Journal of Plant Biology, September 2003, 46(3) : 135-160 **REVIEW**

Chlorophyll Biosynthesis in Higher Plants. Regulatory Aspects of 5-Aminolevulinate Formation

Simon P. Gough*, Tomas Westergren, and Mats Hansson
Department of Biochemistry, Lund University, SE-221 00 Lund, Sweden

Chlorophyll, heme, phytochrome and siroheme biosynthesis in higher plants is regulated by the supply of their precursor 5-aminolevulinate (ALA), which is derived from glutamate. Glutamate conversion to ALA occurs only in the plastid and requires chloroplast coded $tRNA^{Glu}$ and nuclear coded glutamyl-tRNA synthetase, glutamyl-tRNA reductase and glutamate 1-semialdehyde aminotransferase. The chromosomal location in *Arabidopsis thaliana*, rice and barley of these factors are presented with UniGene data for cDNA tissue locations. Similarly also for other nuclear gene products affecting ALA formation: sigma factor sigB; the *A. thaliana Flu* and monocot (*Tigrina-d*) homologs; ξ-carotene desaturase, lycopene synthase and carotenoid isomerase. Cytokinin is a positive regulator of $tRNA^{Glu}$ amounts, which correlate with ALA formation. SigB is required for $tRNA^{Glu}$ transcription. Phytochrome A, cryptochrome and Mg-protoporphyrin repress transcription of glutamyl-tRNA reductase in the dark, which is de-repressed by red and blue light. Post-transcriptional control is little understood but may in *Poaceae* involve messages stabilised by 5'UTR stem-loops. The reductase is inhibited by heme. The Mg-branch has its own inhibitor, *A. thaliana Flu*, encoding a membrane protein, recently found to be identical to barley *Tigrina-d*. It interacts with glutamyl-tRNA reductase through TPR domains mediating Mg-protoporphyrin inhibition. Carotenoid deficient regulatory *tigrina* or their phenocopies point to membranes as a regulatory site or to abscisic acid as a negative regulator.

Keywords: abscisic, Cytokinin, glutamyl-tRNA, glutamyl-tRNA reductase, heme, Mg-protoporphyrin

The autotrophic life of higher plants is dependent on five different tetrapyrroles. These are the closed-ringed molecules chlorophyll, heme, siroheme and Mg-protoporphyrin, and the linear tetrapyrrole phytochromobilin. Chlorophyll is the green pigment of the plant and used for light harvesting and in the photosynthetic process. Heme is the prosthetic group of many different proteins. Among the most well known are cytochromes and leghemoglobin, which participate in electron transfer and nitrogen fixation, respectively. Siroheme is present in two different chloroplast enzymes, nitrite reductase and sulfite reductase, which are involved in assimilation of inorganic nitrogen oxides and sulphur oxides into biological systems. Phytochrome contains phytochromobilin as functional group and mediates red light regulated gene expression. Mg-protoporphyrin is a newly recognised co-repressor and possible blue light receptor.

The five tetrapyrroles have a common biosynthetic pathway, in which eight molecules of 5-aminolevulinate (ALA) are assembled into the tetrapyrrole core structure. The formation of ALA is a key regulated step in the biosynthesis of chlorophyll, heme, siroheme, Mg-protoporphyrin and phytochromobilin. This review will present: a historical introduction to the formation of ALA; the chromosomal location of the genes coding for the enzymes and factors involved; their expression patterns; the enzyme structures and their mechanisms and proteins, which regulate their activity.

THE MYSTERY OF AMINOLEVULINATE FORMATION IN PLANTS

The source of ALA in plants was not known for many years. In early studies, similarities to the heme pathway were sought and apparently found. It was considered that the first, and only, enzyme of the pathway in nature including plants, was ALA synthase, which forms ALA by the condensation of glycine and succinyl-CoA (Fig. 1). ALA synthase had been found both in animals, photosynthetic and non-photosynthetic α-proteobacteria (Jordan and Shemin, 1972). Some studies did not support that glycine and succinyl-CoA were the precursors of ALA (Della Rosa et al., 1953). The breakthrough discovery of the new pathway forming ALA can be attributed to Beale and Castelfranco (Beale and Castelfranco, 1973). A 5-carbon precursor was completely

*Corresponding author; fax +46-46-2224534
e-mail gough@biobase.dk

Abbreviations: ALA, 5-aminolevulinic acid; SAM, S-adenosyl-methionine.

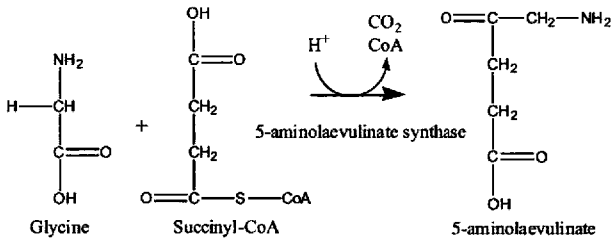

Figure 1. ALA synthase condenses glycine and succinyl-CoA, giving a 6-carbon intermediate which looses CO2 to give ALA.

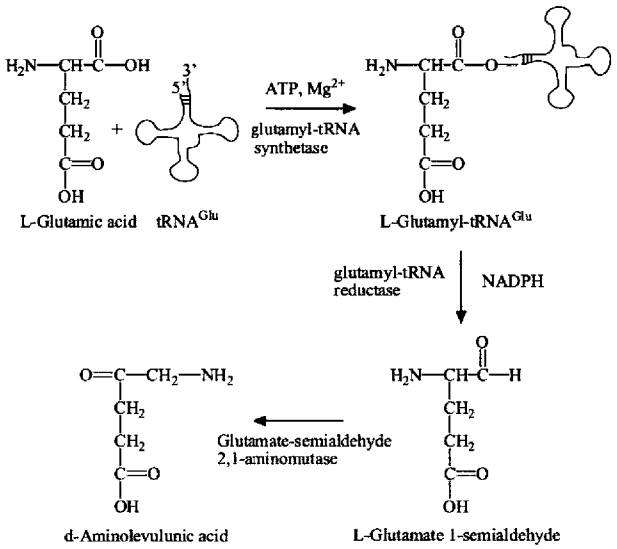

Figure 2. ALA biosynthesis in plants and non-alpha-proteobacteria. Plastid glutamyl-tRNA synthetase activated glutamate and this was followed by a unique reduction of glutamyl-tRNA to glutamate semialdehyde and transamination to give ALA.

incorporated into ALA (Beale et al., 1975; Meller et al., 1975). Early experiments with a regulatory mutant of *Chlamydomonas* indicated that there was only one cellular source of ALA (Wang et al., 1975). ALA was found to be formed, only in the chloroplast and not the mitochondria (Gough and Kannangara, 1976). The first steps and all the enzymes of chlorophyll biosynthesis were shown to be present in the chloroplast (Kannagara and Gough, 1977). Glutamate and not α-ketoglutarate was shown to be the immediate precursor of ALA (Kannagara and Gough, 1977). This led to synthesis and identification of glutamate 1-semialdehyde as an intermediate in glutamate conversion to ALA (Kannagara and Gough, 1978). It soon became clear that at least three separable components were required for ALA formation (Wang et al., 1981). Amazingly one was not a protein, but the chloroplast encoded $tRNA^{Glu}$ (Berry-Lowe, 1987). Thus, precursor ALA for all the chlorophyll, heme, siroheme, Mg-protoporphyrin and phytochromobiline formed in the plant is provided solely by the plastid. Conversion of glutamate to ALA is shown in Figure 2.

It has emerged that the phototropic α-proteobacteria are the only chlorophyll containing organisms to use the ALA synthase route using glycine and succinyl-CoA. Its use is characteristic for members of the α-proteobacteria in general. The possibility that a eukaryotic organism could synthesis ALA via both of the two pathways has been analysed and uniquely found for the one-celled protist *Euglena gracilis*. This ambidextrous cell contains ALA synthase in its mitochondria and the more recently discovered C5-pathway in the chloroplast (Weinstein and Beale, 1983). The malaria parasite *Plasmodium falciparum* contains a plastid like apicoplast organelle with its own DNA. However, the genome of this organism contain only the ALA synthase gene (Wilson et al., 1996). It is interesting to note that in these eukaryotes, ALA synthase functions in the mitochondria (Varadharajan et al., 2002), the organelle considered to be acquired as an α-proteobacterial ancestor by an early evolutionary symbiotic event. Fascinatingly, results from genome sequencing projects suggest that the C5-pathway may also be present in some α-proteobacteria. For example, BLAST searches (www.ncbi.nlm.nih.gov/blast) reveal glutamyl-tRNA reductase from *Magnetospirillum magnetotacticum* (gi:23015681) and from the photosynthetic bacterium *Rhodospirillum rubrum* (gi:22969030). Also, a homolog of glutamate 1-semialdehyde aminotransferase is found in *Sinorhizobium meliloti* (PsymA plasmid, gi:16262971) and *Agrobacterium tumefaciens* (gi:16119351). (Protein homology determined using BLINK in the protein section of http://www.ncbi.nlm.nih.gov/entrez). It will be interesting to see if evidence for enzyme activity can be obtained for these proteins.

ALA synthase identification in any chlorophyll-containing organism apart from the α-proteobacteria and protozoa cannot be confirmed by bioinformatics. However, it has been claimed that this pathway operates in callus tissue from soybean and sugar-beet (Bisbis et al., 1997/1998; Gaspar et al., 1999). We know that in most photosynthetic organisms and most bacteria, glutamate is the precursor of all tetrapyrroles including the chlorophylls. BLAST searches of ALA synthase from *Rhodobacter capsulatus* against the complete plant genome of rice reveal homology to gi:19996295. This protein, however, is a clear endophyte homolog showing 88% identity to gi:15889869 of *A. tumefaciens*. BLAST searches to the database of 28,000 expressed full-length cDNA rice clones (Kikuchi et al., 2003) show no

homologous sequences. In the *Arabidopsis thaliana* genome only an orthologous enzyme, *BioF* (7-keto-8-aminopelargonate synthetase) was found. A search of the NCBI expressed gene database revealed no plant homologs of ALA synthase. Thus, with the exception of the *Euglenaceae* and the α-proteobacteria, it can be concluded that there is no genetic evidence for the existence of any ALA synthase genes in photosynthetic organisms. Since ALA synthase is not present in plants, its inhibition can perhaps be used to combating plant pathogens such as fungus and the α-proteobacteria *A. tumefaciens*, *Sphingomonas melonis*, *Wolbachia*, and others.

