messenger (Reiss and Beale, 1995).

The unicellular algae *C. reinhardtii* seems to regulate its ALA synthesis by controlling levels of the enzyme glutamate semi-aldehyde aminotransferase, as the transcript levels for this enzyme vary 26-fold in response to blue-light, ammonia and/or acetate (Matters and Beale, 1994, 1995; Im *et al.*, 1996). However, details of the regulation of *C. reinhardtii Gtr* have yet to be reported and it is conceivable that it may be similarly regulated. The change in *C. reinhardtii Gsa* transcript levels are mediated via inositol triphosphate-induced Ca^{2+} release which activates calmodulin and a Ca^{2+}/calmodulin dependent protein kinase (Im and Beale, 2000). The similarity between aspects of this transcriptional regulation and that of some plant *Gtr* and *Gsa* genes indicate that this signalling pathway may be conserved between plants and algae although the sensor may have diverged.

B. Magnesium Chelatase

Regulation of magnesium chelatase gene expression has been examined in a number of species. In etiolated barley, expression of ***I*** and ***H*** genes is induced by light, while in green barley seedlings grown in normal day light cycles the ***H*** transcript levels follows a circadian rhythm with maxima in the light phase (Jensen *et al.*, 1996b). The tobacco ***H*** and ***I*** transcripts follow a similar circadian pattern but the ***D*** transcript has an inverse expression pattern with maximal mRNA levels in the dark phase. In *A. thaliana* (Gibson *et al.*, 1996) and *A. majus* (Hudson *et al.*, 1993) the ***H*** transcript is at its maximal level in the dark and is down-regulated in the light. The ***I*** gene in barley and *A. thaliana* is constitutively expressed except during the initial phases of greening. In *C. reinhardtii* all three ***H***, ***I*** and ***D*** genes appear to be regulated the same way. The transcript levels of these genes follow a diurnal regulation with maxima in both the light and dark phases with rapid fall in transcripts at the start of the light phase followed by a rise to a maximum at about 4 hours light and fall to a minimum near the end of the light and an increase to a maxima near the end of the dark phase (Lake and Willows, 2003). Another factor regulating magnesium chelatase activity is the recent finding that an active **I** protein is required to stabilise the **D** protein *in vivo* (Lake *et al.*, 2004). Thus reduction in the amount of **I**

will cause a corresponding loss of the **D** subunit and a reduction of magnesium chelatase activity.

Magnesium chelatase proteins and magnesium protoporphyrin IX have been implicated in chloroplast-to-nuclear signalling. As mentioned previously, the *A. thaliana* genomes-uncoupled mutant, *gun-5*, is a result of a point mutation in the ***H*** gene; a mutation in the ***D*** gene gives a similar phenotype (Mochizuki *et al.*, 2001; Strand *et al.*, 2003). The recently identified GUN-4 protein binds protoporphyrin IX and also interacts with the porphyrin-binding **H** protein. This implicates GUN-4 as one of the downstream signalling components in chloroplast nuclear signalling (Larkin *et al.*, 2003).

C. Protochlorophyllide Oxidoreductase (POR)

Phytochrome, circadian clocks, cytokinin, abscisic acid and leaf age have been implicated in control of *Por* gene expression. The amounts of POR protein and mRNA decrease rapidly in many species when etiolated plants are exposed to light (Forreiter *et al.*, 1990), suggesting phytochrome involvement in this process. Experiments using *A. thaliana* with red and far-red light treatments have confirmed phytochrome A regulates *PorA* mRNA levels (Barnes *et al.*, 1996; Sperling *et al.*, 1997, 1998). Phytochrome has also been shown to regulate the expression of the *Por* gene from a lower plant (*Marchantia paleacea*) (Suzuki *et al.*, 2001). In barley the phytochrome- and/or light-dependent regulation of *PorA* mRNA levels is dependent on a 3′-untranslated region in the mRNA (Holtorf and Apel, 1996). In addition to the reduction in message, a light-dependent degradation of the PORA bound to chlorophyllide, but not protochlorophyllide, occurs and a light-induced protease has been shown to be responsible (C Reinbothe *et al.*, 1995). In contrast, cucumber, which only has a single *Por* gene, shows an increase in *Por* message levels during the de-etiolation process (Kuroda *et al.*, 1995). Moreover, in fully green leaves of cucumber, this gene is expressed at very low levels in the dark and the amount of *Por* message increases dramatically when plants are transferred from dark to light (Kuroda *et al.*, 2000).

The effect of leaf age on *Por* gene expression has been studied in pea (He *et al.*, 1994), barley (Holtorf *et al.*, 1995; Schunmann and Ougham, 1996), wheat (Marrison *et al.*, 1996), and *A thaliana* (Armstrong *et al.*, 1995). In barley and *A. thaliana*, *PorA* mRNA is only expressed in young etiolated tissue while the *PORB* mRNA is expressed throughout development. In light-grown seedlings of pea and wheat the youngest leaves contained the highest POR message levels.

The plant hormones cytokinin and abscisic acid appear to have a role in regulation of *Por* gene expression. The involvement of cytokinin was inferred from the finding that cytokinins overcame the inhibition of greening caused by treatment with cadmium and mercury (Thomas and Singh, 1995, 1996), although cadmium and mercury also have a direct effect on POR enzyme activity (Boddi *et al.*, 1995; Lenti *et al.*, 2002). It was subsequently found that cytokinins directly activated *Por* gene expression in cucumber (Kuroda *et al.*, 2001) and *Lupinus luteus* (Kusnetsov *et al.*, 1998) and that abscisic acid inhibits *Por* gene expression in *L. luteus* (Kusnetsov *et al.*, 1998).

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