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Minireview

Tetrapyrrole regulation of nuclear gene expression

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Abstract

Tetrapyrroles are the structural backbone of chlorophyll and heme, and are essential for primary photochemistry, light harvesting, and electron transport. The biochemistry of their synthesis has been studied extensively, and it has been suggested that some of the tetrapyrrole biochemical intermediates can affect nuclear gene expression. In this review, tetrapyrrole biosynthesis, which occurs in the chloroplast, and its regulation will be covered. An analysis of the intracellular location of tetrapyrrole intermediates will also be included. The focus will be on tetrapyrrole intermediates that have been suggested to affect gene expression. These include Mg-protoporphyrin IX and Mg-protoporphyrin IX monomethyl ester. Recent evidence also suggests a specific signaling role for the H subunit of Mg-chelatase, an enzyme that catalyzes the insertion of Mg into the tetrapyrrole ring. Since gene expression studies have been done in plants and green algae, our discussion will be limited to these organisms.

Abbreviations: ALA – aminolevulinic acid; DP – dipyridyl; Chl – chlorophyll; Chlide – chlorophyllide; CPO – coproporphyrinogen oxidase; GSA – glutamic acid 1-semialdehyde aminotransferase; Mg-PP – Mg-protoporphyrin; Mg-PP-Me – Mg-protoporphyrin monomethylester; PBG – porphobilinogen; PPIX – protoporphyrin IX; UROD – uroporphyrinogen III decarboxylase

Chlorophyll synthesis

In plants, green algae, archaea and most bacteria, the precursor of tetrapyrroles, δ-aminolevulinic acid (ALA), is produced via the C5 pathway. Glutamyl-tRNA^{glu} is the activated form of glutamate (C5) used in tetrapyrrole as well as general chloroplast protein synthesis. Figure 1 outlines the synthesis of tetrapyrroles from activated glutamate. The glutamate residue is reduced by glutamyl-tRNA reductase to form glutamic acid 1-semialdehyde. Glutamyl tRNA reductase, which is encoded by the *HEMA* gene, is predicted to contain a chloroplast transit peptide [Ilag et al. (1994) first cloned the gene from Arabidopsis, see Beale (1999) for others]. Glutamic acid 1-semialdehyde is rearranged in a two-step process to form ALA by glutamic acid 1-semialdehyde aminotransferase (GSA). Genes encoding GSA have been cloned from many different species, and all eukaryotic genes encode proteins with chloroplast transit sequences [Grimm (1990) first cloned the gene from barley, see Beale (1999) for others]. In contrast, in animals, yeast and the α-proteobacteria, ALA is formed through the condensation of glycine and succinyl-CoA by the enzyme ALA synthase.

In a series of three separate reactions, eight molecules of ALA are then converted to uroporphyrinogen III to form a cyclic tetrapyrrole. Two molecules of ALA are condensed to form porphobilinogen (PBG) by PBG synthase (also known as ALA dehydratase). Four molecules of PBG are then condensed by hydroxymethylbilane synthase to form a linear tetrapyrrole, hydroxymethylbilane, which in the third reaction, is immediately cyclized into uroporphyrinogen III by uroporphyrinogen III synthase. In the absence of the synthase, hydroxymethylbilane will

