The retrograde signaling protein GUN1 regulates tetrapyrrole biosynthesis

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The biogenesis of the photosynthetic apparatus in developing seedlings requires the assembly of proteins encoded on both nuclear and chloroplast genomes. To coordinate this process there needs to be communication between these organelles, but the retrograde signals by which the chloroplast communicates with the nucleus at this time are still essentially unknown. The *Arabidopsis thaliana genomes uncoupled* (*gun*) mutants, that show elevated nuclear gene expression after chloroplast damage, have formed the basis of our understanding of retrograde signaling. Of the 6 reported *gun* mutations, 5 are in tetrapyrrole biosynthesis proteins and this has led to the development of a model for chloroplast-to-nucleus retrograde signaling in which ferrochelatase 1 (FC1)-dependent heme synthesis generates a positive signal promoting expression of photosynthesis-related genes. However, the molecular consequences of the strongest of the *gun* mutants, *gun1*, are poorly understood, preventing the development of a unifying hypothesis for chloroplast-to-nucleus signaling. Here, we show that GUN1 directly binds to heme and other porphyrins, reduces flux through the tetrapyrrole biosynthesis pathway to limit heme and protochlorophyllide synthesis, and can increase the chelatase activity of FC1. These results raise the possibility that the signaling role of GUN1 may be manifested through changes in tetrapyrrole metabolism, supporting a role for tetrapyrroles as mediators of a single biogenic chloroplast-to-nucleus retrograde signaling pathway.

retrograde signaling | chloroplast | heme | tetrapyrrole | *gun* mutants

Chloroplasts retain their own reduced genome that encodes for protein subunits that are required for the functional assembly of all of the major photosynthetic complexes (1). However, the majority of photosynthetic proteins are encoded by the nuclear genome and imported into the chloroplast. Thus, the efficient development of chloroplasts and the photosynthetic apparatus requires coordination between the nuclear and chloroplast genomes and this is achieved through reciprocal signaling between the organelles. The nucleus controls the expression of many chloroplast proteins, including those responsible for regulation of transcription of the chloroplast genome, via anterograde signaling pathways (1). Information on chloroplast status can in turn regulate nuclear gene expression via retrograde signaling pathways (2). These signaling pathways are termed biogenic retrograde signaling (3) to distinguish them from the extensive role that signals from mature chloroplasts (termed operational retrograde signaling) play in the response of plants to the environment (2, 4). The expression of a large number of nuclear genes encoding chloroplast proteins is dependent on the presence of functional chloroplasts and mutations in genes affecting chloroplast function or treatments with inhibitors such as norflurazon (NF), an inhibitor of the carotenoid biosynthesis enzyme phytoene desaturase, or the plastid translation inhibitor, lincomycin (Lin), result in the strong down-regulation of many photosynthesis-related genes (5–7). The *genomes uncoupled* (*gun*) mutants that have a reduced ability to coordinate this nuclear response to chloroplast status were identified through the retention of *LHCB1.2* expression after an inhibitory NF treatment (8) and have been the basis for retrograde signaling research for the last 25 y. Of the original 5 *gun* mutants described, *gun2* and *gun3* lack a functional heme oxygenase 1 and phytochromobilin synthase (9), *gun4* led to the identification of the Mg-chelatase regulator GUN4 (10), and *gun5* was mutated in the gene encoding the H subunit of Mg-chelatase (*SI Appendix*, Fig. S1) (9). The GUN1 protein, which is a pentatricopeptide repeat (PPR) protein with a small MutS-related (SMR) domain, is also localized in the chloroplast (5).

In addition to these chloroplast-localized proteins, another class of *gun* mutant has been identified which lacks nuclear-localized components of the signaling pathway. Screens for a *gun* mutant phenotype identified multiple alleles of the blue light photoreceptor cryptochrome 1 (11) and also suggested a role for the red light photoreceptor phytochrome B and the transcription

Significance

The signaling pathway between chloroplasts and the nucleus (retrograde signaling) is important for the correct development of the photosynthetic apparatus of plant seedlings. The pathway is still not understood, but the majority of mutants with altered signaling (*gun* mutants) implicate the tetrapyrrole molecule heme in this process. In this article, we have demonstrated that the major retrograde signaling protein GUN1 can bind tetrapyrroles and regulate the flow through the tetrapyrrole biosynthesis pathway. The results support a role for tetrapyrroles in mediating retrograde signaling and open up the opportunity to develop a unifying hypothesis for this pathway that takes account of all identified *gun* mutants.

