The cell biology of tetrapyrroles: a life and death struggle

Nobuyoshi Mochizuki[1], Ryouichi Tanaka[2], Bernhard Grimm[3], Tatsuru Masuda[4], Michael Moulin[5], Alison G. Smith[5], Ayumi Tanaka[2] and Matthew J. Terry[6]

[1]Department of Botany, Graduate School of Science, Kyoto University, Kitashirakawa, Kyoto 606-8502, Japan

[2]Institute of Low Temperature Science, Hokkaido University, Sapporo 060-0819, Japan

[3]Institute of Biology/Plant Physiology, Humboldt University, Philippstr. 13, Building 12, D 10115 Berlin, Germany

[4]Graduate School of Arts and Sciences, University of Tokyo, Tokyo 153-8902, Japan

[5]Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, United Kingdom

[6]School of Biological Sciences, University of Southampton, Boldrewood Campus, Southampton SO16 7PX, United Kingdom

Corresponding author: Terry, M.J. (mjt@soton.ac.uk).

Tetrapyrroles such as chlorophyll and heme are co-factors for essential proteins involved in a wide variety of crucial cellular functions. Nearly 2% of the proteins encoded by the *Arabidopsis thaliana* genome are thought to bind tetrapyrroles, demonstrating their central role in plant metabolism. Although the enzymes required for tetrapyrrole biosynthesis are well characterized, there are still major questions about the regulation of the pathway, and the transport of tetrapyrroles within cells. These issues are important as misregulation of tetrapyrrole metabolism can lead to severe photo-oxidative stress, and because tetrapyrroles have been implicated in signaling pathways coordinating interactions between plant organelles. In this review, we discuss the cell biology of tetrapyrrole metabolism and its implications for tetrapyrroles as signaling molecules.

The importance of tetrapyrroles

Tetrapyrroles are probably one of the most ancient prosthetic groups in all organisms and, when bound to their apoproteins, exhibit a wide range of chemical properties, including light absorption, electron transfer and oxygen binding. Such properties have been exploited by cells across all Kingdoms of life, and tetrapyrroles are essential components of critical biological processes, including respiration and photosynthesis. However, these life-giving chemical properties of tetrapyrroles can also lead to severe photo-oxidative damage, and cell death under some conditions, because unregulated excitation by light can lead to the generation of free radicals and reactive oxygen species, primarily singlet oxygen [1]. For these reasons, tetrapyrrole metabolism needs to be carefully managed within the cellular environment, and transport of these molecules requires precise regulation. In plants, the tetrapyrroles chlorophyll, heme, siroheme and phytochromobilin are synthesized in plastids, but tetrapyrrole cofactors are widespread, and hemoproteins in particular are found throughout the cell. Tetrapyrroles have also been proposed as signaling molecules that coordinate organelle function within the cell. There are recent reviews on the tetrapyrrole synthesis

pathway itself [2-4] and the putative roles of tetrapyrroles in signalling [5-7]. The aim of this review is to address what is known about the localization and transport of tetrapyrroles within the cell, and how this knowledge can help us to understand their suitability as signaling molecules.

Location of tetrapyrrole-binding proteins

As shown in Figure 1, plants allocate tetrapyrrole-binding proteins virtually everywhere within the cell. In the chloroplast, chlorophyll-binding proteins predominate in the thylakoid membranes, which comprise more than 80% protein by surface area [8]. The light-harvesting antenna proteins bind both chlorophyll *a* and *b*, whereas the reaction centre polypeptides in the photosystems contain chlorophyll *a* only. The reaction centre of photosystem II also contains pheophytin *a*, a modified chlorophyll lacking the central Mg^{2+} ion. In addition, subunit IV of the cytochrome b_6/f complex has been shown to bind a chlorophyll *a* molecule [9]. The cytochrome b_6/f complex also contains heme as the prosthetic group of the two cytochromes: the *c*-type cytochrome (*i.e*. where heme is bound covalently, see below), cytochrome *f*, and cytochrome b_6, which along with cytochrome b_{559} in photosystem II, is a *b*-type cytochrome, with non-covalently bound heme. Nitrite reductase and sulfite reductase in plastids contain another type of tetrapyrrole, siroheme, which mediates electron transfer from the [4Fe-4S] centres of these enzymes for the reduction of nitrite or sulfite, key intermediates in nitrogen and sulfur assimilation. The phytochrome photoreceptor family uses a linear tetrapyrrole chromophore, phytochromobilin, to perceive red and far-red light. Phytochromobilin is assembled with phytochrome apoproteins in the cytosol, but on activation by light, phytochromes become nuclear-localized [10]. Heme has an even more widespread distribution. In mitochondria, heme plays an essential role in the respiratory chain in the cytochrome bc_1 complex, and in cytochrome *c* oxidase, which contains heme *a*. Several types of heme-binding peroxidases and catalase are localized in peroxisomes, with other peroxidases known to function in chloroplasts, and in the extracellular space. Cytochrome P450s constitute the largest

family among the tetrapyrrole-containing proteins in plants. In the genome of *Arabidopsis* (*Arabidopsis thaliana*), a total of 272 cytochrome P450 genes have been identified (including 26 pseudogenes) and over 350 have been found in rice (*Oryza sativa*) [11]. For the majority of these, the subcellular localization is not yet determined although several are known to be targeted to mitochondria and chloroplasts, and at least 18 have been found in the ER of *Arabidopsis* [12]. Identification of heme-binding motifs by analysis of plant genomes has also enabled prediction of many other putative hemoproteins, such as a family of over 100 transcription factors [13]. A conservative estimate of the number of tetrapyrrole binding proteins in *Arabidopsis* is therefore approaching 500, which corresponds to nearly 2% of the 27,379 protein-coding genes in the *Arabidopsis* genome.

Biosynthesis in plants

Despite the various locations of tetrapyrrole proteins, the synthesis of tetrapyrroles is thought to take place almost exclusively in plastids (see later). The pathway is shown in Figure 2, together with our current understanding of the location of the enzymes and intermediates within the organelle. Tetrapyrrole biosynthesis has been extensively explored by both biochemical and molecular genetic analyses. The first committed precursor for all tetrapyrroles is 5-aminolevulinic acid (ALA). In plants, algae, and many bacteria, ALA is synthesized from glutamyl-tRNA^{Glu} (which is also required for plastid protein synthesis) by glutamyl-tRNA reductase and glutamate 1-semialdehyde aminotransferase. Eight molecules of ALA are then assembled into the tetrapyrrole primogenitor uroporphyrinogen III in three enzymatic steps. Uroporphyrinogen III can be converted to siroheme, by methylation and oxidation, and then insertion of Fe^{2+} by the enzyme sirohydrochlorin ferrochelatase. Alternatively, uroporphyrinogen III is oxidatively decarboxylated to form protoporphyrin IX, with the final step by the enzyme protoporphyrinogen oxidase being the

oxidation of the colorless protoporphyrinogen IX, to the fully conjugated (and thereby colored) protoporphyrin IX.

The second branchpoint of the pathway involves the insertion of either Mg^{2+} or Fe^{2+}, by Mg-chelatase and ferrochelatase, respectively, thereby directing protoporphyrin IX into the chlorophyll or heme biosynthetic pathways. In the chlorophyll branch, Mg-protoporphyrin IX is sequentially modified to form protochlorophyllide, which in higher plants accumulates in the dark, because the next enzyme, NADPH:protochlorophyllide oxidoreductase (POR), requires light to reduce protochlorophyllide to chlorophyllide *a*. Chlorophyllide *a* is esterified with a long-chain poly-isoprenol (geranylgeraniol or phytol) by chlorophyll synthase to give chlorophyll *a*, some of which is reversibly converted to chlorophyll *b* via the "chlorophyll cycle" [3].

In the heme branch, ferrochelatase catalyzes the insertion of Fe^{2+} into protoporphyrin IX to produce protoheme (heme *b*). This is the prosthetic group of *b*-type cytochromes, and proteins such as catalase, peroxidase and hemoglobin, where the heme is held noncovalently via coordination with the Fe atom by histidine and/or cysteine residues [14]. In *c*-type cytochromes, protoheme is covalently attached via thioether links between the vinyl groups to cysteines in a characteristic CxxCH motif in the protein. Heme *a* is synthesized from protoheme presumably in the mitochondria, although this has not been characterized in plants. Essentially nothing is known about the assembly of *a*-type or *b*-type cytochromes, whereas we have a good understanding of *c*-type cytochrome biogenesis (reviewed in Ref. [15]). There are three systems that have been characterized. The type I system, found in α- and γ-proteobacteria, including *E. coli*, and in plant mitochondria, involves at least eight protein components, called cytochrome *c* maturation (CCM) proteins. Chloroplasts, cyanobacteria and Gram-positive bacteria use the type II system, comprising of a complex of 2–4 cytochrome *c* synthetase (CCS) proteins. These two prokaryotic-like maturation mechanisms contrast with the type III system found in animal and yeast mitochondria, which involves a single protein, heme lyase. However, despite the differences in protein

components, all three systems operate to transport the heme across a membrane to the site of assembly.

