Running title: Tetrapyrroles as retrograde signals

The role of tetrapyrroles in chloroplast-to-nucleus retrograde signalling

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Abbreviations:

ALA	5-Aminolaevulinic acid
CHLH	MAGNESIUM CHELATASE SUBUNIT H
EX1	EXECUTER 1
FC	FERROCHELATASE
FLU	FLUORESCENT IN BLUE LIGHT
GFP	GREEN FLORESCENT PROTEIN
GLK1	GOLDEN2-LIKE 1
GluTR	Glutamyl-tRNA reductase
GUN	GENOMES UNCOUPLED
HEMA	gene encoding glutamyl-tRNA reductase
HO1	HAEM OXYGENASE 1
HY5	ELONGATED HYPOCOTYL 5
LIN	Lincomycin
lin2	*lesion initiation 2* mutant
LHCB	LIGHT HARVESTING CHLOROPHYLL A/B BINDING PROTEIN
Mg-proto	Mg-protoporphyrin IX
NF	Norflurazon
Pchlide	Protochlorophyllide
Proto	Protoporphyrin IX
PUB4	PLANT U-BOX PROTEIN 4
SIG2	SIGMA FACTOR 2

Abstract:

Chloroplasts contain their own genomes and therefore chloroplast biogenesis requires the co-ordination of both chloroplast and nuclear gene expression. This is achieved by the exchange of signals between the two organelles. The existence of these signals from the chloroplast (chloroplast-to-nucleus retrograde signalling) can be demonstrated by inhibition of chloroplast development through mutation or chemical treatment. This chloroplast damage results in the reduced expression of hundreds of nuclear-encoded genes, including many encoding chloroplast proteins. A classic mutant screen in which nuclear gene expression was retained after chloroplast damage resulted in the isolation of a series of *genomes uncoupled* or *gun* mutants. In five out of six mutants, the mutations resided in components of the tetrapyrrole biosynthesis pathway, resulting in a number of different models for the role of tetrapyrroles as retrograde signals. The current model is that a positive retrograde signal is generated by the activity of the ferrochelatase 1 enzyme suggesting that haem or a product of haem is a signal. The evidence for such a model and the interaction of tetrapyrrole signals with other possible retrograde signals is discussed. In addition, tetrapyrroles can generate singlet oxygen on exposure to light and oxygen and there is accumulating evidence that a tetrapyrrole-derived, singlet oxygen-dependent retrograde signal is important during chloroplast biogenesis and for stress signalling from mature chloroplasts.

Keywords:

Chloroplast (plastid) development, *genomes uncoupled*, gene expression, transcriptional regulation, tetrapyrroles, haem, chlorophyll synthesis, singlet oxygen, retrograde signalling

1. Introduction: Communication between chloroplasts and the nucleus

Plastids are endosymbiotic organelles that have retained their own genome. This genome only encodes 80-250 genes depending on the species (Timmis et al., 2004), a small fraction of the 2000-3000 proteins predicted to be present in one plastid type, the chloroplast, of *Arabidopsis thaliana* (Abdallah et al., 2000). The nucleus therefore controls much of the chloroplast proteome, but all of the major complexes of photosynthesis contain protein components encoded by the chloroplast genome. Therefore, in order to co-ordinate chloroplast development communication is required between chloroplasts and the nucleus (Jarvis & López-Juez, 2013). This communication takes the form of regulation of nuclear-encoded chloroplast protein synthesis and import into chloroplasts to achieve the full complement of proteins required for chloroplast function as well as nuclear control of the expression of chloroplast-encoded proteins. These so-called anterograde signalling pathways enable the co-ordinated expression of both genomes, but require feedback on organelle status for optimal chloroplast function in a process known as retrograde signalling (Chan et al., 2016). Retrograde signalling during the transition from proplastids or etioplasts to chloroplasts (chloroplast biogenesis), the most commonly studied system, is known as biogenic signalling (Pogson et al., 2008). The nature of the signals involved in chloroplast-to-nucleus communication is still unknown, but there is good evidence that tetrapyrroles are involved in this process (Woodson & Chory, 2008; Larkin, 2016). This chapter will explore the evidence for this and provide a model based on the most recent discoveries.

In addition to biogenic signalling, it has also become apparent that chloroplasts have an important role in signalling stress to the rest of the cell and beyond (Chan et al., 2016; Leister & Kleine, 2016; de Souza et al., 2017; Brunkard & Burch-Smith, 2018) something that has been termed operational retrograde signalling (Pogson et al., 2008). Photosynthesis is particularly susceptible and sensitive to environmental stress and the chloroplast fulfils a sentinel role for plant environmental responses. One such operational signal is also tetrapyrrole-related, as singlet oxygen generated by energy transfer from excited chlorophyll molecules at photosystem II as well as by accumulating tetrapyrrole metabolites is important in plant responses to photooxidative stress (Triantaphylidès & Havaux, 2009; Zhang et al., 2014). The role of tetrapyrroles in singlet oxygen signalling and the evidence for singlet oxygen signalling during chloroplast biogenesis will also be discussed.

