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Update on Biochemistry

Regulation of Chlorophyll Biosynthesis in Angiosperms

Steffen Reinbothe* and Christiane Reinbothe

Institute for Plant Sciences, Department of Genetics, Swiss Federal Institute of Technology Zurich (ETH), ETH-Zentrum, Universitätsstrasse 2, CH-8092 Zurich, Switzerland

Light controls plant growth and development in a pleiotropic manner. For example, light dictates a pattern of gene expression that leads to photomorphogenesis (Kendrick and Kronenberg, 1994). In the absence of light, an alternative developmental process, skotomorphogenesis, takes place. Photomorphogenesis and skotomorphogenesis are controlled by several different photoreceptors, such as Pr and Pfr and blue-light-absorbing photoreceptors.

During skotomorphogenesis, the germinating seedling utilizes all of the nutrients contained in the seed to establish conditions that allow the plant to harvest even traces of light. The hypocotyl elongates dramatically just to place the cotyledons above the soil. Within the cotyledons, proplastids differentiate into etioplasts, and a large supply of the tetrapyrrole pigment precursor Pchlide is built up in the prolamellar bodies of the etioplasts. Together with NADPH, Pchlide is bound to the PORA. This ternary complex is photoactive and thus able to immediately photoreduce the Pchlide to Chlide as soon as the cotyledons are exposed to light (for review, see Ryberg and Sundqvist, 1991).

During photomorphogenesis, hypocotyl elongation is suppressed, but cotyledon unfolding and expansion proceed normally. Chloroplast development as well as Pchlide reduction occur in parallel and collectively lead to the rapid greening of the plant.

Pioneering experiments performed in Granick's group almost 40 years ago shed some light on the regulatory mechanisms that govern Chl biosynthesis in higher plants. When angiosperm plants were germinated in the dark, their cotyledons appeared pale yellow. If such etiolated seedlings were incubated with ALA, a common precursor of all tetrapyrrole pigments, they turned greenish (Granick, 1959). This apparent greening was not caused by the synthesis of Chl but was due to the accumulation of excess Pchlide, the immediate precursor of Chlide. Similar to leaf tissues, isolated plastids were found in later studies to accumulate excess Pchlide when they were fed ALA in the dark (Kannangara and Gough, 1977). Taken together, these basic experiments showed that all of the enzymes necessary for the conversion of ALA to Pchlide must be present in dark-grown angiosperm plants, that the entire pathway is likely to operate in the plastid, and that the only known light-requiring reaction for the synthesis of Chl is the reduction of Pchlide to Chlide. It is the aim of this review to summarize our current knowledge on key regulatory steps of Chl biosynthesis in angiosperms.

THE STEPS AND ENZYMES OF CHL BIOSYNTHESIS: THE C_5 PATHWAY

Figure 1 illustrates the C_5 pathway, depicts its various intermediates, and highlights the enzymes of Chl biosynthesis (for review, see also von Wettstein et al., 1995).

ALA is the first committed precursor of Chl. This nonprotein amino acid is formed from the intact carbon skeleton of glutamate in a three-step reaction involving a particular plastid tRNA, $tRNA^{Glu}$. As a next step, two molecules of ALA are condensed to yield porphobilinogen. This reaction is catalyzed by ALA dehydratase. Then, four molecules of porphobilinogen are converted into hydroxymethylbilane by porphobilinogen deaminase. Subsequent reactions include ring closure and simultaneous isomerization of the acetyl and propionyl groups at the pyrrole ring D, leading to uroporphyrinogen III. This compound is then converted into coproporphyrinogen, followed by two successive oxidations, which eventually lead to protoporphyrin IX. In the first reaction, the acetic acid side chains of uroporphyrinogen III are shortened to methyl groups by decarboxylation. Then, two propionic acid side chains are "trimmed" into vinyl groups, followed by oxidation to establish the system of conjugated double bonds in protoporphyrin IX.