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factor ELONGATED HYPOCOTYL 5 (HY5) (11). There is a well-established link between light and retrograde signaling (12, 13) and the involvement of these components reflects this. Other signaling components that have been proposed to have a role in biogenic retrograde signaling, such as PHD TRANSCRIPTION FACTOR WITH TRANSMEMBRANE DOMAINS 1 (PTM1) and ABSCISIC ACID INSENSITIVE 4 (ABI4) have not stood up to scrutiny as other groups have not been able to reproduce the *gun* phenotype of the respective mutants (14, 15). However, overexpression of GOLDEN2-LIKE1, a regulator of chloroplast development (16), does cause a *gun* phenotype (13, 17).

The analysis of *gun2-gun5* resulted initially in the hypothesis that Mg-protoporphyrin (MgProto) is a mobile retrograde signal between the chloroplast and the nucleus (18), but this hypothesis was not supported in further studies in which no correlation was observed between MgProto levels and *LHCB* gene expression (19–21). Instead, the identification of a dominant *gun6* mutant with increased ferrochelatase 1 (FC1) activity (22) led to the proposal that heme synthesized by FC1 is either the signal itself or a precursor of the signal. However, very little progress has been made in further elucidating the signaling mechanism or in establishing whether this is the only biogenic retrograde signal. One barrier to tackling this problem is that the function of the GUN1 protein has remained elusive. GUN1 has been suggested to act independently from the tetrapyrrole-mediated GUN signaling pathway as, in contrast to *gun2-6* mutants, it can also prevent down-regulation of nuclear gene expression after treatment with Lin, an inhibitor of plastid translation (5). A number of contrasting hypotheses have been put forward for the direct role of GUN1, but a common theme is emerging in which plastid protein homeostasis is perturbed (23–26). One proposed role of GUN1 is the regulation of RNA editing in the chloroplast where it interacts with MORF2 to alter transcript maturation for a number of transcripts including *rpoB* and *rpoC1* that encode subunits of the plastid-encoded RNA polymerase (27). Another interacting protein is FUG1, the chloroplast translation initiation factor IF-2, and genetic evidence supports a role for GUN1 as a modulator of plastid protein homeostasis (28). A third direct role proposed recently is in regulating protein import into chloroplasts (29). In this study, GUN1 was shown to interact with the chloroplast chaperone HSC70-1 to promote import of nuclear-encoded chloroplast proteins. When GUN1 is absent, accumulation of preproteins in the cytosol triggers a *gun* phenotype in an HSP90-dependent manner (29). One set of proteins for which import is affected by *gun1* are tetrapyrrole synthesis proteins. The import of glutamyl tRNA reductase (GluTR), the rate-limiting enzyme of aminolevulinic acid (ALA) and tetrapyrrole synthesis (*SI Appendix*, Fig. S1), the GluTR regulatory protein GBP (30), and CHL27, a subunit of the Mg-protoporphyrin monomethyl ester cyclase, were all reduced in the *gun1* mutant after NF or Lin treatment (29). GUN1 has also been reported to interact with tetrapyrrole synthesis proteins. Four tetrapyrrole enzymes were identified by Tadini et al. (24) as interacting with GUN1 in yeast 2-hybrid studies: the D subunit of Mg-chelatase (CHLD), porphobilinogen deaminase (PBGD), uroporphyrinogen III decarboxylase (UROD2), and ferrochelatase I (FC1) (*SI Appendix*, Fig. S1), and these interactions were corroborated in BiFC assays using tobacco-leaf mesophyll cells (24).

Given the importance of tetrapyrrole synthesis in GUN1-mediated retrograde signaling and the observation that GUN1 interacts with and prevents the import of tetrapyrrole proteins, we have tested the impact of GUN1 on tetrapyrrole biosynthesis in *Arabidopsis thaliana*. Here, we show that GUN1 directly binds to heme and other porphyrins, affects flux through the tetrapyrrole biosynthesis pathway, and can increase FC1 activity. These results raise the possibility that the signaling role of GUN1 may be manifested through changes in tetrapyrrole metabolism and support a role for tetrapyrroles as mediators of a single biogenic chloroplast-to-nucleus retrograde signaling pathway.