2. Retrograde signalling during chloroplast biogenesis

In 1979, Bradbeer et al published a landmark paper demonstrating that two barley mutants, *albostrians* and *Saskatoon,* which lacked plastid protein synthesis, resulted in a strong reduction of the nuclear-encoded plastid enzymes, phosphoribulokinase and glyceraldehyde-3-phosphate

dehydrogenase (Bradbeer et al., 1979). The authors concluded that a factor produced by the plastid was required for synthesis of nuclear-encoded plastid proteins. Since that initial study, we have discovered that many other mutants affecting chloroplast function also affect expression of nuclear-encoded proteins as do a number of chemical treatments that target plastids. Mutants lacking proteins involved in plastid transcription, editing, translation or protein import all show this phenotype (see Inaba et al., 2011 for a comprehensive list). The most commonly used chemical treatments to study retrograde signalling are norflurazon (NF), an inhibitor of carotenoid biosynthesis at the phytoene desaturase step that results in photobleached chloroplasts, and lincomycin (LIN), one of a number of plastid translation inhibitors that block plastid development (Gray et al., 2003). We also know that the response is primarily at the level of transcription (or at least transcript abundance). Various microarray studies have shown that hundreds of nuclear genes are down regulated in response to NF (Koussevitzky et al., 2007; Aluru et al., 2009; Page et al., 2017b) or LIN (Koussevitzky et al., 2007; Woodson et al., 2013; Martín et al., 2016) treatment. Many of these genes encode proteins destined for the chloroplast, but not all, and only 34.2% of genes down-regulated after NF treatment were predicted to encode chloroplast-targeted proteins (Page et al., 2017b). Indeed, metabolic processes are affected throughout the cell after chloroplast disruption, illustrating the depth of integration of the chloroplast in cellular function (Aluru et al., 2009). NF or LIN treatments also result in the induction of hundreds of genes (e.g. Koussevitzky et al., 2007; Aluru et al., 2009; Page et al., 2017b) and this probably reflects in large part the consequence of the stress imposed by loss of chloroplast function.

Although retrograde signals cause major changes in transcript abundance, there is also evidence that chloroplast signals can regulate nuclear gene expression post-transcriptionally and post-translationally. For example, the 5' end of the pea plastocyanin-coding *PetE* gene in tobacco is sufficient to confer a post-transcriptional response to NF or LIN treatment (Brown et al., 2005). More recently, post-translational regulation of the transcription factor GOLDEN 2-LIKE1 (GLK1) has been demonstrated (Tokumaru et al., 2017). This transcription factor is a key regulator of photosynthetic development (Waters et al., 2009) and *GLK1* gene expression is transcriptionally down-regulated in response to chloroplast damage (Kakizaki et al., 2009). More significantly perhaps, the GLK1 protein is also degraded via the ubiquitin-proteasome system in response to NF or LIN treatment suggesting it is a critical target for the loss of retrograde signals (Tokumaru et al., 2017).

Although we know quite a lot about the consequences of chloroplast damage or dysfunction, 40 years after the initial discovery of this pathway our understanding of the nature of the signal(s) mediating these changes is still lacking. The early studies that immediately followed the initial discovery of retrograde signalling were consistent with a hypothesis that a (positive) signal produced

by the chloroplast was required for nuclear gene expression (Taylor, 1989). However, as discussed below an inhibitory signal produced by damaged or dysfunctional plastids has also been proposed. It is likely that retrograde signalling during chloroplast biogenesis relies on both positive and inhibitory signals to optimise chloroplast development. These signals may be continuous or intermittent in nature, but it seems probable that communication would be maintained during this process and not initiated only after chloroplast damage.

3. The *genomes uncoupled (gun)* mutant phenotype

What we do know about retrograde signalling results from studies with a different class of mutants affected in this pathway. Susek et al (1993) used a screen in which they took the promoter of the *LIGHT-HARVESTING CHLOROPHYLL-BINDING PROTEIN 1.2* (*LHCB1.2)* gene (still called *CAB3* in the original study), which is strongly inhibited following NF treatment, and linked it to a β-glucuronidase reporter and kanamycin resistance gene. They then screened for mutants that continued to express *LHCB1.2* (and therefore the reporter gene) after NF treatment. These mutants were termed *genomes uncoupled* or *gun* mutants as nuclear gene expression is no longer coupled to chloroplast status. Five *gun* mutants were described initially (Mochizuki et al., 2001; Larkin et al., 2003; Koussevitzky et al., 2007) with a sixth from the same laboratory subsequently (Woodson et al., 2011) and their analysis has provided much of the focus for the field since. One unsubstantiated concern over the *gun* phenotype has been that the *gun* mutants isolated are simply less sensitive to NF treatment and consequently there is less chloroplast damage. However, two recent studies demonstrate that *gun* mutants are more sensitive to NF and that their phenotypes are related to signalling of chloroplast damage and not the amount of damage (Song et al., 2018; Zhao et al., 2018).

As discussed later, GUN1-6 are all chloroplast proteins, but another class of *gun* mutant has also been identified in which mutations in components of light signalling pathways result in elevated nuclear gene expression after NF treatment. Extensive screening for further *gun* mutants using a luciferase-based screen for enhanced *LHCB1.1* expression after NF treatment, identified multiple mutant alleles in the gene encoding the blue-light photoreceptor, cryptochrome 1 (Ruckle et al., 2007). This study also revealed a role for the red light photoreceptor phytochrome B, but only in the absence of GUN1, and the transcription factor ELONGATED HYPOCOTYL 5 (HY5) that has a role in both cryptochrome 1 and phytochrome B regulation of photomorphogenic responses (Ruckle et al., 2007). In this case, HY5 appeared to be functioning in a repressive mode in contrast to its usual role as a positive regulator of light responses. The involvement of these components reflects the strong interaction between retrograde and light signalling that has been observed under various

experimental conditions (Larkin & Ruckle, 2008). Other signalling components that have been proposed to have a role in biogenic retrograde signalling, such as PHD TRANSCRIPTION FACTOR WITH TRANSMEMBRANE DOMAINS 1 (PTM1) and ABSCISIC ACID INSENSITIVE 4 (ABI4) have not stood up to scrutiny (Page et al., 2017a; Kacprzak et al., 2019). Significantly however, overexpression of GLK1, the transcription factor subject to post-translational regulation by chloroplast status (Tokumaru et al., 2017) does cause a *gun* phenotype (Leister & Kleine, 2016; Martín et al., 2016).