Protoheme also serves as the substrate for the formation of phytochromobilin. In chloroplasts, protoheme is oxidatively cleaved by heme oxygenase to form biliverdin IX. Phytochromobilin synthase then converts biliverdin to 3*Z*-phytochromobilin, which is subsequently isomerized to 3*E*-phytochromobilin, the immediate precursor of the bound phytochrome chromophore [16].

Regulation of the pathway

Clearly, given the different roles of the tetrapyrrole cofactors, there will be contrasting demands for these compounds in different tissues of the plant and at different developmental stages. Thus chlorophyll is absent from non-photosynthetic cells but must be synthesized in large amounts during chloroplast biogenesis, for example, during de-etiolation. The combination of substantial changes in demand, together with the severe consequences of photo-oxidation when these molecules are in excess, has resulted in a tight and complex regulatory system for managing the pathway. Considerable progress has been made in recent years in understanding these control mechanisms. The rate-limiting step for the pathway is the synthesis of ALA, or more specifically the activity of glutamyl tRNA reductase (reviewed in Refs [3,4]). More recently attention has focused on the major branchpoint in the pathway at which protoporphyrin IX is directed towards either the chlorophyll or heme branches. Regulation at these steps primarily comprises strong transcriptional control during chloroplast biogenesis together with a range of post-translational mechanisms for rapid regulation of the pathway [2–4,17].

It is now clear that a small subset of tetrapyrrole genes comprise crucial regulatory targets in the pathway. In *Arabidopsis*, a miniarray was used to demonstrate that *HEMA1*, *CHLH*, *CRD1* (*CHL27*) and *CAO* were clustered in a highly regulated group of genes responding strongly to light

and circadian signals [18] (Figure 2). The *GUN4* regulator of Mg-chelatase (see below) is also a member of this group [19]. Co-expression analysis using data from multiple published microarray studies identified *GUN4* as a member of this group, and also led to the addition of two genes *CLA1* (encoding 1-deoxy-D-xylulose-5-phosphate synthase) and *CHLP* (encoding geranylgeranyl pyrophosphate reductase), both required for the synthesis of the phytol tail of chlorophyll [4]. Work is now underway to identify the regulators of these pathway genes during de-etiolation, with the phytochrome-interacting factor proteins, PIF1 and PIF3 [20], and the GLK (Golden2-like) proteins [21] being proposed as major players. Regulation of transcript abundance is also important in mature leaves where *HEMA1* and *CHLH* transcript levels show diurnal and circadian oscillation [22].

The tetrapyrrole pathway is highly regulated at the post-translational level. The major mechanism of product feedback is heme inhibition of glutamyl tRNA reductase (see Ref. [17]). It might be expected that intermediates on the chlorophyll branch such as protochlorophyllide, chlorophyllide or chlorophyll itself would also have a regulatory role. If they are involved, then a potential intermediate would be the regulatory protein FLU [23], which binds GluTR and strongly represses ALA synthesis, but is currently without a known modulator. Another regulator is GUN4, which interacts with Mg-chelatase and stimulates its activity by facilitating substrate binding and/or product release and possibly the interaction of Mg-chelatase with chloroplast membranes [24,25]. Both of these proteins are crucial to plant survival as the absence of FLU is lethal in plants grown under light–dark cycles [23], and *gun4* knockout mutants are entirely chlorophyll deficient under these conditions, even in low light [26].

Redox regulation of CHLI (one of the three subunits of Mg-chelatase) via thioredoxin has been observed, in which the ATPase activity of CHLI is reversibly inactivated by oxidation [27]. Several other tetrapyrrole biosynthesis proteins have also been identified as targets for thioredoxin-regulation via modification of disulfide bonds [28]. Phosphoproteomic analysis of chloroplasts has

indicated that several proteins in the pathway, including CHLI and GUN4, are targets of plastid-localized protein kinases [29]. Regulation through the formation of complexes between adjacent enzymes and the localization of enzymes within the plastid is also thought to be important, and phosphorylation has the potential to modify assembly or localization of tetrapyrrole enzymes as well as their activity. One well characterized example of post-translational regulation is the destabilization of chlorophyllide *a* oxygenase (CAO) in response to over accumulation of chlorophyll *b* [30]. A stretch of ten specific amino-acid residues within the N-terminal domain of CAO has been identified as a degron for destabilization, although the entire N-terminal domain is necessary to respond to chlorophyll *b* [31]. It is likely that these and other mechanisms can account for the numerous examples of post-translational control observed in transgenic plants grown under photo-oxidative conditions (e.g. Refs [32–34]).

Subcellular and intraorganellar localization of biosynthetic pathways

In mammals and yeast, heme synthesis is spatially separated between mitochondria and the cytosol [35]. In higher plants, all the enzymes in the tetrapyrrole pathway (with the exception of the final steps of heme *a* synthesis from protoheme) are present in plastids; however, it has been proposed that a portion of protoporphyrinogen IX is also transferred from plastids to mitochondria and there converted to protoheme by protoporphyrinogen oxidase and ferrochelatase. This hypothesis was based on fractionation studies demonstrating protoporphyrinogen oxidase activity in mitochondrial fractions from pea (*Pisum sativum*) leaves [36], a result supported by subsequent GFP localization studies [37]. In plants, this mitochondrial localization is phylogenetically conserved, but, interestingly, not in the green alga *Chlamydomonas reinhardtii* [38]. If protoporphyrinogen oxidase is present in mitochondria it might be expected that ferrochelatase would also be present to provide a dedicated heme supply for respiratory hemoproteins. However, although ferrochelatase activity has frequently been measured in a mitochondrial membrane fraction (e.g. Refs [39–41]), a variety

of molecular methods have so far failed to provide convincing evidence that ferrochelatase is located there (see Ref. [3] for discussion). Proteomic analyses have also failed to detect either enzyme among 416 Arabidopsis [42] or 322 rice [43] mitochondrial proteins.

The current model for the distribution of the tetrapyrrole pathway in chloroplasts is shown in Figure 2. The trunk pathway through to protoporphyrinogen is localized in the stroma [44], with the enzymes for Mg-porphyrin synthesis, from protoporphyrinogen oxidase through to POR, all associated with both envelope and thylakoid membranes. The situation for Mg-chelatase is particularly complex. Mg-chelatase has been reported to be associated with envelope membranes when the Mg^{2+} concentration is high, but to dissociate from membranes when the Mg^{2+} concentration decreases [45,46]. Membrane-bound activity is also markedly increased by ALA feeding [25]. This complex localization pattern is reflected in the data on the localization of individual subunits. CHLI is always identified in stromal fractions [44] and CHLD has also been considered to be predominantly stromal [45]. However, recent proteomic data have suggested an additional thylakoid localization [44]. In contrast, CHLH is localized in both envelope membranes and the stroma [44, 46]. While this data appear to be difficult to reconcile at the current time, understanding how Mg-chelatase localization is regulated, and the role of GUN4 in this process [25], will undoubtedly be important for understanding the regulation of porphyrin distribution both within the Mg-branch and between the Mg- and Fe-branches of the pathway. There is similarly some uncertainty over the final steps of chlorophyll synthesis, given that chlorophyll synthase is present only in thylakoid membranes [44] whereas CAO has been proposed to be localized in envelope membranes [47]. Unfortunately, the exact location of this enzyme remains elusive due to its low accumulation level [44]. On the heme branch, ferrochelatase is found in thylakoid membranes, whereas the heme oxygenase, HO1, is located in the stroma [44]. There are four heme oxygenase sequences in *Arabidopsis* (three encoding functional enzymes) and all four are localized to the chloroplast [48], which begs the question, how is the majority of cellular heme degraded?

Transport within the cell

As outlined above, as well as tetrapyrrole metabolism within plastids, heme and phytochromobilin are exported to other parts of the cell. However, we know little about the transport of tetrapyrroles around plant cells, a situation that is not much better in animal or fungal systems [49]. For hemoproteins, heme is presumably transported to the appropriate cellular location such as the peroxisomes or ER. Heme might also be transported directly to mitochondria, but the possibility that protoporphyrinogen is transported instead or as well as cannot be disregarded. In addition to the evidence on the location of enzyme activities, there is a considerable body of accumulated data showing that inhibition of protoporphyrinogen oxidase by herbicide treatment results in the production of large quantities of protoporphyrin IX in the cytosol, presumed to be due to non-specific oxidation of accumulated protoporphyrinogen [50]. This results in photo-oxidation and death – the basis of the herbicide action – and underlines the point that a transport mechanism for protoporphyrinogen would have to be extremely carefully managed. There are two additional transfers of tetrapyrroles from plastids: phytochromobilin is exported from the chloroplast to the cytosol for assembly with phytochrome apoproteins that are synthesized in the cytosol (see Ref. [16]); and during chlorophyll degradation a series of catabolic steps leads to the production of non-fluorescent chlorophyll catabolites, which are moved from plastids to vacuoles in a process that probably requires the biggest transport capacity for tetrapyrroles in the cell [51]. Compared with cyclic tetrapyrroles, the linear chlorophyll catabolites present a reduced photo-toxic risk as free molecules, owing to their less rigid and/or conjugated structures.