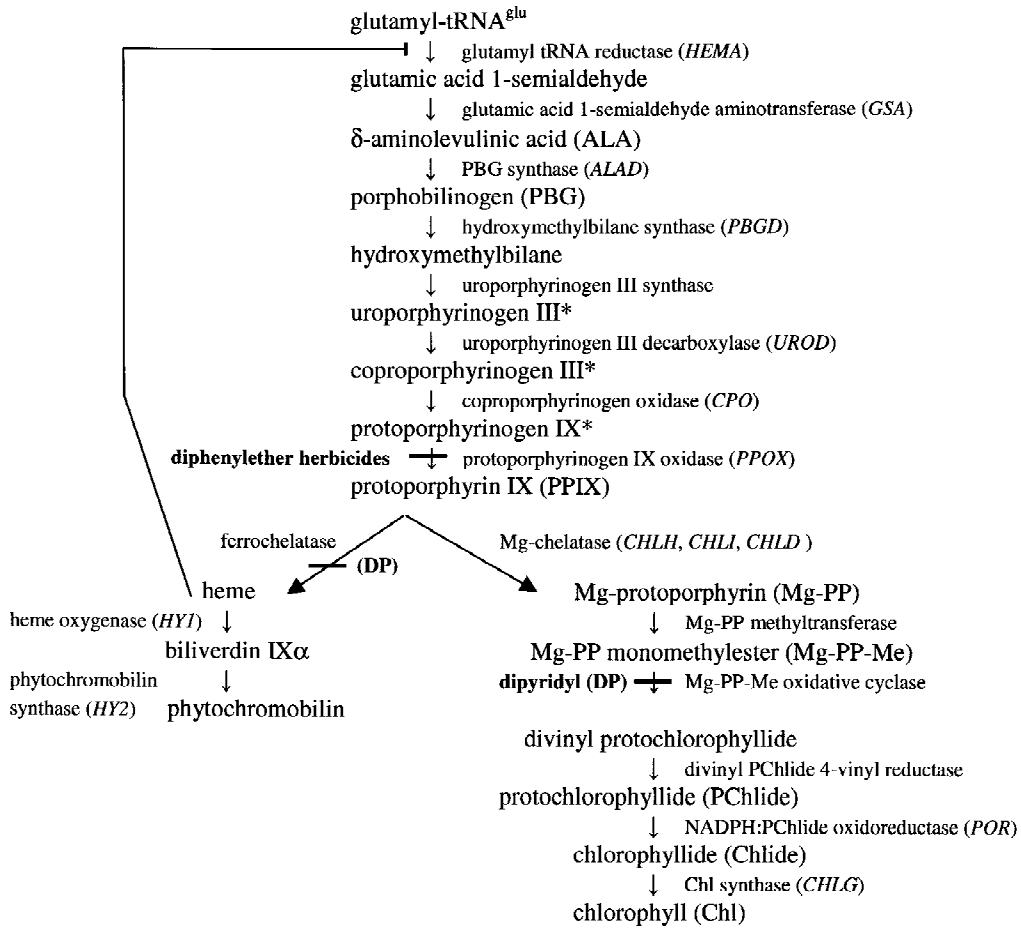

Figure 1. Flow chart of tetrapyrrole synthesis. All of the reactions shown above occur in the chloroplast. When the tetrapyrroles marked with an (∗) accumulate, they are oxidized to photosensitive porphyrins. The postulated feedback inhibition by heme at glutamyl-tRNA reductase is indicated. Inhibitors that have been used in studies of gene expression are also shown.

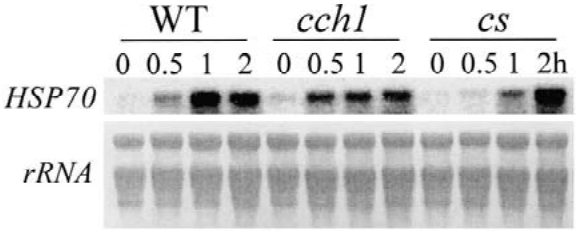

Figure 2. HSP70 mRNA induction by light in dark-adapted *Arabidopsis thaliana*. Fourteen-day-old WT, *cch1* and *cs* plants were placed in the dark for 20 hours, and then exposed to white light (30 μmol photons m^{-2} s^{-1}) for the indicated times. Total RNA was analyzed by RNA gel blot analysis using an *HSP70* probe. The *HSP70* probe was designed from the cytoplasmic-localized *Hsc70-3* (accession Y17053).

spontaneously cyclize to form the non-physiological uroporphyrinogen I.

Uroporphyrinogen III is hydrophilic, photochemically inactive and a poor metal binder. In the next two steps, the acetic acid side chains are trimmed into methyl groups by uroporphyrinogen III decarboxylase (UROD), and two of the propionic side chains are shortened into vinyls by coproporphyrinogen III oxidase (CPO) to produce protoporphyrinogen IX, a more hydrophobic molecule. UROD was cloned from tobacco, and import studies showed that it is transported into the chloroplast, and located in the stroma (Mock et al. 1995). The gene encoding CPO was first isolated from barley (Madsen et al. 1993), and it encodes a protein with a chloroplast transit sequence. The product of CPO, protoporphyrinogen IX, is oxidized by protoporphyrinogen IX oxidase (PPOX), creating a photoactive

tetrapyrrole, protoporphyrin IX (PPIX). Two PPOX genes have been cloned from tobacco; one was imported into plastids while the other was imported into mitochondria (Lermontova et al. 1997).

