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Review

Regulation and function of tetrapyrrole biosynthesis in plants and algae☆

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ABSTRACT

Tetrapyrroles are macrocyclic molecules with various structural variants and multiple functions in Prokaryotes and Eukaryotes. Present knowledge about the metabolism of tetrapyrroles reflects the complex evolution of the pathway in different kingdoms of organisms, the complexity of structural and enzymatic variations of enzymatic steps, as well as a wide range of regulatory mechanisms, which ensure adequate synthesis of tetrapyrrole end-products at any time of development and environmental condition. This review intends to highlight new findings of research on tetrapyrrole biosynthesis in plants and algae. In the course of the heme and chlorophyll synthesis in these photosynthetic organisms, glutamate, one of the central and abundant metabolites, is converted into highly photoreactive tetrapyrrole intermediates. Thereby, several mechanisms of posttranslational control are thought to be essential for a tight regulation of each enzymatic step. Finally, we wish to discuss the potential role of tetrapyrroles in retrograde signaling and point out perspectives of the formation of macromolecular protein complexes in tetrapyrrole biosynthesis as an efficient mechanism to ensure a fine-tuned metabolic flow in the pathway. This article is part of a Special Issue entitled: Chloroplast Biogenesis.

1. Introduction

Tetrapyrroles are macrocyclic molecules, which show distinct structural and functional properties. However, a common characteristic of these molecules is presence of the four pyrrole rings, which are linked by unsaturated methine groups. A pyrrole ring consists of five atoms, four carbons and one nitrogen atom. The different chemical and physical properties of tetrapyrroles are defined by their molecular structure of conjugated double bonds, the variation of substituted side chains, and the chelation of various metal ions. Thus, depending on the molecular structure, tetrapyrroles have either specialized properties to absorb visible light or to accept different redox states. Research on the tetrapyrrole end-products and their physical and biochemical characteristics encompasses a long history of spectacular discoveries. Several Nobel Prize laureates explored tetrapyrroles, their properties and involvement in vital processes: Richard Martin Willstätter (1915), Hans Fischer (1930), Robert Burns Woodward (1965), Hartmut Michel, Johann Deisenhofer, and Robert Huber (1988). Having primarily emphasized the essential functions of tetrapyrroles, which have been also described as the "pigments of life" [1], it is worth to commemorate a few key discoveries and findings with regard to the tetrapyrrole biosynthesis (TBS).

Granick contributed extensively to the elucidation of chlorophyll formation and porphyrin properties by demonstrating accumulation of protoporphyrin (Proto) and Mg-porphyrins in the *Chlorella* mutants, which were not able to synthesize chlorophyll [2,3]. Neuberger and coworkers demonstrated that the pyrroles of heme are derived from glycine [4]. It is also worth to mention that Smith and Kupke described the first protein complex containing chlorophyll precursors, the protochlorophyll holochrome [5]. In 1978, Trevor Griffiths described the protochlorophyll(ide) (PChlide) holochrome as the ternary complex of protochlorophyllide oxidoreductase (POR) with PChlide and NADPH [6]. The first discoveries on 5-aminolevulinic acid (ALA) synthesis referred to the studies performed by Shemin and Russel, who demonstrated that ALA is enzymatically formed by condensation of glycine and succinyl-CoA [7]. In 1953, the first pathway from ALA to Proto was suggested by Bogorad and Granick [8]. Finally, in 1974 Beale and Castelfranco presented an alternative, C-5 pathway of ALA synthesis, which takes place in green plants. The authors described glutamate as the initial substrate of ALA synthesis in photosynthetic eukaryotes [9]. In 1984, an RNA moiety was identified to be involved in ALA synthesis as a unique cofactor [10].

Genetic and biochemical analysis of the pigment mutants paved the way for the identification of the genes involved in chlorophyll biosynthesis and the biochemical characterization of the enzymatic steps. Discovery of the photosynthetic gene cluster in *Rhodobacter* species contributed to the determination of the coding sequences for the enzymes in TBS biosynthesis [11] and, subsequently, to the identification of homologous sequences in higher plants. The first plant cDNA sequence encoding an enzyme of TBS was reported in 1989 for POR [12]. The first 3D crystal structure of an enzyme in TBS of photosynthetic organisms was published for GSAT in 1997 [13]. Among one of the most spectacular phenotypes of pigment synthesis mutants, with either pale green or necrotic phenotypes, the *flu* mutants can be highlighted.

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FLU is the first identified regulatory protein in plant chlorophyll biosynthesis [14], although the molecular mechanism of glutamyl-tRNA reductase inhibition is still not elucidated. Finally, although it does not directly concern the plant TBS, the discoveries of the vitamin B12 biosynthesis pathway by Battersby and his group were groundbreaking and should be acknowledged in a short list of exceptional discoveries in this, in every sense colorful pathway [15]. Although study on TBS encompasses a long history of many extraordinary findings, only a few discoveries can be mentioned in this review.

Many exceptional scientific contributions paved the way to excellent progress in TBS research. Initially, chemical and biochemical analysis shaped the first period of the research on tetrapyrroles and their synthesis, before molecular and genetic analysis helped to identify genes and elucidated transcriptional control of TBS. Presently, the research moves towards biochemistry and structural analyses of the 3D structures of TBS proteins [16–19], which subsequently lead to the exploration of multi-enzymatic protein complexes *in vivo* [20–24], as well as the elucidation of a potent network of metabolic and regulatory interactions [25,26]. Some of these complexes and networks have been proposed years ago [27–29], but the characterization of the physical interactions between particular components still requires elucidation.

2. State of the art and a few aspects of structural and enzymatic properties of tetrapyrrole biosynthesis

The whole TBS pathway in plants is located in plastids, it is branched towards the formation of different tetrapyrrole end-products, and its enzymatic steps involve nuclear-encoded proteins. The pathway is generally highly conserved, although a few catalytic steps involve proteins, which differ in structure and the mode of action between organisms (Fig. 1).

The synthesis of heme and chlorophyll starts with the formation of ALA, the universal precursor for all tetrapyrroles. The ALA biosynthesis in plants, moss, ferns and algae differs from fungi and animals, because of a different phylogenetic origin of this metabolic pathway in these organisms. Mitochondrion-localized ALA synthase originated from the ancestors of γ-proteobacteria and converts glycine and succinyl-CoA into ALA (the so-called C-4 pathway of ALA formation) [30]. The plant ALA synthesis originated from photosynthetic bacteria, most likely the ancestors of cyanobacteria. An exceptional cofactor, a tRNA(Glu), is involved in introduction of glutamate to the pathway. Activated glutamate provided in the form of glutamyl-tRNA(Glu) is reduced to glutamate-1-semialdehyde (GSA) [31]. An amino-group from C2 of GSA is transferred to the neighbor carbon atom to finalize ALA formation (the C-5 pathway) [32].

Eight molecules of ALA form the tetrapyrroles. First a monopyrrole is synthesized by an asymmetric condensation of two ALA units, before four porphobilinogens are consecutively condensed head-to-tail to form the unstable linear molecule of hydroxymethylbilane (Fig. 1). This intermediate is subsequently converted into the cyclic uroporphyrinogen III, which is the substrate either for siroheme synthesis or for porphyrin synthesis. The latter one, which is the dominant pathway, includes decarboxylation and oxidation steps to ultimately form Proto, a substrate at the second branch point and the precursor for the synthesis of chlorophyll and heme. The TBS pathway diverges into the 'iron-branch' leading to the formation of heme and into the 'magnesium (Mg) branch' responsible for the synthesis of chlorophyll. While the porphyrin pathway in the iron branch resembles the metabolic pathway in animals and yeast, the chlorin synthesis is unique for photosynthetic organisms.

The first catalytic step of the chlorophyll branch is the insertion of Mg^{2+} ion into the backbone of Proto. An esterification of a methyl group at C13 protects the propionic acid side chain from further oxidation before the 5th isocyclic ring is formed. Successive reduction steps lead to the addition of two trans-hydrogens at the C17 - C18 double bond and the conversion of a C8-vinyl group to an ethyl group. Esterification with a long hydrocarbon chain to the substituent of C17 completes the synthesis of the chlorophyll *a*, which can be oxidized to chlorophyll *b* by chlorophyll *a* oxygenase, when a methyl group is replaced by a formyl group at C7 (Fig. 1).

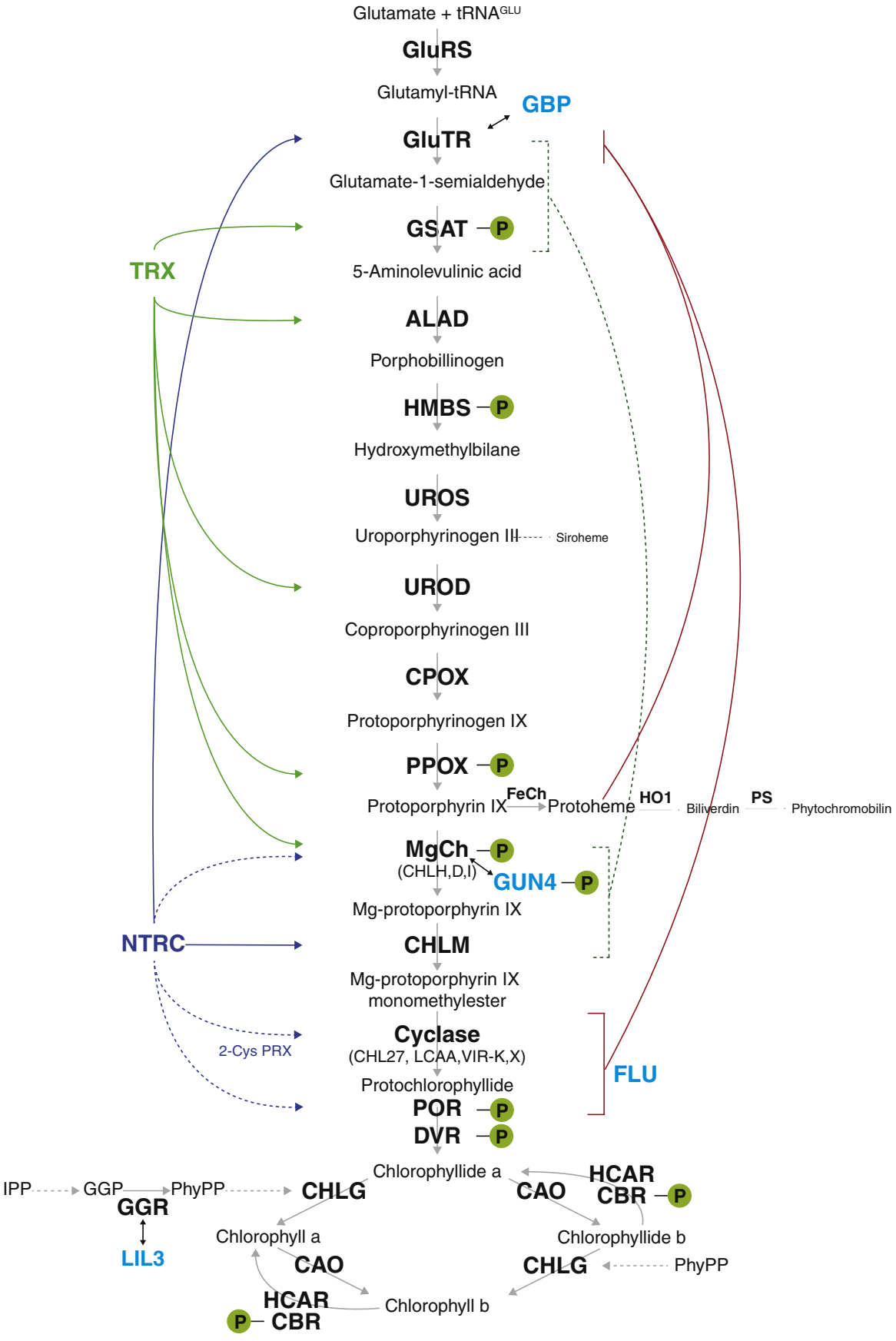
Organisms with an oxygenic photosynthesis mainly synthesize chlorophyll *a* and *b*, thereby many cyanobacteria use only chlorophyll *a*. Anaerobic bacteria require a more complex pathway towards synthesis of different varieties of bacteriochlorophyll (BChl). BChl *a* is often the dominant representative but several other derivatives with varying absorption maxima are found in different bacteria species (for further information on BChl biosynthesis see [33]).