Results

GUN1 Levels Affect Tetrapyrrole Metabolism. To explore the interaction of GUN1 with tetrapyrrole biosynthesis, we first tested whether GUN1 could alter the flow through the tetrapyrrole pathway (31). We performed these experiments using dark-grown seedlings as GUN1 is rapidly degraded in white light (26) and also because we hoped that the absence of the significant changes in tetrapyrrole flow in white light would permit us to observe any GUN1-dependent changes. Fig. 1*A* shows that feeding the precursor ALA to two *gun1* mutant alleles in the dark resulted in increased accumulation of protochlorophyllide (Pchlide) compared to wild type (WT), while seedlings overexpressing GUN1 (24) had reduced accumulation of Pchlide (Fig. 1*A*). Small differences were also observed in the absence of added ALA (*SI Appendix*, Fig. S2*B*) consistent with previous observations of increases in Pchlide synthesis in a *gun1* mutant (32). To verify that this was a consequence of changes in flow rate dependent on GUN1 we incubated isolated seedlings in 0.5 mM ALA and followed Pchlide accumulation over 24 h (Fig. 1*B*). These results demonstrate that even over the first 12 h of Pchlide accumulation there is a significant increase in Pchlide in *gun1* mutants with GUN1 overexpressing lines accumulating significantly less Pchlide. Similar results were seen following incubation with 0.2 mM ALA, although the extent of the differences was reduced (*SI Appendix*, Fig. S2*C*). Interestingly, when fluorescence was measured in samples excited at 405 nm that would preferentially excite protoporphyrin IX (Proto IX) (33), the reduction in fluorescence in the GUN1 overexpressing lines was lost with even higher expression than WT after feeding with 0.5 mM ALA (*SI Appendix*, Fig. S2*C*). These data suggest that Proto IX accumulates following overexpression of GUN1.

As ALA synthesis is the rate-limiting step in the pathway, one possibility to explain the altered Pchlide levels after ALA feeding is that GUN1 is leading to a redistribution of tetrapyrrole to the heme branch. However, analysis of heme levels in dark-grown seedlings showed that *gun1-1* had more heme and GUN1ox lines had less heme than WT (Fig. 1*C*), indicating that GUN1 is impacting on flux through both branches of the pathway. Such a conclusion is consistent with the observation that *gun1* can rescue the reduction of heme caused by the *sig2* mutation (7). As overexpression of GUN1 resulted in reduced heme synthesis and heme is proposed to promote nuclear gene expression, we hypothesized that GUN1ox lines might show a stronger inhibition of gene expression after NF treatment. As shown in Fig. 1*D*, both GUN1ox lines showed a significantly stronger inhibitory response to NF for *HEMA1* and *LHCB2.1* expression than WT seedlings, while, as expected, *gun1* rescued gene expression very strongly. Together these results show that altering GUN1 levels affects tetrapyrrole metabolism and that these changes correlate with changes in nuclear gene expression.

GUN1 Stimulates Zn-Chelatase Activity by Enhancing Substrate Affinity for FC1. Although two FC isoforms are present in the *Arabidopsis* genome, GUN1 interaction was specific to FC1 (24) and since an increase in FC1 activity is associated with increased nuclear gene expression in the *gun6* mutant (22), we hypothesized that GUN1 may affect FC1 activity. To test this possibility, we attempted to express both GUN1 and FC1, but failed to express full-length GUN1 protein (*SI Appendix*, Fig. S3 *A* and *B*). Prediction of the secondary and tertiary structure of GUN1 suggested a highly disordered domain in the N-terminal region containing 231 amino acids (*SI Appendix*, Fig. S4), which may destabilize the GUN1 protein. Removal of this N-terminal domain allowed us to obtain GUN1 protein containing PPR and SMR motifs (PS) that corresponds to amino acids 232 to 918 (GUN1-PS; *SI Appendix*,