At protoporphyrin IX there is a branch point in the C_5 pathway. One route is directed to the synthesis of heme and phytochromobilin (the chromophore of phytochrome; not shown in Fig. 1), whereas the other branch gives rise to the formation of Chl. Ferrochelatase and Mg-chelatase, respectively, catalyze the first unique reactions in the Fe and Mg branches of the C_5 pathway. In the Mg branch subsequent esterification of Mg-protoporphyrin IX leads to Mg-protoporphyrin IX monomethyl ester, followed by the formation of the isocyclic ring of the macrocycle. 3,8-Divinyl Pchlide *a* (alternatively named 2,4-divinyl Pchlide *a*) as the final product of these reactions is then reduced at the vinyl

* Corresponding author; e-mail steffen.reinbothe@ipw.agrl.cthz.ch; fax 41–1201–81–59.

Abbreviations: ALA, δ-aminolevulinic acid; Chl, chlorophyll; Chlide, chlorophyllide; GluTR, glutamyl-tRNA reductase; Pchlide, protochlorophyllide; PORA and PORB, the A and B isoforms of POR; (p)POR, (precursor) NADPH:protochlorophyllide oxidoreductase.

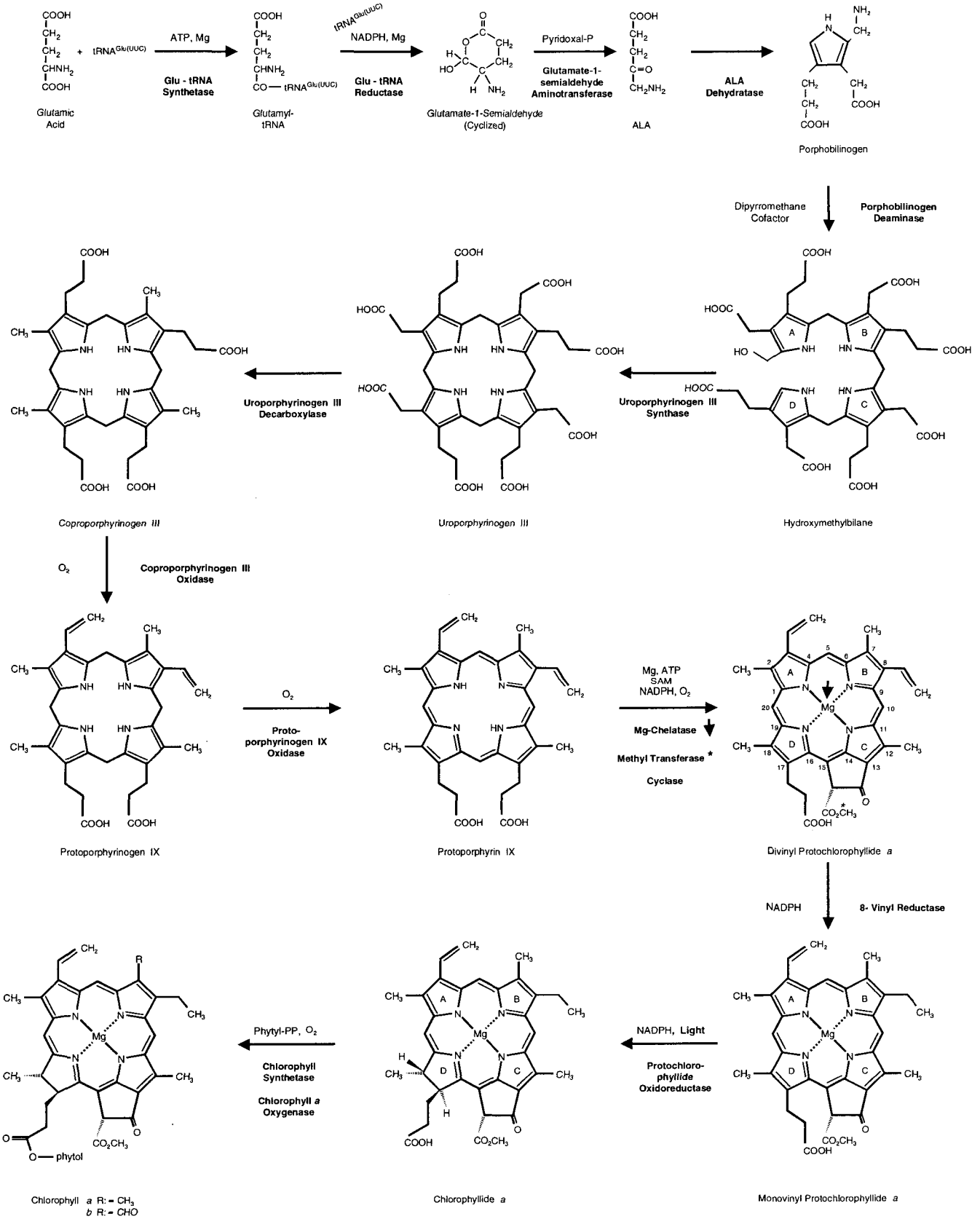

Figure 1. The biosynthetic pathway of Chl. Details are given in the text. Note the isomerization of the acetyl and propionyl groups in ring D during ring closure, giving rise to uroporphyrinogen III.

group at C_8 to yield 3-monovinyl Pchlide *a*. Subsequently, the double bond between C_{17} and C_{18} in ring D of the macrocycle is reduced to yield Chlide *a*. The final steps of Chl biosynthesis include the esterification of Chlide *a* with phytol or earlier alcohol precursors, which must then be reduced to the stage of the phytyl derivative, and oxygenation of the Chl *a* to Chl *b*.

THE COMPARTMENTATION OF THE C_5-PATHWAY ENZYMES