4. The majority of *gun* mutations affect genes required for tetrapyrrole synthesis

Of the chloroplast-localized GUN proteins, five were identified as components of the tetrapyrrole biosynthesis pathway (Mochizuki et al., 2001; Larkin et al., 2003; Woodson et al., 2011). The tetrapyrrole pathway in plants leads to the synthesis of a number of key molecules that are essential for life (Tanaka & Tanaka, 2007; Mochizuki et al., 2010). These include cyclic tetrapyrroles such as haems required in photosynthesis, respiration, cytochrome P450s and a number of other important enzymes such as catalase and peroxidases, and chlorophylls *a* and *b* used for light-harvesting in photosynthesis. In addition, sirohaem is the cofactor for nitrite and sulphite reductases, while the linear tetrapyrrole phytochromobilin serves as the chromophore for the phytochrome family of plant photoreceptors (Figure 1). In terms of regulation, the key steps in the pathway are the synthesis of 5-aminolaevulinic acid (ALA), which is rate limiting for all tetrapyrroles (for more information see Chapter 3 of this volume (Vol 91) by Richter and Grimm) and the main branch point between haem and Mg-porphyrin synthesis. At this stage, Fe^{2+} or Mg^{2+} ions are inserted into protoporphyrin IX (Proto) to give haem or Mg-protoporphyrin IX (Mg-proto), respectively. The principal product feedback regulation of the pathway is the inhibition by haem of the first committed enzyme in the pathway glutamyl-tRNA reductase (GluTR; Cornah et al., 2003). This enzyme is a key regulatory target as its substrate $tRNA^{Glu}$ can also be used for plastid protein synthesis and it is subject to strong transcriptional (Ilag et al., 1994; McCormac et al., 2001) and post-translational (Czarnecki et al., 2011; Apitz et al., 2016; Schmied et al., 2018) regulation. There is also evidence for control of ALA synthesis via the Mg-branch and the regulatory protein FLU (Kauss et al., 2012; Hou et al., 2019).

The first three *gun* mutants to be identified were *gun2*, *gun3* and *gun5*, caused by mutations in haem oxygenase 1 (HO1), phytochromobilin synthase and the H subunit of Mg-chelatase (CHLH), respectively (Mochizuki et al., 2001). This discovery provided a firm basis for a role for tetrapyrroles in retrograde signalling and based on this data a model of retrograde signalling was proposed in which CHLH sensed flow through the tetrapyrrole pathway to alter expression of nuclear genes such as *LHCB1* (Mochizuki et al., 2001). This was not the first time that mutant analysis had implicated

tetrapyrroles in retrograde signalling. The *hy1* mutant (allelic to *gun2* and lacking HO1) had previously been associated with this pathway following its identification in a screen for mutants that underexpressed *LHCB* genes (López-Juez et al., 1998). Furthermore, a study of the genetic interaction between *hy1* and other *gun* mutants had already suggested the possibility for a role for tetrapyrroles in retrograde signalling including that there might be both positive and negative signals (Vinti et al., 2000). As discussed later, this idea is still relevant to current thinking on retrograde signalling.

The conclusions from these papers that tetrapyrroles are involved in retrograde signalling was further developed by Strand et al (2003) who proposed that Mg-proto acted as an inhibitory signal. A number of lines of evidence were proposed. Firstly, there was a massive increase in Mg-proto after NF treatment and this was greatly reduced in *gun2* and *gun5*. The rationale for this was that GUN5 (CHLH) is directly required for Mg-proto synthesis by Mg-chelatase. In fact, there is a good correlation between loss of enzyme activity and increase in nuclear gene expression across a range of *chlh* mutants (Ibata et al., 2016). In addition, the loss of GUN2 (HO1) results in haem accumulation via a reduction in haem degradation (Muramoto et al., 2002) and feedback inhibition of the Mg-branch of the tetrapyrrole pathway (Terry & Kendrick, 1999; Terry et al., 2001). The *gun3* mutant would be expected to have a similar effect on haem levels. Secondly, mutants in trunk pathway enzymes (a porphobilinogen deaminase KO and *lin2*, lacking coproporphyrinogen oxidase; see Figure 1) and the D subunit of Mg-chelatase also showed a *gun* phenotype. Finally, direct feeding of Mg-proto, but not other tetrapyrroles resulted in inhibition of *LHCB* expression (Strand et al., 2003).