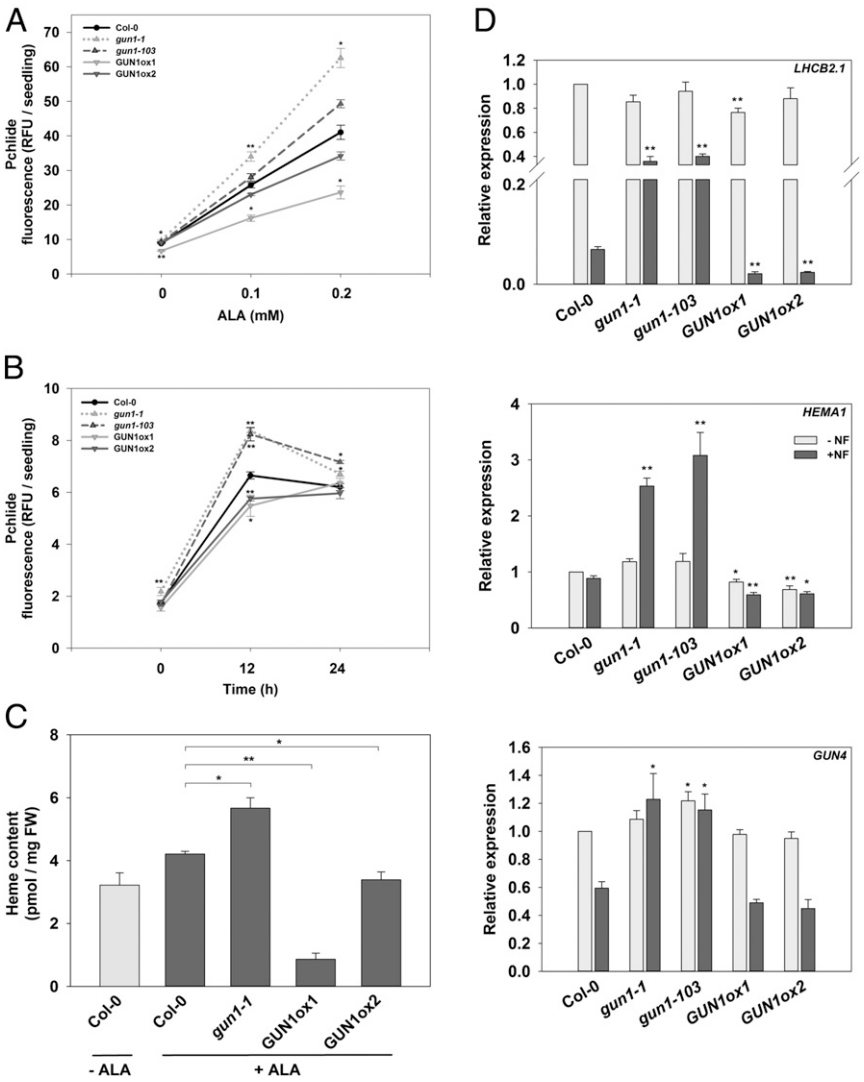

Fig. 1. GUN1 affects tetrapyrrole metabolism. (*A*) Protochlorophyllide accumulation in WT (Col-0), *gun1-1*, *gun1-103* mutants and GUN1ox1 and GUN1ox2 overexpressor lines grown 4 d in the dark with or without 0.1 to 0.2 mM ALA. Thirty seedlings were analyzed for each replicate and fluorescence is shown as relative fluorescence units (RFU). (*B*) Protochlorophyllide accumulation in 4-d-old dark-grown WT (Col-0), *gun1-1*, *gun1-103* mutants and GUN1ox1 and GUN1ox2 overexpressor seedlings incubated in 0.5 mM ALA in the dark for 24 h. Thirty seedlings were analyzed for each replicate. (*C*) Total heme accumulation in seedlings treated with or without 0.2 mM ALA, as described in *A*, was measured as picomole per milligram fresh weight (FW). (*D*) RT-qPCR analysis of *HEMA1*, *LHCB2.1*, and *GUN4* transcript levels in WT (Col-0), *gun1-1*, *gun1-103* and GUN1ox1 and GUN1ox2 seedlings grown with or without 1 μM NF under the following conditions: 2 d dark, 3 d continuous white light (100 μmol m^{-2} s^{-1}). Expression is relative to Col-0 −NF and normalized to *YELLOW LEAF SPECIFIC GENE 8* (*YLS8*, At5g08290). Data shown are means + SEM or ± SEM of 3 independent biological replicates. Asterisks denote a significant difference vs. Col-0 for the same treatment, Student's *t* test (**P* < 0.05; ***P* < 0.01).