Studies on the intraplastidic localization of the enzymes operating in Chl biosynthesis led to a big surprise. Three plastid compartments appear to cooperate during tetrapyrrole synthesis. Although the initial reactions of ALA synthesis as well as the steps leading to protoporphyrinogen IX take place in the stroma, all subsequent steps are catalyzed by membrane-bound or at least membrane-associated enzymes. In particular, the initial and final enzymes and intermediates of the Mg branch of Chl synthesis, such as protoporphyrinogen IX oxidase and protoporphyrinogen IX, or POR and Pchlide, have been detected in isolated envelope membranes of spinach chloroplasts (Joyard et al., 1990; Matringe et al., 1992). By analogy, some of the components of the Mg-chelatase enzyme complex have a pronounced affinity for membranes (Walker and Weinstein, 1991a).

In contrast to these initial steps, all of the later steps of Chl synthesis, such as esterification of Chlide *a* with phytol or earlier alcohol precursors, including their reduction, as well as the final step of Chl *a* oxygenation to give Chl *b*, are supposed to take place in the thylakoid membranes (Block et al., 1980). This spatial separation of the early and late steps of Chl synthesis implies that some intermediate(s) of the C_5 pathway may be transported from the plastid envelope to the thylakoids. Such a pigment transport does not seem to be necessary for the Fe branch of the C_5 pathway, since all of the enzymes operating in heme synthesis are present in the thylakoids (for summary, see Matringe et al., 1994).

REGULATION OF CHL BIOSYNTHESIS

Coarse Control

As pointed out in Granick's work (see, for example, Nadler and Granick, 1970), etiolated angiosperm seedlings contain all C_5-pathway enzymes. Thus, ALA should continuously be channeled through the various steps of the C_5 pathway. However, the only intermediate that accumulates to detectable levels appears to be Pchlide. Its conversion to Chlide is blocked in the dark because POR requires light for activity (Griffiths, 1978).