A role for Mg-proto was initially supported by the identification of GUN4, a new regulator of chlorophyll biosynthesis (Larkin et al., 2003). GUN4 is a soluble protein found in the stroma that can also associate with envelope and thylakoid membranes. It co-purified with Mg-chelatase subunits and was shown to bind to both the product (Mg-proto) and substrate (Proto) of this enzyme to promote its activity (Larkin et al., 2003; Davison et al., 2005; Adhikari et al., 2011). The loss of GUN4 activity would be predicted to reduce the accumulation of Mg-proto and it was proposed that this formed the basis of the *gun4* phenotype (Larkin et al., 2003). Consistent with this interpretation, the *chlI1chlI2* double mutant also shows a *gun* phenotype (Strand et al., 2003; Huang & Li, 2009).

As a porphyrin, Mg-proto might not appear to be an ideal mobile signalling molecule since it is highly light sensitive and like all porphyrins and chlorins generates singlet oxygen in the presence of molecular oxygen (Telfer, 2014). This and other concerns such as the lack of Mg-proto accumulation after NF incubation in Mg-chelatase mutants of barley (Gadjieva et al., 2005) led to a critical re-evaluation of the Mg-proto hypothesis. In particular, two papers, published together, failed to

reproduce the observation that Mg-proto accumulated to high concentrations after NF treatment (Mochizuki et al., 2008; Moulin et al., 2008). In fact, Mg-proto levels were extremely low (Mochizuki et al., 2008; Moulin et al., 2008), consistent with a strong inhibition of tetrapyrrole biosynthesis genes after NF treatment (Moulin et al., 2008). There was also no correlation between the levels of Mg-proto and *LHCB1.2* expression or other retrograde signalling-regulated genes when tetrapyrrole biosynthesis was manipulated either chemically or genetically (Mochizuki et al., 2008; Moulin et al., 2008). Specifically, the *chlm* and *crd1* mutants, that block the Mg-proto methyltransferase and Mg-proto monomethyl ester cyclase steps after Mg-proto synthesis, accumulated large quantities of Mg-proto and its methyl ester, but showed no *gun* phenotype (Mochizuki et al., 2008). The origin of these discrepancies is not known and in particular, the very high accumulation of Mg-proto after NF treatment reported in Strand et al (2003) is hard to explain. Mochizuki et al (2001) had already stated there was no Mg-proto accumulation after NF treatment and accumulation was also not observed in barley seedlings treated with NF (Gadjieva et al., 2005). The explanation suggested was that the peak attributed to Mg-proto represented a contaminating pigment (Mochizuki et al., 2008; Moulin et al., 2008).

5. The requirement for haem synthesis as a promotive retrograde signal

The most significant development in our understanding of the contribution of tetrapyrroles to retrograde signalling came from the characterization of the *gun6-1D* mutant isolated using activation-tagging mutagenesis (Woodson et al., 2011). The dominant *gun6-1D* mutant results from a T-DNA insertion located 8 kb from the gene encoding ferrochelatase 1 (*FC1*) that causes an approximately three-fold induction in *FC1* expression and plastid ferrochelatase activity (Woodson et al., 2011). However, it was not ferrochelatase activity *per se* that caused the *gun* phenotype as overexpression of FC1, but not FC2 resulted in elevated nuclear gene expression after NF treatment. Furthermore, FC1 activity was required, as blocking synthesis of tetrapyrrole precursors blocked the *gun* phenotype caused by FC1 overexpression, as did treatment with the iron chelator dipyridyl that inhibits ferrochelatase activity (Woodson et al., 2011). Dipyridyl is also known to increase Mg-porphyrins (Terry & Kendrick, 1999) through inhibition of Mg-proto monomethyl ester cyclase providing further evidence that Mg-proto is not an inhibitory signal. When tetrapyrrole production was genetically inhibited in the trunk pathway leading to haem via mutations in *hema1*, *hema2* or *lin2* (see Figure 1), no *gun* phenotype was observed (Woodson et al., 2011). The *lin2* result was in contrast to that observed previously (Strand et al., 2003).

The model proposed was that haem or a product of haem synthesized by FC1 was a positive signal promoting expression of nuclear-encoded chloroplast proteins (Woodson et al., 2011). The model

was consistent with the expected consequences of the other *gun* mutants, such as *gun2* and *gun3*-inhibiting haem degradation and mutations in GUN4 and GUN5 blocking the Mg-branch of the pathway, thus allowing Proto to be re-directed to FC1 (see Figure 1). This model is plausible for a number of reasons. Firstly, haem and its bilin derivatives are less photoactive than Mg-porphyrins and therefore make more suitable signalling molecules. Secondly, there is ample precedent for haem as a signalling molecule controlling gene expression in prokaryotes and eukaryotes (discussed in Terry & Smith, 2013). This includes photosynthetic organisms where both haem (von Gromoff et al., 2008) and bilins (Duanmu et al., 2013) have been implicated as retrograde signals in the green alga *Chlamydomonas reinhardtii*. As an aside, the role of tetrapyrroles as signals in algal systems may be quite varied as Mg-proto has also been implicated in retrograde signalling in *Chlamydomonas* (Voß et al., 2011) and in co-ordinating DNA replication in the red alga *Cyanidioschyzon merolae* (Kobayashi et al., 2011). Thirdly, chloroplasts provide all of the tetrapyrroles required by plant cells including for the numerous haem-binding proteins located throughout the cell (Mochizuki et al., 2010). Haem export from the chloroplast has been demonstrated previously (Thomas & Weinstein, 1990) as has export of linear tetrapyrroles derived from haem such as phytochromobilin, the phytochrome chromophore (Terry & Lagarias, 1991). It is unlikely, however, that phytochromobilin itself is the signalling molecule as its synthesis is blocked in *gun2* and *gun3*. Fourthly, FC1 is already associated with providing this non-photosynthetic haem (Nagai et al., 2007). Interestingly, *HEMA2* shows similar expression patterns to *FC1* (Ujwal et al., 2002; Nagai et al., 2007) suggesting it too is responsible for non-photosynthetic tetrapyrrole production. Expression of *FC1* and *HEMA2* (as well as *PROTOPORPHYRINOGEN OXIDASE 2* (*PPO2*) and *UROPORPHYRIN III METHYLASE 1* (*UPM1*) is actually elevated after NF treatment in contrast to the strong downregulation of the rest of the pathway (Moulin et al., 2008). This is likely to be a stress-induced response (Nagai et al., 2007). The observation that HEMA2 overexpressing lines also show a *gun* phenotype (Woodson et al., 2011) supports the idea that the synthesis of haem destined to be exported from the plastid is the primary positive retrograde signal. Interestingly, longstanding ideas around a separate route to non-photosynthetic haem have had some support from studies on post-translational regulation of ALA synthesis (Czarnecki et al., 2011).