Fig. S3 *A* and *D*). This is slightly smaller than the mature GUN1 protein used by Tadini et al. (24) for their pull-down assays. We also expressed recombinant *Arabidopsis* FC1 as a GST fusion protein. The obtained GST-FC1 showed enzyme activity as measured by an increase in Zn-protoporphyrin (ZnProto) fluorescence (Fig. 2*A*) (34). We then evaluated the effect of GUN1-PS on the ability of FC1 to catalyze Zn chelation. The addition of GUN1-PS to FC1 enhanced the Zn-chelatase activity linearly with increasing concentration of GUN1-PS (Fig. 2*A*). In comparison, the same concentration of bovine serum albumin (BSA) had only a slight effect on activity, suggesting GUN1-PS did not merely stabilize FC1 (Fig. 2*A*). GUN1-PS itself had no Zn-chelating activity. Analysis of Michaelis–Menten kinetics revealed that addition of GUN1-PS decreased K_M values for Proto IX from 26.5 μM to 4.5 μM (Fig. 2*B*), suggesting GUN1-PS stimulated Zn-chelatase activity by enhancing substrate affinity for FC1.

GUN1-PS Binds to Heme through PPR Motifs. It has previously been shown that GUN4 can enhance Mg-chelatase activity by directly binding MgProto, the product of this reaction (10). To examine whether a similar stimulating mechanism is employed in GUN1-dependent enhancement of FC1 activity, we tested the ability of GUN1-PS to bind heme using hemin-agarose beads. As shown in Fig. 3*A*, GUN1-PS demonstrated hemin-binding activity, and a series of GUN1 truncations were constructed to identify the region of the GUN1 protein required for binding (*SI Appendix*, Fig. S3*A*). Fig. 2*B* shows that GUN1 proteins containing the PPR motifs (PPR1 and PPR2) showed significantly more hemin binding than those containing only the SMR motifs (SMR1 and SMR2). During the heme-binding assay, we observed that the color of the hemin solution changed upon GUN1-PS binding (Fig. 3 *C*, *Inset*), suggesting that changes in the hemin spectrum had occurred on binding. Spectrophotometric analysis showed that the GUN1-hemin

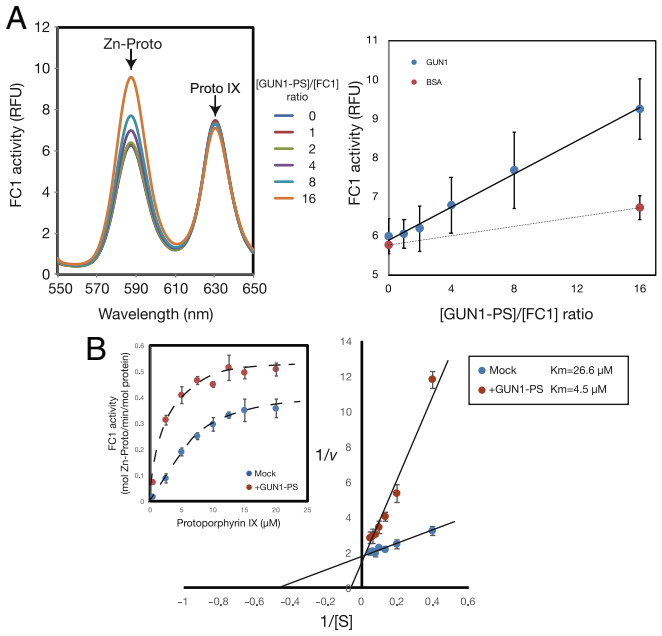

Fig. 2. GUN1-PS enhances FC1 activity. (*A*) *Arabidopsis* FC1 protein expressed as a GST-fusion protein showed Zn-chelatase activity. Addition of GUN1-PS enhanced the formation of Zn-protoporphyrin IX (Zn-Proto; measured as relative fluorescence units [RFU]) from protoporphyrin IX (Proto IX) linearly with increasing concentration of GUN1-PS. BSA was used as a negative control. (*B*) Double reciprocal plot analysis of Zn-Proto formation by FC1 in the presence or absence of GUN1-PS. *Inset* shows Michaelis–Menten plot of the same data. K_M values of FC1 in the presence or absence of GUN1-PS are shown. Data shown are means + SEM (or ± SEM) of 3 independent replicates.