Many variations in structure and catalytic mechanisms of different enzymatic steps of TBS developed during the evolution. Thereby, an oxygen availability and the light-dependent control are responsible for modifications of enzymatic reactions in TBS. Reactions of coproporphyrinogen oxidase (CPO), protoporphyrinogen oxidase (PPOX) and the Mg-protoporphyrin monomethylester (MgProtoME) cyclase can be catalyzed by an oxygenic and/or an anoxygenic form of the enzyme. In organisms that carry both forms of PPOX, both enzymes are encoded in the same genome and catalyze the same reaction, but have a different protein structure. However, in most instances the mechanisms behind regulation of the expression and activity still remain unknown.

Under microaerobiosis, the conversion of coproporphyrinogen III to Protogen proceeds via a [4Fe-4S] anoxygenic coproporphyrinogen III oxidase encoded by *HemN*, while oxidative decarboxylation of the substrate requires oxygen and is catalyzed by HEMF, an enzyme without any structural and functional resemblance to HemN [34]. The reaction of PPOX is catalyzed by three different types of proteins, indicating that at least three fundamentally different classes of protoporphyrinogen (Protogen)-oxidizing enzymes have evolved. Two enzymes have been shown to depend on flavin as a cofactor. In *Escherichia coli*, a small soluble flavin mononucleotide (FMN)-containing enzyme of the long chain flavodoxin family, namely HemG, has been found to catalyze the oxidation of Protogen. Homologous sequences of this bacterial-type PPOX are restricted to γ-proteobacteria [35]. The 3D structure of HemG was determined and models for the FMN-mediated electron transfer via quinones, which allow synthesis of heme under aerobic and anaerobic growth conditions, have been proposed [36]. In eukaryotes and a few Gram-negative bacteria, the PPOX is 50 kDa protein, which belongs to the FAD super-family [37]. This oxygen-dependent eukaryotic-type of PPOX is encoded by two homologous genes in plants, PPOX I and PPOX II. PPOX I is targeted exclusively to plastids and the product of its activity is used as a substrate for heme or chlorophyll biosynthesis. In tobacco PPOX II was detected only in the mitochondria [38], but in spinach it is present in mitochondria and in the envelope membrane of plastids [39]. A third type of PPOX enzyme, HemJ, encoded by *slr1790* in cyanobacteria, resembles the M subunit of NADH dehydrogenase and it was proposed to be an alternative Protogen oxidation enzyme [40].

Another example of an oxygen-sensitive enzymatic step is the cyclization reaction of MgProtoME. Two different mechanisms were described for the cyclization reaction, which involves different proteins [41]. A di-iron subunit has been reported to be involved in the oxygen-dependent conversion of MgProtoME to divinyl PChlide (see below). An anaerobic mechanism, active during photosynthesis under low oxygenation, requires activity of the [4Fe-4S] cluster containing CHLE/BchE protein [41].

A lot of consideration was given to the characterization of a light- and a dark-operating POR. The dark-operating/light-independent POR consists of three subunits, CHLB, CHLN, and CHLL, which are encoded by genes located in the chloroplast genome [42–44]. Analysis of the crystal structure of these POR subunits indicated that this protein complex has a structural similarity to the components of nitrogenase [45–48], which explains also the oxygen sensitivity of the dark-operating POR [49]. In fact, during evolution both enzymes evolved from a common ancestral enzymatic system. With the exception of


Glutamate + tRNA^GLU
GluRS
Glutamyl-tRNA
GBP
GluTR
Glutamate-1-semialdehyde
GSAT
P
TRX
5-Aminolevulinic acid
ALAD
Porphobillinogen
HMBS
P
Hydroxymethylbilane
UROS
Uroporphyrinogen III
Siroheme
UROD
Coproporphyrinogen III
CPOX
Protoporphyrinogen IX
PPOX
P
Protoporphyrin IX
FeCh
Protoheme
HO1
Biliverdin
PS
Phytochromobilin
MgCh
P
(CHLH,D,I)
GUN4
P
Mg-protoporphyrin IX
NTRC
CHLM
Mg-protoporphyrin IX monomethylester
2-Cys PRX
Cyclase
(CHL27, LCAA,VIR-K,X)
FLU
Protochlorophyllide
POR
P
DVR
P
Chlorophyllide a
IPP
GGP
PhyPP
CHLG
HCAR
CAO
CBR
P
GGR
LIL3
Chlorophyll a
Chlorophyllide b
CAO
CHLG
PhyPP
HCAR
P
CBR
Chlorophyll b

angiosperms, all eukaryotic photosynthesizing organisms use both types of POR enzymes, enabling chlorophyll biosynthesis in light and dark. Thus, the angiosperms rely on the light-dependent POR, which might be economically beneficial for this group of plants, but with the drawback to down-regulate TBS in darkness, to avoid accumulation of photoreactive chlorophyll precursors (see chapter 7.1) [50,51].

Apart from the structural and enzymatic distinction of enzymes with different sensitivity for oxygen, it was surprising that the 8-vinyl reductase, responsible for the reduction of the C-8 vinyl group to an ethyl group in different chlorophyll precursors, is encoded by non-homologous genes in *Arabidopsis* [52] and *Synechocystis* sp. PCC 6803 (*slr1923*; [53]). The structurally unrelated plant and cyanobacterial enzymes use either NADPH or ferredoxin as two different reductants. Interestingly, the cyanobacterial 8-vinyl-reductase is the homologous protein to 7-hydroxymethyl chlorophyll *a* reductase (HCAR) of higher plants [54].

Catalysis of several enzymatic steps is facilitated when different proteins form macromolecular complexes, as already indicated for the dark-operating POR. The reaction catalyzed by MgCh received a lot of attention, due to the complexity of the mechanism of inserting Mg^{2+} into Proto. MgCh consists of a multi-enzymatic complex of three different subunits, CHLH, CHLI, and CHLD, in the respective stoichiometry of 1:6:6, and constitutes a highly regulated step in TBS [55]. While CHLH is essential for substrate binding and catalysis, CHLI provides the energy for the chelation reaction by ATP hydrolysis. Although CHLD carries amino acid sequence similarities to CHLI, no ATPase activity was observed [55].

The MgProtoME cyclase catalyzes the closure of the fifth ring to form chlorin. It was proposed, that the functional enzyme is formed by a protein complex, which is composed of at least three subunits, all essential for the cyclization reaction [56]. Two of these subunits are bound to the chloroplast membranes, while the third subunit was suggested to be localized in the soluble plastid fraction [56]. The first membrane-bound subunit is most likely the catalytic one, designated as the COPPER RESPONSE DEFECT1 (CRD1) and related COPPER TARGET HOMOLOG 1 (CTH1) *in C. reinhardtii* [57], the AEROBIC CYCLIZATION SYSTEM F-CONTAINING subunit (ACSF) in *Rubrivivax gelatinosus* [58], CHL27 [59] and XANTHA-L [56], and $ChlA_I$/CycI and $ChlA_{II}$/CycII in *Synechocystis* sp. PCC6803 [60,61]. Recently, the hypothetical chloroplast open reading frame 54 (YCF54) protein was identified as a novel membrane-associated factor, required for the cyclase reaction in *Synechocystis* sp. PCC6803 [62], followed by the identification of its homolog in *N. tabacum*, named LOW CHLOROPHYLL ACCUMULATION A (LCAA; [62,63]). LCAA carries a DUF2488 domain of unknown function, common in Ycf54 homologs, and interacts with CHL27 *in vivo*. A precise function is currently difficult to assign to LCAA and it is proposed that it is the second membrane bound subunit, responsible for the coordination of the multimeric cyclase complex at the chloroplast membrane, thus possibly playing a role of a scaffold protein [63]. Up to date, a reducing activity required for the catalytic reaction could not be assigned to any of the subunits of the MgProtoME cyclase.

3. Transcriptional control of TBS genes in plants

Genes encoding enzymes of the TBS pathway are controlled by multiple environmental stimuli and endogenous effectors. These signal emitters coordinate also the transcriptional control of all of the genes associated with the TBS metabolism in a tissue- and developmental stage-dependent manner [64,65]. Due to the space limitations, the transcriptional control of TBS will be described only briefly in this review.

Dependently on the organism, several enzymatic steps of TBS are encoded by a small gene family, often consisting of 2 to 3 homologous genes. However, the functions of the proteins encoded by these sets of genes have been examined only in a few cases. Generally, the pattern seems to be emerging that one gene encodes the protein for a continuous, but low expression under normal conditions, enabling a constant supply of metabolic products, while a second gene encodes the protein required at different and highly regulated situations, such as changes in light or other environmental conditions.

The distinct transcriptional control of each member of the *Arabidopsis POR* gene family [66], the two genes *HEMA1* and *HEMA2* encoding glutamyl-tRNA reductase (GluTR), the enzyme at the rate limiting step of TBS [67], or the *CRD1* and *CTH1*, two genes encoding homologous subunits of the *Chlamydomonas reinhardtii* MgProtoME cyclase with a reciprocal gene regulation depending on the availability of Cu^{2+} and O_2 [57] are striking examples that reflect the complexity of the regulation of certain enzymatic steps, often catalyzed by different isoforms.

The TBS genes were classified into four clusters, based on their expression profiles [68]. The next step in exploration of the transcriptional control in TBS was to analyze factors transforming the endogenous and environmental stimuli into a transcriptional response. Several different sets of transcription factors are responsible for a balanced gene expression for photomorphogenesis and plastid development, including also TBS. The function of these transcription factors have been described either during extended etiolation or during de-etiolation of seedlings.

Light-dependent stimulation of genes is one of the most extensively studied mechanisms of gene expression during plant development, especially in processes determining chloroplast biogenesis. The basic leucine-zipper transcription factor LONG HYPOCOTYL-5 (HY5) [69] acts on the stimulatory effect of red and blue light receptors-mediated transcriptional control, while it is continuously degraded by CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1)-mediated proteolysis in darkness, leading to repression of the light-inducible genes [70]. Many of the TBS genes are controlled by HY5, e.g. GluRS, URO2, PPOI, CHLH, GUN4, CHL27, DVR, PORC, CAO, CHLP, and HO1, but surprisingly not HEMA1 [71]. Therefore, it is possible that the control of HEMA might be PHYTOCHROME-INTERACTING FACTOR (PIF)-dependent [72]

Members of the PIF family have been identified as important negative transcription regulators for light-dependent chloroplast biogenesis, and PIF1 and PIF3 are found to be actively participating in transcriptional control of many TBS genes [73,74]. These factors act as negative regulators in coordinated phytochrome-induced gene expression of photomorphogenesis. Involvement of PIF-controlled repression of the expression of many TBS genes became appealing in a quadruple *pifq* mutant of *Arabidopsis*, which showed abnormal transcriptional up-regulation of TBS genes in etiolated seedlings [75,76].