In a subsequent study, a reduction in $tRNA^{Glu}$ synthesis in the *Arabidopsis sig2* mutant lacking SIGMA FACTOR 2 also led to lower levels of haem (and of other tetrapyrroles) and reduced expression of a number of nuclear genes including *LHCB* (Woodson et al., 2013). In this system feeding of 5-aminolevulinic acid (ALA), the *gun5* mutation or FC1 overexpression all increased haem levels (though not directly tested for FC1 overexpression) and promoted *LHCB* expression (Woodson et al., 2013). However, although the hypothesis that haem is the primary positive regulator is gaining

traction, not all data agree with this model. In particular, where measured, haem levels cannot account for the retrograde signalling phenotypes (Voigt et al., 2010; Espinas et al., 2012). This may reflect the difficulty in measuring regulatory haem pools against a background of the bulk haem synthesized for photosynthesis and other cellular processes. We are a long way from being able to distinguish haem synthesized by FC1 and FC2. In another study, it was observed that ALA synthesis rates were reduced in *gun* mutants (consistent with elevated haem levels), but that, in disagreement with Woodson et al (2011), chemical inhibition of ALA synthesis enhanced expression of nuclear gene (Czarnecki et al., 2012). Resolving these discrepancies will be key to understanding how tetrapyrroles contribute to retrograde signalling.

6. Does GUN1 also regulate tetrapyrrole synthesis?

Since most of the chloroplast-localized GUN proteins affect tetrapyrrole biosynthesis, one obvious question to ask is whether GUN1 could also act via a tetrapyrrole-mediated pathway. This is an especially pertinent question as the *gun1* mutant probably exhibits the strongest *gun* phenotype in many studies (Vinti et al., 2000; McCormac & Terry, 2004). Since GUN1 was first described (Koussevitzky et al., 2007) progress on understanding its mode of action has been slow with attention initially on whether GUN1 exhibited nucleotide-binding activity typical of pentatricopeptide repeat proteins. Most recently, attention has focused on three different models for GUN1 function. It has been proposed to have role in protein import with retrograde signalling mediated by precursor proteins of unimported chloroplast proteins (Wu et al., 2019). Alternatively, it may function in protein homeostasis (Colombo et al., 2016). Finally, it is proposed to function in chloroplast gene editing (Zhao et al., 2019). It should be remembered that the *gun1* mutant can rescue nuclear gene expression following both Lin and NF treatments in contrast to *guns2-6* that can only rescue after NF (Koussevitzky et al., 2007). This suggests that GUN1 operates in a different pathway to GUN2-6 that may not be related to the tetrapyrrole pathway. This idea is supported in some genetic studies (Vinti et al., 2000; Mochizuki et al., 2001; McCormac & Terry, 2004), but comparison of transcriptional profiles of GUN1-dependent and GUN5-dependent retrograde signalling suggested they reside on the same pathway (Koussevitzky et al., 2007). If the GUN5-dependent pathway is tetrapyrrole related it might therefore be expected that GUN1 also affects this pathway and there is some evidence to support this idea. *HEMA1* expression is elevated in darkness in a *gun1* mutant (McCormac & Terry, 2004) resulting in elevated protochlorophyllide (Pchlide) synthesis (Xu et al., 2016) and presumably providing the explanation for the increased sensitivity of *gun1* to a far-red block of greening treatment (McCormac & Terry, 2004; Page et al., 2017b) and the seedling greening phenotype initially observed by (Mochizuki et al., 1996). Consistent with this possibility, GUN1 has been shown to interact with a number of tetrapyrrole