Either an Fe or Mg ion can be inserted into the center of the PPIX tetrapyrrole ring, resulting in the production of heme or chlorophyll, respectively. Mg chelation is catalyzed by Mg-chelatase, a three-subunit enzyme. Although the three subunits are predicted to be soluble, membrane fractions of isolated chloroplasts have been shown to be essential for activity (Walker and Weinstein 1991). The three subunits are called H (120–155 kDa), I (37–46 kDa), and D (60–87 kDa), and the optimum ratio for *in vitro* activity is 4:2:1 (Jensen et al. 1998). The H subunit, which binds PPIX and Mg, is activated by the I and D subunits (Walker and Willows 1997). The D subunit has been shown to interact with both I and H in the yeast two-hybrid system and in *in vitro* reconstitution assays (Gräfe et al. 1999). The crystal structure of the I subunit reveals structural homology to AAA-type ATPases and that the I subunits assemble into hexamers (Fodje et al. 2001). These are likely to interact with the D subunits via an integrin I domain. Both the I and H-subunits have conserved integrin I binding motifs, and the authors proposed that D subunits first bind the I hexamer which prevents ATP hydrolysis. This complex then binds to the H subunit, in the presence of Mg^{2+}, which results in a conformational change that allows ATP hydrolysis and metal chelation of PPIX.

Plants that have reduced levels of Mg-chelatase by antisense (Papenbrock et al. 2000a, b) have reduced levels of PPIX, the substrate of Mg-chelatase. In these plants, the ALA synthesizing capacity has been decreased by feedback inhibition. The advantage of regulating Chl synthesis early in the pathway is that the accumulation of photosensitive tetrapyrrole intermediates is prevented. The mechanism of this feedback inhibition is controversial, but one possibility could be a transient increase in PPIX, which would be shunted towards heme production by ferrochelatase. Most heme is bound by proteins, but in plants accumulation of excess free heme inhibits the formation of ALA by inhibition of glutamyl tRNA-reductase (Gough and Kannagara 1979). In plants and Chlamydomonas, glutamyl tRNA-reductase has a 31–34 amino acid extension, downstream of the chloroplast leader (Vochknecht et al. 1998; A. Srivastava and S. Beale, unpublished results). When this region of the barley sequence was deleted, the recombinant protein expressed in *E. coli* was no longer feedback inhibited by heme. Heme regulation of ALA biosynthesis was first proposed for the ALA synthase pathway (Lascalles and Hatch 1969), but appears to function in the C5 pathway as well. Heme has been shown to be a negative regulator of glutamyl-tRNA reductase protein stability in *Salmonella typhimurium* (Wang et al. 1999) and a positive regulator of iso-1-cytrochrome c gene transcription in yeast (Guarente and Mason 1983). Chl synthesis is also regulated via a heme-independent mechanism that involves the membrane-bound plastid-localized protein FLU (Meskauskiene et al. 2001).

The product of Mg chelation, Mg-protoporphyrin IX (Mg-PP), is then methylated by a soluble enzyme to produce Mg-PP monomethylester (Mg-PP-Me). In the next three steps, an isocyclic ring (E) is formed below ring C, resulting in the production of protochlorophyllide (PChlide). The first enzymatic step requires O_2 and Fe, and iron deficiency results in the accumulation of Mg-PP and Mg-PP-Me (Spiller et al. 1982). Treatment of plants and algae with 2′2′-dipyridyl (DP), an iron chelator, also results in the accumulation of Mg-PP-Me (Duggan and Gassman 1974).