Function of PIFs was demonstrated during seedling development right after the emergence from the soil and exposure to the first light illumination. Analysis of the etiolated *pif* mutants, which are normally phenotypically distinguishable by PChlide-accumulation, indicates the potential role of PIFs in the control of *POR* expression [73]. Other TBS genes, like *FERROCHELATASE II* (*FCII*), or *HEME OXYGENASE* (*HO3*), are also controlled by PIFs [77]. Recently, an interaction of PIF3 and REDUCED POTASSIUM DEPENDENCY 3/HISTONE DEACETYLASE-type was shown to be required for repression of chlorophyll biosynthesis genes in etiolated seedlings [78].

The PIF- and phytochrome-independent control of chlorophyll synthesis genes was reported for two members of the GATA transcription factor family, GNC (GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED) and GNL/CGA1 (GNC-LIKE/CYTOKININ-

Fig. 1. Scheme of the plant tetrapyrrole biosynthetic pathway. The coloring highlights the redox regulation by NTRC with blue and by TRX with green lines, respectively. Red lines show the feed-back loop for regulation of ALA-synthesis in response to heme and PChlide. Post-translational regulators or scaffold proteins are indicated in cyan. Evidence for phosphorylation of TBS enzymes are denoted by 'P' inside green circles. Solid lines refer to verified interactions and dotted lines to potential targets of post-translational regulation and regulatory connected parts of the pathway. Further details and the abbreviations of the enzymes are given in the text.

RESPONSIVE GATA FACTOR1 [79]). These factors are up-regulated in light and in response to cytokinin signaling, while down-regulated by gibberellins, and stimulate the expression of *GUN4, HEMA1, PORB and PORC* [80]. The GOLDEN-LIKE 1 and 2 (GLK1 and GLK2) transcription factors are also required for light-dependent chloroplast biogenesis and control of the gene expression for chlorophyll biosynthesis [81].

Under the conditions when light is not the dominant inducer of the nuclear gene expression for chloroplast development, a hormonal modulation of gene expression becomes more apparent. Application of cytokinin triggers photomorphogenic development in darkness, while deregulated cytokinin signaling affects, among others, also the expression of chlorophyll synthesis genes [82,83]. Ethylene acts on PChlide accumulation in etiolated seedlings by stimulation of *PORA* and *PORB* expression. This ethylene effect is mediated by the transcription factor ETHYLENE INSENSITIVE 3 (EIN3) which content is reduced in a light-responsive manner [84,85].

Antagonistic effects of auxin on cytokinin-stimulated plastid development in dark were observed through the AUX/IAA-factors, which can simulate photomorphogenic processes in darkness [82,86,87]. Finally, gibberellin signaling acts negatively on light-dependent transcriptional control and its modulatory input was studied in etiolated mutants. It was shown that DELLA, the repressor of gibberellin signaling, interacts with PIF and de-represses the photomorphogenic transcriptional control of the chlorophyll synthesis genes [88]. The gibberellin-triggered negative effect of PIF was demonstrated on promoters of genes associated with photosynthesis and chlorophyll biosynthesis. Apparently, gibberellin has also a regulatory effect on chlorophyll accumulation during de-etiolation [89] and acts directly or indirectly on the COP-mediated proteolysis of HY5 [88].

It is fascinating when elaborated explorations uncover the multilayer transcriptional control of genes encoding tightly regulated enzymes at the key steps of TBS. An orchestrated cooperation of the regulatory factors result in stability or degradation of transcriptional regulators in darkness and upon light exposure, to ensure an adjusted gene expression for TBS.

4. Redox Regulation of TBS Enzymes by thioredoxins and NADPH-dependent thioredoxin reductase C

Regulation of the activity and stability of proteins by redox regulators is an important post-translational mechanism. Enzymatic activity and the tertiary structure of proteins are often determined by the redox state of the redox-active amino acid residues, like cysteines, which undergo a thiol switch leading to the oxidized or reduced cysteine residues groups. Depending on the strength of the oxidizing environment and the presence of a second cysteine, intramolecular or intermolecular disulfide bridges can be formed.

In plastids of higher plants, at least two different reducing systems control TBS. The first mechanism involves the light-dependent activity of thioredoxins (TRXs), which in turn are reduced by electrons derived from the photosynthetic electron transport chain via the ferredoxin-thioredoxin reductase [90,91]. The multigene family of chloroplast-localized TRXs consists of ten members, which are classified according to their structure, localization, and target proteins [92]. A second mechanism for TRX reduction involves the light-independent activity of NADPH-dependent reductases (NTR). While mitochondria and the cytosol contain two dually localized NTRs (NTRA and NTRB; [93]), chloroplasts harbor the NTRC [94,95]. NTRC differs structurally from NTRA and B by the appendix of a C-terminal TRX domain. Hence, NTRC reduces its own TRX domain [96] enabling NTRC to act independently from both free TRX and the light-driven activity of the photosynthetic complexes. NTRC was shown to serve as a reductant for 2-cysteine-peroxiredoxins A and B (2-CysPRXA/B), which are small H_2O_2 reductases [95]. Apart from other potential metabolic targets, NTRC activity has been reported also to be involved in the redox regulation of starch synthesis [97–99].

The multiple TRX isoforms and NTRC turned out to be essential for maintaining a constant flow of intermediates through the TBS pathway and, therefore, are indispensable for proper function of chlorophyll and heme-requiring processes [100,101]. Activity of ALA biosynthesis enzymes of photosynthetically active organisms is coordinated with the demand for chlorophyll. Therefore, control of the TBS pathway, including ALA synthesis, is tightly correlated with the availability of light. Because plastid-localized TRX activity depends on the presence of light, the redox regulation enables a fast and reliable activation of the pathway upon the transition from dark to light. Using TRX-dependent proteomic approaches, such as affinity chromatography, GSAT, ALAD, UROD, PPOX and MgCh subunit CHLI were found to interact with TRX*f* and TRX*m* [102,103]. All of these proteins contain conserved cysteines and future work will verify whether thiol switches modulate the activity, conformation and stability of the enzymes and, consequently, the metabolic flow in TBS.

Experimental evidence for the redox regulation of the chelation reaction by MgCh was published by Jensen and co-workers (2000). Recombinant MgCh subunits treated with N-ethylmaleimide (NEM), a thiol-reactive chemical, resulted in reduced MgCh activity, which was attributed to a decreased ATPase activity of CHLI. Therefore, the CHLI redox status was suggested to be crucial for the activity of the MgCh complex [104]. These results were further supported by the finding that recombinant TRX*m* and TRX*f* are competent reductants for an intramolecular disulfide bond at the CHLI C-terminus, and that the reduction of disulfides leads to the stimulated ATPase activity of CHLI [105]. Ultimately, the physical interaction of CHLI with TRX*f* was confirmed using yeast-two-hybrid and bi-molecular fluorescence complementation assays. Upon virus-induced silencing of *TRXm* and *TRXf* genes, pea plants contained less of reduced CHLI, resulting in compromised MgCh activity, regardless of the expression of NTRC [106]. Although these results indicate TRX-dependent regulation of MgCh, a NTRC-dependent redox modification of CHLI was proposed by Stenbaek and Jensen [107]. In another study, recombinant NTRC was used to reduce cysteines of CHLI and to examine a stimulatory effect on CHLI-ATPase activity. It was demonstrated that NTRC is at least as efficient in CHLI activation as additional TRXs [108]. In contrast, *in vitro* experiments with recombinant NTRC and CHLI from pea did not reveal stimulation of MgCh activity by NTRC [106]. These seemingly conflicting results of stimulatory impact of NTRC on CHLI-ATPase activity without significant effect on total MgCh activity could be explained if CHLI assembled into the MgCh complex is not accessible for NTRC-dependent reduction. In that case, modification of the entire MgCh complex cannot be observed. In other words, the 'free' CHLI can be reduced by both TRX and the TRX-domain of NTRC, while integration into the MgCh complex prevents the interaction with NTRC. This argumentation is further supported by the fact, that the double knock-out of TRX*m* and TRX*f* cannot sufficiently be complemented by endogenous NTRC [106]. In addition, *ntrc* mutants do not show reduced MgCh activity [101,109]. Thus, because *ntrc* and *trxm*/*trxf* mutants contain lower amounts of CHLI protein [106,108], it could be assumed that the redox regulation of conserved CHLI cysteine residues is responsible for the stability of CHLI and the entire MgCh *in vivo*. The *in vitro* stimulation of CHLI activity by reductants underlines the need of the reduced cysteines during ATP hydrolysis, and argues for a redox-dependent activation of MgCh.

Biochemical analysis of *Arabidopsis ntrc* mutant lines revealed evidence for the redox regulation of GluTR, MgProto methyltransferase (CHLM) and POR. The *ntrc* seedlings exhibit a pale green coloration and a retarded growth phenotype, which is similar to other chlorophyll synthesis mutants [94,100,101]. Although the expression of genes involved in TBS is not altered in NTRC-deficient plants, the amount of GluTR, CHLM and POR is decreased compared to wild type. In the *ntrc* mutant GluTR forms oligomeric complexes under non-reducing conditions. GluTR interacts with NTRC *in vivo*, therefore it seems plausible that a stabilized GluTR is kept in the reduced form by NTRC and contributes to elevated ALA synthesizing activity [101].

First indication for the redox regulation of CHLM was obtained with the ALA-fed *ntrc* mutant, which accumulates MgProto [109]. The *ntrc* seedlings contained lower CHLM content than the wild-type control [110]. *In vivo* interaction of CHLM with NTRC and additional biochemical analysis supported the redox regulation of CHLM by NTRC [101]. *Arabidopsis* CHLM contains two cysteines at position 111 and 115 and a cysteine in the C-terminal region of the protein at position 177 [101]. These cysteines were thought to be essential for either the catalytic activity, structural integrity, or the interaction of CHLM with other proteins. Recently, the elucidation of the three-dimensional structure of *Synechocystis* sp. PCC6803 ChlM indicates that the peptide backbone of cysteine-115 is directly involved in the binding of the co-factor S-adenosyl methionine, while cysteine-111 lies within the first beta-sheet adjacent to the co-factor binding motif. The cysteine at position 177 is located at the end of another beta-sheet [111]. Based on the structure, it was suggested that the cysteines are buried inside the protein, but cysteine-115 undergoes a conformational change when no cofactor is present [111]. However, diminished CHLM content in *ntrc* suggests a post-translational stabilization of the protein by the redox control. Because the recombinant CHLM with substituted cysteines shows lower enzymatic activities *in vitro* (unpublished results, Richter et al.), the *chlm* with substituted cysteines should be analyzed in the *chlm* knock-out background.

The future scope of research focused on the redox regulation of the TBS pathway will certainly uncover the specific roles of TRX and NTRC in regulation of other TBS enzymes. The ability of NTRC and TRX to functionally compensate for each other can be avoided in the future studies by taking advantage of TRX/NTRC knock-out mutants.