enzymes (Tadini et al., 2016). Although rather promiscuous in its protein interactions, in pulldown assays using a GUN1-GFP expression line, CHLD, porphobilinogen deaminase (PBGD), uroporphyrinogen III decarboxylase (UROD2) and FC1 were all shown to interact with GUN1 in both yeast 2-hybrid assays and when using bimolecular fluorescence complementation (Tadini et al., 2016). There was some specificity in these results as a comprehensive list of other tetrapyrrole proteins, including GUN2-5, FC2 and CHLI1 and CHLI2, showed no interactions (Tadini et al., 2016). Very recently, more direct evidence has been presented to show that GUN1 affects tetrapyrrole synthesis (Shimizu et al., 2019). GUN1 was shown to be able to bind to both haem and Zn-proto and to directly affect FC1 activity. Physiological experiments also demonstrated that GUN1 could repress tetrapyrrole synthesis even after ALA feeding (Shimizu et al., 2019). The repression of tetrapyrrole synthesis can also explain the observation that the *gun1* mutation could rescue the inhibition of haem synthesis in the *sig2* mutant (Woodson et al., 2013) and thus, potentially, inhibition of gene expression due to reduced haem synthesis. This recent evidence therefore suggests the possibility that all *gun* mutants mediate recovery of nuclear gene expression via a common, tetrapyrrole-related pathway (see Figure 2). A role for GUN1 in modulating tetrapyrrole synthesis would also fit with recent observations showing that the GUN1 protein is rapidly degraded in the light (Wu et al., 2018). This would allow an immediate rise in tetrapyrrole synthesis capacity at exactly the time it would be required.

7. Tetrapyrrole-mediated singlet oxygen signalling

An important property of porphyrins and chlorins, such as chlorophyll, is that they are highly light absorbent. This is of course critical during photosynthesis, where the energy absorbed is passed onto the reaction centres to drive charge separation and ultimately the synthesis of energy rich-compounds. However, for chlorophyll biosynthetic intermediates energy absorption is a problem. Excited porphyrins can transfer their energy to molecular oxygen to produce singlet oxygen, a highly reactive molecule that can cause extensive cellular damage (Apel & Hirt, 2004). Thus, the tetrapyrrole pathway is under extremely tight regulatory control (Brzezowski et al., 2015). The *flu* mutant that lacks the FLU repressor of ALA synthesis exhibits excessive synthesis of unbound Pchlide leading to a burst of singlet oxygen on transfer to light (Meskauskiene et al., 2001). This results in induction of nuclear genes involved in stress responses, such as oxylipin-regulated genes, (op den Camp et al., 2003) and activation of cell death (Danon et al., 2005). Since singlet oxygen has a short half-life and is confined to the chloroplast, the activation of nuclear gene expression is the consequence of a retrograde signalling pathway. Two chloroplast proteins, EXECUTER1 (EX1) and EX2 have been implicated in mediating this response as mutants lacking these proteins show rescue of cell death (Wagner et al., 2004; Lee et al., 2007). The function of EX1 and EX2 remains unknown,

but they do not repress singlet oxygen production (Kim et al., 2012) and EX1 degradation by the FtsH2 metalloprotease is required for signal transduction (Dogra et al., 2017) where it is implicated in photosystem II repair (Wang & Apel, 2018).

As described above, the current hypothesis for retrograde signalling is that haem synthesis acts to promote a positive retrograde signal that in turn promotes the expression of tetrapyrrole biosynthesis genes such as *HEMA1.* One problem with this hypothesis is that there is a risk of over accumulation of tetrapyrrole pathway intermediates and thus severe damage or even cell death. Direct feedback inhibition of GluTR activity by haem (Cornah et al., 2003) may be one way to prevent this. Whether this is the same haem pool as the putative retrograde signal is not known, but both nuclear gene expression and feedback inhibition are promoted in the HO1 mutant *gun2* suggesting at least some co-influence of the two pools. It might also be hypothesized that an inhibitory retrograde signal could exist that inhibits excess production of intermediates. Evidence for such a signal comes from experiments in which singlet oxygen was produced by transferring seedlings pre-treated with far-red light to induce Pchlide accumulation (and inhibit Pchlide oxidoreductase (POR) expression) into white light. Under these conditions a burst of singlet oxygen production results in a rapid down-regulation of the expression of nuclear genes encoding photosynthesis-related proteins (Page et al., 2017b). In particular, the expression of *HEMA1* and *GUN4* genes was reduced within 30 mins with approximately half of this response dependent on EX1 and EX2 (Page et al., 2017b). The presence of an additional inhibitory pathway activated by excess porphyrin synthesis (see Figure 2) fits in well with earlier ideas of the control of nuclear gene expression being determined by the balance of the two main tetrapyrrole branches (Vinti et al., 2000).

An alternative regulatory pathway dependent on tetrapyrrole-derived singlet oxygen was identified by examining the consequence of Proto accumulation in the *fc2* mutant of *Arabidopsis* (Woodson et al., 2015). A screen for mutants that were able to green after transfer to white light identified an ubiquitin ligase PUB4 that mediated ubiquitination of chloroplasts, presumably via surface proteins, and their subsequent degradation through a non-autophagic pathway. As the singlet oxygen signalling pathway was unaffected by the loss of EX1, a different damage-limitation signalling pathway is presumably in operation (Woodson et al., 2015). Singlet oxygen is also produced at the reaction centre of photosystem II, in particular under conditions of stress, and contributes to photoinhibition and signalling to the nucleus (Triantaphylidès & Havaux, 2009). This pathway is also independent of the EX proteins and has instead been proposed to be mediated via carotenoid derivatives such as the β-carotene-derived β-cyclocitral (Ramel et al., 2012) and dihydroactinidiolide (Shumbe et al., 2014). Whether these carotenoid breakdown products also contribute to signalling

during de-etiolation is unknown, but carotenoids are present in significant quantities in etiolated seedlings (Park et al., 2002).