Superimposed on these effects, a yet undetermined control mechanism restricts the level of Pchlide in the dark. Barley mutants have been identified that are impaired in this regulatory mechanism (von Wettstein et al., 1974). Among them, the *tigrina* mutants display striking differences in leaf color when grown in the dark or in the light (for summary, see von Wettstein et al., 1995). Although the photoreduction of Pchlide to Chlide can proceed normally in light-grown plants, excess Pchlide accumulates if plants are germinated in the dark. If such etiolated plants are transferred to the light, excited Pchlide molecules that are not bound to POR dissipate their excitation energy by interacting with triplet oxygen to form singlet oxygen. This compound is potentially harmful to the plant because it causes membrane lipid peroxidation, protein denaturation, and pigment bleaching. These effects collectively lead to photooxidative damage of the plastid compartment. *tigrina* mutants grown in dark/light cycles hence show the typical green-white leaf banding. In barley, at least four genes normally prevent excess Pchlide accumulation in the dark. The identity of these genes remains to be determined.

Etiolated seedlings of angiosperms such as barley respond to illumination with a massive accumulation of Chl. This large increase is due in part to an enhanced rate of ALA synthesis. However, transcription of neither the plastid-encoded $tRNA^{Glu}$ gene nor of the nuclear glutamyl-tRNA-synthetase, GluTR, and glutamate-1-semialdehyde aminotransferase genes appear to change upon illumination of barley seedlings (Berry-Lowe, 1987; Grimm, 1990). This implies that posttranscriptional steps might be involved in promoting ALA synthesis in the light.

The light-stimulated increase in the rate of ALA synthesis is accompanied by a general increase in the flow of metabolites through the C_5 pathway. mRNA levels of ALA dehydratase and ferrochelatase were raised approximately 20- and 5-fold, respectively, in etiolated seedlings of pea and *Arabidopsis thaliana* upon illumination (Witty et al., 1993; Smith et al., 1994). These findings suggest that the major part of protoporphyrin IX is channeled through the Mg branch of the C_5 pathway. Surprisingly, the mRNA of one of the putative components of the Mg-chelatase, OLIVE (OLI), declines during the day in *Antirrhinum majus* seedlings grown under 12-h dark/12-h light cycles (Hudson et al., 1993). During the night, however, *oli* transcript levels recovered. The *oli* gene product, by analogy to its bacterial counterpart *bchH*, is thought to bind protoporphyrin IX (Hudson et al., 1993; Gibson et al., 1995), whereas the actual Mg insertion step seems to be catalyzed by other components of the Mg-chelatase enzyme complex. One might therefore postulate that depressing the level of OLI protein in the light could relieve a block in the flow of protoporphyrin IX through the Mg branch of the C_5 pathway that normally operates in the dark.

Similar to *oli* transcripts, the mRNA level of another enzyme of the C_5 pathway was also found to decline upon illumination. *porA* transcripts are highly abundant in etiolated seedlings but vanish rapidly from illuminated plants (for summary, see Forreiter et al., 1990). Superimposed on this effect, light operating via phytochrome depresses transcription of the *porA* gene (Mösinger et al., 1985). It has been proposed that the PORA protein might be expressed for the specific purpose of establishing the paracrystalline structure of the prolamellar body of the etioplast (for reviews, see Ryberg and Sundqvist, 1991; von Wettstein et al., 1995). Because PORA does not seem to act as an ordinary enzyme but instead acts as a suicidal enzyme that retains the product of catalysis, Chlide (Reinbothe et al., 1995c),

and thereby becomes susceptible to attack by plastid proteases (Reinbothe et al., 1995a, 1995b), one might assume that this protein must be removed from illuminated plants in order for rapid formation of the thylakoid membrane pigment-protein complexes to take place.

Fine Control

Feedback inhibition of ALA synthesis by an end product of the Fe branch or Mg branch of the C_5 pathway has long been favored to be a key regulatory step of Chl biosynthesis. Preliminary evidence suggests that heme inhibition of GluTR could be such a site of control. Indeed, heme inhibited GluTRs purified from *Synechocystis* sp. PCC 6803 and barley (for summary, see Pontoppidan and Kannangara, 1994).