The D tetrapyrrole ring of PChlide is reduced in a light-dependent step by NADPH-protochlorophyllide oxidoreductase (POR). In etiolated plants, PChlide and NADPH bound to POR accumulate (Sperling et al. 1998), and upon exposure to light, the PChlide is converted to chlorophyllide (Chlide). A phytol tail is added to the carboxyl group of ring D by chlorophyll synthase (Oster et al. 1997). The completed Chl molecules then immediately bind to reaction center or light harvesting proteins, and do not accumulate unbound in the thylakoid membrane. In higher plants and green algae, a proportion of Chlide, which has been synthesized in the *a* form, is converted to Chlide *b* by chlorophyllide *a* oxygenase (Tanaka et al. 1998; Espineda et al. 1999; Oster et al. 2000).

Where are tetrapyrroles located within the cell?

Chl and heme are synthesized and incorporated into proteins within the chloroplast, but heme is also required for electron transport in the mitochondria and as a cofactor for many cytoplasmic enzymes. Mitochondria contain PPOX (Jacobs et al. 1982; Lemontova et al. 1997) and ferrochelatase (Chow et al. 1997), but do not contain enzymes for the earlier steps of tetrapyrrole biosynthesis, suggesting

that the substrate protoporphyrinogen IX must be exported from the chloroplast. One series of experiments documented the intracellular location of porphyrins (Jacobs and Jacobs 1993). In these studies, isolated barley chloroplasts were treated with ALA to allow detectable amounts of porphyrins to accumulate. Fifty percent of the PPIX was found in the supernatant fraction, while much smaller fractions of uroporphyrin III and coproporphyrin were detected in the supernatant. In these experiments protoporphyrinogens were non-specifically oxidized to the porphyrin form, and thus these results support the export of either protoporphyrinogen or PPIX from the chloroplast. When intact chloroplasts were incubated with ALA in the presence of a diphenyl ether herbicide, which specifically inhibited PPOX, PPIX was not detected. However, if the herbicide-treated chloroplasts were incubated with *E. coli* membranes, which contain a herbicide-resistant PPOX, then PPIX was detected at high levels. The *E. coli* enzyme could only oxidize exported protoporphyrinogen, and thus these experiments demonstrate protoporphyrinogen export from the chloroplast. PPOX is located on the thylakoid and chloroplast envelope, and it is possible that the PPIX formed at the envelope is preferentially exported (Matringe et al. 1992). When intact leaves were treated with diphenyl ether herbicides, PPIX was shown to directly correlate to cellular damage in light-grown plants (Becerril and Duke 1989). The intracellular location of PPIX was not determined, but is likely to be outside of the chloroplast where protoporphyrinogen oxidation could occur by non-specific herbicide-resistant peroxidases (Jacobs et al. 1991).

Recently, an Arabidopsis mutant, *laf6*, was isolated that has a two-fold increase in PPIX as well as long hypocotyls when grown under far red light (Møller et al. 2001). The intracellular location of PPIX was not determined, but interestingly, the *LAF6* gene was found to encode a soluble ABC transporter protein that likely interacts with a membrane-bound partner. LAF6 protein has a chloroplast leader and was localized to the periphery of the chloroplast. It is possible that the LAF6 transporter is involved in porphyrin import or export, but more detailed measurements of additional porphyrin products besides PPIX, as well as identification of the membrane-bound channel, are needed before a function can be assigned.

Tetrapyrroles can prevent light induction of *Lhcb1* mRNA

In 1973, Hoober and Stegman first proposed that tetrapyrroles could affect gene expression. It was found that synthesis of peptide c (now called Lhcb1) did not occur under conditions where PChlide would accumulate, such as in darkness or when seedlings were exposed to light of wavelengths greater than 675 nm. This repression of peptide c synthesis was eliminated when cells were treated with chloramphenicol, a chloroplast protein synthesis inhibitor. A chloroplast-encoded repressor, which required PChlide as a co-repressor, was proposed to reduce the transcription of the peptide c gene. At the time this model was proposed, it was not known that peptide c was encoded by a nuclear gene, and therefore this model is unlikely to be correct.

PChlide was also suggested to regulate gene expression in *Chlamydomonas reinhardtii* strains (*ya12* and *ac40*) that do not synthesize Chl in the dark. These lines have reduced levels of *Lhcb1* mRNA in the dark when compared to WT. It was speculated that accumulated PChlide enhanced turnover of *Lhcb1* mRNA because ^{32}P-pulse experiments suggested that rates of *Lhcb1* transcription were equivalent in the WT and mutants (Herrin et al. 1992).