8. Conclusion

Over nearly three decades since a role for tetrapyrroles in chloroplast-to-nucleus retrograde signalling was first proposed the evidence to support this hypothesis has been steadily accumulating. Although we still do not have a clear idea of the signalling pathway nor even the nature of the retrograde signal itself, there has been a coalescence of ideas around a role for haem as a positive retrograde signal indicating a requirement for chloroplast proteins. The phenotype of almost all mutants and transgenic lines tested for retrograde signalling are consistent with this hypothesis and the recent observation that GUN1 alters tetrapyrrole metabolism gives us hope that the effects of changes in chloroplast protein synthesis on nuclear gene expression can be reconciled in a single model. Nevertheless, there are a number of major unanswered questions. What is the exact nature of the tetrapyrrole signal? What is the receptor for the signalling molecule? And how is its production controlled? Some of this information will come from more careful analyses of transgenic plants in which haem synthesis is modified, but until we can measure haem more precisely progress may remain slow.

9. References

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Figures

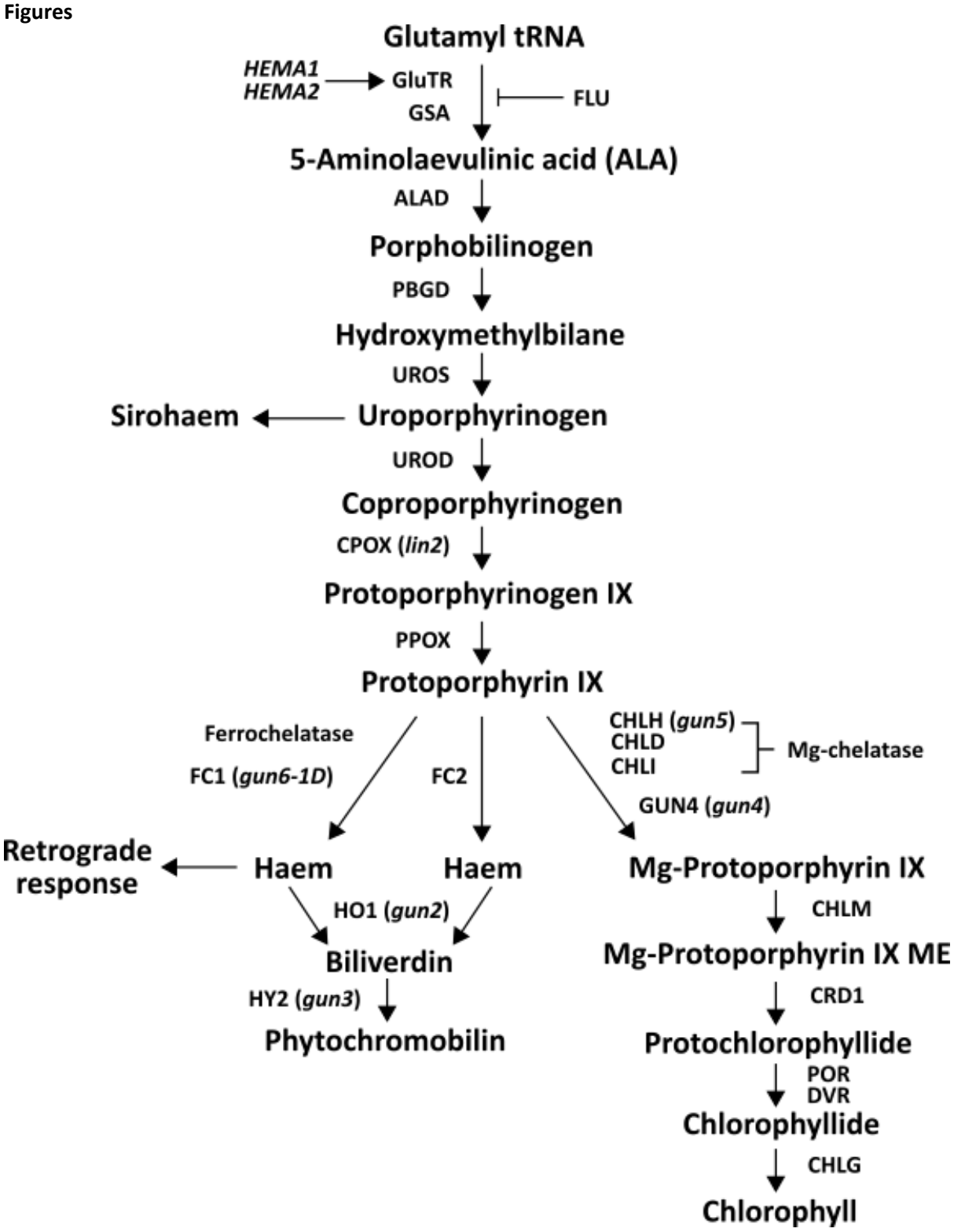

Figure 1: The tetrapyrrole biosynthesis pathway in plants. In this pathway, two separate pools of haem are shown. The first is produced by FC1. Haem from this pool has been proposed to act as a

positive retrograde signal in plants. In contrast, there is currently no evidence that the pool of haem produced by FC2 is involved in a retrograde response. Abbreviations: ALAD, 5-aminolaevulinate dehydratase; CAO, chlorophyllide *a* oxygenase; CHLD, Mg-chelatase D subunit; CHLI, Mg-chelatase I subunit; CHLH, Mg-chelatase H subunit; CHLM, Mg-proto IX methyltransferase; CPOX, coproporphyrinogen III oxidase; CRD1, subunit of Mg-proto IX monomethylester cyclase; DVR, divinyl protochlorophyllide reductase; FC, ferrochelatase; FLU, FLUORESCENT IN BLUE LIGHT; GluTR, glutamyl-tRNA reductase encoded by *HEMA* genes; GSA, glutamate-1-semialdehyde 2,1-aminomutase; *gun, genomes uncoupled*; HO1, haem oxygenase 1; HY2, phytochromobilin synthase; *lin2, lesion initiation 2*; PBGD, porphobilinogen deaminase; POR, NADPH:protochlorophyllide oxidoreductase; PPOX, protoporphyrinogen IX oxidase; UROD, uroporphyrinogen III decarboxylase; UROS, uroporphyrinogen III synthase.