Very little is known about covalent modifications that could influence the activities and/or levels of the enzymes operating in Chl biosynthesis. There is only one well-characterized example of a higher-plant C_5-pathway enzyme that undergoes pronounced changes in abundance during chloroplast development. PORA is rapidly degraded in etiolated seedlings upon illumination. If complexed with its product, Chlide, PORA becomes highly sensitive to proteolysis (Reinbothe et al., 1995a, 1995b). A light-induced plastid protease activity degrades the POR-Chlide complex. The POR-degrading protease, which is composed of several constituents comprising both Asp-type and Cys-type proteinases, is not found in etioplasts but is highly active in chloroplasts (Reinbothe et al., 1995a, 1995b). Some of its constituents are encoded by light-responsive nuclear genes (Reinbothe et al., 1995a). Proteolytic fragments of PORA similar to those detected in vitro have been proposed to act as vehicles for the integration of freshly formed Chlide into the developing thylakoid membranes of etiochloroplasts (Reinbothe et al., 1995a).

Metabolite Channeling between Different Plastid Compartments

The unexpected observation that Pchlide synthesis occurs in the plastid envelope, whereas functional pigment (Chlide) is required in the thylakoids for the rapid buildup of the photosynthetically active membrane complexes, has long been considered to be a contradiction to well-established concepts of Chl biosynthesis (e.g. Nadler and Granick, 1970). However, recent studies have in part solved this problem (summarized in Figure 2A). The plastid import pathway of the cytosolic precursor of PORA (pPORA) of barley was found to depend on its substrate, Pchlide (Reinbothe et al., 1995d). This pigment triggers the actual translocation step by which pPORA crosses the plastid envelope membranes. The interaction between Pchlide and pPORA as well as ATP appear to be the driving forces for the translocation step. During import, a pPORA-Pchlide-NADPH complex is formed in the stroma (Reinbothe et al., 1995b, 1995c). Then, the transit peptide is removed from the precursor protein. The resulting PORA-Pchlide-NADPH complex persists in the dark but rap-