It is probable that an energy-dependent transport mechanism is required to move heme and other more hydrophilic tetrapyrrole metabolites through or out of the lipid bilayer [49]. At present, with the exception of the movement of chlorophyll catabolites, little is known about these processes. However, given the need to avoid accumulation of non-metabolized porphyrins with the potential

for photo-oxidative damage, these transporters are likely to play a pivotal role in the biology of the cell. One interesting candidate for a porphyrin transporter is the membrane-bound, tryptophan-rich sensory protein, TSPO, first identified in *Rhodobacter sphaeroides.* This protein is a homolog of the protoporphyrin-binding mammalian benzodiazepine receptor that is involved in the interaction with dicarboxylic porphyrins and is essential for the homeostasis and excretion of uroporphyrinogen III [52]. The *Arabidopsis* TSPO has been functionally linked to protoporphyrin IX uptake in a recombinant *E. coli* protoplast system [53] and a *Physcomitrella patens tspo* mutant also shows distinct changes in the protoporphyrin IX levels [54]. Although a good candidate as a heme transporter, recent evidence has shown that *Arabidopsis* TSPO is localized to the secretory pathway, where it might be involved in responses to drought stress [55].

In other Kingdoms several heme and porphyrin-binding proteins with a function in intracellular trafficking have been reported [49,56]. The heme-carrier protein HCP1 and the heme exporter FLVCR (feline leukemia virus subgroup C cellular receptor) have been described in mammalian stem or cancer cells [57,58]. ABCB6, a member of the half-molecule ABC (ATP-binding cassette)-transporter subfamily ATM was identified as a mitochondrial outer membrane transporter for translocation of coproporphyrinogen from the cytosol for completion of the final steps of mammalian heme biosynthesis [59]. The heme-efflux protein ABCG2/BCRP (breast cancer resistance protein) is involved in the translocation of a wide variety of pharmacological substances and is found in all organisms [60]. A mouse *brcp1* knock-out mutant suffered from accumulation of the phototoxic chlorophyll catabolite pheophorbide *a* derived from the diet [61]. In yeast a plasma membrane-localized protein, PUG1 (protoporphyrin IX uptake gene I), enables protoporphyrin IX uptake in exchange for heme efflux [62]. However, deletion of PUG1 does not affect the uptake of heme or protoporphyrin IX, indicating that other loci also encode heme and protoporphyrin IX transporters.

These findings suggest possible candidates for tetrapyrrole transporters in plants. ABC transporters are encoded in plant genomes in large gene families [63], and it has already been demonstrated that the *Arabidopsis* vacuolar ABC transporters AtMRP1-3, which are similar to the glutathione-conjugate transporters MRP1 (human) and YCF1 (yeast), can transport non-fluorescent chlorophyll catabolites to the vacuole during chlorophyll degradation [64,65]. It is likely that ABC transporters can fulfill similar functions in intracellular heme and porphyrin transport in plants. One candidate is the *Arabidopsis* plastid ABC protein LAF6, which was reported to accumulate protoporphyrin IX [66]. However, further analysis has shown that its phenotype is likely to result from a defect in Fe–S cluster biosynthesis [67], resulting in accumulation of 7-hydroxymethyl chlorophyll, an intermediate of the chlorophyll cycle [68].

In addition to transport through membranes, it is also assumed that there must be tetrapyrrole carrier proteins that are responsible for tetrapyrrole trafficking in cells because tetrapyrroles are poorly soluble in aqueous solutions under physiological conditions. Indeed given the relatively hydrophobic nature of many tetrapyrroles such as heme it is possible that carrier proteins might be sufficient for transport in some circumstances, acting to recruit tetrapyrroles directly from membranes. In animal cells, the cytosolic proteins, p22HBP and SOUL, have been isolated from mouse liver [69] and chicken retina and pineal gland extracts [70], respectively. These 22 kDa proteins are ~40% identical at the amino acid level and show high affinity for porphyrins. EST database searches have revealed rice, tobacco (*Nicotiana tabacum*), and *Arabidopsis* genes with sequence similarity to the SOUL/p22HBP family, and thus it is possible that these gene products are functioning in heme trafficking in plant cells. Takahashi *et al*. [71] characterized *Arabidopsis* cytosolic heme-binding proteins (cHBPs) homologous to the p22HBP/SOUL family. Recombinant cHBP proteins bind to a range of porphyrins (including both Fe- and Mg-porphyrins) with dissociation constants (K_d) of less than 1 μM, suggesting that their properties are suitable for tetrapyrrole carrier proteins in plant cells [71].

Coordination of tetrapyrrole metabolism with other cellular processes

The function of tetrapyrroles is not limited to their roles as prosthetic groups; they can also serve as signaling molecules. In mammals and yeast, heme has several important signaling roles. For example, in yeast it binds to the transcription factor HAP1, mediating responses to oxidative stress [72]. In mammals, heme coordinates regulation of metabolism with the circadian clock via the Rev-erb heme sensors [73,74]. In these organisms, heme also controls its own synthesis at the level of ALA synthase [35], a single enzyme that catalyses the synthesis of ALA directly from succinyl CoA and glycine. Heme has been demonstrated to regulate expression, enzyme activity and even mitochondrial import of ALA synthase. It also regulates pri-miRNA processing of heme synthesis genes via the heme-binding protein DGCR8 (DiGeorge critical region-8) [75]. In bacteria, heme has a well-established role in the iron-dependent control of gene expression in α-proteobacteria [76]. Other tetrapyrroles have also been linked with signaling functions. In the red alga *Cyanidioschyzon merolae*, synchronization of nuclear DNA replication with organellar DNA replication has been shown to be mediated by Mg-protoporphyrin [77]. Similarly, there is evidence for tetrapyrrole regulation of gene expression in *Chlamydomonas* where feeding exogenous Mg-protoporphyrin to cultures has been shown to substitute for light in inducing nuclear *HSP70* expression [78]. More recent experiments using Mg-chelatase mutants suggest that heme might also be an active signaling molecule in this system [79]. In bacteria there is also the intriguing hypothesis that phytochrome photoreceptors originated as bilin sensors [80].

A regulatory role for tetrapyrroles in higher plants is currently uncertain. The coordination of genome replication by Mg-protoporphyrin seen in *Cyanidioschyzon* has also been observed in tobacco BY-2 cell cultures [77], and pheophorbide *a* has been shown to induce programmed cell death in *Arabidopsis*, although the mechanism of this light-independent signaling is unknown [81]. Perhaps the most discussed role for a tetrapyrrole in plant signaling is the proposal that Mg-

protoporphyrin acts as a signal mediating communication between plastids and the nucleus [82].

Coordinated expression of photosynthesis-related genes encoded in the plastid and nuclear genomes is important for photosynthetic eukaryotes, and can be achieved by both anterograde (nucleus-to-plastid) and retrograde (plastid-to-nucleus) signaling [6,7]. Although originally discovered through the analysis of mutants with defective chloroplast biogenesis, retrograde signaling has typically been demonstrated using inhibitors of chloroplast function, such as the chloroplast protein synthesis inhibitor lincomycin, or the photobleaching herbicide Norflurazon (NF). Treatment with these compounds leads to the loss of expression of many hundreds of genes [82,83] (A.C. McCormac and M.J. Terry, unpublished), the best studied of which are the *Lhcb* genes encoding light-harvesting chlorophyll *a/b*-binding proteins associated with photosystem II. Several studies have led to the suggestion that tetrapyrroles are plastid signals in higher plants. Early experiments using pathway inhibitors had already implicated Mg-porphyrins in regulating nuclear gene expression (e.g. Ref. [84]), but it was the identification of *Arabidopsis* mutants with an altered response to NF-treatment that led to the model of Mg-protoporphyrin as a mobile signal. A reporter-based screen isolated five *genomes uncoupled* (*gun*) mutants that showed a partial recovery of *Lhcb1* expression following NF treatment [85,86]. Four of these mutants were in genes involved in tetrapyrrole metabolism: *gun2*, *gun3*, *gun4* and *gun5* were deficient in heme oxygenase, phytochromobilin synthase, GUN4 and CHLH, respectively [24,86]. In contrast, GUN1 is a plastid-localized, nucleic acid-interacting protein [87], which appears to act independently of the tetrapyrrole pathway. Each of the tetrapyrrole-related *gun* mutants would be expected to have impaired ability to synthesize Mg-protoporphyrin, *gun4* and *gun5* because of a direct effect on Mg-chelatase, and *gun2* and *gun3* because their inability to metabolize heme would be expected to down regulate ALA synthesis. Similarly, a POR-overexpressing line also exhibits a *gun* phenotype [88]. Further analysis of the *gun* mutants led to the report that NF treatment caused accumulation of Mg-protoporphyrin in wild type, but not in *gun2* and *gun5* mutants, and that increasing Mg-

protoporphyrin in *gun2* and *gun5* suppressed their *gun* phenotype [82]. Moreover, addition of Mg-protoporphyrin, but not heme or other tetrapyrrole intermediates, could repress *Lhcb1* expression in leaf protoplasts [82]. Finally, the increase in Mg-protoporphyrin after NF treatment was also reported using laser-scanning confocal microscopy, but only if accumulation of Mg-protoporphyrin had been enhanced by the addition of ALA [89].