Later, tetrapyrroles were suggested to play a role in the light-induced expression of *Lhcb1* in synchronized cultures of Chlamydomonas (Johanningmeier and Howell 1984; Johanningmeier 1988). Treatment of Chlamydomonas cultures with the Fe chelator dipyridyl (DP) resulted in the accumulation of Mg-PP-Me. When DP-treated cultures were transferred to the light, complete repression of *Lhcb1* expression was observed, however rRNA levels were also lowered. Nuclear run-on assays demonstrated that *Lhcb1* transcription was completely abolished while *rRNA* transcription was reduced only eight-fold (Jasper et al. 1991). In addition, a general loss of nuclear and plastid mRNAs was excluded because *rbcS2* and *rbcL* mRNA levels were normal in DP-treated cultures. Supporting a specific role of Mg-PP-Me was the observation of partial restoration of DP-repressed *Lhcb1* mRNA levels when cultures were treated with hemin or levulinic acid in addition to DP. These compounds inhibit Chl synthesis prior to or just after ALA synthesis, and therefore reduce the accumulation of Mg-PP-Me. In cress (Kittsteiner et al. 1991) and Arabidopsis (A. Linford and J. Brusslan, unpublished data), DP completely repressed *Lhcb1* mRNA accumulation as

well. Mutant studies in Chlamydomonas indicated that *Lhcb1* mRNA was not expressed in the brown, br_s mutant, which accumulates PPIX, but cannot accumulate the downstream compound, Mg-PP-Me (Crawford et al. 1982). Together, these studies implicated both PPIX and Mg-PP-Me as inhibitors of *Lhcb1* gene expression. PPIX was later excluded as a cytosol-localized repressor when it was shown that diphenyl ether herbicides, which result in the accumulation of high levels of PPIX, did not block light-induced *Lhcb1* mRNA induction at concentrations that inhibited Chl synthesis (Jasper et al. 1991).

Etiolated cress seedlings were also treated with β-thujaplicin, a Chl biosynthesis inhibitor that was shown to greatly increase levels of Mg-PP-Me (Oster et al. 1996). Treated seedlings displayed a 50% inhibition in *Lhcb1* mRNA levels after four hours of continuous far red light or after a two-minute red light pulse. Nuclear run-on experiments indicated that repression occurred at the level of transcription. These experiments support the role of Mg-PP-Me as a repressor of *Lhcb1* gene transcription. Also supporting a role for Mg-PP is the inability to induce *Lhcb1* mRNA by one minute of dim light in barley seedlings grown with the lycopene biosynthesis inhibitor, amitrole (La Rocca et al. 2001). In these lines, Mg-PP levels were increased five-fold, however, these etiolated seedlings were unable to form a prolamellar body, and thus the affects of amitrole were pleiotropic.

Additional experiments suggest that altering the levels of tetrapyrroles can result in reduced *Lhcb1* mRNA levels. Transgenic tobacco plants with antisense constructs to UROD or CPO displayed light-dependent necrosis (Kruse et al. 1995; Mock and Grimm 1997; Hu et al. 1998). Reductions in UROD and CPO activity were correlated to increased levels of uroporphyrin III or coproporphyrin, respectively. The intracellular location of these photosensitizing porphyrins was not determined, but it is likely that the porphyrinogen form entered the cytoplasm where it was non-specifically oxidized to the phototoxic porphyrin form. *Lhcb1* mRNA levels were found to be decreased in the CPO antisense lines (Mock and Grimm 1997). Antisense inhibtion of the H subunit of Mg-chelatase resulted in pale tobacco plants that had lower levels of PPIX, and thus were not photosensitive. In these plants, *Lhcb1* mRNA levels were also reduced. The H subunit antisense results indicate that changing the flux through the tetrapyrrole biosynthetic pathway can affect *Lhcb1* gene expression, and that reductions in *Lhcb1* gene expression are not solely a result of porphyrin photodamage. In contrast, reduced flux through the pathway by antisense expression of a *GSA* gene in tobacco did not affect *Lhcb1* gene expression although the plants had a 90% reduction in Chl content (Höfgen et al. 1994). A further complication in these lines of studies is the observation that *Lhcb1* mRNA levels were reduced when the *laf6* mutant, which has elevated PPIX levels, was grown under high fluence far red light, but that *Lhcb1* mRNA levels remained normal when the mutant was grown under white, blue, red or low fluence far red light (Møller et al. 2001). These results suggest that there is crosstalk between porphyrins and phytochrome A.