THE INTERMEDIATES OF THE TETRAPYRROLE BIOSYNTHETIC PATHWAY AFTER ALA

As the regulation of ALA formation is intimately connected with the entire tetrapyrrole biosynthetic pathway a brief description of the pathway will be given. Figure 3 shows the enzymes and intermediates of the pathway of chlorophyll biosynthesis. These intermediates after ALA were revealed by the pioneering work of Granick (Granick and Beale, 1978). He demonstrated that ALA dehydratase catalysed the conversion of two ALA molecules to the mono-pyrrole, porphobilinogen. He also isolated chlorophyll deficient mutants of *Chlorella*, which accumulated the oxidised form of the intermediates uro-, copro- and protoporphyrinogen, some of which were also seen in mammalian mutants with genetic defects in heme biosynthesis called porphyrias. Porphobilinogen is converted by porphobilinogen deaminase and uroporphyrinogen synthase, to uroporphyrinogen III. This intermediate has four acetyl groups, which are changed to methyl groups by uroporphyrinogen decarboxylase to coproporphyrinogen III. This is then converted by coproporphyrinogen oxidase to protoporphyrinogen. An anaerobic and aerobic version of coproporphyrinogen oxidase is known, which form the dicarboxylic protoporphyrinogen IX. Another oxidase converts protoporphyrinogen to the first wine-coloured intermediate, protoporphyrin IX. Porphyrinogens are easily oxidised by air to give aromatic porphyrins. These highly coloured hydrophobic compounds, as well as later intermediates and products of the pathway, absorb light energy. The energy can activate oxygen molecules, leading to photodynamic damage, particularly of membranes. Carotenoids are necessary to take up this energy and dissipate it as heat, thus protecting the pigments. In contrast to single cells, multicellular plants have evolved

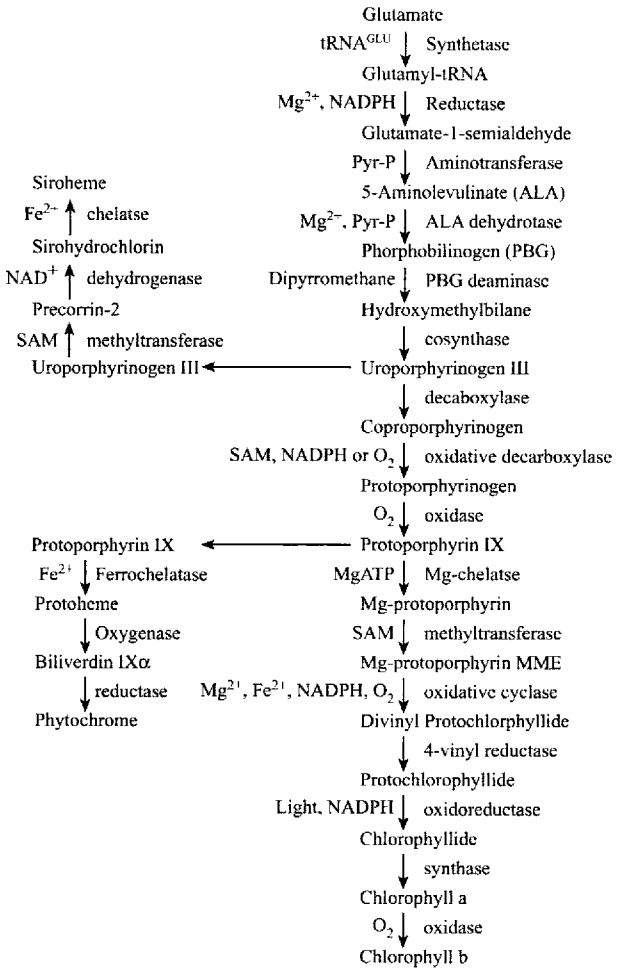

Figure 3. The enzymes and intermediates of the pathway of chlorophyll biosynthesis.

a mechanism that normally prevents the accumulation of large amounts of intermediate porphyrins. As we shall see, higher plants mutants are known where this mechanism is suspended thus revealing regulatory mechanisms.

Granick proposed a shared pathway until the stage where either Mg^{2+} or Fe^{2+} was inserted into protoporphyrin to give Mg-protoporphyrin or heme, respectively. This was later confirmed by the discovery of the enzymes Mg-chelatase and ferrochelatase. Other *Chlorella* mutants accumulated the substrate of the methyl transferase (Mg-protoporphyrin), the cyclase (Mg-protoporphyrin monomethyl ester) and the oxidoreductase (protochlorophyllide) (Granick and Beale, 1978). It should be noted that, in contrast to lower plants, the protochlorophyllide oxidoreductase of angiosperms requires light for the conversion of protochlorophyllide to chlorophyllide. After the formation of uroporphy-

rinogen the hydrophobic character of chlorophyll is gradually achieved by removal of the eight carboxylic acid side chains of the strongly hydrophilic uroporphyrinogen molecule. Two carboxylates remain in protoporphyrin; these are esterified, as methyl and phytyl esters, so that chlorophyll becomes a hydrophobic molecule. Chlorophyll synthase is responsible for the addition of phytol to chlorophyllide (Rudiger et al., 1980). Chlorophyll b synthase (Tanaka et al., 1998) (chlorophyll a oxidase) activity has been demonstrated in a few higher plants and *Prochlorothrix* the sole prokaryont known to form chlorophyll b.

Later discoveries were a side pathway leading from uroporphyrinogen to siroheme, the important cofactor of nitrite and sulfite reductase. Note that in bacteria the cobalamin ring is formed from uroporphyrinogen. There is no evidence for cobalamin formation in higher plants. Phytochromobilin is formed from heme by two enzymes, heme oxygenase and bilirubin IXα reductase.

REGULATION: PRODUCTS, INTERMEDIATES, COFACTORS AND LIGHT RESPONSIVE FACTORS REGULATING THE TETRAPYRROLE BIOSYNTHETIC PATHWAY

Heme plays an important role in regulation of the tetrapyrrole synthetic pathway, both at the enzyme level and as a precursor of light-sensitive phytochrome that regulates transcriptional events. The siroheme containing sulfite reductase regulates transcription of chloroplast DNA (Sekine et al., 2002).

Knowledge about the enzymes of the chlorophyll branch of the pathway has benefited enormously from the early genetic work on the photosynthetic gene operon of the *R. capsulatus* (Suzuki et al., 1997). Gene names are in many cases derived from this work. This work led to the discovery of the Mg^{2+} inserting heterotrimeric Mg-chelatase coded by the *bchI, D* and *H* genes, which is presently the subject of intense research activity. Mg-protoporphyrin is emerging as an important regulatory molecule affecting plant development as revealed from studies of *A. thaliana gun* mutants (Larkin et al., 2003; Strand et al., 2003). Limitation of its methylation to Mg-protoporphyrin monomethyl ester by cytoplasmically synthesised S-adenosyl-methionine (SAM) (Ravanel et al., 1998), points to this cofactor as an important regulator. A di-iron protein Crd1 that plays a role in the Mg-protoporphyrin monomethyl ester cyclase reaction binds iron (Pinta et al., 2002). Iron limitation prevents chlorophyll formation at this step as indicated by inhibition with iron chelators (Granick, 1961). All angiosperm possess a light dependent protochlorophyllide oxidoreductase. Light is therefore necessary for chlorophyll formation. Only factors affecting ALA formation will be considered here. Much interest was centred on the formation of ALA. Feeding of ALA to cut dark-grown angiosperms turned them green. This showed that negatively regulated formation of ALA was one main reason for the yellow colour of these plants (Granick, 1959). Mutants affecting the regulation of chlorophyll biosynthesis overproduced ALA in the dark and are green in the dark (Gough and Kannangara, 1979). Equally responsible for the yellow colour is the light requirement of the penultimate step of chlorophyll biosynthesis, protochlorophyllide oxidoreductase (Smith and Kupke, 1956). Angiosperms become green only in the light. By providing substrate for heme and chlorophyll biosynthesis ALA has profound effects on plant development.

In Vivo Regulation of ALA Formation and Chlorophyll Synthesis by Light and Dark

Etiolated shoots exposed to light begin to form chlorophyll. When the light is turned off chlorophyll formation stops and a small amount of protochlorophyllide is formed, which is bound to protochlorophyllide oxidoreductase. Early experiments with ALA feeding of cut etiolated shoots in the dark led to protoporphyrin and protochlorophyllide accumulation in a ratio of about 1:10 (Gough, 1972). Recent similar experiments with rice, using more accurate fluorescent detection of intermediates, show that Mg-protoporphyrin also accumulates (Gough et al., in preparation). This suggests that four steps prevent chlorophyll synthesis in etiolated leaves in the dark. These rate limiting steps are: 1) ALA formation 2) Mg-chelatase 3) Mg-protoporphyrin monomethyl ester transferase and 4) protochlorophyllide oxidoreductase that requires light to function.

Evidence for in Vivo Inhibition by Heme and Its Reversal by Light

The rate of ALA formation can be studied in cut shoots by incubating them in the dark for a time on levulinate, a competitive inhibitor of the conversion of ALA to porphobilinogen. The ALA formed accumulates in a pool, which can be measured. When light was subsequently turned on, the shoots accumulated ALA. Surprisingly, when the shoots were light induced and then returned to the dark, they formed ALA equivalents to a much greater extent than normally seen in protochlorophyllide (Gough, 1978). A similar

effect was seen in cucumber chloroplasts (Huang and Castelfranco, 1989). Levulinate may therefore interfere with feedback control of ALA formation, which normally operates. A flow of ALA in the dark, towards another end product such as heme, Mg-protoporphyrin, in addition to protochlorophyllide, is suggested. Heme inhibition is supported by the dark increase in ferrochelatase activity observed in barley grown under circadian conditions (Papenbrock et al., 1999).

The Mg-chelatase mutant *xantha-f*[10] provided evidence for feedback inhibition, which could be due to heme and not to intermediates or end products of the Mg-branch. The mutant *xantha-f*[10] contains no protochlorophyllide. Light did not cause the light induced increase in ALA accumulation in the presence of levulinate seen in the wild type. However this mutant contains normal dark levels of enzyme and tRNA able to convert glutamate to ALA in vitro (Gough et al., unpublished observations). The enzymes are therefore inactive in vivo. No intermediates are formed. The plant remains yellow in the light. This is consistent with the hypothesis that, in the dark: ALA formation is inhibited by heme (formed from protoporphyrin) and that phytochrome (made from heme) is involved in transcriptional repression of the reductase. Other evidence for heme as a feedback inhibitor was provided by observations of low protochlorophyllide levels in phytochrome biosynthesis mutants. Treatment of dark grown plants with the iron-chelator, α,α-dipyridyl, stimulates endogenous ALA formation leading to larger amounts of protochlorophyllide. This is attributed to interference with heme feedback. In the phytochrome mutants this effect was attenuated, suggesting a larger pool of inhibitory heme, because the mutations block heme conversion to phytochrome (Terry and Kendrick, 1999).

It had been suggested by (Lascelles and Hatch, 1969), that heme regulated Bacteriochlorophyll biosynthesis. Similarly, when light converts protochlorophyllide to chlorophyllide, the intermediates then flow without accumulation, through the pathway to chlorophyll. Protoporphyrin is then no longer diverted to heme, which no longer inhibits ALA formation.

Several studies have shown light induced rate increases of ALA formation using levulinic acid (Harel and Klein, 1972; Gough, 1978). In barley the activity of enzymes converting glutamate to ALA increased three times during greening (Kannangara and Gough, 1979). In cucumber the activity of the glutamyl-tRNA^{Glu} reductase was increased 3-times during greening (Masuda et al., 1996).

The question is: is this due to increased amounts of enzyme as well as the release from feedback inhibition operating in the dark? The following evidence shows that the *activity* of enzymes involved in ALA formation increase during greening and is regulated post-transcriptionally.

The Regulation of ALA Formation and Chlorophyll Synthesis by Light and Dark at the Post-Transcriptional Level

Cycloheximide, an inhibitor of cytoplasmic protein synthesis inhibited light-induced ALA accumulation in levulinate treated shoots, by about 90%. Cytoplasmic protein synthesis was therefore required for the increase in activity (Gough, 1978). Enzymes that could form ALA at a quarter the rate of light-induced plants could also be isolated from dark-grown shoots.

It seems clear that translational control operates to regulate the expression of messages for ALA forming enzymes. This is because transcript levels do not always correspond with protein levels for many enzymes involved in chlorophyll biosynthesis (Papenbrock et al., 1999). In the barley albostrians mutant glutamyl-tRNA reductase transcript levels were not correlated with protein determined by Western blot (Yaronskaya et al., 2003). This indicates that post-transcriptional control mechanisms are operating.

Few clues to the mechanism of translational control of the ALA biosynthesis enzymes have emerged. One clue is the stem-loop structures observed in the 5′ region of two barley *hemA* promoter regions. Such regions are also found in the 5′ region from other genes of the *Poaceae* family (Hansson, 2001). This suggests that these messages may act as ribo-switches. Cofactors such as cobalamin, thiamine and SAM bind to, and regulate the expression of the 5′ untranslated region of messages from all classes of organism, including plants (Winkler et al., 2003). In this way Mg-protoporphyrin could regulates the translation of messages involved in chlorophyll biosynthesis.

No data is presently available about translational control of any of the messages involved in ALA biosynthesis. However, details of translational control mechanism are emerging in plants. Recently it has been shown that norflurazon can also inhibit translation of mRNA (Tamada et al., 2003). Wheat germ, translation of the mRNA for SAM decarboxylase, a polyamine biosynthetic enzyme, is inhibited by polyamines (Raney et al., 2002). Light regulated translation control of expression of the small subunit of ribulose-1,5-bisphosphate carboxylase and catalase have been shown (Skadsen and Scandalios, 1987; Berry et al., 1990).