Although this evidence might seem compelling there are some inconsistencies. Firstly, *Arabidopsis cs* and *ch42* mutants defective in the CHLI1 subunit of Mg-chelatase do not show a *gun* phenotype, even though production of Mg-protoporphyrin is greatly reduced [86]. Furthermore, Mg-protoporphyrin accumulation was not detected in barley (*Hordeum vulgare*) seedlings treated with NF [90]. Recently, the role of Mg-protoporphyrin in plastid signaling after NF treatment was re-examined in detail in two complementary reports, which both concluded that Mg-protoporphyrin is not an accumulating mobile signal that mediates plastid signaling [83,91]. Both studies investigated the accumulation of Mg-protoporphyrin following NF treatment, either by using a liquid chromatography-mass spectrometry (LC/MS) system [83], or by conventional high-performance liquid chromatography (HPLC) with fluorescence detection [91] (see Box 1 for a discussion about the analysis of tetrapyrroles in plant tissues). In both cases, no accumulation of Mg-protoporphyrin was observed, but rather there was a strong reduction of several intermediates including Mg-protoporphyrin after NF-treatment. These data were supported by microarray analysis, which demonstrated a strong downregulation of all tetrapyrrole synthesis genes after NF treatment [83]. Furthermore, both studies showed no correlation between tetrapyrrole intermediates (including Mg-protoporphyrin) and *Lhcb1* expression levels regardless of whether tetrapyrrole levels were manipulated by light and chemicals [83], or genetically [91]. These conclusions were further supported by Voigt *et al*. [92] who also found no consistent effect of the *gun* mutations on total heme accumulation.

Are tetrapyrroles involved in plastid signaling?

Although evidence now points away from a tetrapyrrole such as Mg-protoporphyrin functioning as a mobile signaling molecule, we still require an explanation for the connection between tetrapyrroles and plastid signaling demonstrated by the *gun* mutants. One possibility is that signals only accumulate transiently and locally and are therefore beyond detection. This makes sense because release of Mg-protoporphyrin would result in many of the same problems of photo-toxicity discussed earlier for protoporphyrinogen. Chlorophyll, it should be noted, is completely detoxified before release from the chloroplast. Potential alternative signaling candidates include heme and phytochromobilin, both of which are known to leave the plastid. As discussed in Box 1 we are now developing better methods for measuring tetrapyrroles and this will be crucial for detecting local, transient signals. The measurement of heme and, in particular, distinguishing unbound protoheme from non-covalently bound heme pools, is a particular problem for the field. Moreover, given that much of the research on plastid signaling to date has focused on *Arabidopsis* seedlings, other plant systems also need to be explored. For example, analysis of mature tobacco plants in which tetrapyrrole enzymes have been depleted using anti-sense technology has also shown a strong relationship between manipulation of the tetrapyrrole pathway and changes in nuclear gene expression [93,94].

One possibility for a tetrapyrrole-generated signal is the production of reactive oxygen species (ROS) such as singlet oxygen [1] or, indirectly, hydrogen peroxide [95]. Indeed, most plastid signaling experiments were carried out under intense light, where changes in tetrapyrrole intermediates levels might affect ROS levels. As discussed earlier, however, there is no simple correlation between the accumulation of tetrapyrrole intermediates (and therefore likely ROS production) and changes in gene expression after NF treatment [83,91], and any involvement of ROS is likely to involve more subtle temporal and spatial signals. The perturbation of tetrapyrrole synthesis might also impact on other known plastid signaling pathways [6]. For example, the redox

state of plastids also contributes to the regulation of nuclear gene expression and altered tetrapyrrole metabolism is likely to affect redox status [96]. It could also impact on translation efficiency, either through photo-oxidative damage, or perhaps via changes in glutamyl-tRNA availability [97].

Conclusions and key areas for future research

From the discussions above, it is clear that tetrapyrrole metabolism is at the heart of plant cellular function. Early studies concentrated on elucidating and characterizing the biosynthetic enzymes, whereas the advent of genome sequencing led to considerable focus on the regulation of tetrapyrrole synthesis and the identification of components important for some aspects of tetrapyrrole protein assembly. We now need to turn full circle and extend careful biochemical analyses to tease apart the processes that are important in localization and assembly. The new methodologies for tetrapyrrole analysis that have been established, including unambiguous identification of intermediates using mass spectrometry, and sophisticated imaging technologies, coupled with single-cell sampling approaches such as laser capture microscopy, will be important in this endeavor. Similarly, a full understanding of signaling processes, and tetrapyrrole metabolism more generally, requires detailed knowledge of where and how tetrapyrroles are transported within and between organelles. Candidate transporter protein genes, as well as the means to track these molecules through the cell will need to be the subject of scrutiny over the next few years. If this is the case, it is likely that plant tetrapyrrole metabolism will continue to provide surprises for years to come.

Acknowledgments

Research of tetrapyrroles by the authors is supported by the UK Biotechnology and Biological Sciences Research Council (MJT), EU FPV and the Gatsby Charitable Foundation (AGS) and Deutsche Forschungsgemeinschaft (DFG) grant FOR 804: GR 936/12-1 (BG).

Disclosure

The authors declare no conflicting interests.

References

1 op den Camp, R.G. *et al.* (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. *Plant Cell* 15, 2320-2332

2 Eckhardt, U. *et al*. (2004) Recent advances in chlorophyll biosynthesis and breakdown in higher plants. *Plant Mol. Biol.* 56, 1-14

3 Tanaka, R., and Tanaka, A. (2007) Tetrapyrrole biosynthesis in higher plants. *Annu. Rev. Plant Biol.* 58, 321-346

4 Masuda, T., and Fujita, Y. (2008) Regulation and evolution of chlorophyll metabolism. *Photochem. Photobiol. Sci.* 7, 1131-1149

5 Brusslan, J.A. and Peterson, M.P. (2002) Tetrapyrrole regulation of nuclear gene expression. *Photosyn. Res.* 71, 185-194

6 Nott, A. *et al.* (2006) Plastid-to-nucleus retrograde signaling. *Annu. Rev. Plant Biol.* 57, 739-759

7 Kleine, T. *et al*. (2009) Plastid signalling to the nucleus: messengers still lost in the mists? *Trends Genet.* 25, 185-192

8 Kirchhoff, H (2008) Molecular crowding and order in photosynthetic membranes. *Trends Plant Sci.* 13, 201-207

9 Stroebel, D. *et al.* (2003) An atypical haem in the cytochrome b_6f complex. *Nature* 426, 413-418

10 Rockwell, N.C. *et al.* (2006) Phytochrome structure and signaling mechanisms. *Annu. Rev. Plant Biol.* 57, 837-858

11 Nelson, D.R. *et al*. (2004) Comparative genomics of rice and Arabidopsis. Analysis of 727 cytochrome P450 genes and pseudogenes from a monocot and a dicot. *Plant Physiol.* 135, 756-72.

445 12 Dunkley, T.P.J. *et al*. (2006) Mapping the *Arabidopsis* organelle proteome. *Proc. Natl. Acad. Sci.*
446 *USA* 103, 6518-6523
447 13 Aubourg, S. (2000) In *Arabidopsis thaliana*, 1% of the genome codes for a novel protein family
448 unique to plants. *Plant Mol. Biol.* 42, 603–613
449 14 Schneider, S. *et al.* (2007) Diversity and conservation of interactions for binding heme in b-type
450 heme proteins. *Nat. Prod. Rep*. 24, 621–630
451 15 Kranz, R.G. (2009) Cytochrome *c* biogenesis: mechanisms for covalent modifications and
452 trafficking of heme and for heme-iron redox control. *Microbiol. Mol. Biol. Rev*. 73, 510–528
453 16 Terry, M.J. *et al.* (2002) Making light of it: the role of plant haem oxygenases in phytochrome
454 chromophore synthesis. *Biochem. Soc. Trans.* 30, 604-609
455 17 Cornah, J.E., *et al.* (2003) Green or red: what stops the traffic in the tetrapyrrole pathway?
456 *Trends Plant Sci.* 8, 224-230
457 18 Matsumoto, F. *et al.* (2004) Gene expression profiling of the tetrapyrrole metabolic pathway in
458 Arabidopsis with a mini-array system. *Plant Physiol.* 135, 2379-2391
459 19 Stephenson, P.G. and Terry, M.J. (2008) Light signalling pathways regulating the Mg-chelatase
460 branchpoint of chlorophyll synthesis during de-etiolation in *Arabidopsis thaliana*. *Photochem.*
461 *Photobiol. Sci.* 7, 1243–1252
462 20 Stephenson, P.G. *et al.* (2009) PIF3 is a repressor of chloroplast development. *Proc. Natl. Acad.*
463 *Sci. USA* 106, 7654–7659
464 21 Waters, M.T. *et al.* (2009) GLK Transcription Factors Coordinate Expression of the
465 Photosynthetic Apparatus in *Arabidopsis. Plant Cell* 21, 1109-1128
466 22 Papenbrock, J. *et al.* (1999) Expression studies in tetrapyrrole biosynthesis: Inverse maxima of
467 magnesium chelatase and ferrochelatase activity during cyclic photoperiods. *Planta* 208, 264–
468 273