Tetrapyrroles can induce HSP70 mRNA

Tetrapyrroles have also been implicated in the induction of genes. In Chlamydomonas, light can induce the expression of two heat shock genes, *HSP70A* and *HSP70B*. Exogenous addition of Mg-PP and Mg-PP dimethyl ester to dark-grown Chlamydomonas cultures substituted for the light signal, and induced *HSP70A* mRNA (Kropat et al. 1997). Addition of other tetrapyrrole precursors, PPIX, PChlide and Chlide, did not result in the accumulation of *HSP70A* mRNA. Accumulation of *HSP70A* mRNA was blocked in the br_c mutant that has high levels of PPIX, and this block in *HSP70A* expression was reversed by addition of Mg-PP. These data clearly support the role of Mg-PP and Mg-PP dimethyl ester as positive modulators of nuclear gene expression. However, as described in the previous section, Mg-PP has the opposite effect on *Lhcb1* gene expression. Further support was the observation that Mg-PP and Mg-PP-Me levels increased prior to *HSP70A* mRNA induction when algae were transferred to light. In addition, the light-induced increase in Mg-PP and Mg-PP-Me could be blocked by the cytoplasmic protein synthesis inhibitor cycloheximide, and a concomitant loss of *HSP70A* induction was observed. Interestingly, feeding dark-grown cells with PPIX did not result in *HSP70A* expression, even though levels of downstream products Mg-PP and Mg-PP-Me did increase. It was proposed that light is required to release these compounds from the chloroplast into the cytoplasm (or nucleus) where they can affect nuclear gene expression. Feeding Mg-PP or Mg-PP-Me to dark-grown cells placed these tetrapyrroles in the cytoplasm (or nucleus) directly, and thus light was not required for *HSP70A* induction (Kropat et al. 2000).

Our laboratory has investigated the role of Mg-PP-Me on *HSP70* gene expression in Arabidopsis. We used defined mutations (*cch1* and *cs*) in the genes that respectively encode the H and I subunits of Mg-chelatase (Koncz et al. 1990; Mochizuki et al. 2001). These lines are yellow-green because Chl synthesis is attenuated by the mutated Mg-chelatase. Plants were grown for 2 weeks, and then placed in the dark for 20 hours. They were then returned to low light (30 μmol photons m^{-2} s^{-1}) for 30 min, one hour or two hours. A representative RNA gel blot as well as control rRNA levels are shown in Figure 2. Similar induction kinetics were obtained in two other independent experiments. Results were quantified and normalized as described in Espineda et al. (1999). In low light, *HSP70* mRNA levels increased linearly for one hour, and the rate of increase slowed for the second hour. *HSP70* mRNA induction kinetics were similar in the H subunit mutant (*cch1*), but were significantly delayed in the I subunit mutant (*cs*), with a linear rate of increase occurring after one hour. Our results suggest that the I subunit may play a role in mediating rapid *HSP70* mRNA induction.

Tetrapyrrole regulation of gene expression during photooxidative stress

Another area of gene expression in which tetrapyrroles have been suggested to play a role is the repression of *Lhcb1,* and other mRNAs under conditions of photooxidative stress. *Lhcb1* mRNA levels were found to correlate to carotenoid levels in a series of albino maize mutants (Mayfield and Taylor 1984). The carotenoid biosynthesis inhibitor, Norflurazon (Nf), phenocopied these mutants; Nf-treated seedlings grown in dim light had normal levels of *Lhcb1* mRNA, but under higher light photobleaching conditions, the *Lhcb1* transcripts were completely absent. Time course studies indicated that *Lhcb1* mRNA was undetectable by RNA gel blot analysis after 4 h of high light exposure. Nuclear run-on assays of the same seedlings showed that *Lhcb1* transcription rates were reduced by 80% after 4 h (Burgess and Taylor 1988). These observations indicate that the complete loss of *Lhcb1* mRNA is a result of repression at the level of transcription and also suggest that active *Lhcb1* mRNA degradation is occurring under photooxidative stress. Also supporting post-transcriptional regulation is the observation of a 5 h half-life of *Lhcb1* mRNA in aging mustard cotyledons, longer than the 4 h required for complete loss of *Lhcb1* mRNA during photooxidative stress (Oelmüller et al. 1986).