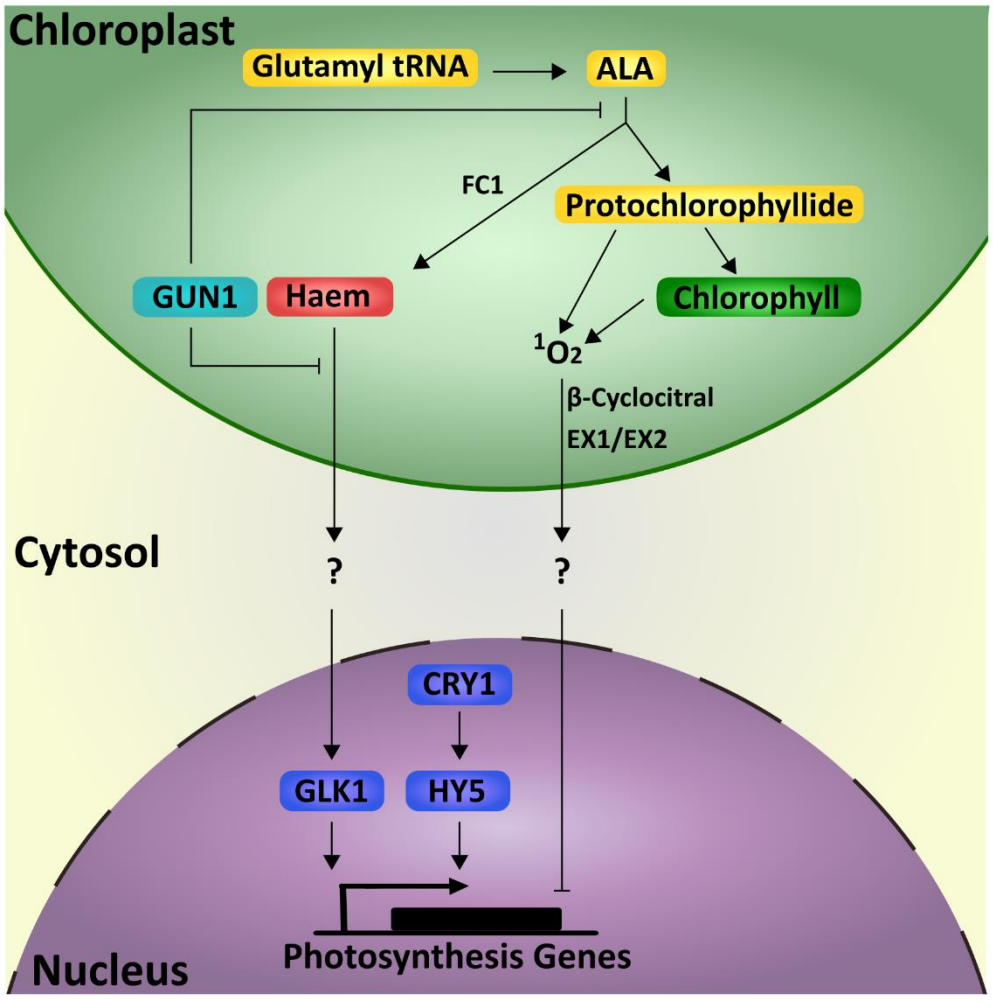

Figure 2: The current model for biogenic chloroplast retrograde signalling in plants. In this model there are two proposed signals. The first is a positive signal that is thought to originate from a pool of haem produced by FERROCHELATASE 1 (FC1). This positive signal is implicated in the promotion of photosynthesis-associated gene expression, although it is unknown if haem itself acts as the messenger, or if there is a secondary messenger that conveys the signal to the nucleus. There is evidence that several nuclear-localised factors may be involved, and act as signalling candidates in this process, these include CRYPTOCHROME 1 (CRY1), ELONGATED HYPOCOTYL 5 (HY5), and GOLDEN2-LIKE1 (GLK1). The second signal is proposed to be inhibitory and originate from the production of the reactive oxygen species singlet oxygen ($^1O_2$). Tetrapyrroles are the source of this signal, as excess porphyrin accumulation can lead to production of $^1O_2$ when exposed to light. Production of $^1O_2$ in the chloroplast is known to lead to a down-regulation of nuclear-encoded photosynthesis-associated genes, in a partly EXECUTER (EX1/EX2) dependent manner. However, $^1O_2$ has a short half-life and is unable to leave the chloroplast and a secondary messenger will be required to transmit the signal from the chloroplast to the nucleus. GENOMES UNCOUPLED 1 (GUN1) is also known to play a role in this pathway, although its function remains largely unknown. One possibility is that GUN1 also exerts its effect on nuclear gene expression via the tetrapyrrole pathway, while others have placed it downstream of the tetrapyrrole synthesis as an integrator of multiple signals.

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