23 Meskauskiene, R. *et al.* (2001) FLU: a negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* 98, 12826-31

24 Larkin, R.M. *et al.* (2003) GUN4, a regulator of chlorophyll synthesis and intracellular signaling. *Science* 299, 902-906

25 Adhikari, N.D. *et al.* (2009) Porphyrins promote the association of GENOMES UNCOUPLED 4 and a Mg-chelatase subunit with chloroplast membranes. *J. Biol. Chem.* 284, 24783- 24796

26 Peter, E. and Grimm. B. (2009) GUN4 is required for posttranslational control of plant tetrapyrrole biosynthesis. *Mol Plant* 2, 1198-1210

27 Ikegami, A. (2007) The CHLI1 subunit of *Arabidopsis thaliana* magnesium chelatase is a target protein of the chloroplast thioredoxin. *J. Biol. Chem.* 282, 27, 19282–19291

28 Balmer, Y. *et al*. (2006) A complete ferredoxin/thioredoxin system regulates fundamental processes in amyloplasts. *Proc. Natl. Acad. Sci. USA* 103, 2988-2993

29 Reiland, S. *et al.* (2009) Large-scale Arabidopsis phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. *Plant Physiol.* 150, 889-903

30 Yamasato, A. *et al*. (2005) The N-terminal domain of chlorophyllide *a* oxygenase confers protein instability in response to chlorophyll *b* accumulation in Arabidopsis. *Plant Cell* 17, 1585-1597

31 Sakuraba, Y. *et al.* (2009) Determination of a chloroplast degron in the regulatory domain of chlorophyllide *a* oxygenase. *J. Biol. Chem*. 284, 36689-36699

32 Papenbrock, J. *et al.* (2000) Decreased and increased expression of the subunit CHL I diminishes Mg chelatase activity and reduces chlorophyll synthesis in transgenic tobacco plants. *Plant J.* 22, 155-164

33 Franklin, K.A. *et al*. (2003) Misregulation of tetrapyrrole biosynthesis in transgenic tobacco seedlings expressing mammalian biliverdin reductase. *Plant J.* 35, 717-728

493 34 Hedtke, B. *et al*. (2007) *HEMA* RNAi silencing reveals a control mechanism of ALA
494 biosynthesis on Mg chelatase and Fe chelatase. *Plant Mol. Biol.* 64, 733–742
495 35 Ponka, P. (1999) Cell biology of heme, *Am. J. Med. Sci.* 318, 241-256
496 36 Smith, A.G. *et al.* (1993) Investigation of the subcellular location of the tetrapyrrole-biosynthesis
497 enzyme coproporphyrinogen oxidase in higher plants. *Biochem. J.* 292, 503-508
498 37 Watanabe, N. *et al.* (2001) Dual targeting of spinach protoporphyrinogen oxidase II to
499 mitochondria and chloroplasts by alternative use of two in-frame initiation codons. *J. Biol.*
500 *Chem* .276, 20474-20481
501 38 Oborník, M. and Green, B.R. (2005) Mosaic origin of the heme biosynthesis pathway in
502 photosynthetic eukaryotes. *Mol. Biol. Evol*. 22, 2343-2353.
503 39 Jacobs, J.M. and Jacobs N.J. (1995) Terminal enzymes of heme biosynthesis in the plant plasma
504 membrane, *Arch. Biochem. Biophys.* 323, 274-278
505 40 Papenbrock, J, *et al.* (2001) Impaired expression of the plastidic ferrochelatase by antisense
506 RNA synthesis leads to a necrotic phenotype of transformed tobacco plants. *Plant J*. 28, 41-
507 50
508 41 Cornah, J.E., *et al.* (2002) Measurement of ferrochelatase activity using a novel assay suggests
509 that plastids are the major site of haem biosynthesis in both photosynthetic and non-
510 photosynthetic cells of pea (*Pisum sativum* L.). *Biochem. J.* 362, 423-432
511 42 Heazlewood, J.L. *et al*. (2004) Experimental analysis of the Arabidopsis mitochondrial proteome
512 highlights signalling and regulatory components, provides assessment of targeting prediction
513 programs, and indicates plant-specific mitochondrial proteins. *Plant Cell* 16, 241-256
514 43 Huang, S. *et al*. (2009) Experimental analysis of the rice mitochondrial proteome, its biogenesis
515 and heterogeneity. *Plant Physiol.* 149, 719-734
516 44 Joyard, J. *et al*. (2009) Chloroplast proteomics and the compartmentation of plastidial isoprenoid
517 biosynthetic pathways. *Mol Plant.* 2, 1154-1180

45 Guo, R., *et al*. (1998) Magnesium-chelatase from developing pea leaves. Characterization of a soluble extract from chloroplasts and resolution into three required protein fractions. *Plant Physiol.* 116, 605-615

46 Nakayama, M. *et al.* (1998) Cloning and expression of the soybean *chlH* gene encoding a subunit of Mg-chelatase and localization of the Mg^{2+} concentration-dependent chlH protein within the chloroplast. *Plant Cell Physiol.* 39, 275-284.

47 Eggink, L.L. *et al*. (2004) Synthesis of chlorophyll *b*: localization of chlorophyllide *a* oxygenase and discovery of a stable radical in the catalytic subunit. *BMC Plant Biol*, 4, 5

48 Gisk, B. *et al.* (2010) Characterization of the haem oxygenase protein family in *Arabidopsis thaliana* reveals a diversity of functions. *Biochem. J.* 425, 425-434

49 Severance, S. and Hamza, I. (2009) Trafficking of heme and porphyrins in metazoan. *Chem. Rev.* 109, 4596-4616.

50 Matringe, M. *et al.* (1989) Protoporphyrinogen oxidase as a molecular target for diphenyl ether herbicides. *Biochem. J.* 260, 231-235

51 Hortensteiner S. (2006) Chlorophyll degradation during senescence. *Annu. Rev. Plant Biol.* 57, 55-77

52 Yeliseev, A. A. and Kaplan, S. (1999) A novel mechanism for the regulation of photosynthesis gene expression by the TspO outer membrane protein of *Rhodobacter sphaeroides* 2.4.1 *J. Biol. Chem.* 274, 21234–21243

53 Lindemann, P. *et al.* (2004) A novel *Arabidopsis thaliana* protein is a functional peripheral-type Benzodiazepine receptor. *Plant Cell Physiol.* 45, 723-733

54 Frank, W. *et al*. (2007) A mitochondrial protein homologous to the mammalian peripheral-type benzodiazepine receptor is essential for stress adaptation in plants. *Plant J.* 51, 1004-1018

55 Guillaumot, D. *et al.* (2009) The Arabidopsis TSPO-related protein is a stress and abscisic acid-regulated, endoplasmic reticulum-Golgi-localized membrane protein. *Plant J.* 60, 242-256

543 56 Krishnamurthy, P. *et al.* (2007) The role of transporters in cellular heme and porphyrin
544 homeostasis. *Pharmacol. Ther.* 114, 345-358
545 57 Shayeghi, M. *et al.* (2005) Identification of an intestinal heme transporter. *Cell* 122, 789-801
546 58 Quigley, J.G. *et al.* (2004) Identification of a human heme exporter that is essential for
547 erythropoiesis. *Cell* 118, 757-766
548 59 Krishnamurthy, P. C. *et al.* (2006) Identification of a mammalian mitochondrial porphyrin
549 transport. *Nature* 443, 586-589
550 60 Latunde-Dada, G. *et al.* (2006) Recent advances in mammalian haem transport. *Trends Biochem.*
551 *Sci.* 31, 182-188
552 61 Jonker, J.W. *et al*. (2002) The breast cancer resistance protein protects against a major
553 chlorophyll-derived dietary phototoxin and protoporphyria. *Proc. Natl. Acad. Sci. USA*, 99,
554 15649-15654
555 62 Protchenko, O. *et al.* (2008) Role of PUG1 in inducible porphyrin and heme transport in
556 *Saccharomyces cerevisiae*. *Eukaryot. Cell* 7, 859-871
557 63 Rea, P.A. (2007) Plant ATP-binding cassette transporters. *Annu. Rev. Plant Biol.* 58, 347-375
558 64 Lu, Y.P. *et al.* (1998) AtMRP2, an Arabidopsis ATP binding cassette transporter able to
559 transport glutathione *S*-conjugates and chlorophyll catabolites: functional comparisons with
560 AtMRP1. *Plant Cell* 10, 267-282
561 65 Tommasini, R. *et al.* (1998) An ABC-transporter of *Arabidopsis thaliana* has both glutathione-
562 conjugate and chlorophyll catabolite transport activity. *Plant J*. 13, 773-780
563 66 Møller, S.G. *et al.* (2001) A plastidic ABC protein involved in intercompartmental
564 communication of light signaling. *Genes Dev.* 15, 90-103
565 67 Xu, X., *et al.* (2005) AtNAP1 represents an atypical SufB protein in Arabidopsis plastids. *J. Biol.*
566 *Chem.* 280, 6648-6654