Another possible post-transcriptional regulatory mechanism involves chloroplast uptake and concerns 14-3-

3 proteins. Transgenic potatoes over-expressing a 14-3-3 protein contained more chlorophyll and had delayed senescence (Szopa, 2002). 14-3-3 and hsp70 proteins assist in uptake into the chloroplast (Jarvis and Söll, 2002), interact with key metabolic enzymes in plants including stromal glutamine synthetase (Comparot et al., 2003).

So the activity of the ALA forming system increases during greening. This increase is regulated post-transcriptionally but it is not known how. More details of the regulatory process have emerged through study of the components required to convert glutamate to ALA. The first is chloroplast glutamyl-tRNA.

CHLOROPLAST GLUTAMYL-tRNA

Fractionating ALA synthesis components from green chloroplast stroma gave one fraction that contained no protein. This fraction contained RNA. In a glutamate tRNA activating assay catalysed by another fraction, the RNA was activated with glutamate. This could subsequently be used to form ALA. The RNA hybridised to the chloroplast genome (Kannangara et al., 1984). Sequencing of chloroplast DNA indicated an identical sequence to the barley chloroplast $tRNA^{Glu}$ gene (Berry-Lowe, 1987). Only one $tRNA^{Glu}$ species was found in dark grown or greened barley chloroplasts and its level was not affected by light (Schön et al., 1986; Hansson et al., 1997). In *Synechocystis* the dual role of the tRNA in protein and ALA synthesis has been shown directly (O'Neill and Söll, 1990).

Characterisation of a pale green *A. thaliana* mutant abc1 has shown that the expression of the $tRNA^{Glu}$ is dependent on a chromosome 1, regulatory, nuclear encoded, sigma factor (sigB) required by the plastid encoded polymerase. The tRNA gene is increasingly expressed during the first 12 h of germination. SigB transcripts are expressed in seedlings of *Arabidopsis*. UniGene information is so far only available for a few rice clones (Kanamaru et al., 2001) (Table 1). This gene demonstrates nuclear control of the plastid DNA coded $tRNA^{Glu}$ required for tetrapyrrole biosynthesis.

Chlorophyll biosynthesis is also limited by reduced plastid glutamyl-tRNA transcript level in white leaves of the barley *albostrians* nuclear gene mutant (Hess et al., 1992). Similar limitation is also seen in the *albina-f*[17] barley mutant (Gough et al., unpublished observations). In *albostrians* segregants with green, striped and white leaves are seen. The increase in ALA formation in the light in levulinate imbibed green leaves was not seen in white leaves. Total heme levels were decreased and carotenoids were very low. Etiolated white leaves could convert very little exogenous ALA to Mg-protoporphyrin compared to wild type. Interestingly glutamyl-tRNA reductase transcript levels were not correlated with protein levels determined by Western blot (Yaronskaya et al., 2003).

Cytokinin Increases Dark Formation of ALA by Its Effect on $tRNA^{Glu}$ Transcript Levels in the Chloroplast

Recently it has also been shown that ALA mimics the hormonal effects of cytokinin and auxin on rooting and shooting of callus (Bindu and Vivekanandan, 1998). Cytokinins are required for differentiation of plant callus to chlorophyll containing shoots (Kim et al., 1992). Cytokinin levels decrease under many kinds of stress and stimulated chlorophyll formation during recovery from stress or wounding (Hare et al., 1997). Benzyl adenine treatment of cucumber cotyledons increased the level of RNA two-fold, including $tRNA^{Glu}$ in plastids in the dark or during greening. The two-fold increase in rate of ALA formation in the dark was attributed solely to an increased amount of $tRNA^{Glu}$ (Masuda et al., 1995). This increase can be attributed to the increased amount of $tRNA^{Glu}$ because reductase activity is directly proportional to the amount of tRNA added to the assay (Kannangara et al., 1984).

These results show that cytokinin increases levels of $tRNA^{Glu}$, thus increasing the amount of ALA formed in the dark and during greening. It will be interesting to determine how.

Glutamyl tRNA is the substrate of the synthetase and available information will be considered next.

CHLOROPLAST GLUTAMYL-tRNA SYNTHETASE

Genes and Their Transcription

There are three or four glutamyl-tRNA synthetases in the species shown in Table 1. Only the gene coding for the chloroplast synthetase involved in ALA biosynthesis is considered here. The identity of these synthetase genes as homologs is dependent on the sequence determined for the single barley enzyme present in greening chloroplast stroma, from where it was first isolated and cloned. This enzyme has a N-terminal signal sequence predicted to direct it to the chloroplast (Andersen, 1992a). Its gene is found on barley chromosome 6H (Hansson et al., 1998). In *A. thaliana* the glutamyl-tRNA synthetase most similar to the barley

chloroplast synthetase is on chromosome 5. Expressed sequences for these chloroplast synthetase genes have been found in roots and seedlings leaves in *A. thaliana* and in the monocots considered here, in seed tissue (Table 1). During greening expression of the glutamyl-tRNA synthetase message is not significantly increased by light. After 4 h it begins slowly to decline until 8-10 hours where it is reduced more rapidly. After 24 h there is hardly any message left (Andersen, 1992b). In cucumber no change in the activity of the synthetase and amount of protein determined by Western blot levels were found in the light (Masuda et al., 1996). ALA formation was not correlated with synthetase transcription by the barley *tigrina-d*[12] mutant in the dark, which forms 2-3 times more ALA (Gough and Kannangara, 1979). Synthetase transcripts were increased

Table 1. The chromosomal location of genes important for ALA biosynthesis, UniGene cDNA cluster names and the tissue of expression of these cDNAs in *A. thaliana*, rice and barley. Chromosomal locations (*ChrX*) are given in italic. UniGene cluster names are underlined. The chromosomal locations of the *A. thaliana* genes are followed by the locus tag in the Entrez data base (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein). Barley gene location were from (Bougri et al., 1996; Hansson et al., 1998; Hansson, 2001). Expressed sequence data (ESTs) are from the UniGene section of the Entrez nucleotide database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). If UniGene data were not available, ESTs identical/similar to the protein were found by BLAST searches. Rice map data were from the full length clones reported by (Kikuchi et al., 2003) at http://cdna01.dna.affrc.go.jp/cDNA/. ND, not determined. Map locations not found in these references were determined by BLAST searches at http://www.ncbi.nlm.nih.gov/BLAST/.

	Arabidopsis (5 chromosomes)	Rice (12 chromosomes)	Barley (7 chromosomes)
Chloroplast glutamyl-tRNA synthetase	*Chr 5 (At5g64050)* At.8457, roots, seedling	*Chr 2* Os.7227, leaf, panicles, endosperm	*Short arm of Chr 6H* Hv.8709, seedling shoot, spike, embryo
$tRNA^{Glu}$	*Chloroplast genome (gi:7525012, trnE)*	*Chloroplast genome (gi:11466763, trnE)*	*Chloroplast genome (gi:11600)*
Plastid sigma factor	*Chr 1 (At1g08540)* At.23349, above ground organs, green siliques, seedling	*Chr. ND* Os.28459	*Chr ND*
Glutamyl-tRNA reductase	*Chr 1 (At1g58290, hemA1)* At.241, seed, flower, green seed-pod, light induced	*Chr 10* Os.11129, rice blast infected leaf	*Long arm of Chr 1H (hemA1)* Hv.117, light induced, germinating seeds, shoots, leaves, flower, spike, endosperm
	Chr 1 (At1g09940, hemA2) At.27711, roots, *E. cichoracearum* infected leaf, seed-pod/flower		*Short arm of Chr 1H (hemA2)* Hv.8961, light induced, shoots, leaves, after inoculation with *B. graminis*
	Chr 2 (At2g31250, hemA3) At.38245, green seed-pods		*Chr ND (hemA3)* Hv.118, root, seedling leaf, leaves, after inoculation with *B. graminis*
Flu (mag2.7) /Tigrina-d	*Chr 3 (At3g14110, Flu (mag2.7))* At.22594, roots, green seed-pods, seedlings leaf and root, flowers	*Chr 1* Os.9779, leaf, stem, callus	*Chr 5H* Hv.4022, etiolated leaves, shoot, leaves, spike
Tigrina-o (probablelycopene-β-cyclase)	*Chr 3 (At3g10230)* At.1532, seedling, seed-cases, seed, flowers	*Chr.7 (gi:19927417)*	*Chr 5H* Hv.6557, caryopsis, leaves
Tigrina-b (probable ç-carotene desaturase)	*Chr 3 (At3g04870)* At.24092, roots, above ground organs, siliques, seed	*Chr.7* Os.7230, leaf, callus, stem	*Chr. ND* Leaf, seedling, roots, spike, flowers, after inoculation with *Blumeria graminis*
Tigrina-n (probable carotenoid isomerase)	*Chr 1 (At1g06820)* At.25584, seedling, flower, green seed coats, leaf, roots seedling hypocotyls, seed, rosette leaf	*Chr 11 (gi: 30349118)*	*Chr ND* Leaf, germinating seeds, after inoculation with *B.* graminis
	Chr 1 (At1g57770) At.28533, many stages of development		
Glutamate 1-semialdehyde aminotransferase	*Chr 5 (At5g63570, gsa-1)* At.27758, seeds, root, leaf, flower	*Chr 8* Os.10144, leaf, stem	*β-arm of Chr 7H* Hv.186, seed, root, shoots, leaves, flower, spike
	Chr 3 (At3g48730, gsa-2) At.19963, seed, leaf, flower, green seed-pod		

four-fold but only a slight increase in enzyme activity is seen. This was the only change in the in vitro activity of the soluble enzymes or $tRNA^{Glu}$ involved in ALA formation seen in this mutant (Hansson et al., 1997).

However, when chlorophyll formation ceases and it is broken down, another picture is seen. Chlorophyll decreases during development of the anthocyanin containing red canopy of the ornamental *Euphorbia pulcherrima*. The reduction is correlated with lowered mRNA and Western blot levels of the synthetase and also the reductase, and Mg-chelatase H (olive) and I (ch42) subunits (Kannangara and Hansson, 1998).

There is therefore no evidence for a requirement for transcription of the synthetase, increase in activity or amount by Western blot to account for the increased ALA synthesis in the light. However information about the enzyme itself suggests that we should be cautious about dismissing the synthetase as an important regulator of ALA formation.

Chloroplast Glutamyl-tRNA Synthetase. Enzyme Activity

This enzyme activates glutamate using Mg-ATP to form glutamyl-$tRNA^{Glu}$. Only one enzyme has been isolated from barley chloroplasts (Bruyant and Kannangara, 1987). The pH optimum is 6.8. 4 mM $MgCl_2$ is required for optimal activity. The enzyme does not require high Mg^{2+} concentration (Bruyant and Kannangara, 1987). However concentrations of $MgCl_2$ up to 25 mM do not inhibit it (Gough, unpublished). High concentrations of Mg^{2+} are used in coupled assays for glutamate conversion to ALA. Chloroplast glutamyl-tRNA synthetase provides substrates for 3 different processes. The synthetase is therefore considered to be very important for plant development because its activity is necessary for chlorophyll, heme provision to the whole plant and chloroplast protein synthesis. An additional function of the synthetase is to regulate the entrance of glutamine into plastid proteins by its role in loading glutamate to $tRNA^{Gln}$. As in many bacteria and *Chlamydomonas reinhardtii* the glutamyl-$tRNA^{Gln}$ is then transamidated to give glutaminyl-$tRNA^{Gln}$ (Schön et al., 1988; Chen et al., 1990). The synthetase may therefore play a role in coordinating tetrapyrrole and protein synthesis in the plastid.

The Structure of Glutamyl-tRNA Synthetase

Structures of the *Thermus thermophilus* enzyme with its unmodified tRNA and with a substrate analog glutamol AMP have been determined (Sekine et al., 2003) (Table 2). This enzyme carries the tRNA like a man carrying a large sack over his shoulder. In contrast to the barley synthetase this synthetase is of the discriminating class, which selectively loads $tRNA^{Glu}$ and not $tRNA^{Gln}$. This is achieved using the single R358 residue that is in contact with the 5'-cytosine anticodon base of the $tRNA^{Glu}$ (Sekine et al., 2001). In the barley synthetase this residue is replaced with the non-discriminatory G406. (This is a useful way to discriminate organellar from cytoplasmic synthetases.) However, since four out of the other five residues that contact the anticodon region are conserved or similar (Fig. 4), the bacterial structure would allow modelling of the plant glutamyl-tRNA synthetases.