5. Phosphorylation – a new layer of posttranslational regulation of TBS

Almost all cellular processes, including metabolic pathways, are controlled by protein kinase-catalyzed phosphorylation [112]. In this process, the gamma-group of a phosphate donor, e.g. ATP or GTP, is transferred to a phosphorylatable amino acid residue, namely serine, threonine, aspartate, histidine, or tyrosine. Alteration to a negatively charged phosphorylated amino acid residue modifies the tertiary structure and, thus, biochemical and physical properties of the protein, including function, activity, stability and the ability to interact with other proteins. Since the identification of the first plastid-localized phosphorylated polypeptides, which belong to the light-harvesting complexes (LHCs) [113], it took almost three decades to identify the responsible kinase(s) and to understand the regulation and physiological consequence of LHC phosphorylation in more detail [114,115]. The thylakoid bound-state transition kinases STN7 and STN8 are responsible for a complex regulation of antenna proteins of photosystem II by redox-modification and phosphorylation, which affects the energy distribution between the photosystems [116–119]. STN7 and STN8 are presently the best studied protein kinases of the chloroplasts. The actual list of unambiguously confirmed plastid protein kinases contains 15 candidates, including soluble CASEIN KINASE 2 (CKII), the CHLOROPLAST SENSOR KINASE (CSK) and the THYLAKOID-BOUND KINASE 1 (TAK1). In contrast to the number of already confirmed plastid-localized protein kinases, statistical and bioinformatic analysis predicts at least 80 nuclear-encoded kinases with plastids localization [120]. The expectation of a large number of protein kinases in plastids correlates with a still increasing number of newly identified phosphorylatable proteins in this organelle. The development of new mass spectrometry techniques increased the detection of phosphorylated plastid-localized proteins from 197 [121] to 294 [122] within the last 5 years. Moreover, a chip with 905 peptides representing the state of the art *phosphome* of the chloroplast is available to identify new kinase-substrate relations [123]. Phosphorylated proteins are found in all functional clusters of intra-plastidic processes, like DNA-/RNA-, primary- and secondary metabolism. Hence, phosphorylation seems to be of a high importance for the general function of chloroplasts (a comprehensive overview about phosphorylation networks is given by Baginsky and Gruissem [124], and by Schönberg and Baginsky [125]).

5.1. Phosphorylation of the TBS enzymes – an emerging research area

Very little is known about the phosphorylation targets in TBS and the physiological significance of this process. As it was emphasized above, the efforts were undertaken to identify phosphorylated proteins from chloroplast preparations by phosphoproteomic approaches. These attempts revealed an initial list of TBS enzymes containing phosphorylation sites (p-site, Table 1). The candidates for kinase-dependent regulation are assigned either to the early steps of TBS, like GSAT, or to the enzymes acting downstream of ALA-synthesis, such as hydroxymethylbilane synthase (HMBS), UROD and PPOX. In case of UROD, the identified phosphopeptide aligns to the predicted transit peptide of the protein. Phosphopeptides were also identified for enzymes of the Mg-branch, namely MgCh subunits CHLH and CHLI, but also GUN4, PORB, DVR and chlorophyll b reductase (CBR; Table 1).

The sites of phosphorylated amino acid residues identified in TBS enzymes resemble the p-sites found in proteins of different plant organelles, with a serine phosphorylation at a frequency of approx. 80% and a threonine phosphorylation at approx. 20% (Fig. 2; [122,124]). Although there are some indications for tyrosine phosphorylation [122,126], the experimental evidence for plastid-localized protein phosphorylation at the tyrosine residues are limited. Hence, the analysis of Baginsky and Gruissem [124] were extended and new motif alignments of these enzymes were proposed (Fig. 2). The recognition of a phosphorylation target by a kinase is mainly determined by the chemical properties of the amino acids adjacent to the p-site. For example, the CKII has a clear preference for acidic amino acids adjacent to the phosphorylated amino acid [124]. In case of the TBS enzymes, the consensus motif of amino acids surrounding the identified p-sites is characterized by a high chemical complexity. Among these potential p-sites, a single protein kinase recognition motif is not enriched in the alignment of peptide domains, suggesting that phosphorylation of these enzymes is likely catalyzed by different kinases. However, when p-sites of enzymes of the Mg-branch are aligned, then a clear preference is predicted for a serine phosphorylation within a consensus motif of K/RxxxxxxxS (Fig. 2). It is worth mentioning that two enzymes from different parts of the TBS share almost identical consensus motifs. HMBS (encoded by *HEMC*) and one of the identified p-sites of MgCh subunit CHLH-1 from *Arabidopsis* harbor a common phosphorylation motif of xxxILSxxx in a stretch of almost identical amino acid residues, with similar chemical and structural properties (Fig. 2).

5.2. Regulation of TBS by phosphorylation – the missing link?

At present, complex regulatory networks, including feed-forward and feed-back regulation of enzymatic activities within the pathway, leading to a fine-tuning of the flow of intermediates, remain poorly understood. One of these regulatory circuits is the connection of ALA synthesis enzymes located at the beginning of the pathway and the activities of enzymes located at the branching point towards Mg-porphyrin and heme synthesis (see also chapter 7.1). Analyses of transgenic plants with a reduced expression of genes, encoding enzymes involved in either ALA-synthesis or MgCh reaction, revealed a close regulatory connection of both parts of TBS. From these studies, it can be concluded that constitutive or induced knock-down of *HEMA1* and *GSA* (encoding GSAT) influences activities of enzymes from the Mg-branch [127,128]. On the other hand, knock-down of *CHLH, CHLI* and *CHLM* genes leads to inactivation of ALA synthesis, which prevents the accumulation of the porphyrin intermediates metabolized by the affected enzymes [129–131]. The simultaneous reduction of enzymatic activities at the beginning of TBS and in the chlorophyll synthesis part cannot be

explained by modified nuclear gene expression alone. Therefore, post-translational mechanisms, such as protein phosphorylation, are presumed to play a major role in regulation of TBS, providing rapid adjustments to changing conditions.

As none of these phosphorylation events have been addressed yet in a biochemical approach, there are many potential regulatory mechanisms to be elucidated. The main scope of the future research on post-translational regulation of TBS is likely to be on the analysis of metabolic implications of phosphorylated TBS proteins. The putative phosphorylation sites should be confirmed by a specific kinase assay using recombinant wild-type and mutant proteins. A complementation experiment of knock-out lines with gene constructs encoding wild-type and mutant proteins should uncover the *in vivo* significance of the phosphorylation of TBS enzymes.

6. Post-translational regulation by complex formation

Organisms evolved regulatory mechanisms preventing the accumulation of free tetrapyrrole intermediates, which due to their photodynamic properties may pose a threat to the cell. Avoidance of metabolic perturbation can be achieved by degradation of transiently accumulating tetrapyrrole intermediates through disposal pathways, feedback-controlled inactivation of early steps of the pathway, or efficient substrate channeling between enzymatic steps of the TBS pathway. Formation of multi-enzyme complexes is a beneficial strategy, because several enzymatic activities are instantaneously controlled in locally restricted subcellular compartments. Scaffold, adaptor or anchor proteins contribute to protein complex formation without a direct involvement in catalysis, but for maintaining the structural integrity of the metabolic pathway consisting of multi-enzymatic complexes. While oligomeric complexes are assembled to form functional enzymatic units (e.g. MgCh and MgProtoME cyclase), macromolecular complexes in TBS are proposed. Although still often hypothetical, such complexes would improve continuous flow of the intermediates through the pathway, optimizing enzymatic reaction and connecting distant metabolic reactions [29].

6.1. GluTR interactions with other proteins

GluTR initiates the rate limiting step of ALA synthesis by catalysis of the introductory regulatory key step of TBS. A first suggestion of an interaction between GluTR and GSAT arose when a three-dimensional structure of dimeric GluTR from *Methanopyrus kandleri* [16] and dimeric GSAT from *Synechococcus* [13] merged in a model structure that faces the catalytic sites of both enzymes in close proximity [16]. Two GluTRs interacting through their C-terminal domain form a V-shaped dimer and provide a potential cleft for an interaction with the GSAT dimer [13,16,132,133]. Finally, direct physical interaction of GluTR and GSAT was recently demonstrated in *E. coli* by biochemical approaches [22], and it is plausible that plants may also benefit from close physical interaction of both enzymes, because of the facilitated substrate channeling.

Formation of another protein complex was demonstrated to repress GluTR activity in darkness, to prevent the accumulation of phototoxic intermediates. Chlorophyll synthesis of angiosperms depends on light due to the strictly light-dependent conversion of PChlide to Chlide. After transition from light to dark, the membrane-bound FLU protein mediates dark repression of ALA synthesis by interaction with GluTR [14,134]. The loss of the FLU-dependent repression of GluTR correlates with accumulation of PChlide in the dark phase, which leads to the programmed cell death upon subsequent light exposure [14,135]. Interestingly, only GluTR1 encoded by *HEMA1* interacts with the tetratricopeptide-repeat domain-containing FLU [136]. Mass spectrometry data of plastid protein fractions of dark-adapted plants indicated FLU and GluTR to be in complex with CHL27 and POR; however, in light GluTR was not detected in the complex. This implies that ALA synthesis undergoes a shift from an 'open' (active) in light to a 'closed' (inactive) pathway in darkness [137], although low-level synthesis of ALA in the dark is expected for the supply of heme.

It is worth mentioning that the *flu* mutant not only show higher ALA synthesis capacity in darkness but also in light compared to wild type [14]. Thus, the regulatory function of FLU in ALA synthesis in light cannot be excluded. The molecular mechanisms behind the FLU-mediated inactivation of ALA synthesis, including sensing the ALA requirements, are poorly understood. It is plausible that these mechanisms might be underlying swift adjustments of metabolic activities in plants upon rapid changes in the environment. Additional regulatory fine-tuning of the ALA synthesis may occur by the redox regulation (see above).

A membrane-bound GluTR-BINDING PROTEIN (GBP, [138] formerly designated as PROTON GRADIENT REGULATION 7 (PGR7), [139]) was shown to bind a small amount of GluTR at the thylakoid membrane. It is suggested that this membrane-bound GluTR ensures a minimum of ALA synthesis in a separate plastidic subcompartment, distant from a dominant ALA synthesis in the stroma, and thus prevents a complete FLU-mediated GluTR inactivation, when heme synthesis is still required. The *pgr7* mutant is characterized by reduced growth, slightly decreased chlorophyll level, and impaired photosynthetic electron transport through the cytochrome b_6f complex (Cytb_6f; [139]). Stronger reduction in heme than the chlorophyll content in *pgr7* [140] is in line with observed malfunctions of the Cytb_6f complex, due to the insufficient supply of heme for photosynthetic electron transport [140]. The recently published crystal structure of GluTR-GBP dimers shows GBP binding to the N-terminus of GluTR [141]. The authors presented a combined enzymatic assay for ALA formation from glutamyl-tRNA *in vitro*, using recombinant purified enzymes of the ALA synthesizing pathway and *E. coli* tRNA(glu), and demonstrated a stimulated ALA synthesis in the presence of GBP [141]. However, *gbp* mutants are not considerably altered in their ALA-synthesizing capacity and their chlorophyll content compared to wild type [139,140]. Future studies should help to elucidate how GBP contributes to stimulation of the GluTR activity and a spatio-temporal regulation of ALA synthesis.

Characterization of the mutants with deficiency in heme breakdown and *in vitro* experiments with purified GluTR revealed a regulatory impact of heme on ALA synthesis. It is assumed that a pool of heme involved in that regulation accumulates to a certain threshold under different physiological conditions, before it exerts the inhibitory effect on GluTR [143]. An N-terminal truncated GluTR of barley is insensitive to heme-dependent inhibition, suggesting that the N-terminal domain harbors a heme-responsive element, which is not essential for catalysis [144] or for FLU-mediated dark repression of ALA-synthesis [67]. Cell-free extracts of *Chlorella*, *Synechocystis,* and *Chlorobium* show a reduced GluTR-activity after addition of heme in the micromolar range [145–148]. GluTR purified from barley leaves shows a 50% reduced catalytic activity after the exogenously applied heme in a millimolar range [149]. ALA-synthesis in isolated barley chloroplasts is inhibited upon addition of an excess of heme [150]. However, these studies have a certain ambiguity due to the possibility of unspecific effects observed with an excess of heme in enzymological studies [151]. However, posttranslational feedback regulation by end-products is a very common control mechanism. This may also be the case for plant tetrapyrrole biosynthesis, as it was demonstrated in heme biosynthesis in *E. coli*, yeast, and mammals [152,153].