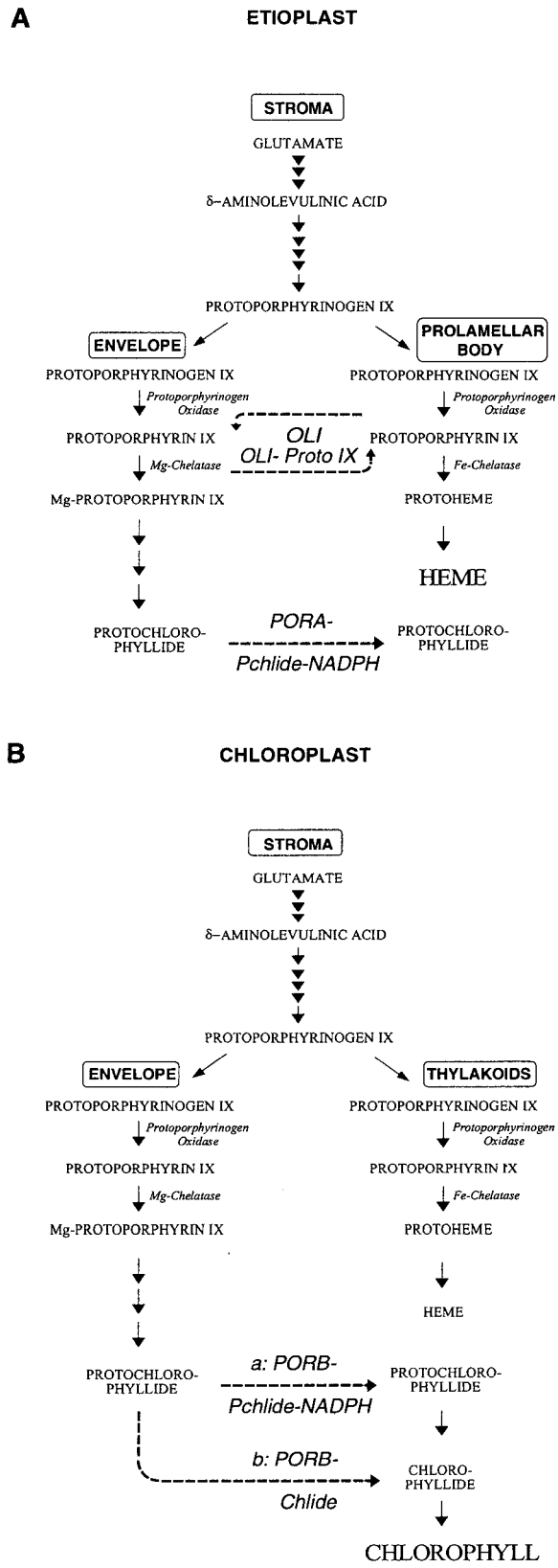

Figure 2. A putative model of pigment transport between different compartments of etioplasts (A) and chloroplasts (B). In chloroplasts the fate of imported PORB in the stroma is different in the dark (*a*) and in the light (*b*), as specified in the text.

idly converts its Pchlide to Chlide if the organelles are shifted to the light. In vitro, the PORA-Chlide complex in turn undergoes rapid proteolytic degradation in chloroplasts but remains stable in etioplasts, since the POR-degrading protease is absent (Reinbothe et al., 1995a, 1995b).

MULTIPLE POR ENZYMES SUPPORT CHL SYNTHESIS IN ETIOLATED AND GREENING PLANT TISSUES

The rapid decline in the plastidic Pchlide concentration, which impairs the translocation of the pPORA, and the lack of resynthesis of the cytosolic precursor of PORA, which is due to the drastic reduction in *porA* gene transcription, should lead to a depletion of thylakoid-bound Chlide in etiolated plants soon after the beginning of illumination. However, Chl synthesis can still proceed at a time when virtually no PORA enzyme is present in such illuminated plants. This apparent paradox has recently been solved by the discovery of a second Pchlide-reducing enzyme. In angiosperms such as barley (Holtorf et al., 1995) and *A. thaliana* (Armstrong et al., 1995), this enzyme, termed PORB, is present after both dark and light treatments. PORB protein and mRNA are also abundantly expressed in light-adapted plants. The actual PORB protein concentration appears to be adjusted to the varying needs of such plants for Chl by coupling continuous turnover of PORB-Chlide binary complexes in the plastids to enhanced rates of pPORB in the cytosol. This mechanism can operate because the cytosolic precursor of PORB, although not dependent on Pchlide as a specific cofactor for translocation, traps this pigment during transport through the plastid envelope membranes and in turn converts the pigment to Chlide (Reinbothe et al., 1995b) (see Fig. 2B, route *b*). Changes in *porB* mRNA concentration thus appear to be sufficient to compensate for the preferential loss of the enzyme in the light.

In the dark, the rate of PORB enzyme synthesis seems to be rather low. However, the traces of freshly synthesized and imported PORB enzyme can stably accumulate because Pchlide molecules resynthesized throughout the night period (Griffiths et al., 1985) bind to PORB, giving rise to protease-resistant PORB-Pchlide-NADPH ternary complexes (Reinbothe et al., 1995b) (Fig. 2B, route *a*). Thus, pronounced diurnal fluctuations in PORB protein abundance do not occur in angiosperm plants (e.g. Holtorf et al., 1995).

A MODEL OF CONCERTED TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL CONTROL OF THE C_5-PATHWAY ENZYMES

Based on the results summarized in this review, a model of concerted transcriptional and posttranscriptional control of the C_5-pathway enzymes can be proposed. According to this model, ALA synthesis would be low in etiolated angiosperm seedlings but would increase once such seedlings have been exposed to light. Both in the dark and in the light, ALA would be converted into protoporphyrin IX. By regulating the actual amount of this pigment in the thylakoids and the envelope, the flow of metabolites could be adjusted to the varying needs of dark- and light-grown plants for heme and Chl.