Feedback Regulation of Glutamyl-tRNA Synthetase

In *Synechocystis* the synthetase is not inhibited by heme (10 μM) or protochlorophyllide (2 μM) (Rieble and Beale, 1991). In barley the enzyme is not sensitive to heme. Protochlorophyllide (up to 1.7 μM) in prolamellar bodies isolated from dark grown barley failed to inhibit glutamate conversion to ALA (Gough et al., 1981). When the *Chlamydomonas* enzyme was purified it was reported to be strongly inhibited by 5 μM heme (Chang et al., 1990). In the mutant *C2A* of *Scenedesmus*, which required light to green; 2 μM protochlorophyllide inhibits the synthetase by 60% (Dörnemann et al., 1989). Thus, feedback regulation of glutamyl-tRNA synthetase is not important in barley or *Synechocystis* but is so in algae.

Table 2. Structures of ALA biosynthetic enzymes. Structures should be viewed by downloading the program Swiss PDB-Viewer from http://www.expasy.org/spbbv. Structures are available at http://www.rcsb.org/pdb

Enzyme and organism	Substrate/inhibitor	Cofactor	Structure accession no.
Glutamyl-tRNA synthetase (*T. thermophilus*)	Glutamol AMP	$tRNA^{Glu}$ ATP, Mg^{2+}	1N77, 1N78 (Sekine et al., 2003)
Glutamyl-tRNA reductase (*M. kandleri*)	Glutamyl-puromycin analog	No	1GPJ (Moser et al., 2001)
Glutamate 1-semialdehyde aminotransferase (*Synechococcus* sp.)	Gabaculin	Pyridoxal phosphate	3GSB (Hennig et al., 1997)

```
T. thermophilus   355 MRPRFDTLKEFPEKARYLFTEDYPV-----SEKAQRKLEEGLPLLKELYPRLRAQEEWTE
                       ++   D + +       L +  YP+     S++A+  +E+ L  +           E  +
H. vulgare        403 LKEGIDLITDADAALCKLLS--YPLHETLSSDEAKSVVEDKLSEVASGLISAYDSGELDQ

T. thermophilus   410 AALEA------LLRGFAAEKGVKLGQVAQPLRAALTGSLETPGLFEILALLGK 456
                        A  E        ++ F      K   +  PLR  LTG L  P +   + L+ K
H. vulgare        461 ALAEGHDGWKKWVKSFGKTHKRKGKSLFMPLRVLLTGKLHGPAMDSTVILVHK 513
```

Figure 4. Comparison of the residues of *T. thermophilus* glutamyl-tRNA synthetase (pdb 1N77) within 3 Å of the anticodon region (underlined) aligned with the barley chloroplast glutamyl-tRNA synthetase. R358 is the discriminatory residue in *T. thermophilus* replaced by G406 in the non-discriminating barley enzyme. Four of the five anticodon contacting residues are also conserved or semi conserved in barley (underlined).

Regulation of Cytoplasmic Glutamyl-tRNA Synthetase by 13-3-3 Proteins

It is however very likely that the synthetase is regulated by other factors. It is of interest that an *A. thaliana* cytoplasmic glutamyl-tRNA synthetase (as determined by its discrimator residue) is associated with 14-3-3 proteins in *A. thaliana* suspension cells where growth on sugar inhibits chloroplast development (Cotelle et al., 2000). Binding of 14-3-3's to targets such as nitrate reductase is stimulated by cations such as Mg^{2+} that alters the conformation of the target-binding groove. Polyamines bind to the same groove at much lower concentrations, stimulating interaction between proteins. This could modulate the activity of the target (Roberts, 2003). It will be interesting to see if a 14-3-3 protein or other factor modulates glutamyl-tRNA synthetase activity in the chloroplast.

Regulatory Interactions with Glutamyl-tRNA Synthetases in Other Organisms

In *Bacillus subtilis* binding of glutamate and ATP is stimulated by association of the synthetase with the purine biosynthetic enzyme, adenylosuccinate lyase (Gendron et al., 1992). Binding of $tRNA^{Glu}$ is also stimulated by Arc1p in yeast (Simos et al., 1996). The aminoacyl minihelix binding domain of Arc1p is found in other RNA-binding proteins and when cleaved out has cytokine signalling activity affecting e.g. angiogenesis (Kaminska et al., 2000; Yang et al., 2002). Also this kind of interaction could be of importance for the chloroplast synthetase.

GLUTAMYL-tRNA REDUCTASE: GENES AND TRANSCRIPTION

The *hemA* gene encoding glutamyl-tRNA reductase is very important for tetrapyrrole biosynthesis in plants, especially from a regulatory point of view. Three glutamyl-tRNA reductase genes are found in barley and in *A. thaliana*. Only one has so far been found in rice and two in cucumber. Table 1 shows the gene location as well as the tissues where transcripts occur. Table 3 summaries environmental factors, which have been reported to affect transcription. Their transcription patterns have been studied in detail only in *A. thaliana*, barley and cucumber. The sequence of the *hem1* (*hemA*) gene for the protein catalysing the glutamyl-

Table 3. Factors affecting glutamyl-tRNA reductase transcription. Data obtained from (Ilag et al., 1994; Bougri et al., 1996; Tanaka et al., 1996; McCormac and Terry, 2002; Ujwal et al., 2002). FRc, continuous far red light.

Factor	Barley	*A. thaliana*	Cucumber
Light induced	*hemA1* (gi:1041424, bha1)	*hemA1* (gi:20141560)	*hemA1* (gi:1015319)
	hemA2 (gi:1666078, bha13)		*hemA2* (gi:1694926)
Repressed by sugars in the light		*hemA1* (gi:20141560)	
Repressed by FRc		*hemA1* (gi:20141560)	
Circadian oscillation. Induced by cytokinin-in etiolated leaves	*hemA1* (gi:1041424, bha1)		
Inhibited by glucose or sucrose	*hemA1* (gi:1041424, bha1)	*hemA2* (gi:1346263) Low levels in roots and flowers	*hemA2* (gi:1694926)
	hemA3 (gi:2967443)		
Expressed in roots		*hemA3* (gi:15224641)	
Expressed in root, leaf, stem and flower tissues		*hemA1* (gi:20141560)	

tRNA reductase reaction was first found in *Escherichia coli* (Li et al., 1989). The *Synechocystis* enzyme is a homolog of the *hem1* gene of *E. coli* and was the first isolated from a photosynthetic organism by rescuing an *E. coli* mutant of this gene (Grimm, 1992). The homologous nature of bacterial and higher plant enzymes was confirmed by purification and N-terminal sequencing of the barley glutamyl-tRNA reductase (Pontoppidan and Kannangara, 1994).

Glutamyl-tRNA Reductase Genes and Their Transcriptional Expression in *A. thaliana*

In *A. thaliana hemA1* and *hemA2* are located on chromosome 1 and *hemA3* on chromosome 3 (Table 1). The deduced polypeptides are all predicted to have N-terminal chloroplast signal sequences. It is of interest that the calculated isoelectric points of the three proteins increase from 6.5, 7.6 to 8.5. The cloned *hemA* gene of *A. thaliana* was shown to have glutamyl-tRNA reductase activity (Ilag et al., 1994). *A. thaliana* expressing antisense RNA of the light induced *hemA1* gene, were deficient in chlorophyll and non-covalently bound hemes (Kumar and Söll, 2000). The *hemA2* transcripts were expressed only in low levels in roots, leaf, stem and flowers (Ilag et al., 1994). This *hemA2* transcription was not affected by either red, blue or uv light, but was inhibited by glucose or sucrose but not fructose (Table 3) (Ujwal et al., 2002). The *hemA2* mRNA has been found in leaves infected with fungus (Table 1).

The *hemA1* transcripts are strongly induced by light (Table 3) (Ilag et al., 1994). Further studies showed that the *hemA1* promoter was necessary for norflurazon induced bleaching (Ujwal et al., 2002). This, together with other evidence, reviewed below, indicated regulation by two repressors, phytochrome A and Mg-protoporphyrin (Ujwal et al., 2002). The *hemA1* expression was also inhibited by sugars but only in the light (Ujwal et al., 2002). Light regulated development of plants has been shown in *A. thaliana* to be extremely complex. At least five phytochromes, two blue-light phototropins and two cryptochrome photoreceptors are involved (Briggs and Olney, 2001). The phytochrome transduction pathway involves cGMP, calcium and down-stream components. Recently, cGMP calmodulin regulated ion channels have been described in plants. They may regulate Ca^{2+} and K^{+} fluxes in the cell (Talke et al., 2003). In *A. thaliana*, studies with phytochrome and cryptochrome mutants showed that both light receptors were involved in regulating transcription of the *hemA* gene and its expression as a *hemA1* promoter-*gus* reporter (McCormac and Terry, 2002).

Phytochrome A Regulation of *hemA1* Gene Expression in *A. thaliana*

After 3 days growth under continuous far red light (FRc), followed by white light, *A. thaliana* seedlings are unable to form chlorophyll. Dark grown plants of the same age can green. FRc repression of *hemA1* resulting in failure to green is mediated by phytochrome A. This is shown by the loss of repression of *hemA1::gus* in the *phyA* mutant under FRc and subsequent white light. Norflurazon represses *hemA1* in an additive way to FRc. Norflurazon was still active in repression of the *phyA* mutant construct showing that this inhibitor of carotenogenesis acts at a second site (McCormac and

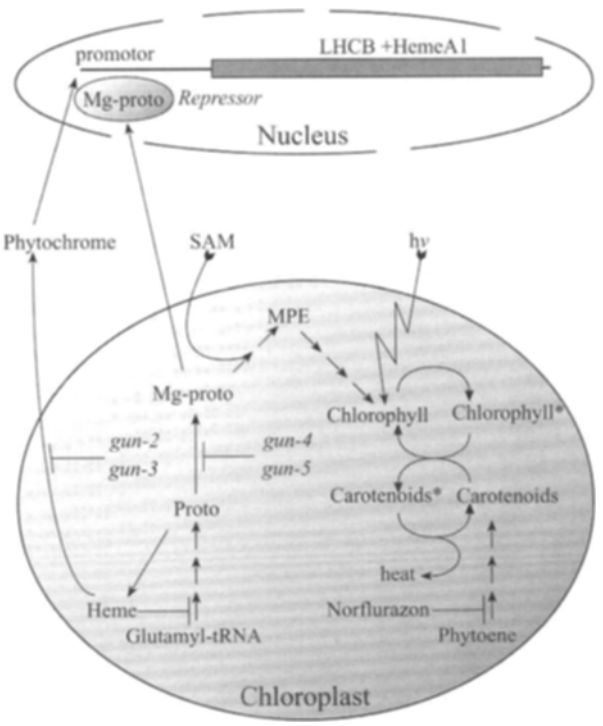

Figure 5. Model for repression of LHCB and *hemA1* transcription by the plastid signals Mg-protoporphyrin and phytochrome A. In the dark Mg-protoporphyrin accumulates, diffuses from the plastid and represses transcription. Light photo-converts protochlorophyllide leading to continuous chlorophyll formation, depletes Mg-protoporphyrin intermediate levels, and derepression occurs. Norflurazon causes membrane destruction. Membrane bound enzymes converting Mg-protoporphyrin to chlorophyll become depleted and Mg-protoporphyrin accumulates again repressing transcription. In *gun*, *xantha* and other mutants blocked before Mg-protoporphyrin formation, Mg-protoporphyrin cannot accumulate and derepression occurs. Mutants (*gun*) blocked in phytochrome biosynthesis, also release the repression by this signal transduction system. Proto, protoporphyrin; Mg-proto, Mg-protoporphyrin; MPE, Mg-protoporphyrin monomethylester; Pchlide, protochlorophyllide.