567 68 Nagane, T., *et al*. (2010) Involvement of AtNAP1 in the regulation of chlorophyll degradation in
568 *Arabidopsis thaliana*. *Planta* 231, 939–949
569 69 Taketani, S., *et al.* (1998) Molecular characterization of a newly identified heme-binding protein
570 induced during differentiation of urine erythroleukemia cells. *J. Biol. Chem* .273, 31388-
571 31394
572 70 Zylka, M.J. and Reppert, S.M. (1999) Discovery of a putative heme-binding protein family
573 (SOUL/HBP) by two-tissue suppression subtractive hybridization and database searches.
574 *Brain Res. Mol. Brain Res.* 74, 175-181
575 71 Takahashi, S. *et al.* (2008) Characterization of cytosolic tetrapyrrole-binding proteins in
576 *Arabidopsis thaliana*. *Photochem. Photobiol. Sci.* 7, 1216-1224
577 72 Zhang, L. and Hach, A. (1999) Molecular mechanism of heme signaling in yeast: the
578 transcriptional activator Hap1 serves as the key mediator. *Cell. Mol. Life Sci.* 56, 415-426
579 73 Yin, L. *et al.* (2007) Rev-erbα, a heme sensor that coordinates metabolic and circadian pathways.
580 *Science* 318, 1786-1789
581 74 Raghuram, S. *et al.* (2007) Identification of heme as the ligand for the orphan nuclear receptors
582 REV-ERBα and REV-ERBβ. *Nature Struct. Mol. Biol.* 14, 1207-1213
583 75 Faller, M. *et al*. (2007) Heme is involved in microRNA processing. *Nature Struct. Mol. Biol.* 14**,**
584 23-29
585 76 Small, S.K. *et al.* (2009) Heme-dependent metalloregulation by the iron response regulator (Irr)
586 protein in *Rhizobium* and other alpha-proteobacteria. *Biometals* 22, 89-97
587 77 Kobayashi, Y. *et al*. (2009) Tetrapyrrole signal as a cell-cycle coordinator from organelle to
588 nuclear DNA replication in plant cells. *Proc. Natl. Acad. Sci. USA* 106, 803-807
589 78 Kropat, J. *et al.* (1997) Chlorophyll precursors are signals of chloroplast origin involved in light
590 induction of nuclear heat-shock genes. *Proc. Natl. Acad. Sci. USA* 94**,** 14168-14172

79 von Gromoff, E.D. *et al.* (2008) Heme, a plastid-derived regulator of nuclear gene expression in *Chlamydomonas*. *Plant cell* 20, 552-567

80 Montgomery, B.L. and Lagarias, J.C. (2002) Phytochrome ancestry: sensors of bilins and light. *Trends Plant Sci.* 7, 357-366

81 Hirashima, M. *et al*. (2009) Light-independent cell death induced by accumulation of pheophorbide *a* in *Arabidopsis thaliana*. *Plant Cell Physiol.* 50**,** 719-729

82 Strand, A. *et al.* (2003) Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. *Nature* 421, 79-83

83 Moulin, M. *et al.* (2008) Tetrapyrrole profiling in *Arabidopsis* seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-protoporphyrin IX accumulation. *Proc. Natl. Acad. Sci. USA* 105, 15178-15183

84 La Rocca, N. *et al.* (2001) Amitrole treatment of etiolated barley seedlings leads to deregulation of tetrapyrrole synthesis and to reduced expression of *Lhc* and *RbcS* genes. *Planta* 213, 101-108

85 Susek, R.E. *et al.* (1993) Signal transduction mutants of Arabidopsis uncouple nuclear *CAB* and *RBCS* gene expression from chloroplast development. *Cell* 74, 787-799

86 Mochizuki, N. *et al.* (2001) Arabidopsis genomes uncoupled 5 (GUN5) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. *Proc. Natl. Acad. Sci. USA* 98, 2053-2058

87 Koussevitzky, S. *et al.* (2007) Signals from chloroplasts converge to regulate nuclear gene expression. *Science* 316, 715-719

88 McCormac, A.C. and Terry, M.J. (2002) Loss of nuclear gene expression during the phytochrome A-mediated far-red block of greening response. *Plant Physiol.* 130, 402-414

89 Ankele, E. *et al*. (2007) In vivo visualization of Mg-protoporphyrinIX, a coordinator of photosynthetic gene expression in the nucleus and the chloroplast. *Plant Cell* 19**,** 1964-1979

90 Gadjieva, R. *et al*. (2005) Analysis of *gun* phenotype in barley magnesium chelatase and Mg-protoporphyrin IX monomethyl ester cyclase mutants. *Plant Physiol. Biochem.* 43**,** 901-908

91 Mochizuki, N. *et al.* (2008) The steady-state level of Mg-protoporphyrin IX is not a determinant of plastid-to-nucleus signaling in Arabidopsis. *Proc. Natl. Acad. Sci. USA* 105, 15184-15189

92 Voight, C. *et al.* (2010) In-depth analysis of the distinctive effects of norflurazon implies that tetrapyrrole biosynthesis, organellar gene expression and ABA cooperate in the GUN-type of plastid signalling. *Physiol. Plant.* 138, 503–519

93 Papenbrock, J. *et al.* (2000) Role of magnesium chelatase activity in the early steps of the tetrapyrrole biosynthetic pathway. *Plant Physiol.* 122, 1161-1169

94 Alawady, A.E. and Grimm B. (2005) Tobacco Mg protoporphyrin IX methyltransferase is involved in inverse activation of Mg porphyrin and protoheme synthesis. *Plant J.* 41, 282-290

95 Kimura, M. *et al*. (2003) Identification of *Arabidopsis* genes regulated by high light-stress using cDNA microarray. *Photochem. Photobiol.* 77, 226-233

96 Biehl, A. *et al*. (2005) Analysis of 101 nuclear transcriptomes reveals 23 distinct regulons and their relationship to metabolism, chromosomal gene distribution and co-ordination of nuclear and plastid gene expression. *Gene* 344**,** 33-41

97 Levican, G. *et al.* (2005) A tRNA(Glu) that uncouples protein and tetrapyrrole biosynthesis. *FEBS Lett.* 579, 6383-6387

98 Inskeep, W.P. and Bloom, P.R. (1985) Extinction coefficients of chlorophyll *a* and *b* in *N,N*-dimethylformamide and 80% acetone. *Plant Physiol.* 77, 483-485

99 Stiegler, J.C. *et al.* (2004) Comparison of acetone and N,N-dimethylformamide for pigment extraction in turfgrass. *Commun. Soil Sci. Plant Anal.* 35, 1801-1812

100 Smith, A.G. and Witty M., eds (2002) *Heme, Chlorophyll and Bilins*, Humana Press

101 Tripathy, B.C. and Rebeiz, C.A. (1985) Chloroplast biogenesis 49: Chloroplast biogenesis - quantitative determination of monovinyl and divinyl Mg-protoporphyrins and protochlorophyll(ides) by spectrofluorometry. *Anal. Biochem.* 149, 43-61

102 Kopetz, K.J. *et al.* (2004) Chloroplast biogenesis 89: development of analytical tools for probing the biosynthetic topography of photosynthetic membranes by determination of resonance excitation energy transfer distances separating metabolic tetrapyrrole donors from chlorophyll a acceptors. *Anal. Biochem.* 329, 207-219

103 Yaronskaya, E. *et al.* (2006) Cytokinin effects on tetrapyrrole biosynthesis and photosynthetic activity in barley seedlings. *Planta* 224, 700-709

104 Kolossov, V.L. *et al.* (2003) Chloroplast biogenesis 87: Evidence of resonance excitation energy transfer between tetrapyrrole intermediates of the chlorophyll biosynthetic pathway and chlorophyll a. *Photochem. Photobiol.* 78, 184-196

105 Stillman, L.C. and Gassman, M.L. (1978) Protoheme extraction from plant tissue. *Anal. Biochem.* 91, 166-172

106 Pandey, A.V. *et al.* (1999) A colorimetric assay for heme in biological samples using 96-well plates. *Anal. Biochem.* 268, 159-161

107 Huy, N.T. *et al.* (2005) An improved colorimetric method for quantitation of heme using tetramethylbenzidine as substrate. *Anal. Biochem.* 344, 289-291

108 Thomas, J. and Weinstein, J.D. (1990) Measurement of heme efflux and heme content in isolated developing chloroplasts. *Plant Physiol.* 94, 1414-1423

109 Masuda T, and Takahashi, S. (2006) Chemiluminescent-based method for heme determination by reconstitution with horseradish peroxidase apo-enzyme. *Anal. Biochem.* 355, 307-309