complex exhibited a red shift of the Soret band and Q-band peaks compared to unbound hemin, consistent with specific binding (Fig. 3*C*). To determine the affinity of GUN1 for hemin, GUN1-PS was incubated with increasing hemin concentrations and binding determined by differential spectrophotometry (Fig. 3*D*). The increase in absorbance of the shifted Soret peak was plotted against porphyrin concentration (Fig. 3*E*) and a dissociation constant (K_D) for the binding of GUN1-PS to hemin was estimated to be 6.08 ± 1.11 μM using nonlinear regression analysis and assuming a 1-site binding model. This value sits within the range measured for a variety of heme-binding proteins (35). Similar analyses for GUN1 binding to other metal porphyrins resulted in estimated K_D values for MgProto and ZnProto of 8.65 ± 1.80 μM, and 3.10 ± 0.86 μM, respectively (*SI Appendix*, Fig. S5). Furthermore, analysis by size exclusion chromatography of GUN1-PS and Proto IX mixtures showed that GUN1-PS is also able to bind to Proto IX (Fig. 3*F*). Differential spectrophotometry was used to confirm the saturated binding of Proto IX to GUN1-PS (Fig. 3 *G* and *H*). The K_D value for Proto IX was 4.42 ± 0.56 μM and therefore GUN1-PS has similar binding affinities for all 4 porphyrins tested.

GUN1 in Plant Extracts Binds to Heme. Finally, to test whether GUN1 can bind to hemin in plant extracts, we constructed *Arabidopsis* lines expressing FLAG-tagged GUN1 under the control of its own promoter in the *gun1-102* mutant background (lines A3022 and A3026). As a control we also expressed FLAG-tagged GUN5 (line cchZ 3-17) in the *cch GUN5*-deficient mutant (36). The *gun1-102* phenotype was complemented by *GUN1* expression with derepression of *LHCB1.2*, *RBCS1A*, and *CHLH* expression by Lin restored in these lines (*SI Appendix*, Fig. S6). Since GUN1 accumulates detectable levels only at very young stages of leaf development (26), proteins were extracted from 4-d-old seedlings and subjected to the hemin binding assay (Fig. 3*I*). FLAG-tagged GUN1 was enriched in the fraction bound to hemin, while the GUN5 protein, which also binds porphyrins (37), showed only a very faint band (Fig. 3*I*), indicating that hemin binding is specific for GUN1 at least in comparison to GUN5.

Discussion

GUN1 has proved to be the most enigmatic of the proteins identified to date with a major role in retrograde signaling. The ability of *gun1* to rescue the inhibition of nuclear gene expression after both NF and Lin treatment (5) as well as various other mutations that affect retrograde signals (7, 24, 38) has resulted in the hypothesis that it functions as an integrator of retrograde signals. This includes a tetrapyrrole signal that is also rescued by the other *gun* mutants (9, 10). A signal integrator should act downstream of these different signals, but instead GUN1 seems to have a variety of roles in maintaining plastid function during early chloroplast development (27–29). Of the recently proposed roles for GUN1, only one includes a mechanism for *gun1* maintenance of nuclear gene expression. In this model, GUN1 promotes plastid protein import under conditions of plastid damage and its absence leads to the accumulation of chloroplast preproteins, which in turn induce expression of chloroplast genes (29). However, a *gun* phenotype is not seen for other mutants with reduced chloroplast import and it is not clear why failure to import proteins into damaged chloroplasts should induce more expression of these preproteins. As most mutants in chloroplast proteins that result in a *gun* phenotype are involved in tetrapyrrole biosynthesis, an alternative hypothesis is that the *gun1* mutant enhances or protects a tetrapyrrole signal (39, 40). This tetrapyrrole signal could be one of a number of biogenic signals or could function as the primary signal responding to chloroplast status. To support such a hypothesis there is an increasing body of evidence that GUN1 does affect tetrapyrrole biosynthesis. It has been shown to interact with tetrapyrrole biosynthesis proteins in two studies (24, 29), albeit with different proteins identified, and import of tetrapyrrole proteins was also reported to be affected in *gun1* (29).