More recent experiments examining the heme effects have been performed with more caution by using genetic tools and analysis of measurable endogenous heme contents. *Chlorobium vibrioforme* GluTR was shown to bind heme when it was applied in a micromolar range [24]. In this context, an additional protein factor for heme inhibition of GluTR activity was proposed in *C. reinhardtii* [154]. The GluTR-binding protein of *Arabidopsis* (GBP) was identified as a putative candidate for this proteinaceous factor [138]. Biochemical studies led to the assumption that heme binds to GluTR in a 1:1 ratio and that GBP and heme

Table 1
Overview of enzymes with phosphorylated peptide domains from the TBS pathway, identified by phosphoproteomic approaches. P-sites are indicated in brackets where a specific p-site was assigned. In case of DVR and CBR the identified peptide carries more than one putative phosphorylated amino acid. The phosphopeptide identified for UROD (encoded by *HEME2*) was found to be localized in the predicted transit peptide.

Name	ATG	identified phosphopeptide	Reference
GSA2 (glutamate-1-semialdehyde 2,1-aminomutase 2/ GSAT)	AT3G48730	AG(**pS**)GVATLGLPDSPGVPK	Lohrig et al. (2009)
HEMC (hydroxymethylbilane synthase/ HMBS)	AT5G08280	IL(**pS**)QPLADIGGK	Reiland et al. (2009)
HEME2 (uroporphyrinogen decarboxylase/UROD)	AT2G40490	M(**pS**)ILQVSTSSLSSSTLLSI(**pS**)PR	Lohrig et al. (2009)
HEMG1 (protoporphyrinogen IX oxidase/ PPOX1)	AT4G01690	LPKPQGQ(**pT**)VGSFR	Sugiyama et al. (2008)
		MLIKPN(**pS**)TDPLK	PhosPhat4.0
		(**pT**)ECLIDGELKGFGQLHPR	PhosPhat4.0
CHLH-1 (magnesium chelatase H subunit)	AT5G13630	GSDKGIL(**pS**)DVELLK	Engelsberger and Schulze (2012)
CHLH-2 (magnesium chelatase H subunit)		QLQDMYL(**pS**)RK	PhosPhat4.0
CHLI1 (magnesium chelatase I-1 subunit)	AT4G18480	VC(**pS**)ELNVDGLR	Reiland et al. (2009)
CHLI2 (magnesium chelatase I-2 subunit)	AT5G45930	KDPLESMD(**pS**)GILVTEK	Sugiyama et al. (2008)
GUN4 (genomes uncupled 4)	AT3G59400	VFKTNY(**pS**)F	Reiland et al. (2009)
PORB (protochlorophyllide oxidoreductase)	AT4G27440	GYV(**pS**)ETESGKR	Wang et al. (2013)
DVR (divinyl reductase/ PCB2)	AT5G18660	YYAAESMLILDPETGEYSEEK	Sugiyama et al. (2008)
CBR (chlorophyll b reductase/ NYC1)	AT4G13250	VENLEMVFSSVAVQIAR	Engelsberger and Schulze (2012)

have an antagonistic effect on GluTR activity [141]. However, the X-ray analysis of the quaternary structure of the interacting homodimers of GluTR and GBP did not reveal heme binding to any of these proteins [141].

Interestingly, *ulf3*, a second side revertant of *flu*, carries a mutation in the *heme oxygenase* gene (*HY1*) and shows a suppression of PChlide accumulation in *flu* [67]. The *flu* mutants are only viable in continuous light conditions [14]. In contrast, *ulf3* accumulates less PChlide in darkness suggesting a negative impact of the *hy1* mutation on ALA-synthesis in the *flu* background. It was proposed that *flu*/*hy1* double mutants transiently accumulate heme, which in turn represses ALA-synthesis [67]. Although not proven experimentally, the negative impact of heme was suggested to become dominant over the missing FLU-mediated repression of the GluTR activity in *flu*/*hy1*. However, when the heme-breakdown is altered, the amounts of intermediates downstream of heme, such as phytochromobilin, are also changed. Phytochromobilin plays a role of a chromophore in phytochrome and it is essential for light-induced expression of GluTR encoded by *HEMA1* [155]. However, the exact mechanism behind the heme-dependent regulation of transcriptional and posttranslational ALA biosynthesis still remains to be elucidated.

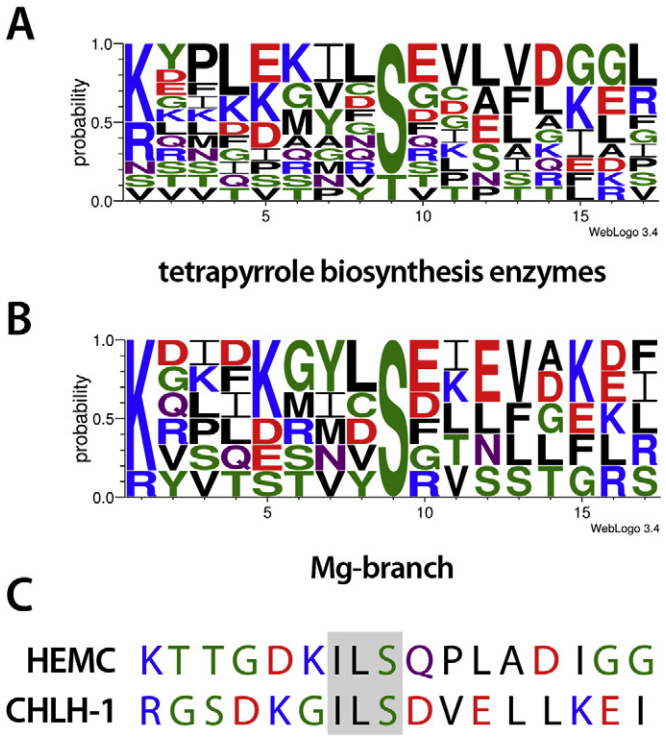


Fig. 2. Overview of putative phosphorylation targets within TBS. (A) Alignment of all identified phosphopeptides of TBS enzymes. Sequences of the *A. thaliana* proteins eight amino acids up- and downstream of the putative p-site were aligned using the WebLogo3 algorithm [246]. (B) Alignment of the putative p-sites (+/- 8 aa) from enzymes of the Mg-branch of TBS. (C) Alignment of the putative p-site of HMBS (encoded by *HEMC*) and one of the identified p-sites of MgCh H subunit (CHLH-1). Alignments were performed with identified phospho-peptide sequences from *A. thaliana* only.

The multifaceted regulation of ALA synthesis enzymes is extended with an additional type of regulation. GluTR has been suggested to be a substrate for the plastidic caseinolytic peptidase (Clp) system. Pulldown experiments with the substrate selector subunit (ClpS1) of Clp revealed that GluTR may interact with ClpS1. Consequently, a *clps1* mutant has increased amounts of GluTR [142]. Future analyses are expected to determine the significance of the Clp-dependent GluTR degradation in the context of multiple post-translational regulation mechanisms of ALA synthesis, and correlation between the GluTR interactions with multiple protein complexes and its activity.

6.2. Protein-protein Interactions among enzymes of the Mg-branch

Interaction of the CHLH subunit of MgCh with the regulatory protein GENOMES UNCOUPLED 4 (GUN4) results in a stimulatory, but dispensable effect on MgProto formation [156–159]. GUN4 was primarily identified in a mutant screen for defects in the retrograde signaling [156, 160]. Further studies revealed wider effects of the GUN4 activity on the TBS pathway, because the *gun4* knock-down mutant is characterized by reduced MgCh activity and, directly or indirectly, ALA synthesis capacity, which result in a pale green and growth-retarded phenotype [156,158]. A *gun4* knock-out of *A. thaliana* is lethal under photoperiodic growth, but able to produce small amounts of chlorophyll in continuous dim light [156,158].

In vitro enzyme assays provided additional indications for the regulatory mechanisms. GUN4-triggered stimulation of MgCh activity depends on the Proto and MgProto binding of GUN4 at limiting substrate concentrations with a lower *K*d for MgProto than for Proto [161]. Preincubation of GUN4 with CHLH and Proto results in higher MgCh activities and, therefore, GUN4 assists MgCh by providing the substrate Proto or releasing the product MgProto from MgCh [156, 161]. These potential mechanisms would contribute to overall metabolic flow through the TBS pathway by substrate channeling into the Mg branch and further towards the MgProto methyltransferase (CHLM). Interestingly, the interaction of CHLH with CHLM was found to stimulate the activity of the methyltransferase [162–164].

Furthermore, transgenic plants with inactivated expression of either MgCh subunits or CHLM show a reduced enzymatic activity of ALA synthesis, apart from lower activity in the Mg branch. It was hypothesized

that upon attenuation of activities in the Mg branch both transcriptional and post-translational feedback regulation mechanisms contribute to modulation of metabolic activities within TBS, affecting ALA synthesis [129,163]. The mode of action is still a matter of debate, but may involve interaction between MgCh/GUN4 and ALA synthesis enzymes or a negative feedback control by heme.

Stabilization of another enzyme by a protein, which does not directly belong to the tetrapyrrole metabolism, was illustrated by the interaction of the two isoforms of LIGHT-HARVESTING-LIKE PROTEIN 3 (LIL3:1 and LIL3:2) with geranylgeranyl reductase (GGR, encoded by *CHLP*). GGR catalyzes the reduction of geranylgeranyl pyrophosphate to phytyl pyrophosphate, providing the reduced phytol side chain of chlorophyll molecules. LIL3 belongs to a group of proteins harboring different numbers of membrane spanning domains, of which one or two are designated as light-harvesting complex (LHC) domains [165]. LIL3 was initially assigned to protein complexes containing protochlorophyll in light-exposed etioplasts [166]. Because this complex did not contain any other chlorophyll-binding protein, it was proposed that LIL3 is a first candidate that enables binding of newly synthesized chlorophyll, and is responsible for the delivery of chlorophyll to the photosynthetic complexes [166]. However, lil3:1/lil3:2 double mutants accumulate geranylgeranylated chlorophyll *a* and *b*. Although the *CHLP* transcript levels are not altered in the double mutant, the GGR amounts are diminished below the detection level [165]. Hence, membrane-bound LIL3 are essential for the stabilization of GGR. It is proposed that binding of GGR to thylakoid membranes is essential for proper function and the transmembrane domain of LIL3 is responsible for the maintenance of the GGR-containing high-molecular-weight complexes [167]. In the same line, Cell growth defect factor 1 (Cdf1), recently renamed as CHAPERONE-LIKE PROTEIN of POR 1 (CPP1) was shown to be required for the accumulation of POR. Lack of CPP1 leads to accelerated POR instability, as well as to an enhanced denaturation and degradation of POR under stress conditions [168]. Although this chaperone function may prevent photooxidative damage of POR, in particular during de-etiolation and reorganization from prolamellar bodies to thylakoid membranes, future work has to address how this role can be accomplished in POR oligomers, which seems to be the favorable formation for enzymatic reduction of PChlide to Chlide [169].