110 Takahashi, S. and Masuda, T. (2009) High-throughput heme assay by detection of chemiluminescence of reconstituted horseradish peroxidase. *Combi. Chem. High Throughput Screen.* 12: 532-535

111 Halket, J.M. *et al.* (2005) Chemical derivatization and mass spectral libraries in metabolic profiling by GC/MS and LC/MS/MS. *J. Exp. Bot.* 56, 219-243

112 Gough, S.P. *et al.* (2000) Anaerobic chlorophyll isocyclic ring formation in *Rhodobacter capsulatus* requires a cobalamin cofactor. *Proc. Natl. Acad. Sci. USA* 97, 6908-6913

113 Bu, W. *et al.* (2003) Simultaneous determination of six urinary porphyrins using liquid chromatography-tandem mass spectrometry. *J. Chrom. B Anal. Tech. Biomed. Life Sci.* 783, 411-423

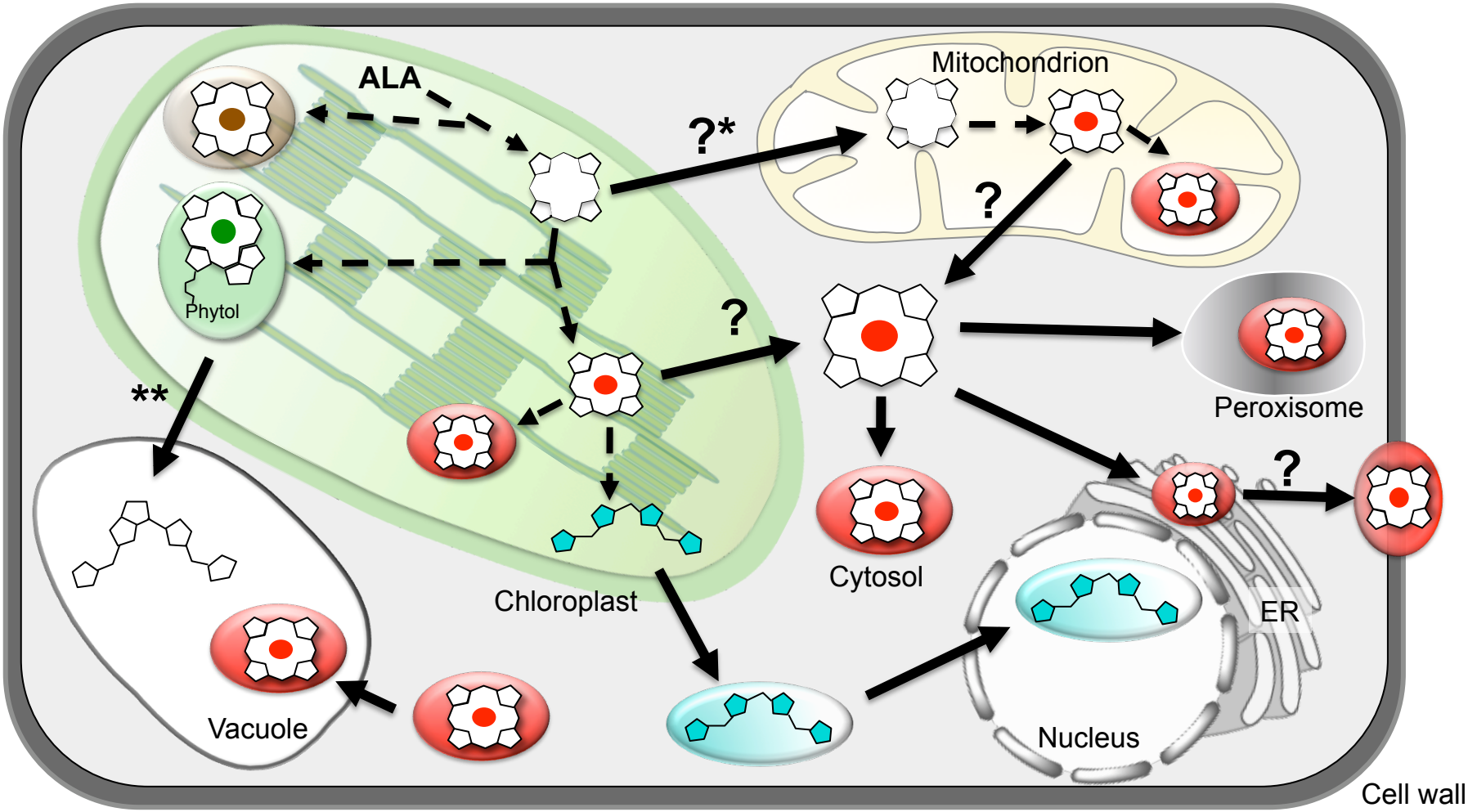
114 Airs, R.L. *et al.* (2001) Development and application of a high resolution liquid chromatographic method for the analysis of complex pigment distributions. *J. Chrom. A* 917, 167-177

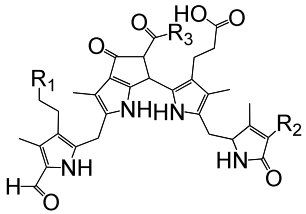
115 Kristiansen, K.A. *et al.* (2009) Non-invasive method for *in vivo* detection of chlorophyll precursors. *Photochem. Photobiol. Sci.* 8, 279-286

Box 1. Measuring tetrapyrroles in plant tissue

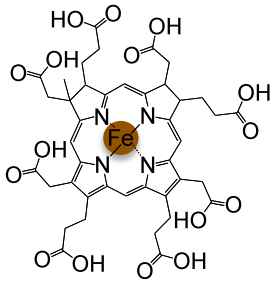
Several methods have been used to measure tetrapyrroles in plant samples. Chlorophylls are the most abundant and can easily be measured by spectrophotometry after extraction with either acetone or *N*,*N*-dimethylformamide, followed by calculation using empirical formulae [98]. Although extraction with the two solvents is similar, it has been shown that *N*,*N*-dimethylformamide is more efficient for chlorophyll extraction and does not require a grinding step [99]. Tetrapyrrole intermediate levels remain difficult to measure because of their low abundance in plants and interference by chlorophyll. They have been analyzed by spectrofluorimetry or spectrophotometry in crude acetone extracts [100]. Determination by fluorescence is made by using specific wavelengths for excitation and emission at 77 K, concentrations being calculated by empirical formulae [101,102]. A better way of measuring tetrapyrroles is separation by high-performance liquid chromatography (HPLC). After acetone extraction, and phase separation with hexane to remove chlorophylls, tetrapyrrole intermediates are separated with a gradient of acetonitrile/methanol, coupled to a spectrofluorimeter [93,94,103]. Even so, this method can lead to misidentification or overestimation of intermediates (such as Mg-protoporphyrin and Chlide *a*) owing to the extraction solution and coelution [91]. The work-up required in all these methods, including derivatization to facilitate HPLC separation, can lead to differential losses and is time consuming. In addition fluorescence detection can also lead to misidentification of related pigments that have similar fluorescence properties at room temperature [104]. A major inconvenience is that heme cannot be detected by these methods because it does not fluoresce. Heme determination uses an acidic extraction method [105] that is not suitable for Mg-porphyrins because this induces the loss of the Mg^{2+} ion. Instead, there are several colorimetric methods that have been developed [106,107], and methods based on the ability of the horseradish peroxidase apo-enzyme to reconstitute with heme to form an active enzyme have been developed [108] that use a chemiluminescence-based measure of activity [109,110].

A completely different approach has arisen through the advent of nuclear magnetic resonance (NMR) and mass spectrometry (MS) for measuring metabolites [111]. Tetrapyrroles can be detected by ^{1}H NMR spectroscopy [112], but the sensitivity of the detectors remains too low for determining endogenous tetrapyrroles in plants. By contrast, MS is much more sensitive and is commonly used in medical science to study porphyrin disorders in humans [113], and for isolating and characterizing transformation products of chlorophyll pigments in sediments [114]. Recently, we showed that tetrapyrrole intermediates in crude plant extracts can be detected and quantified by HPLC coupled to an electrospray MS (LC-ESI MS/MS) [83]. Each chlorophyll biosynthetic intermediate was identified according to its mass and its fragmentation pattern, enabling them to be distinguished unequivocally. With the exception of NMR, the methods described above are efficient and sensitive enough to measure quantitatively tetrapyrrole intermediates in plant extracts. Nonetheless, these methods require extraction of the intermediates before analysis and thus are *de facto* destructive. The development of microscopy techniques now offers the possibility to detect these compounds in a non–invasive manner. So far two different methods have been described. Ankele *et al*. [89] reported the use of single photon laser excitation in combination with confocal scanning microscopy to detect Proto IX, Mg-Proto IX Mg-Proto ME, although these were only detectable after feeding plant tissues with 5-aminolevulinic acid (ALA). In contrast, an improved method using multi-photon microscopy reported the detection of endogenous levels of all tetrapyrrole intermediates from Proto IX to Chlide [115]. Multi-photon microscopy also appears to be more reliable for detecting Pchlide because it uses pulsed infrared light instead of a UV light source and prevents the photo conversion of Pchlide to Chlide [115].