Photooxidative stress was shown to affect *Lhcb1* mRNA, but not all RNA species. *RbcS* mRNA levels were variably affected while mRNAs encoding aldolase and PEP carboxylase were unaffected by Nf treatment in high light (Taylor 1989). Chloroplast-encoded transcripts encoding the D1 protein and the P700 apoprotein, as well as 16S rRNA were greatly reduced by Nf treatment in the dark and in the light (Batschauer et al. 1986). Photooxidative damage was also shown to reduce the level of mRNA encoding the photorespiratory enzyme glycolate oxidase (Barak et al. 2001) as well as *HEMA1* mRNA encoding glutamyl-tRNA reductase (McCormac et al. 2001). Both of these mRNAs were regulated at the transcriptional level as demonstrated by a loss of GUS activity using promoter::GUS fusions. Activities of chloroplast-localized enzymes, NADP-glyceraldehyde-3-phosphate dehydrogenase and nitrite reductase, were reduced when chloroplast development was blocked by the plastid protein synthesis inhibitor, chloramphenicol (Oelmüller et al. 1986). The activities of a number of cytoplasmic enzymes were unaffected by the same treatment. These included phenylalanine ammonia-lyase, chalcone synthase, NAD-glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and NAD-dependent malate dehydrogenase.

It was proposed that a plastid signal exists that maintains high levels of *Lhcb1* mRNA, and that under photooxidative stress, this signal is destroyed resulting in the loss of *Lhcb1* mRNA. An alternative hypothesis is that a repressor of nuclear gene expression is induced during photobleaching. A genetic screen was designed to isolate mutants in *Arabidopsis thaliana* affecting the plastid factor (Susek et al. 1993). The *Lhcb1*1* promoter was fused to *uidA* which encodes GUS and also to *hph* which encodes resistance to hygromycin, and this double construct was transformed into plants. Transgenic seedlings homozygous for the double transgene were hygromycin-sensitive when grown with Nf, due to the repression of *Lhcb1*1::hph*. Mutants that were resistant to hygromycin in the presence of Nf should have incomplete repression of the *Lhcb1*1* promoter, and these were selected from the progeny of EMS-treated transgenic seeds. GUS levels were measured as a secondary screen, and genomes uncoupled (*gun*) mutants were recovered. Compared to wildtype, *gun* mutants had elevated levels of *Lhcb1*

mRNA when grown with Nf in white light. *gun* mutants must either express the plastid signal constitutively or they may have defects in the repressor system induced under photooxidative stress.

Four GUN genes have been identified at the molecular level. *GUN2* and *GUN3* are alleles of *HY1* and *HY2*, respectively, and encode heme oxygenase (*HY1*) and phytochromobilin synthase (*HY2*), enzymes involved in the biosynthesis of phytochromobilin from heme (Vinti et al. 2000; Mochizuki et al. 2001). These mutants are pale due to decreased turnover of heme which is proposed to feedback inhibit ALA synthesis (Terry and Kendrick 1999). *GUN4* encodes a novel chloroplast protein (Larkin et al. 2000), while *GUN5* encodes the H subunit of Mg-chelatase (Mochizuki et al. 2001). Interestingly, the *l*-Blandy4 mutant of maize was shown to have elevated *Lhcb1* mRNA levels in the presence of Nf. It is also defective in the conversion of PPIX to Mg-PP (Burgess and Taylor 1988), so it may be a *GUN5* homologue. These four Arabidopsis *gun* mutants have decreased Chl accumulation while *gun1* has a normal green phenotype. *GUN1* has not yet been defined at the molecular level.