7. Tetrapyrrole-derived retrograde signaling

7.1. The concept of the retrograde signaling

Several components of the chloroplast have been proposed to function as the signaling molecules, or to be involved in multiple retrograde signaling (organelle-to-nucleus) pathways (Fig. 3). The classification of the retrograde signaling is based on the series of discoveries, in which an abnormal expression of the nuclear genes was demonstrated to be independent from changes in the chloroplast status. Based on these observations, five main pathways have been proposed, which were grouped dependently on the source of the signal in the chloroplast: (i) plastid-localized protein synthesis [170]; (ii) intermediates of tetrapyrrole and carotenoid biosynthesis [160,171–173]; (iii) ROS, including $^1O_2$ and H_2O_2 [174–176]; (iv) redox status of the photosynthesis components [177,178] and (v) changes in the pool of metabolites [179,180].

An alternative classification of the chloroplast retrograde signaling is based on the developmental stage of chloroplasts [181]. In this case, signals are divided into two groups: (i) 'biogenic control' and (ii) 'operational control'. The 'biogenic control' includes processes taking place at the early stages of the chloroplast development, such as plastid gene expression, protein and pigment biosynthesis, and assembly of the photosynthetic apparatus. In turn, the 'operational control' is a group of retrograde signals that originate in mature chloroplasts. The 'operational control' plays a role in continuous adjustment of the chloroplast status to the environmental fluctuations, by exerting changes on nuclear gene expression. However, it is expected that multiple signaling pathways are triggered simultaneously in response to various stimuli, while alterations in nuclear gene expression are always a result of the converging effect of these signals [139].

7.2. Retrograde signaling derived from tetrapyrrole biosynthesis pathway

Thirty years ago, it was proposed that the chlorophyll synthesis precursors may be involved in the regulation of the nuclear gene expression in *C. reinhardtii* [182]. The authors' conclusion was based on the observation, that the chlorophyll precursor accumulation negatively affects *CHLOROPHYLL A/B-BINDING (CAB, presently LHCB)* transcript levels [182]. Later discoveries of the involvement of the carotenoid and tetrapyrrole intermediates in the chloroplast retrograde signaling were based on the apparent impairment of the signaling, understood as a deficiency in a negative signals down-regulating expression of the nuclear genes encoding chloroplast proteins, despite dysfunctional chloroplasts. Damage to the chloroplast was caused by photooxidative stress due to carotenoid deficiencies, which in turn were caused by mutations [171] or treatment with norflurazon (NF), a non-competitive inhibitor of carotenoids biosynthesis [160]. Nevertheless, in such conditions chloroplasts emit signals which are capable to suppress expression of a specific subset of the nuclear genes encoding chloroplast-localized proteins [171]. Experiments involving NF treatment led to the discovery of a series of mutants impaired in the chloroplast retrograde signaling, named *genomes uncoupled* (*gun*), in which nuclear gene expression was no longer dependent on the chloroplast status [160] (Fig. 3). More specifically, due to the impairment in the retrograde signaling in the *gun* mutants (*gun1* - *gun6*) upon the NF treatment, transcriptional activation of certain photosynthesis-associated nuclear genes (PhANGs), including *LHCB* and *RBCS2*, is less repressed compared to wild type [160,172,183].

GUN1 encodes a chloroplast-localized pentatricopeptide-repeat protein [184], which is not directly related to the retrograde signals potentially originating in the TBS [172]. However, GUN1 seems to be integrating several signals originating in the chloroplast, acting as a master switch inducing ABSCISIC ACID INSENSITIVE 4 (ABI4), which is the APETALA 2 (AP2)-type transcription factor, and by these means blocking expression of the photosynthetic genes in the nucleus [184].

The *gun2* and *gun3* [160] were determined to be allelic to *long hypocotyl 1* (*hy1*) and *hy2* mutations, respectively, which were initially identified by a long hypocotyl and pale-green phenotype [172,185]. The *gun2* and *gun3* mutants are affected in the expression of the *HEME OXYGENASE* [186,187] and the *PHYTOCHROMOBILIN SYNTHASE* gene, respectively [188] (Fig. 3). Thus, both *gun2* and *gun3* carry mutations in the genes encoding proteins involved in the heme catabolic pathway. However, only *gun2* mutation seems to result in heme accumulation [173] (Fig. 3). Defects in phytochromobilin biosynthesis compromise phytochrome function and phytochrome-mediated expression of TBS genes. It was demonstrated that disruption in heme degradation causes repression of ALA biosynthesis, so that the Mg branch is also affected [67]. Additionally, studies on interactions of *hy1* and *gun* mutants showed that these mutations might be in fact affecting the same signaling pathway, with possible interactions between HY1 and GUN4 or GUN5 [185]. However, a direct evidence for such interaction is still lacking.

Studies of the *gun4* mutants of *C. reinhardtii* revealed multiple, but likely pleiotropic effects of the mutation, e.g. alterations in PSI and PSII composition, impaired photosynthetic electron transfer, accompanied by the enhanced activity of PLASTID TERMINAL OXIDASE (PTOX), and significant alterations in the nuclear transcriptome [189]. Most recent studies of a different *C. reinhardtii gun4* and the *chlD-1* strain overexpressing GUN4, pointed to a possibility of GUN4 being involved in sensing accumulating Proto (Fig. 3). It was proposed that GUN4-bound Proto is shielded from collisions with O_2, which reduces Proto-triggered photosensitisation resulting in generation of $^1O_2$. This also

suggests a more direct function of GUN4 in mediating $^1O_2$-dependent retrograde signaling, triggered by a defect in MgCh [190] (Fig. 3).

The *gun5* mutation was identified to be located in the gene encoding the CHLH subunit of MgCh (Fig. 3). This discovery is remarkable because *cs* and *ch42* mutants of *A. thaliana*, carrying defects in CHLI subunit of MgCh, do not show the *gun* phenotype [172]. It was proposed that CHLH, presumably located at the inner membrane of the chloroplast envelope, measures the flux at the beginning of the chlorophyll synthesis branch, and sends a retrograde signal, which might be modulated by binding the ligands [172]. Because CHLI and CHLD are able to interact with free, as well as Proto- or MgProto-bound CHLH, both subunits were suggested to play the roles of such ligands [172]. Additionally, it was suggested that the CHLH-dependent signal could be also indirectly modulated, through *gun2*, *gun3*, and *gun4*, affecting the ligand levels or causing changes in the level of CHLH subunit itself [172]. However, the direct evidence for the CHLH subunit playing the role of such sensor, passing the retrograde signaling information to the nucleus about the flux through the chlorophyll branch, is still lacking and requires further studies.

7.3. Putative involvement of Mg chelatase, porphyrins and Mg-porphyrins

Over the years, Proto, MgProto, and MgProtoME were proposed to be involved in the chloroplast retrograde signaling. Early indications of the possible involvement of Mg-porphyrins in regulation of the nuclear gene expression came from studies involving TBS inhibitors, such as α,α-dipyridyl [182], thujaplicin [191], amitrole [192], S23142 [193], or aforementioned NF, but also from studies of *gun* mutants and Mg-porphyrins feeding experiments [160,172,192–195]. However, with time, it became increasingly uncertain whether these intermediates can act as the signaling molecules per se. It was suggested that their accumulation or deficiency might be triggering, directly or indirectly, different and independent signaling pathways.

The mutation in *long after far-red 6* (*laf6*) of *Arabidopsis* causes an impaired hypocotyl growth in response to far-red light. The *LAF6* gene encodes the ATP-binding cassette (ABC) protein, named AtABC1 [196]. ABC proteins belong to a superfamily of solute transporters, found in organisms of all kingdoms [197] and LAF6 was suggested to be responsible for transport of Proto from the envelope into the stroma [196]. It was reported that *laf6* accumulates Proto (simultaneous Protogen accumulation cannot be excluded) and it was proposed that Proto cannot be transported into the stroma of chloroplasts, making it less accessible for subsequent metabolic steps of TBS [196]. It was therefore suggested, that accumulating Proto acts as a negative signal, affecting nuclear gene expression and photomorphogenesis, in the response specific to the far-red light [196]. However, it is expected that the inaccessibility of Proto would impair the whole TBS pathway, including ALA synthesis. Such deficiency would have potential consequences on photomorphogenic as well as on the gene expression responses in *laf6*, which would be just more apparent under the far-red light than under the white-light conditions. Furthermore, far-red light is not sufficient to activate POR, therefore PChlide accumulation would be also expected [50]. This may result in $^1O_2$ generation due to the PChlide photosensitizing effect (Fig. 3), triggering ROS-stress and most likely the ROS-signaling responses [175].

In turn, Kropat and co-workers proposed that the tetrapyrrole intermediates may act as positive signals in the light-induction of nuclear gene expression [194,195]. The initial studies revealed that the *brs-1* mutant of *C. reinhardtii*, blocked in MgCh, is also defective in light-induced *HSP70A* and *HSP70B* expression. However, upon feeding of MgProto or MgProtoME to dark-grown cultures, the inducibility of the *HSP70* genes could be observed [194], as it was suggested at that time, due to the induction of a light-responsive promoter element in the dark [194]. These results suggested that in *C. reinhardtii* MgProto feeding can circumvent the block caused by the *brs-1* mutation, or induce expression of the heat-shock protein in cells that normally do not express these genes in a specific developmental stage. In turn, the rescue of the *HSP70A* and *HSP70B* inducibility was not observed upon feeding of Proto, PChlide or Chlide to the mutant cells. Therefore, it was concluded that MgProto and/or MgProtoME could play a role of the signaling molecules, with a positive effect on nuclear gene expression [194,195]. Nevertheless, the allelic *brs-1* and *chl1* of *Chlamydomonas* [198,199] carry defects in synthesis of the CHLH subunit of MgCh [199], which was previously identified as *gun5* in *A. thaliana* (Fig. 2). It is therefore more likely that the CHLH subunit is involved in retrograde signaling, rather than MgProto and/or MgProtoME.

In fact, it was demonstrated that the *HSP70A* promoter contains at least three different regions that respond to tetrapyrroles [200,201], $^1O_2$, and H_2O_2, respectively [202]. The region which was shown to be inducible by tetrapyrroles [200,201] does not seem to respond to ROS [202], pointing to a highly specific system of *HSP70A* expression regulation. If the induction in response to $^1O_2$ or H_2O_2 requires a protein factor, as it was suggested by Shao et al. [202], then the specificity of the transcriptional activation of *HSP70* may depend on the site of the signal emission, which in case of a putative tetrapyrrole-mediated signaling might involve proteins interacting with heme or MgProto.

7.4. Involvement of porphyrins and Mg-porphyrins in the retrograde signaling revisited

More recent studies appear to contradict the hypotheses of Mg-porphyrins involvement in the retrograde signaling, because there seem to be no correlation between Mg-porphyrin steady-state levels and PhANG expression [203,204]. Involvement of Mg-porphyrins have been revised, at least in part, because of more precise methods to measure accumulation of given tetrapyrrole intermediates. Thus, it was shown that the *chlm* knock-out mutant of *A. thaliana* accumulates MgProto, which correlates with a repression of the *LHCB* expression [205]. Indeed, it was reported that MgProto and MgProtoME accumulate in the cytoplasm of *A. thaliana* cells treated with NF, but only under the condition of exogenously applied ALA, which was causing increased flux through the pathway [206]. Nevertheless, such cytoplasmic Mg-porphyrin accumulation is more likely due to chloroplast leakage than an intended transport. Such chloroplast leakage of porphyrins is expected due to the damage to the membrane lipids caused by ROS generated by accumulating tetrapyrroles. It was demonstrated, that upon NF treatment, accumulation of MgProto, or in fact any other tetrapyrrole intermediate, could not be detected, measured either by liquid chromatography-mass spectrometry (LS/MS) [204] or by high-performance liquid chromatography (HPLC) [203]. On the contrary, in response to NF, a strong reduction in the tetrapyrrole intermediates was observed, including MgProto [203,204]. When the MgProto level was artificially increased by exogenously applied ALA, the expression of the nuclear genes was not repressed, but induced [204]. In turn, transcriptome analysis of the NF-treated plants revealed a strong down-regulation of the genes encoding components of the TBS pathway [204]. It was suggested that perhaps not the tetrapyrroles themselves but ROS, or changes in the plastid redox-state resulting from perturbed TBS, might be responsible for generating the retrograde signal exerting changes on the nuclear gene expression [204].