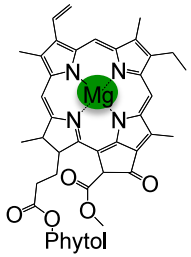




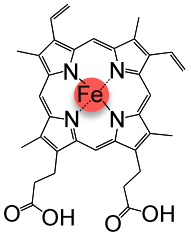
NCCs



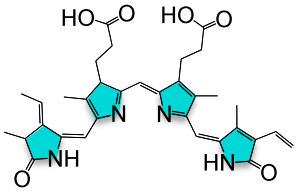
Siroheme



Chlorophyll



Heme



Phytochromobilin

Figure 1. Localization of tetrapyrroles in higher plant cells. The known tetrapyrrole-binding proteins are color-coded according to their respective tetrapyrrole cofactor: green, chlorophyll; brown, siroheme; blue, phytochromobilin; red, heme. Tetrapyrroles are represented schematically and their exact structure is shown in the lower part of the figure. *It has been proposed that protoporphyrinogen (indicated by a tetrapyrrole ring without color coding) might be exported from plastids to mitochondria and for heme synthesis. **Chlorophylls are degraded to non-fluorescent chlorophyll catabolites (NCCs) and exported to the vacuole. Broken arrows indicate biosynthetic pathways; solid arrows indicate tetrapyrrole transport; and question marks have been added when evidence for that transport pathway is limited (see main text).

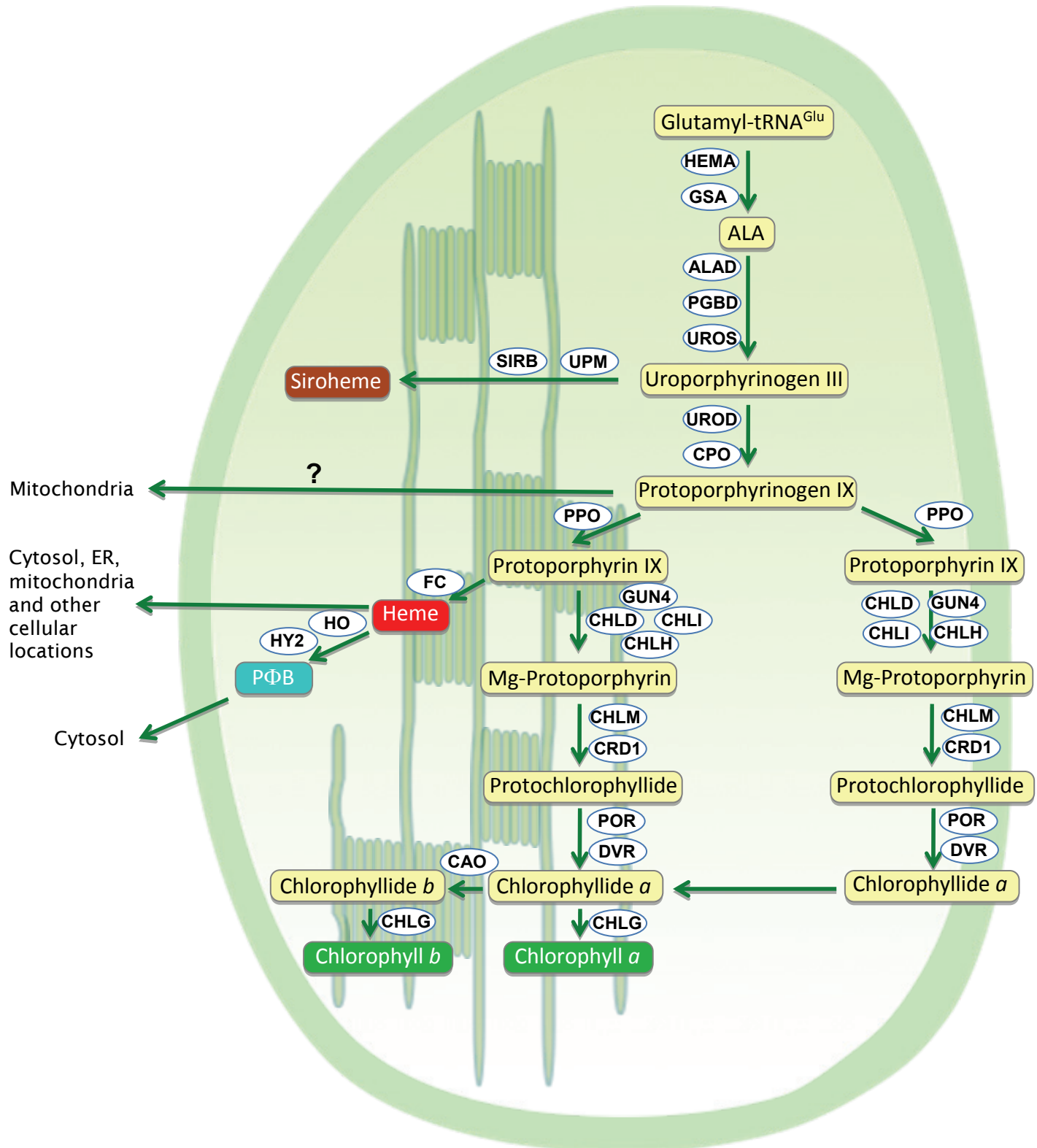

Glutamyl-tRNA^{Glu}
HEMA
GSA
ALA
ALAD
PGBD
UROS
Uroporphyrinogen III
SIRB
UPM
Siroheme
UROD
CPO
Protoporphyrinogen IX
?
Mitochondria
PPO
PPO
Protoporphyrin IX
Protoporphyrin IX
FC
Heme
HO
HY2
PΦB
Cytosol, ER, mitochondria and other cellular locations
Cytosol
GUN4
CHLD
CHLI
CHLH
CHLD
GUN4
CHLI
CHLH
Mg-Protoporphyrin
Mg-Protoporphyrin
CHLM
CRD1
CHLM
CRD1
Protochlorophyllide
Protochlorophyllide
POR
DVR
POR
DVR
CAO
Chlorophyllide b
Chlorophyllide a
Chlorophyllide a
CHLG
CHLG
Chlorophyll b
Chlorophyll a

Figure 2

Figure 2. Localization of tetrapyrrole synthesis in chloroplasts. Location of tetrapyrrole enzymes and biosynthetic intermediates in the chloroplast is shown based on proteomic analyses [44] and other data as outlined in the main text. In some cases (UPM, SIRB and CAO), there is currently no experimental data to indicate sub-organellar localization, and locations of these enzymes have been predicted. The location of enzymes represents their major sub-organellar localizations (stroma, envelope or thylakoids), but differences between grana- or stroma-thylakoids are not intended to be indicated. The localization of the subunits of Mg-chelatase is particularly difficult to indicate precisely (see main text), but their arrangement reflects what is currently known about the sub-chloroplast localization [25,44]. Enzymes are indicated by the following gene codes: HEMA, glutamyl-tRNA reductase (At1g58290, At1g09940, At2g31250); GSA, glutamate-1-semialdehyde 2,1-aminomutase (At5g63570, At3g48730); ALAD, 5-aminolevulinate dehydratase (At1g69740, At1g44318); PBGD, porphobilinogen deaminase (alternatively, hydroxymethylbilane synthase (At5g08280); UROS, uroporphyrinogen III synthase (At2g26540); UROD, uroporphyrinogen III decarboxylase (At3g14930, At2g40490); CPO, coproporphyrinogen III oxidase (At1g03475, At4g03205); PPO, protoporphyrinogen IX oxidase (At4g01690, At5g14220); CHLH, Mg-chelatase H subunit (At5g13630); CHLI, Mg-chelatase I subunit (At4g18480, At5g45930); CHLD, Mg-chelatase D subunit (At1g08520); GUN4, regulator of Mg-chelatase (At3g59400); CHLM, Mg-proto IX methyltransferase (At4g25080); CRD1, Mg-proto IX monomethylester cyclase (At3g56940); POR, NADPH:protochlorophyllide oxidoreductase (At5g54190, At4g27440, At1g03630); DVR, divinyl-protochlorophyllide reductase (At5g18660); CHLG, chlorophyll synthase (At3g51820); CAO, chlorophyllide *a* oxygenase (At1g44446); UPM, uroporphyrinogen III methylase (At5g40850); SIRB, sirohydrochlorin ferrochelatase (At1g50170); FC, protoporphyrin IX ferrochelatase (At2g30390, At2g26670); HO, heme oxygenase (At2g26670, At2g26550, At1g69720, At1g58300); HY2,

phytochromobilin synthase (At3g09150). Note: CHLP (geranylgeranyl-diphosphate reductase, At1g74470), which is also essential for chlorophyll biosynthesis, catalysing the formation of the phytol side chain, localizes primarily on thylakoid membranes [44]. PϕB, phytochromobilin.