Terry, 2002). Norflurazon was also shown to strongly inhibit reductase activity induction by white light (Kumar et al., 1999). As we shall see, in the section on *gun* mutants below, these results suggests that *hemA1* expression is repressed both by phytochrome A and by Mg-protoporphyrin, perhaps via a repressor protein (Fig. 5).

The Genome Uncoupled, *gun*, Mutants of *A. thaliana*

Mutants with the genome uncoupled *gun* phenotype are defective in the carotenoid deficiency-linked repression of nuclear coded light induced genes (Susek et al., 1993). Mutants with a *gun* phenotype express transcripts for LHCB, in the light even when carotenoid biosynthesis is inhibited by norflurazon. Many chlorophyll biosynthesis enzyme transcripts are also repressed (Strand et al., 2003). Transcription in the light of *hemA1* is repressed by norflurazon (Ujwal et al., 2002). Barley mutants of the *xantha-f, -g* and *-h* loci, unable to form Mg-protoporphyrin have a *gun* phenotype, accentuated by the presence of norflurazon (Batschauer et al., 1986)(Gadjieva et al. unpublished). However, the cyclase mutant *xantha-l*^{81}, which can form Mg-protoporphyrin does not have a *gun* phenotype (Gadjieva et al., unpublished). This agrees with a previous report, where light induced LHCP transcripts were also reduced in this mutant (Batschauer et al., 1986). These experiments are important because they suggested that a Mg-branch intermediate I probably regulates light expression and dark repression of the *hemA1* glutamyl-tRNA reductase gene. Investigations discussed next have shown that Mg-protoporphyrin is probably this intermediate.

Mutants Blocked in Mg-Protoporphyrin and Phytochrome Biosynthesis Are also *gun* Mutants

Mutants *gun-2* and *gun-3* are defect in biosynthetic enzymes converting heme to phytochrome. Mg-chelatase is a three subunit enzyme which inserts Mg^{2+} into protoporphyrin forming Mg-protoporphyrin (Willows, 2003). The mutant *gun-5* is a mutant of the Mg-chelatase H-subunit and *gun-4* is a regulatory subunit, associated with the H subunit (Mochizuki et al., 2001; Larkin et al., 2003).

In a recent, illuminating paper, mutants of the Mg-chelatase D-subunit, or earlier enzymes of the pathway, which are therefore deficient in the formation of Mg-protoporphyrin, were shown to be *gun* mutants. Norflurazon treated wild-type plants accumulated Mg-protoporphyrin whereas Mg-protoporphyrin levels in *gun-2* and *gun-5* were 4 to 20 times lower levels. Dipyridyl treatment, which could cause Mg-protoporphyrin and its methyl ester accumulation to wild-type levels, eliminated the *gun* phenotype. Treatment of wild-type protoplasts with Mg-protoporphyrin depressed expression of a LHCB gene. Further evidence was provided by the following experiments. LHCB1:: Luciferase constructs with and without a mutated G3M light-regulated binding site for transcriptional regulators, were introduced into the *gun-5* mutant. Only the wild-type G3M element showed expression of luciferase on norflurazon treatment (Strand et al., 2003).

Plastids from norflurazon treated leaves are totally devoid of inner membrane systems (La Rocca et al., 2000). Since biosynthetic enzymes after the Mg-chelatase are found in these membranes (Willows, 2003), this could explains why Mg-protoporphyrin accumulates in norflurazon treated leaves in the light.

These experiments suggested that norflurazon repression of light induced genes functions by causing Mg-protoporphyrin accumulation in the plastid from where it diffused out to act as corepressor (Fig. 5). The norflurazon inhibition of chlorophyll synthesis suggests an analogy with repression of hemA expression in darkness. Chlorophyll synthesised in the light is protected by carotenoids and incorporated into chlorophyll proteins. Mg-protoporphyrin does not accumulate. During darkness, chlorophyll synthesis ceases, because of the light requirement for protochlorophyllide reductase. Mg-protoporphyrin diffuses out of the chloroplast and represses or derepresses many chloroplast protein genes.

These experiments suggest that Mg-protoporphyrin or a derived product acts as the signal that is sent from the plastid and acts as a co repressor of the LHCB. By inference this signal also core presses the light induced, norflurazon repressed, *hemA1* gene (Fig. 1)

Light Responsive Promoters of the *hemA* Genes

The G3M promoter is a CUF1 G-box motif CACGTA. This contains the bZip binding ACGT element, whose flanking regions determine its specificity (Ramachandran et al., 1994). It is related to the G-box CACGTG, which is found also in light responsive promoters e.g. GBF binding of the ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit promoter (Giuliano et al., 1988) and cG-1 binding of the chalcone synthase promoters (Staiger et al., 1989). 14-3-3 proteins are known to be involved in transcriptional control of light and hormonally regulated expression by binding to bZip G-box binding-factors (Siberil et al., 2001).

The –199/+200 promoter region of the *hemA1* gene of *A. thaliana* in a *gusA* construct was enough to confer

light regulated, phytochrome A repressed, expression. This region contains GT-1/I-box (GATAAA) and CCA-1 binding sites implicated as the light-responsive cis elements. Norflurazon abolished the promoter construct expression indicating that a plastid factor was necessary (McCormac et al., 2001).

Inspection of the sequences shows that the GT-1 is immediately followed by a CUF1-like sequence AACGTGT. Looking in the 1000 bp upstream of the other *hemA* genes, CUF-like sequences were found in most cases. In *A. thaliana hemA2* AACGTGT is found but in *hemA3* not at all. In rice *hemA*, AACGTGA is also found in the upstream 5' 1000 bp. In barley *hemA1* TTCGTGG and in *hemA2* CTCGTGG are found. Both *A. thaliana hemA2* and rice and barley *hemA*'s have GT-1 boxes but they are further away from the CUF1-like motif.

This suggests that it is very likely that the *hemA* genes, except *A. thaliana hemA3*, can be regulated by Mg-protoporphyrin co-repression. Correlation of the promoter structure with the light induced expression will be interesting to see.

The Identity of the Putative Mg-Protoporphyrin Binding Repressor Is Not Known

If the Mg-protoporphyrin interacts with the promoter region of the *hemA* genes, this interaction is presumably mediated by a repressor protein factor in addition to bZip. Its identity is unknown. Search for nuclear transit (using PSOR at http://psort.nibb.ac.jp/form.html) sequences in proteins known to bind Mg-protoporphyrin suggests that the Mg-chelatase H subunit might play a role as a co-repressor. The *A. thaliana* H subunit sequence at position 200, KRKKQ, is rich in basic amino acids and is fairly well conserved in all higher plant H subunits (Gough, unpublished).

In summary, in *Arabidopsis*, the *hemA1* gene is repressed by Mg-protoporphyrin, phytochrome A, cryptochrome and sugars. Phytochrome A and Mg-protoporphyrin act on contiguous GT-1 and bZip binding promoters. Light reverses these repressions. Sugars also regulated the non-light responsive *hemA1* gene which has non-contiguous GT-1 and bZip elements. The root expressed *hemA3* gene lacks 5-upstream bZip elements.

Glutamyl Reductase Genes and Their Expression in Barley

In barley *hemA1* and *hemA2* are assigned to chromosome 1H (Table 1) (Hansson et al., 1998). The location of the *hemA3* is unknown (Bougri et al., 1996). The three barley genes have chloroplast signal sequences. Maximum levels of transcripts during greening and under circadian conditions corresponded to the activity of ALA formation measured using levulinate in shoots (Bougri and Grimm 1996; Papenbrock et al., 1999). Transcripts of all three genes have been found in seedling leaves (shoots) and leaves; *hemA1* is expressed in flowers, spikes and seeds (Table1) and *hemA3* in roots (Table 3) (Tanaka et al., 1997). Both *hemA1* and *hemA2* are induced by light but *hemA1* is present in roots and induced by cytokinin in etiolated leaves in the dark (Bougri et al., 1996).

This is a somewhat arrangement to *Arabidopsis* except that both *hemA1* and *hemA2* are light induced.

Glutamyl-tRNA Reductase Genes and Their Expression in Rice and Cucumber

In rice transcripts of the *hemA* gene on chromosome 10 were so far found only in rice blast infected leaves (Table 1). In cucumber it should also be mentioned that three *hemA* genes are found. The *hemA1* is expressed in cotyledons and leaves and was the only one strongly induced by light (Table 3). The *hemA2* is found in all tissues (Tanaka et al., 1996) and *hemA3* is expressed primarily in roots (Tanaka et al., 1997). A chloroplast signal is not predicted for *hemA1* but this may be due to a sequencing error.

The number, location and expression pattern the cucumber hemA genes are similar to the other dicot *Arabidopsis*.

Transcripts of *hemA* in Fungally Infected Leaves of *A. thaliana*, Rice and Barley

A. thaliana hemA2 transcripts have been reported in leaves infected with *Erysiphe cichoracearum* (Table 1). Barley *hemA2* and *hemA3* are also found in leaves infected with *Blumeria graminis*. As blast (*Magnaporthe grisea*)-infected rice leaves also transcribe *hemA*, the tetrapyrrole pathway may be important in resistance to fungal attack. One suggestion is to provide heme for the oxidative burst NADPH oxidase and protective catalases and peroxidases (Møller, 2001).

In conclusion, *hemA* genes in plants are known to be repressed by Phytochrome A, cryptochrome and Mg-protoporphyrin. This repression is released by light. Repression by sugars and induction by cytokinin also regulates hemA transcription. We may note that glucose and cytokinin signalling are antagonistic.

An important unanswered question is: whether enzyme

activity, during greening, correlates with the amount of protein determined by Western blot. The answer to this question will indicate whether other factors may be affecting the activity of the reductase. The following results provide fuel for this view.

GLUTAMYL-tRNA REDUCTASE: ENZYME ACTIVITY

In cucumber glutamyl-tRNA reductase activity increases three fold during greening (Masuda et al., 1996). Similarly in barley the activity of the enzymes converting glutamate to ALA increase three-fold in barley during greening (Kannangara and Gough, 1979). In barley glutamyl-tRNA reductase requires high concentrations of Mg^{2+} and reduces glutamyl-tRNA with NADPH to give glutamate-1-semialdehyde (Kannangara et al., 1988). Enzyme activity converting glutamate via glutamate semialdehyde to ALA is best measured by the devised HPLC method employing reverse phase C18 separation of these compounds (Wang et al., 1981). Similar enzyme activities have been shown in many other plants, algae, photosynthetic and non-photosynthetic bacteria.

Mg^{2+} stimulated ALA formation by barley stroma 16-fold at the optimum pH 7.9 maximally at the unphysiological concentration 20 mM (Gough and Kannangara, 1977). High Mg^{2+} concentrations gave maximal activity also in *Chlorella* (12 mM) (Weinstein and Beale, 1985). *Euglena* and *Synechocystis* (15 mM) (Mayer et al., 1987; Rieble and Beale, 1988). Maximal activity could also be observed with 15 mM Mn^{2+} but not Zn^{2+} or Ca^{2+}. Mg^{2+} stabilises the folded form of tRNA by eliminating charge interaction between phosphates (Misra and Draper, 2002). The effectiveness of low concentration of Mg^{2+} was increased by Ca^{2+}. In cucumber the stimulatory effect of red light and cytokinin on chlorophyll formation has been shown to be related to an influx of Ca^{2+} (Reiss and Beale, 1995). In spinach chloroplasts light increases the stromal Mg^{2+} concentration from 0.5 mM to 2 mM. Ca^{2+} channel blockers prevented this increase (Ishijima et al., 2003). In spinach stromal Mg^{2+} increased from 3 - 4 mM to 8 - 10 mM during development and was involved in stabilizing RNA (Horlitz and Klaff, 2000). So it is possible that chlorophyll formation is regulated to some extent by light induced increases in stromal Mg^{2+} concentration, which increase the activity of the reductase. However, as we have seen the activity of the reductase in vitro can be greatly increased by non-physiological concentrations of Mg^{2+}. This suggests that, in plants, an unknown positive regulatory factor remains to be discovered.