We speculate that the OLI protein could act as an inhibitor of the envelope-bound protoporphyrinogen IX oxidase. In the dark this inhibition would lead to accumulation of protoporphyrinogen IX in the stroma and its subsequent channeling into the thylakoid-associated Fe branch of tetrapyrrole synthesis. Consistent with such a model would be the fact that the light-induced decrease in the expression of the OLI protein correlates with the higher activity of protoporphyrinogen IX oxidase activity in chloroplast envelope membranes compared to etioplast membranes (Matringe et al., 1992). Those traces of protoporphyrinogen IX that could still be oxidized by protoporphyrinogen IX oxidase in the envelope would give rise to Pchlide. Raising the level of protoporphyrin IX experimentally by feeding ALA to leaf tissues or isolated plastids in the dark would cause an increase in the rate of Pchlide synthesis, because excess protoporphyrin IX could bind to the OLI protein and thereby release this protein from the envelope-bound protoporphyrinogen IX oxidase (Fig. 2A). An OLI-protoporphyrin IX complex could be formed in the stroma and could then travel to the prolamellar body of the etioplast. At this place, OLI could be "discharged" by the binding of its protoporphyrin IX to the Fe-chelatase. Finally, OLI would move back to the envelope to bind a new molecule of protoporphyrin IX. Excess protoporphyrin IX not converted into Mg-protoporphyrin IX could thus be transferred from the plastid envelope to the thylakoids. In non-ALA-treated samples, the rate of Pchlide synthesis would be kept low in the dark, however, because an excess of free OLI protein would exist. If such etiolated plants would be illuminated, the drastic decrease in OLI expression would finally lead to an increase in the activity of the envelope-bound protoporphyrinogen IX oxidase. Together with the increase in the actual Mg-chelatase activity (Koncz et al., 1990; Walker and Weinstein, 1991b), this effect would favor channeling of protoporphyrinogen IX into Chl synthesis. As a result, less heme would be formed in the thylakoids, inhibition of ALA synthesis by heme would be relieved in the stroma, and a further increase in the flow of metabolites through the Mg branch of the C_5 pathway would be observed. To compensate for a potential depletion of heme, the expression level of ferrochelatase would then have to be increased, in concert with the activation of the thylakoid-associated protoporphyrinogen IX oxidase. By establishing a new steady-state level of heme, ALA synthesis would finally be adjusted to the actual need for tetrapyrroles in the light.

SUMMARY AND PERSPECTIVES

During the last few years, major clues in identifying the various steps of Chl synthesis of higher plants have come from different experimental approaches. Classical biochemical techniques to purify and characterize the enzymes of tetrapyrrole synthesis have recently been complemented by studies using mutants that are impaired in certain steps of Chl biosynthesis. In particular, the identi-

fication of many of the genes necessary for bacteriochlorophyll biosynthesis in *Rhodobacter capsulatus*, an anoxygenic photosynthetic purple bacterium that synthesizes a close relative of Chl, has helped to elucidate the steps of tetrapyrrole synthesis (Bollivar et al., 1994). Except for a few steps that are unique to the biosynthesis of bacteriochlorophyll, most reactions in *Rhodobacter* and higher plants appear to be very similar if not identical. Mutants of *Rhodobacter* in which certain biosynthetic genes have been disrupted can thus be exploited to identify by functional complementation the genes that govern Chl biosynthesis in higher plants. Even in those cases in which the plant and bacterial counterparts were expected to be different (due to their different requirements and sensitivities to oxygen, respectively), heterologous functional complementation has been used successfully to study the effects of mutations on substrate binding and active site residues in the key enzymes of Chl biosynthesis of higher plants (e.g. Wilks and Timko, 1995).

Also in plants, mutants have been identified that are either defective in enzymes operating in Chl biosynthesis or impaired in the regulatory circuits that control the flow of metabolites through the C_5 pathway (for review, see von Wettstein et al., 1995). Such mutants will be the tools of choice to identify biosynthetic and regulatory genes and to confirm their role in Chl biosynthesis as well as chloroplast and leaf development. Overexpression of such genes can be expected to restore the wild-type situation, whereas expression of antisense probes in wild-type backgrounds should produce mutant phenotypes. Pioneering work by Höfgen et al. (1994) and recent work by Kruse et al. (1995) have highlighted the importance of antisense approaches for the study of Chl biosynthesis in higher plants.

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