Subsequent study in *Hordeum vulgare* L. showed that the *xantha-f*, *-g*, and *–h* mutants, defective in *CHLH*, *CHLD* and *CHLI* genes, respectively, display the *gun* phenotype upon the NF treatment [207]. The *xantha-l* mutant, defective in a gene encoding the MgProtoME cyclase subunit, did not show the *gun* phenotype upon treatment with NF, which was argued to be consistent with the lack of accumulation of MgProto and MgProtoME [207]. Moreover, without the NF treatment, the *LHC* transcription was increased in *xantha-l*, which was accompanied by accumulation of the high amount of MgProtoME [207]. In contrast, CHLM antisense and sense RNA-expressing tobacco lines showed decreased and elevated *LHCB* transcript contents, respectively, but hardly any changes in Mg-porphyrin levels [163]. These observations were

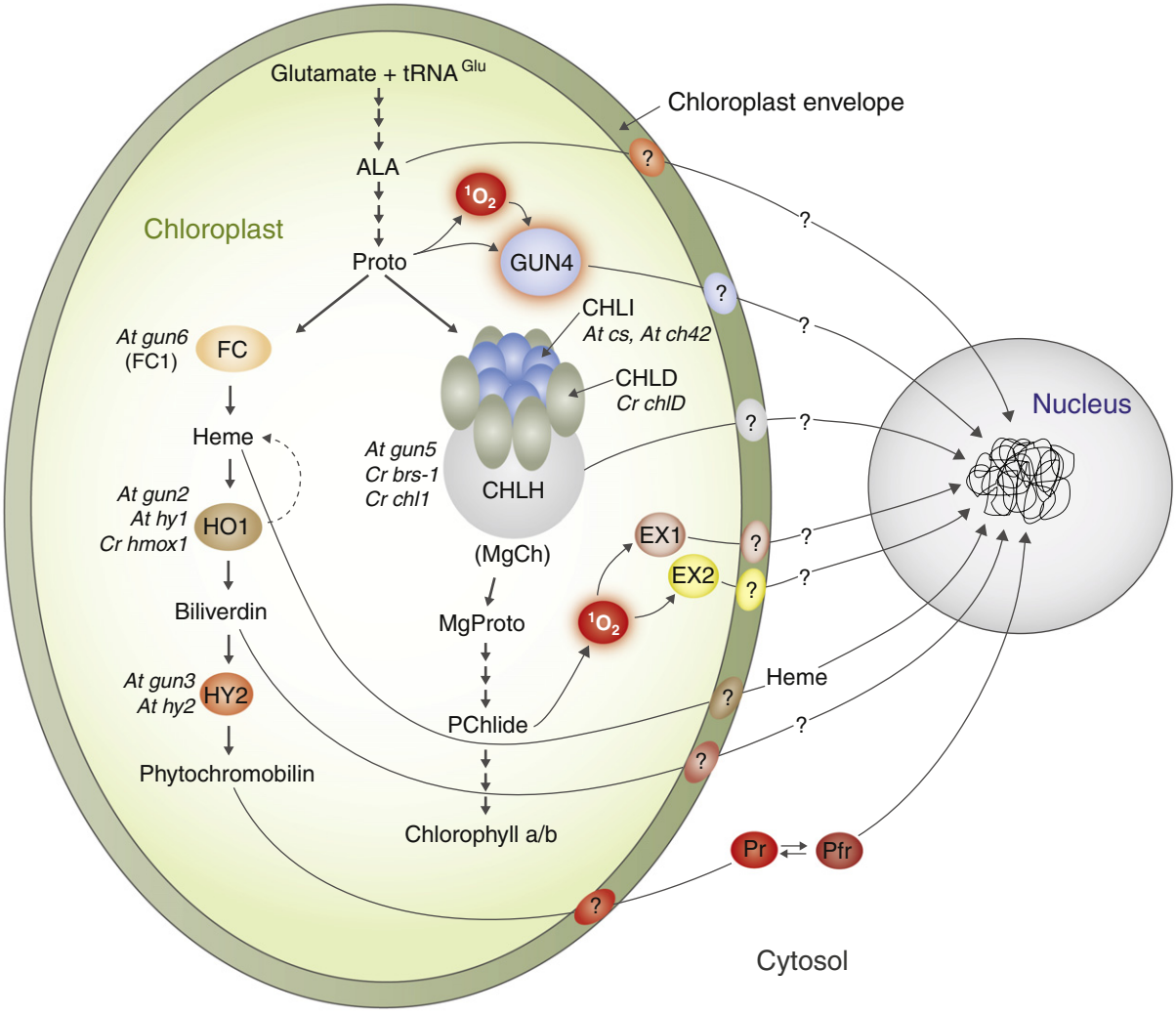


Fig. 3. A general model for the tetrapyrrole-mediated chloroplast retrograde signaling in eukaryotic photoautotrophs. The model does not reflect the chloroplast retrograde signaling in any particular organism, but it reflects current understanding of confirmed or hypothetical signaling pathways. Indicated *A. thaliana* and *C. reinhardtii* mutant names are preceded with respective abbreviations. The formation of 5-aminolevulinc acid (ALA) was demonstrated to be able to exert changes on nuclear gene expression [247]. The *genomes uncoupled* (*gun*) mutations, identified as components of the TBS are indicated. The *gun2* mutant, originally identified as *long hypocotyl 1* (*hy1*), is allelic to the gene encoding HEME OXYGENASE 1 (HO1,) [186,187]; *gun3* is allelic to the gene encoding PHYTOCHROMOBILIN SYNTHASE (HY2) [188]. The *gun6* mutant overexpresses FERROCHELATASE1 (FC1), which was suggested to lead to an increased activity of FC [173]. Heme accumulation in *gun2* is illustrated by dashed arrow. In *gun6*, *gun2*, and *gun3*, heme was suggested to play a role of a positive signal controlling PhANG expression [173,201]. Heme is required in multiple cellular processes and therefore must be exported from the chloroplast [241]. Analysis of *hmox1* mutant of *C. reinhardtii* demonstrated that biliverdin is also able to affect nuclear gene expression, as it was suggested, to prevent ROS generation during transitions from dark to light [243]. GUN4 is thought to shield Proto from collisions with O_2 and/or to act as a sensor for Proto-generated $^1O_2$ [190]. The *gun5* mutant of *A. thaliana*, as well as *brs-1* and *chl1* of *C. reinhardtii*, all carry defects in the gene encoding CHLH subunit of Mg chelatase, which is also thought to be implicated in the retrograde signaling [172]. In the *flu* mutant of *A. thaliana*, which accumulates PChlide resulting in $^1O_2$ generation in light, EXECUTER 1 (EX1) and EX2 are required for mediating $^1O_2$-triggered signal transduction to the nucleus [214]. In higher plants, phytochromobilin is exported form the chloroplast to function as the chromophore component of the phytochrome (Pr). Transition between Pr (red) and Pfr (far-red), as well as its signaling function are indicated. Any possible transport systems responsible for transferring the signals outside of the chloroplast, depicted by colored ovals in the envelope area, still remain unknown. There is no clear evidence for Proto or Mg-porphyrins involvement in the chloroplast retrograde signaling; therefore, it is not depicted in the Figure. *A. thaliana* mutants, *cs* and *ch42*, with defects in CHLI subunit, as well as *C. reinhardtii chlD* mutant with defect in CHLD subunit of MgCh, are also indicated, although these mutants do not show the *gun* phenotype.

explained by the thorough feed-forward regulation of ALA synthesis in response to deregulated CHLM.

It was recently reported [208] that the NF-treatment results in accumulation of MgProto, with or without ALA feeding, and that it is always accompanied by *LHCB* repression (compare to [206]). Using dimethylthiourea (DMTU) and Tiron as ROS scavengers, the authors obtained only a partial reversion of the NF-induced repression of *LHCB,* which led to the conclusion that the *gun* phenotype cannot be attributed merely to ROS [208]. However, DMTU and Tiron are known scavengers of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$), respectively. Meanwhile, carotenoid deficiency may lead to the energy transfer from the excited chlorophyll in a triplet state (^{3}Chl) to O_2 at the reaction centre of PSII [209], which results in generation of $^1O_2$. Therefore, it is very likely that the main ROS produced in response to NF-treatment is $^1O_2$. In fact, the $^1O_2$ is also the major ROS generated by photosensitizing tetrapyrroles [174,202,210]. Additionally, the reactivity of $O_2^{\bullet-}$ and H_2O_2, unlike $^1O_2$, is not sufficient to oxidize polyunsaturated fatty acids [211] and thus cellular membranes are not vulnerable to direct damage caused by these ROS. Therefore, it would be interesting to determine whether reversion of the *LHCB* gene repression following NF-treatment could be observed after application of $^1O_2$-specific scavengers, such as diphenylamine.

The most recent report [131] also questions the involvement of tetrapyrrole intermediates in chloroplast retrograde signaling. In this study, an inducible RNAi system was applied to examine the immediate

effect of independently induced silencing of *CHLH*, *CHLM*, and *CHL27* on the nuclear gene expression. This approach allowed to eliminate the pleiotropic effects or interference of other signaling pathways. The silencing of *CHLH*, *CHLM*, and *CHL27* led to the reduced activity of MgCh, MgProto methyltransferase, and MgProtoME cyclase, respectively, and caused changes in the content of tetrapyrrole intermediates [131]. No instantaneous effect on the chlorophyll content was detected, indicating that the photosynthesis and other physiological processes were functional. Using a transcriptomic approach, it was demonstrated that no effect on the nuclear gene expression could be observed as the sole consequence of the modified TBS [131]. In the same study, a long-term deactivated expression of *CHL27* led to the altered expression of PhAN and ROS-stress responsive genes, while silencing of *CHLH* and *CHLM* caused a slight elevation in expression of PhAN and TBS genes [131]. It was concluded that any change in nuclear gene expression examined in this study can be observed only in a long-term process and represents a set of a secondary, likely photooxidative effects, which do not directly correlate with the deactivation of the given enzymatic steps [131].

The hypothesized role of Mg-porphyrins as the signaling molecules generates the question about the possible way of their transport across the chloroplast membranes (Fig. 3). With this notion however, the risk of the phototoxicity remains, but is not anymore confined to the chloroplast. Therefore, it is very unlikely that the retrograde signaling involving tetrapyrrole intermediates could be based on porphyrins per se as the molecules passing the information to the nucleus. Additionally, the actual evidence for possible transport of Proto or Mg-porphyrins across the chloroplast envelope is still lacking.

7.5. Alternatives to Mg-porphyrins

In the light of the increasing evidence that porphyrins and Mg-porphyrins do not act as the signaling molecules, it is worth to consider other possibilities. Due to their physicochemical characteristics, unbound chlorophyll and its accumulating intermediates may lead to the photooxidative stress [212]. Metabolites of the TBS pathway, downstream from uroporphyrinogen, become gradually more hydrophobic and photoreactive, capable to undergo intersystem crossing to the triplet state and interact with triplet molecular oxygen to produce $^1O_2$ [213].