Interestingly it was initially reported that for optimal activity 10 mM $MgCl_2$ was also necessary in *E. coli* (Moser et al., 2001). However, in bacteria it has now been shown that glutamyl-$tRNA^{Glu}$ prepared at high Mg^{2+} concentration (15 mM Mg^{2+} in the presence of 25 mM K^+) is effective as a substrate for the reductase in a solution without metal ions (Moser et al., 1999). In this connection we note that in the absence of Mg^{2+} the tRNA can assume a hairpin structure with two internal loops (Madore et al., 1999). It is therefore possible that it binds to the reductase in quite a different way than tRNA to a synthetase. The difference between the situations on the one hand in plants and photosynthetic bacteria and the other hand non-photosynthetic bacteria is quite reasonable. Photosynthetic organisms require a large amount of chlorophyll, but only a relatively small amount of heme is necessary in non-chlorophyllaceous bacteria.

Are their any candidates for the positive regulator mentioned above?

Because polyamines decrease during greening (Andreadakis and Kotzabasis, 1996) they are unlikely candidates for the role of the physiological. Another candidate would be 14-3-3 proteins, which are important regulators of plant development. Over-expression of 14-3-3 in transgenic potato has been shown to increase chlorophyll content (Szopa, 2002). 14-3-3 is a positively charged protein rich in lysine and arginine and interacts with phosphorylated serine groups on its target proteins (Roberts, 2003).

Another type of positive regulation would be to physically separate an inhibitor from the reductase. Increased ion concentrations in the light could do just this by dissociating the reductase from its regulation by the membrane located inhibitors, which are discussed in the next sections.

Feedback Regulation of Glutamyl-tRNA Reductase by Heme and Mg-Protoporphyrin

An endogenous inhibitor, resembling protoheme, from thylakoid membranes inhibited ALA formation by intact cucumber plastids (Castelfranco and Zeng, 1991). With soluble enzymes from greening barley plastids, glutamate conversion to ALA was completely inhibited by heme (50 µM), partially by Mg-protoporphyrin (50 µM) and not at all by protoporphyrin (Gough et al., 1981). In barley, heme inhibition of in vitro ALA formation acts on glutamyl-tRNA reductase (Kannangara et al., 1988). Removal of endogenous heme by addition of apoperoxidase increased in vitro ALA formation (Thomas and Weinstein, 1992). Purified *Syn-*

echocystis reductase was inhibited 50% by 5 μM heme (Rieble and Beale, 1991). In *Chlorella* heme inhibits the reductase 50% at 1.2 μM (Weinstein and Beale, 1985). In cell free extracts of *Chlorella*, heme inhibition of ALA formation was increased 10-times in the presence of glutathione (Weinstein et al., 1993). Tobacco plants expressing antisense RNA for the ChlH subunit of Mg-chelatase had reduced Mg-chelatase activity, chlorophyll levels and growth rate. Glutamyl-tRNA reductase activities and transcript levels were also decreased. Because Mg-chelatase activity was genetically blocked, this suggested that heme formed from protoporphyrin inhibited glutamyl-tRNA reductase activity (Papenbrock et al., 2000). When barley glutamyl-tRNA reductase is cloned by rescue of an *E. coli hemA* mutant, N-terminal deletion of barley reductase is always observed. It has been shown that the N-terminal of cloned barley glutamyl-tRNA reductase is involved in heme inhibition. N-terminal truncated enzyme is almost insensitive to 1.5 μM heme, which inhibits full-length enzyme by 50% (Vothknecht et al., 1998). The truncation removed the H residue that is suggested to be one of the heme binding residues (see discussion of the structure below).

Regulation by Turnover of the Glutamyl-tRNA Reductase

In *E. coli* the turnover of glutamyl-tRNA reductase is decreased from 300 min when heme is limiting to 20 min when it is not. The ClpA chaperone, together with ClpP was required for turnover. The N-terminal 18 amino acid residues were suggested to constitute a degradation tag but a construct containing these amino acids linked to LacZ is not correctly regulated by heme (Wang et al., 1999). The 18 residues include the strongly conserved H10 suggested below to bind heme. *E. coli* glutamyl-tRNA reductase is inhibited by heme in crude extracts (Javor and Febre, 1992). It is therefore likely that the *E. coli* reductase is also degraded when it binds heme. A similar heme dependent turnover of the plant glutamyl-tRNA reductase is also a possibility.

The Structure of Glutamyl-tRNA Reductase

The reductase conversion of the glutamate bound to glutamyl-tRNA to glutamate 1-semialdehyde has been shown to proceed through an enzyme bound thioester intermediate. The following findings summarise the arguments for this.

The enzyme has one conserved cysteine and a 3-D structural prediction suggested an enzyme bound

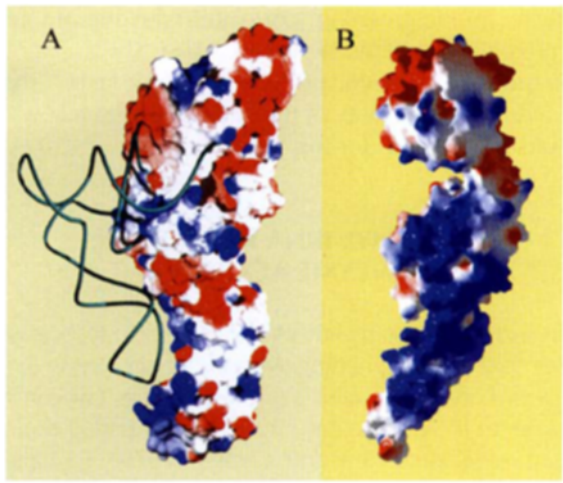

Figure 6. Electrostatic potentials mapped to the surface of: **A.** *T. thermophilus* glutamyl-tRNA synthetase. The tRNA with T-loop is shown as a black line. **B.** A model of barley glutamyl-tRNA reductase. Model based on *M. kandleri* glutamyl-tRNA reductase structure (1gpj) and prepared, together with potential maps, using Swiss-Modeller. (http://www.expasy.org/spdbv). Blue, positive charge; red, negative charge.

thioester intermediate. A NADPH binding Rossman fold was also predicted (Brody et al., 1999). Site directed mutagenesis of the conserved cysteine, resulting in loss of activity also supported a thioester intermediate (Moser et al., 1999). A crystal structure for the *Methanopyrus kandleri* enzyme with a glutamycin inhibitor but without the cofactor NADPH or the tRNA has been published (Moser et al., 2001). There are three domains; the middle domain which is the substrate binding domain, the top domain which is the presumed NADPH binding, Rossman fold, domain, and a bottom domain consisting of α-helices (Fig. 6). All cysteines, including one in the active site, in the enzyme were converted to serine to enable crystallization (Moser et al., 2001). The pdb 1gpj structure shows the glutamycin, which is an analog of the 3'-end of the glutamyl-tRNA in a position close to the presumed position of the active site cysteine. This further supported the idea of a thioester intermediate. Glutamate from glutamyl-tRNA was transferred to the reductase in the absence of NADPH thus providing evidence for the existence of an enzyme linked glutamyl-intermediate. Since a mutant lacking the only conserved cysteine is inactive this suggests an enzyme bound glutamyl-thio-cysteine intermediate (Schauer et al., 2002). A single critical note about this excellent structure is worth mentioning. The replacement of the non-polar cysteine by polar serine can lead to structural changes in enzymes (Gangloff et al., 2001). This is particularly of concern when

the cysteine is in the active site.

How the $tRNA^{Glu}$ binds to the enzymes is a matter of debate. A *Euglena* chloroplast $tRNA^{Glu}$ mutation in the T-loop does not affect protein synthesis but inhibits chlorophyll synthesis. The T-loop residue is therefore important for recognition by the reductase (Stange-Thomann et al., 1994). The barley glutamyl-tRNA reductase model has an area of positive charge on one side of the C-terminal (Fig. 6B). This is not the case for the *M. kandleri* enzyme which has a negative surface charge. In glutamyl-tRNA synthetase it is known that the tRNA binds to a positively charged area on the domain with the T-loop facing away from the protein (Fig. 6A). However, the inactivity of the *Euglena* mutant tRNA mentioned above suggests that in contrast to the glutamyl-tRNA synthetase, the T-loop of the tRNA docks against the positively charged (blue in Fig. 6B) domain of the reductase. This agrees with the suggestion that the A53-U61 (last nucleotides before the T-loop), among other nucleotides of barley $tRNA^{Glu}$ are required for recognition of the barley glutamyl-tRNA reductase (Willows et al., 1995). However the high $Mg2^{+}$ concentrations used in plant reductase assays do not inhibit tRNA loading, as mentioned above. This suggests that the tRNA conformation is similar for synthetase and reductase.

It may however bind to the reductase in a different way than to the synthetase.

The structure (pdb 1gpj) also reveals that the totally conserved H84 is 8.6 Å from H11.They are ideally situated to bind a feedback inhibitor between them. H11 is strongly conserved in all glutamyl-tRNA reductases, though sometimes replaced with Y. In *M. kandleri* the inhibitor could be cobalamin or coenzyme F430. This remains to be tested. Binding of inhibitor would interfere with glutamyl-tRNA from binding. In higher plants where the two histidine residues are totally conserved, the inhibitors heme or Mg-protoporphyrin could bind here.

All residues within 3.5 Å of the glutamyl-adenylate analog glutamycin are conserved in all three isozymes of glutamyl-tRNA reductase in *A. thaliana* as are the two histidine residues suggested to bind the metalloporphyrin inhibitors. This suggests that there are no functional differences between the three forms.

The NADPH binding Rossmann fold is in the middle domain of the reductase (Moser et al., 2001). Fitting NADPH from the structure of S-adenosylhomocysteine hydrolase 1 (pdb 1b3r) to this domain shows that the nicotinamide ring is 19 Å from the glutamyl-thioester intermediate (Fig. 7). Movement of this domain relative to the N-terminal glutamyl-thioester domain would be required for thioester reduction to glutamate semialdehyde.

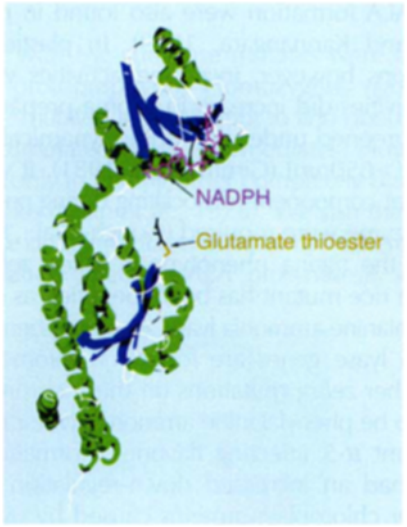

Figure 7. A model of the barley glutamyl-tRNA reductase showing the glutamyl-thioester and the NADPH, whose nicotinamide ring is 19 Å from the thioester. Modelling as Figure 6B. NAD coordinates were taken from the structure of S-adenosylhomocysteine hydrolase (pdb 1b3r), which has a very similar Rossman fold to the reductase. Methods as Figure 6. GTR, glutamyl-tRNA reductase; other abbreviations as Figure 5.

Finally, it should be noted that complex formation between the synthetase, the tRNA and the reductase has been demonstrated (Jahn, 1992).

REGULATORS OF GLUTAMYL-TRNA REDUCTASE GENES AND THEIR TRANSCRIPTION

The *tigrina* Mutants of Barley

The *tigrina* phenotype is characterised by transverse alternating, necrotic or bleached and green bands on the leaf. The banding is correlated with the day and night growth cycle and absent in mutants grown in continuous light. Leaves of the mutants of the *Tigrina-d, -b, -o* and *-n* loci have an excess protochlorophyllide content in dark grown leaves. The phenotype was attributed to accumulation of excess photodestructive protochlorophyllide in the dark. The severity of the phenotype is in the order *Tigrina-d, -b, -o*. The excess protochlorophyllide accumulation has been shown to be due to unregulated ALA formation in the dark. This is probably also the case for *tigrina-n* mutants with a less extreme phenotype. In *tigrina-d*, increased

rates of ALA formation were also found in etioplasts (Gough and Kannangara, 1979). In plastid stroma preparations however, increased activities were not seen. Activities did increase in stroma prepared from *tigrina-d* greened under non-photodynamically active red light (>650nm) (Girnth et al., 1981). It was concluded that components dissociating during preparation of the enzyme were required (Gough et al., 1981).