Double mutant studies indicated that Nf-grown *gun1*,*gun4* and *gun1,gun5,* had higher *Lhcb1* mRNA levels than single mutants, and are therefore additive for plastid signaling. These results suggest that *GUN4* and *GUN5* function in a separate pathway than *GUN1*. *gun4,gun5* double mutants were extremely pale indicating that they are synergistic with respect to tetrapyrrole biosynthesis, but *Lhcb1* mRNA levels have not yet been measured in the lethal double mutant. In addition, a null allele of *hy1* (*hy1-6.2*) was used in double mutant studies (Vinti et al. 2000). Both *hy1,gun4* and *hy1,gun5* were additive for Chl biosynthesis, but neither combination was additive for *Lhcb1* mRNA levels in the presence of Nf. These data place *HY1*, *GUN4* and *GUN5* in the same pathway. *HY1* is related to this pathway as a result of decreased tetrapyrrole levels and not as a result of decreased phytochrome signaling because *phyB,gun5* double mutants were not synergistic with regards to Chl accumulation or *Lhcb1* mRNA levels in the presence of Nf (Vinti et al. 2000).

The plastid signaling pathway defined by *HY1*, *GUN3*, *GUN4* and *GUN5* suggests that tetrapyrroles are important for repression of *Lhcb1* during photooxidative stress. Since *GUN5* encodes the H subunit of Mg-chelatase, reduced Mg-PP and Mg-PP-Me levels would be expected in *gun5* mutants, implicating these tetrapyrroles as plastid signaling molecules. This is consistent with the results described earlier in this review, but is contradicted, however, by the observation of normal repression of *Lhcb1* mRNA levels in the *cs* and *ch42* mutants which have defective Mg-chelatase I subunits. These lines should also have reduced levels of Mg-PP and Mg-PP-Me. Also contradicting the idea that Mg-PP (or Mg-PP-Me) is the signaling molecule is the observation that *Lhcb1* mRNA levels are similar in strong (*cch1*) and weak (*gun5*) H subunit mutants, which presumably have different Mg-chelatase activities. Together, these observations suggest that the signaling function of the H subunit is uncoupled from the catalytic function (Mochizuki et al. 2001). The H subunit binds PPIX (Gibson et al. 1995) and Mg^{2+}, and after chelation, is likely to bind Mg-PP (Walker and Willows 1997), and it is possible that any of these forms are important for signaling. The H subunit of Mg-chelatase is imported into the stroma, but becomes associated with the chloroplast inner membrane at higher, yet physiological concentrations of Mg^{2+} (Gibson et al. 1996; Nakayama et al. 1998). Thus the H subunit, perhaps when bound to Mg^{2+}, is in the proximity of the cytoplasm where it could act as a signaling molecule for nuclear gene expression. Alternatively, the H subunit is first synthesized in the cytoplasm before it is imported into the chloroplast, and it is possible that the cytoplasmic form of the H subunit has a signaling role. The interesting finding that mutations in the I subunit have normal repression of *Lhcb1* gene expression, suggests that the H subunit can act in the signaling pathway prior to its interaction with the I subunit.

Conclusions and future studies

The participation of tetrapyrroles in the regulation of nuclear gene expression is beginning to be unraveled through biochemical feeding, inhibitor and genetic analyses. Many of the results from these different studies converge on Mg-PP, Mg-PP-Me and Mg-chelatase, and therefore this portion of tetrapyrrole biosynthesis should be the focus of future studies. The crystal structure of the H subunit of Mg-chelatase will provide insights into function. Also, suppressors of *gun5* may define the molecular intermediates that are proposed to function in plastid signaling. In addition, a role for tetrapyrroles in transcriptional as well as post-transcriptional regulation of *Lhcb1* mRNA levels during photooxidative stress has yet to

be considered. Induction of *HSP70* mRNA is mediated by increased levels of cytoplasmic Mg-PP and Mg-PP-Me in Chlamydomonas, suggesting that tetrapyrroles can play a signaling role outside of the chloroplast. Continued studies on intracellular location of tetrapyrroles will he needed in the future. The emerging role that tetrapyrroles and Mg-chelatase play in regulating gene expression is ready for exploration.

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