The conditional *flu* mutant of *A. thaliana* became one of the best studied cases of the ROS-triggered retrograde signaling. It was demonstrated that the expression of several genes was modulated after shifting *flu* seedlings from dark to light. It is widely accepted that the signaling in *flu* is based on the generated $^1O_2$, mediated by the chloroplast-localized EXECUTER1 (EX1) and EXECUTER2 (EX2) proteins [214], but not by the accumulating PChlide itself (Fig. 3). Ultimately, it is plausible that not the porphyrins themselves, but $^1O_2$ and possibly also other ROS, generated by the photosensitizing TBS intermediates, are responsible for triggering the retrograde signaling. It is also possible that additional signals, considered to be originating from TBS, converge on a common pathway. Because of the short half-life of $^1O_2$ [215], and thus a short distance it may travel in the cell [216], but also because of the high reactivity with membrane lipids [217,218], it is likely that $^1O_2$ itself acts as an emitter rather than a signaling molecule.

Three different mechanisms were proposed to mediate in the signaling pathways triggered by $^1O_2$: (i) direct oxidation of the true signaling component, (ii) oxidation products reacting with such molecules, and (iii) imbalanced oxidation state of the chloroplast, including higher proportion of the oxidized signaling components [219]. Some results indicate that $^1O_2$-signaling might be mediated by oxidation of the membrane lipids [220,221]. Additionally, spatial separation of processes generating ROS, which in turn affect other components of the cell on-site, might be triggering the true signaling molecules. This would ensure specificity of the signal in terms of the transcriptional response, as it was suggested for the GUN4 protein in *C. reinhardtii* [190] (Fig. 3). In summary, despite identification of the several components possibly involved in the chloroplast retrograde signaling, whether it was proposed to originate from the TBS or from any of the other proposed pathway, the true signaling molecule(s) passing the information from the chloroplast to the nucleus still remains elusive.

7.6. Ferrochelatase and heme

Ferrochelatase (FC) catalyzes the ultimate step of heme biosynthesis by inserting a ferrous ion into Proto. Plants possess two FC genes, which follow a diverse tissue- and development-dependent expression pattern. It was proposed that *FERROCHELATASE 1 (FC1)* is constitutively expressed at a low level, but dominantly in root tissue. *FC1* is induced upon stress and adverse environmental conditions, while *FC2* is the dominant gene in above-ground tissue and serves for heme-containing enzymes in photosynthetically-active cells [222–224]. It was proposed that heme synthesized specifically by an increased activity of FC1 but not FC2, modulates nuclear gene expression by retrograde signaling from the chloroplast to the nucleus [173] (Fig. 3). Although the mitochondrial localization of ferrochelatase has been recently demonstrated in red algae *Cyanidioschyzon merolae* [225], according to the present knowledge, in higher plants both FC isoforms localize to the chloroplast [173,226,227], which questions former findings of FC mitochondrial activity [228–230].

Heme is an indispensable cofactor of multiple proteins acting in primary metabolism, e.g. the redox-reactions of electron transport chains, detoxification of xenobiotics (P450 enzymes) and oxidants (catalase, peroxidase), as well as regulatory factors in expression and signaling. It was demonstrated and widely accepted that heme is implicated in regulation of the transcription factors and the signal transducers in non-photosynthetic prokaryotic and eukaryotic organisms, e.g. IRON REGULATORY PROTEIN (IRR) in *Bradyrhizobium japonicum* [231], HEME ACTIVATOR PROTEIN (HAP1) in *Saccharomyces cerevisiae* [232], or transcriptional repressor BACH1 [233] and RAS-MAPK signaling pathway [234] in mammalian cells. Heme functions as a signaling molecule in these organisms have been reviewed elsewhere, e.g. in [235]. These findings are also important for investigation of the heme involvement in plastid retrograde signaling in photosynthetic organism, because they might be indicative of the evolutionary conserved function of this tetrapyrrole.

An indication of heme involvement in chloroplast retrograde signaling came from the analysis of the *gun6* mutant of *A. thaliana*, a gain-of-function mutant overexpressing *FC1* (Fig. 3). The *gun6* mutant displays an enhanced flux through the heme branch of TBS, which correlates with an increased expression of PhANGs [173]. While the NF-treated wild-type seedlings showed a decrease in expression of typical PhANG representatives, in *gun6* lower repression of the PhANG expression was observed, e.g. of genes encoding carbonic anhydrase, chloroplast protein 12, LHCB, plastocyanin and the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase [173]. Interestingly, it was demonstrated that only FC1 but not FC2 activity has such effect on PhANG expression when chloroplast development was blocked, suggesting specificity of the response to the pool of heme produced by FC1 [173] (Fig. 3).

Similarly to other tetrapyrroles, unbound heme may also act photodynamically and generate ROS, mainly in association with lipid membranes, which results in changes in their permeability, as indicated by the more thorough studies in the animal systems [236–238]. Additionally, Fe^{2+} released during heme degradation may act as the catalyst in Fenton-type or in Haber-Weiss reactions with $O_2^{\bullet-}$ or H_2O_2, leading to the production of highly toxic hydroxyl radicals ($OH^{\bullet}$) [239]. Nonetheless, exogenously applied heme does not repress the *LHCB* expression, unlike it was suggested for MgProto [193]. Microarray analysis showed a transient change in the expression of almost 1000 genes upon heme and MgProto feeding, although not followed by changes in the level of the corresponding proteins [240]. Expression of only a few PhANGs

was altered in response to exogenous heme or MgProto, and thus it was concluded that these tetrapyrroles have rather a more general effect on the nuclear gene expression [240]. Surprisingly, no changes in *LHC* genes expression was observed upon feeding with either of these tetrapyrroles [240].

Nevertheless, heme seems to be a better candidate than Proto or Mg-porphyrins for a molecule playing the signaling role, mainly because of the documented export from the chloroplasts [241,242] (Fig. 3) and the obvious need of heme in all cellular compartments. Thus, a model was proposed in which heme is exported from the healthy chloroplast to up-regulate PhANGs expression, although the mechanisms for these processes remain unknown [173] (Fig. 3).

Linear heme catabolites, biliverdin and/or phycocyanobilin, have been also proposed to play a role in the chloroplast retrograde signaling [243]. The analysis of the heme oxygenase mutant *hmox1* of *C. reinhardtii* (Fig. 3) showed that bilin metabolites might be responsible for triggering mechanisms preventing ROS generation during transition from dark to light [243]. The mechanism involves induction of the expression of a specific subset of genes, while suppressing expression of PhANGs [243]. Thus, because according to the present knowledge *C. reinhardtii* does not possess phytochromes, or any bilin-based photoreceptors, these linear tetrapyrroles seem to constitute a light-independent signaling pathway, also capable of exerting changes on nuclear gene expression (Fig. 3).

8. Future perspectives

In a few final comments we would like to pinpoint some perspectives for promising topics, which will likely be addressed in the future and will provide advancements in forthcoming research. Activation, enduring activity and stability of TBS enzymes were found to depend on additional post-translational mechanism. Due to new technologies with high throughput or high resolution and the academic demands to include multifaceted aspects of regulation and metabolism in future studies, the research will continue to explore the multiple posttranslational modifications of enzymes in TBS and mechanisms governing the supply of end-products for photosynthesis and heme-requiring proteins and reactions.

Also, new insights are expected with regards to the transcriptional control, with identification of new transcription factors controlling genes of TBS, while the regulatory mechanism of recently described transcription factors will be unraveled [73,80]. These future investigations will help to narrow the gaps in our understanding of environmental and endogenous mechanisms of the TBS control at the transcriptional level. Other regulatory mechanisms, such as the circadian clock or plastid-derived retrograde signals have often emphasized how deep TBS is embedded in the regulatory network. Seedlings, either at the early stages of development, or etiolated, are often most suitable for the experiments designed to decipher the multiple aspects of the transcriptional control of specific genes involved in TBS, under control of specific hormonal signaling pathways.

Studies of the TBS in other organisms may also provide interesting results. *C. reinhardtii* seems to be a good candidate, mainly because of the ability to grow heterotrophically. Therefore, even mutants with a complete chlorophyll deficiency and accumulating high amounts of TBS intermediates can be maintained in darkness and studied. Thus, *C. reinhardtii* may provide answers to several crucial aspects of TBS and related processes, e.g. unknown enzymatic subunits and regulatory proteins, transcriptional-, translational- and posttranslational regulation, regulatory connection to TBS with the assembly of chlorophyll in the photosynthetic apparatus, and mechanisms behind apparent TBS-derived retrograde signaling. However, several differences in biochemistry and regulation are expected between *C. reinhardtii* and plants, e.g. regulatory functions of the homologs of FLU/FLP and GUN4 seem to differ in plants and green algae due to the apparent lack of the instantaneous down-regulation of ALA synthesis in case of impaired metabolic flow in the Mg branch [14,156,190,244]. Additionally, there are differences in homologous gene copy numbers for enzymatic steps between model algae and plants, while other differences in TBS may be revealed because of the ability of *C. reinhardtii* to produce chlorophyll in the dark. On the other hand, several aspects of TBS biochemistry and regulation are similar, which often makes the knowledge gained on studies on *C. reinhardtii* and model plants complementary.

Identification of transporters for heme, phytochromobilin, protoporphyrinogen or other intermediates will be important to better understand the retrograde signaling. Even more essential will be to describe the distribution of tetrapyrrole metabolites to all cellular compartments and organelles. It will be of a great interest to determine whether ABC transporters identified in yeast and animals, have their homologues in plants responsible for heme and porphyrin transport. The need for heme transport from plastids into the cytoplasm and the cellular organelles is obvious, because of the multiple heme-dependent proteins in subcellular compartments. Identification of the porphyrin transporters may help to confirm or refute the presence of the second heme synthesis pathway in mitochondria.

Meanwhile, all enzymatic steps are being thoroughly analyzed. However, many questions still remain unanswered, e.g. the MgProtoME cyclase reaction is still intriguing, because it is expected that this enzymatic step requires additional subunits, which are necessary to reconstitute an *in vitro* cyclase reaction. While chlorophyll degradation has been intensively investigated and many substantial reports were published over the last twenty years, very little is known about the fate of heme degradation products, downstream from biliverdin in plants.

A few exciting highlights were recently reported, where the crosstalk and the regulatory interactions between connected metabolic pathways have been addressed [245]. It is expected that more details of the regulatory linkage between the methylerythritol phosphate (MEP) biosynthesis pathway, the terpenoid synthesizing pathway, and the carotenoid synthesis will be discovered at the transcriptional and post-translational level. These regulatory interactions probably exist through still unknown retrograde signaling mechanisms, and are responsible for transcriptional control of genes contributing to the interrelated metabolic pathways.

More recently, genetic and biochemical approaches helped to explore the important interrelation between chlorophyll synthesis and the integration and assembly of chlorophyll to the chlorophyll binding proteins. It is a great scientific contribution, when auxiliary factors, such as LIL3 or OHP/HLIPs (one-helix-protein/high-light-induced proteins), or other chaperones, are identified to transfer chlorophyll to the assembly sites of PSI and PSII and their antenna complexes. Moreover, it will be also challenging to unravel the process of co-translational integration of plastid-encoded chlorophyll-binding proteins, such as D1 into thylakoid membranes, because it is very likely that this mechanism requires simultaneous integration and assembly of pigments. Assembly factors will be required for these processes and it is expected that the future research on the biogenesis of the assembly of photosynthetic units will make a substantial contribution to characterization of the highly regulated and balanced process of chloroplast membrane biogenesis.

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