In rice the *tigrina* phenotype is called *zebra*. The zb8 *zebra* rice mutant has been identified as deficient in phenylalanine ammonia lyase. As other phenylalanine ammonia lyase genes are found on chromosomes 2 and 4, other *zebra* mutations on these chromosomes could also be phenylalanine ammonia lyase mutations.

A mutant *tt-5* affecting flavonoid formation in *A. thaliana* had an increased down-regulation of RNA coding for chloroplast proteins caused by uvB radiation. The phenotype was the same in *gun* mutants indicating that Mg-protoporphyrin was not involved (Jordan et al., 1998). In the grasses the extension growth and greening tissue is at the base of the leaf and a flavonoid deficient mutant, may therefore have *zebra* phenotype due to the different sensitivity of tissue developed in the night and day time.

Flu - the Regulator of Protochlorophyllide Accumulation

Isolation of an *A. thaliana* mutant *flu* which accumulated excess protochlorophyllide in the dark like the regulatory *tigrina*, led to the identification of the Flu protein (Table 1). The *Flu* gene is on *A. thaliana* chromosome 3. It does not affect transcription of the reductase or aminotransferase genes. It is a small protein (316 aa) tightly associated with plastid membranes and contains three C-terminal tetratricopeptide (TPR) repeats (Meskauskiene et al., 2001). The repeat is a structural domain composed of about 34 amino acids. Yeast two-hybrid studies showed that the TPR repeat domain allows association with glutamyl-tRNA reductase but not glutamate-1-semialdehyde aminotransferase. Mutation of *flu* within the TPR domain prevented association with the reductase (Meskauskiene and Apel, 2002). The *Flu* protein has not yet been reported to affect in vitro reductase activity. Homologs of *Flu* are present in rice and barley. Expression of their cDNA has been reported in many different tissues; roots, seedlings and flowers of *A. thaliana*, leaf, stem and callus of rice and etiolated leaves, shoots, leaves and spikes of barley (Table1). As we have seen, this variety of expressed tissue sites is also exhibited by reductase genes. The expression pattern shows the importance of *Flu* at many different developmental stages.

A membrane associated TPR protein required for chlorophyll synthesis under low light and heterotrophic growth conditions has been found in *Synechocystis* (Kong et al., 2003). It is not structurally related to *Flu* except for the TPR domains

There are several suggestions for how Flu regulates the reductase. Most likely, is that Flu regulates the activity of glutamyl-tRNA reductase by linking it, through the TPR domains, to the membrane where inhibition by protein-bound intermediates and end products can take place. As mentioned above the membrane fraction of chloroplasts inhibited glutamate conversion to ALA. Another possibility is that the Flu TPR domains lead to association of the reductase with chaperones, which mediate refolding to an inactive configuration or facilitate turnover. This would be dependent on the presence of an inhibitor such as Mg-protoporphyrin. TPR proteins function as signal transducers by bringing together regulatory proteins and chaperone hsp90. Disruption of the interaction leads to degradation of the proteins (Pratt, 1998). Hsp90 is well known as a mediator of heme binding to proteins (Lee et al., 2003; Osawa et al., 2003), which can be mediated by TPR proteins (Shao et al., 2002). A third alternative is that Flu regulates chloroplast uptake of reductase. TPR proteins involved in chloroplast uptake have been described (Sohrt and Söll, 2000; Chou et al., 2003). Further deduction about the nature of Flu is possible from the following interesting facts.

Location of a Group of Chlorophyll Biosynthetic Related Genes on Chromosomes 3 of *A. thaliana* and Chromosome 1 of Rice Linked to *Flu*

It is notable that five chlorophyll biosynthesis related proteins are found both on one arm of chromosome 3 of *A. thaliana* and chromosome 1 of rice (Table 1). Four of these can be considered to be regulatory genes: Bilirubin IXα reductase forms phytochromobiline of the phytochrome chromophore, mutants (*tigrina-o*) of lycopene β-cyclase and *Flu* affect glutamyl-tRNA reductase and *Crd1* is involved in iron regulation of the Mg-branch of the chlorophyll biosynthetic pathway. The gene order is identical in *A. thaliana* and rice except for the fifth gene encoding uroporphyrinogen decarboxylase.

The *Tigrina-d* and *Tigrina-o* genes are known to be linked and close to the centromere of chromosome 5H (Hagberg and Lehman, 1978). Genes close to centromeres are more often syntenous (Kurar et al., 2003). The mutant *tigrina-o* also accumulates lycopene. It is

Table 4. Genes affecting chlorophyll biosynthesis on *A. thaliana* chromosome 3 are also found on rice chromosome 1. Data from the NCBI genome database. Rice chromosome 1 data (http://rgp.dna.affrc.go.jp/cgi-bin/statusdb/statassign.pl? chr=1&lab=RGP&sort=date).

Gene	*A. thaliana* (gene order)	Rice chromosome 1 (blast score)	Rice, Chr 1 Centimorgan	Barley, Chr 5H
Bilirubin IX α -reductase	At3g09150	B1147A04.9 (4e-54)	167.2-169.5	
Lycopene β or ε-cyclase	At3g10230 (β-cyclase)	B1097DO5.4 (7e-77)	93.1 (ε-cyclase)	*Tigrina-o* Chr.5H (Hagberg and Lehman, 1978)
			73.2 centromere (Round et al., 1997; Sasaki et al., 2002)	62cM centromere
Flu	At3g14110	P0520B06 (1e-84)	73.4	*Tigrina-d* Chr 5H (Hagberg and Lehman, 1978)
Uroporphyrinogen decarboxylase	At3g14930	P0501G01.7 (1e-163)	102.3	
Crd1	Atg356940 (centromere region)	P0003H10 (0.0)	52.4	

therefore probably defective in the β-lycopene cyclase gene coded by a single gene in *Arabidopsis* on the same chromosome (3) as the *Flu* gene (Table 4). On rice chromosome 1, lycopene ε-cyclase and the *Flu* homolog are found. The ε-cyclase has a somewhat similar structure to the β-cyclase and possibly the enzymatic function of this gene could have changed during evolution. The lycopene β-cyclase gene is found on rice chromosome 7 (Table 1).

The chromosomal location of the *Tigrina-b* gene is not known in barley. It is however closely linked to the *tigrina-n and xantha-u* mutant locus (Kahn et al., 1976). In rice genes homologous to the lycopene β-cyclase and the ξ-carotene desaturase are found on chromosome 7, whereas a carotenoid isomerase is found on chromosome 10 (Table 1).

The *Flu* and *Tigrina-d* Genes are Homologous

A map-based clue to the identity of this component emerged recently. The rice *Flu* and ε-cyclase on chromosome 1 are tightly linked to the centromere of chromosome 1 like the barley *Tigrina-d and tigrina-o*, which are on chromosome 5H. As shown in Table 4,

A. thaliana Flu and the carotene β-cyclase are linked on chromosome 3. These facts strongly suggested that *Flu* and *Tigrina-d* are identical. Also that the dissociating factor in *tigrina-d*, which causes increased rate of ALA formation in vivo and *in organello*, is the mutated form of the membrane bound barley *Flu* homolog, the *Tigrina-d* protein. Because the pleiotrophic carotenoid deficiency in the regulatory *tigrina-o* is absent in *flu*, it was not considered as candidate.

Very recent exciting news is that the *Flu* gene has been cloned and sequenced from *tigrina-d*[12]. It is indeed a frame shift mutation of the barley *Flu* homolog causing the last two TPR repeats to be deleted (Lee et al., in press). The implications of this are discussed in the third section below, on double mutants. First some other regulatory mutants have to be described.

Some Regulatory *tigrina* Mutants Have Significant Pleiotrophic Defects

Three of the four regulatory *tigrina* mutants are pleiotrophic. Etioplasts exhibit membrane abnormalities: the feature common to all were crystalloids and undulating osmophilic sheets, similar to those seen in chloroplasts and in plants treated with carotenoid biosynthesis inhibitors (section below) (Nielsen, 1974). Defects in β-carotene biosynthesis were found in these mutants in the dark. In *tigrina-o*, lycopene accumulated; in *tigrina-b*, ξ-carotene and in *tigrina-n*, polycis-lycopene (Nielsen and Gough, 1974). This suggests that *tigrina-b* is defective in ς-carotene desaturase also present on *A. thaliana* chromosome 3 but on rice chromosome 7 (Table 1). Mutants at the *tigrina-n* and *xantha-u* loci are both deficient in β-carotene biosynthesis at the stage of the carotenoid isomerization. This step is necessary for carotenoid formation in plants (Park et al., 2002). Therefore they are probable part of the same locus, as also evidenced by the lack of crossing over between them. The *xantha-u* mutant is also pleiotropic. It is also blocked in chlorophyll biosynthesis, but only when fed ALA does it accumulate uroporphyrin and protoporphyrin. Two copies of carotenoid isomerase are present on chromosome 1 of *A. thaliana*. The *ccr-1 and ccr-2* mutations, as has been shown in *xantha-u*, accumulate cis-carotenoids in the dark, which can be non-enzymatically isomerised in the light. They have

defective prolamellar bodies of which carotenoids are constituents (Park et al., 2002). The mutants are probably identical to the two carotenoid isomerase-like genes on chromosome 1. Table 1 shows that numerous transcripts in many different tissues are found for the first of these two genes; the data is not specific for the second. In barley, BLAST searches indicate expression of lycopene β-cyclase during germination, in leaves and after Blumeria infection (Table 1). It is a puzzle how deficiency in these different carotenoid biosynthetic genes can all lead to deregulation of ALA formation. A recent discovery has shed new light on this question.

A Phenocopy of Carotenoid Deficient Regulatory Mutants

In etiolated barley amitrole inhibits carotenoid biosynthesis causing lycopene accumulation. In addition elevated levels of non-photoactive protochlorophyllide accumulate. Norflurazon treated plants did not make more protochlorophyllide. The deregulation of ALA formation in amitrole treated plants was suggested to be related to the disarrangement of the prolamellar body caused by amitrole. Exogenous ALA treatment of amitrole or norflurazon treated plants led to twice as much protochlorophyllide accumulation. This was attributed to a defective regulation later in the pathway (La Rocca et al., 2001a). Note that norflurazon treated plants are blocked in the conversion of Mg-protoporphyrin to protochlorophyllide (Strand et al., 2003).Excess protochlorophyllide accumulation also occurred in barley treated with the lycopene cyclase inhibitor CPTA (N,N-diethyl-N-[-2-(4-chlorophenylthio)-ethyl]amine (La Rocca et al., 2001b). However, the deregulation of ALA biosynthesis was not related to disarranged prolamellar bodies in *tigrina-b* or *tigrina-n*. There are two factors, which connect the mutants and the phenocopies. The first is the structural disturbances in membrane structure observed: abnormal presence of stacked membrane, crystalloids and undulating osmophilic membranes (Nielsen, 1974). The second is defective abscisic acid formation.

Abscisic acid biosynthesis from violoxanthin, takes place entirely in the plastid (Milborrow, 2001) and is inhibited both in norflurazon treated plants and in mutants blocked in carotene biosynthesis (Zeevaart and Creelman, 1988; Singh et al., 2003). In dark-grown *tigrina-o*, violoxanthin levels are only 25% of wild-type levels (Casadoro et al., 1983). During greening abscisic acid levels are known to slowly decline (Symons and Reid, 2003). Cytokinin and abscisic acid have an antagonistic effect on leaf senescence (Thomas and Stoddart, 1980). Because, as mentioned previously, cytokinin has been shown to increase ALA formation in etiolated cucumber by raising the level of $tRNA^{Glu}$ (Masuda et al., 1995), it may be suggested that the regulatory *tigrina* mutants with carotenoid deficiencies have increased levels of $tRNA^{Glu}$. However, there is a problem with this suggestion. Carotene deficient barley mutants with a *tigrina* phenotype exist, which do not over-accumulate protochlorophyllide, e.g. *tigrina-f*, which accumulates phytoene, phytofluene and zeta-carotene and *tigrina-m*, which accumulates lycopene.

Experiments with Double Mutants Suggest an Inhibitory Role of Heme and Mg-Protoporphyrin in Membranes on Glutamyl-tRNA Reductase Activity

Experiments with double mutants of the regulatory *Tigrina* loci with mutants of the chlorophyll pathway have yielded interesting insights (Table 5). The *xantha-f^{35}* mutant cannot efficiently convert Mg-protoporphyrin monomethyl ester to protochlorophyllide. It has a deficiency in the cyclase activity. However, this mutant does not accumulate Mg-protoporphyrin unless exogenous ALA is provided. Similarly, when ALA is provided, mutants of the Mg-chelatase subunits I, D, and H, form only protoporphyrin. The completely blocked mutants have no protochlorophyllide at all, showing that the ALA formation can be inhibited even when Mg-porphyries cannot be formed (Gough, 1972).

Endogenous accumulation of these intermediates

Table 5. Patterns of porphyrin accumulation by double mutants of regulatory and structural genes of chlorophyll biosynthesis. Adapted from (von Wettstein et al., 1974; Kahn et al., 1976).

Mutant genotype	% Protoporphyrin	% Mg-protoporphyrin	% Protochlorophyllide
tig-d/tig-d xan-f^{35}/xan-f^{35}	0	55	45
tig-d/tig-d xan-f^{10}/xan f^{10}	0	0	0
tig-d/tig-d xan-f^{28}/xan-f^{28}	0	0	0
tig-b/tig-b xan-f^{35}/xanf^{35}	0	30	70
tig-b/tig-b xan-g^{28}/xan-g^{28}	100	0	0
tig-b/tig-b xan-f^{10}/xan-f^{10}	100	0	0
tig-o/tig-o xan-f^{35}/xan-f^{35}	0	35	65
tig-o/tig-o xan-f^{10}/xan-f^{10}	100	0	0

was however seen but only in certain specific combinations of double *tigrina* and *xantha* mutants and not in others (Table 5). Double mutants of *tigrina-d, -b or -o* with *xantha-l*[35] all accumulated Mg-protoporphyrin precursors. In contrast, only double mutants of the carotenoid deficient *tigrina-b* or *tigrina-o* with Mg-chelatase subunit mutants (*xantha-g*[28] or *xantha-f*[10]) accumulated protoporphyrin. No double mutants of *tigrina-d* with Mg-chelatase mutants were found that formed protoporphyrin. Homozygous *tigrina-d* was therefore mutated in an attempt to find double mutants that accumulated other intermediates. However, this resulted only in finding other completely blocked *xantha-l* alleles (Kahn et al., 1976). We can therefore say that these double mutants point to two different aspects of regulation affecting ALA biosynthesis leading to

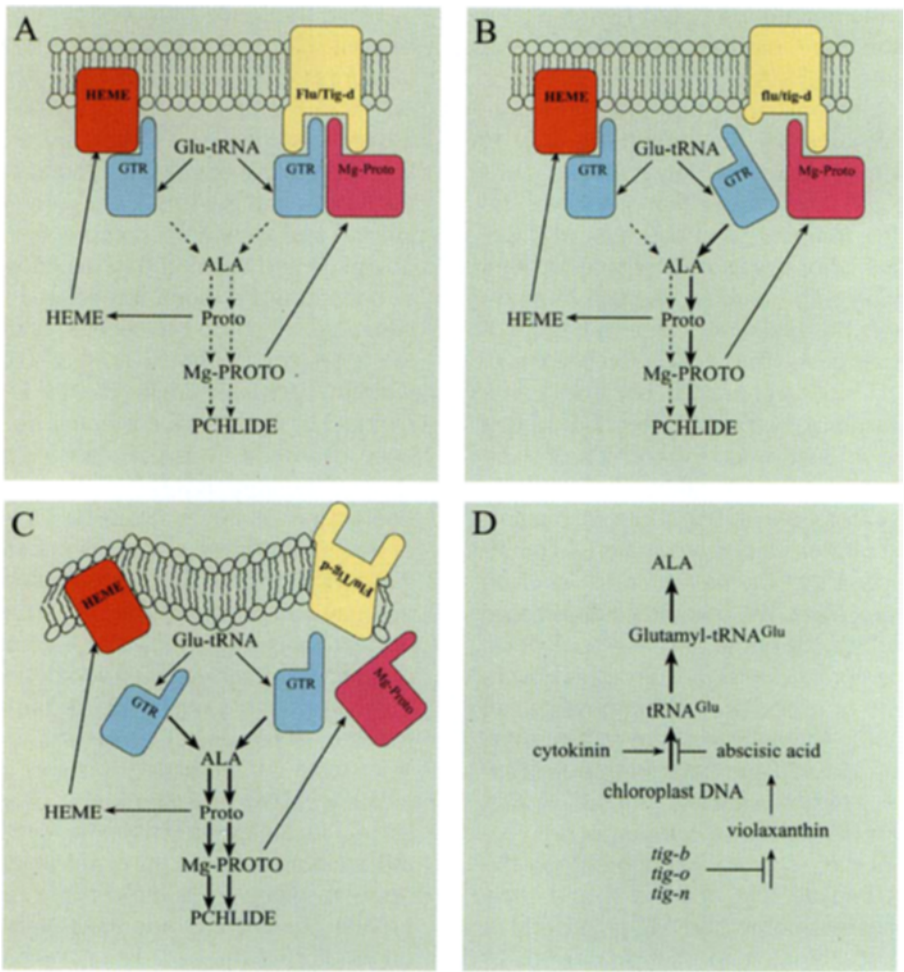

Figure 8. A. Wild-type regulation of the glutamyl-tRNA reductase in the dark. The glutamyl-tRNA reductase is associated with the membrane and regulated by heme and the Flu/Tigrina-d protein (through its TPR domains) mediating Mg-protoporphyrin inhibition. Heme present in the membrane or stroma inhibits the reductase. Only small amounts of protochlorophyllide can be made. **B.** In the *flu/tigrina-d* mutant, the glutamyl-tRNA reductase is dissociated from the *flu/tigrina-d* mutant protein that has deficient TPR domain. Excess protochlorophyllide can be made or Mg-protoporphyrin (monomethyl ester) in cyclase (*xantha-l*) double mutants with *flu/tigrina-d*. Protoporphyrin in Mg-chelatase double mutants (*xantha-f, -g* or *-h*) cannot be made because heme regulation works normally. **C.** Scenario 1 for the carotenoid deficient *tigrina-b, -o* and *-n* mutants. Membrane structure is defective. Therefore association of the glutamyl-tRNA reductase to the membrane bound inhibitors heme and flu/tigrina-d protein associated Mg-protoporphyrin is also defective. Excess protochlorophyllide can accumulate. Both Mg-chelatase mutants and cyclase mutants can accumulate protoporphyrin and Mg-protoporphyrin (monomethyl ester), respectively, in double mutants with *flu/tigrina-d*. **D.** Scenario 2 for the carotenoid deficient *tigrina-b, -o* and *-n* mutants. Abscisic acid deficiency causes increased transcription of $tRNA^{Glu}$. The reductase is more active in the dark. More protochlorophyllide accumulates. Both Mg-chelatase mutants and cyclase mutants can accumulate protoporphyrin and Mg-protoporphyrin (monomethyl ester), respectively.

protochlorophyllide. In double mutants with *tigrina-b* and *tigrina-o* both protoporphyrin and Mg-protoporphyrin can accumulate. However, with *tigrina-d* only Mg-protoporphyrin accumulation is possible. In *tigrina-d* the lesion only affects regulation by Mg-protoporphyrin or Mg-protoporphyrin monomethyl ester one or both of which intermediates therefore also regulates ALA formation. We now know that the mutations *tigrina-d* and *flu* are in homologous genes (Lee et al., in press). The membrane associated Tigrina-d (Flu) protein would therefore mediate inhibition of the glutamyl-tRNA reductase by Mg-protoporphyrin and/or its methyl ester. Only small amounts of Mg-protoporphyrin or protochlorophyllide are formed (Fig. 8A). As mentioned above, increased in ion concentrations caused by light could be the factor that dissociates the reductase from the inhibitors and activates ALA formation. The mutant *flu* or *tigrina-d* membrane protein's defective association with the reductase fails to mediate Mg-protoporphyrin inhibition. Larger amounts of Mg-protoporphyrin or protochlorophyllide can be formed. Protoporphyrin cannot accumulate because heme regulation functions normally (Fig. 8B). This does suggest that the pathway to heme and protochlorophyllide must be sequestered from each other. The evidence provided by the carotenoid deficient *tigrina* mutants and their phenocopies produced by carotenoid inhibitors now also suggest that the membrane is involved in the regulatory processes. The membrane disturbance makes regulation by both heme and Mg-porphyrins, present in the membrane, defective. Protoporphyrin, Mg-protoporphyrin or excess protochlorophyllide can accumulate (Fig. 8C). Alternatively, these mutants may be deficient in abscisic acid and have an up-regulated formation of the $tRNA^{Glu}$ leading to increased ALA formation. In turn in mutants, protoporphyrin, Mg-protoporphyrin, or excess protochlorophyllide in the wild-type can accumulate. (Fig. 8D).This would imply a competition between heme and Mg-protoporphyrin and $tRNA^{Glu}$ for binding to the glutamyl-tRNA reductase as suggested in the discussion on the structure of this enzyme.

GLUTAMATE 1-SEMIALDEHYDE AMINOTRANSFERASE: GENES AND ENZYME

The enzyme converts glutamate semialdehyde to ALA. The requirement of a pyridoxal phosphate cofactor was suggested by the inhibitory effect of the nerve toxin gabaculin on glutamate conversion to ALA in *Chlorella* extracts (Weinstein and Beale, 1985). Gabaculin inhibits many pyridoxal phosphate dependent enzymes (Soper and Manning, 1982). Glutamate 1-semialdehyde is converted by the enzyme via a diaminovaleric acid intermediate (Smith et al., 1991). The pyridoxal requirement was confirmed by the structure of glutamate 1-semialdehyde aminotransferase with bound gabaculin-pyridoxal (Hennig et al., 1997).

The two *A. thaliana* glutamate 1-semialdehyde aminotransferase genes (*Gsa*) are on chromosome 3 and 5, while the single monocot gene is found on chromosome 8 (rice) and 7H (barley) (Table 1). Barley transcripts are found in all tissues but in rice, so far, only in stem and leaf. *A. thaliana Gsa-1* transcripts found in seed, root, leaf and flowers are strongly induced by light while those of *Gsa-2* are found similarly except for the root (Ilag et al., 1994). *Gsa-2* is repressed by nitroflurazon and so may be corepressed by Mg-protoporphyrin (Strand et al., 2003). In barley, similar amounts of transcripts are found in etiolated tissue and during greening, which do not change, until a decrease after 10 hours. At 24 hours no transcript was detected (Grimm, 1990). A similar picture is seen in soybean (Frustaci et al., 1995). Post-transcriptional control has been described in several species. In *A. thaliana* the aminotransferase is one of the genes repressed by norflurazon (Strand et al., 2003). However the amount of aminotransferase protein is not decreased by norflurazon (Kumar et al., 1999). Post-transcriptional control may also occur in barley. Although transcript levels are unchanged (Grimm, 1990) enzyme activity from greening leaves increases, only to decrease after 24 hours (Kannagara and Gough, 1978). Barley transcripts are found in all tissues but in rice, so far, only in stem and leaf (Table 1). In barley enzyme activity does not increase during greening but is decreased in mature leaves. In barley grown under circadian conditions, the aminotransferase transcript level oscillated in an opposite phase to glutamyl-tRNA reductase, but the protein content did not show substantial oscillation under diurnal and circadian growth conditions (Kruse et al., 1997). In cucumber the activity of the aminotransferase and Western blot levels were unchanged compared to dark levels during greening (Masuda et al., 1996). Cucumber enzyme levels were also unchanged by the cytokinin, benzyl adenine (Masuda et al., 1995). The enzyme was induced 5-fold by water stress in maize (Riccardi et al., 1998). The consensus is that glutamate 1-semialdehyde aminotransferase activity does not regulate chlorophyll or heme synthesis in higher plants.

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