To test the hypothesis that GUN1 affects the tetrapyrrole pathway directly, we examined the effect of GUN1 on flow through the pathway and its ability to bind tetrapyrroles. Our results suggest that GUN1 has two possibly independent functions in modifying tetrapyrrole metabolism (Fig. 4). First, it was able to restrict flow through both branches of the tetrapyrrole pathway such that feeding ALA resulted in reduced accumulation of both Pchlide and heme in dark-grown seedlings. The mechanism for this restriction is unknown but may be related to the observation here that GUN1 can bind porphyrins and/or that it can interact with the tetrapyrrole enzymes PBGD and UROD2 that catalyze shared steps in the tetrapyrrole pathway (24). There are some preliminary data to suggest that Proto IX might accumulate in the presence of excess GUN1, which suggests that restriction is later in the pathway. Whatever the target, GUN1 is not a very abundant protein (26) and it is unlikely that any restriction of the flow of tetrapyrroles is due to sequestration by binding to GUN1 as the ratio of GUN1 protein to tetrapyrrole molecules would be very low. Rather, we propose that the mode of action of GUN1 is regulatory. Moreover, the rapid degradation in the light (26) would permit an increased flow of tetrapyrroles at a time when the demand for Chl synthesis is greatest. We also saw a small effect of GUN1 on Pchlide synthesis in the absence of ALA feeding as observed previously (32). While there is evidence that expression of *HEMA1*, encoding the rate-limiting enzyme GluTR, is elevated in dark-grown *gun1* seedlings compared to WT (41), it was reported that import of GluTR was reduced in *gun1* (29), suggesting the effects of GUN1 could be posttranslational.

The second molecular function of GUN1 identified in this study is the enhancement of FC1 activity through a more than

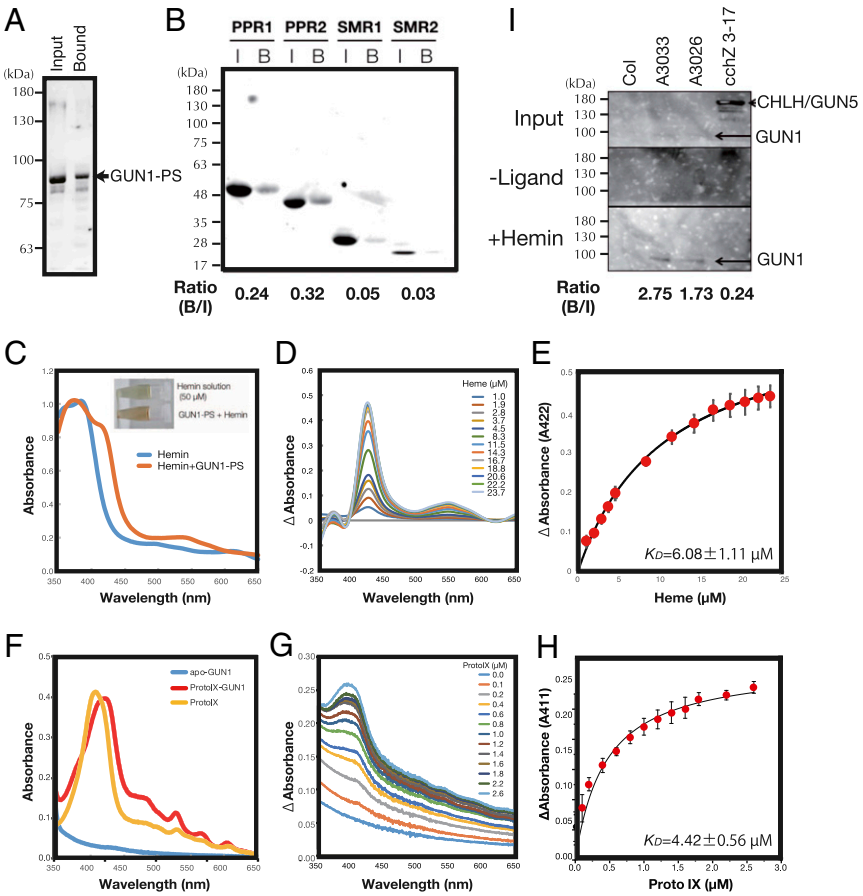

Fig. 3. Recombinant GUN1 protein binds to heme through PPR motifs. (*A*) Binding of GUN1-PS to hemin-agarose beads. (*B*) Binding of a truncated series of GUN1 proteins (*SI Appendix* and Fig. 2*A*) to hemin-agarose beads. GUN1-PS was detected by Western blot analysis using polyclonal His-tag antibodies. I, input; B, bound. Ratio (B/I) indicates ratio of band intensities of bound fraction to input fraction. (*C*) Absorption spectra of hemin and hemin-GUN1-PS complexes. (*Inset*) Photograph of hemin solution (50 μM) and hemin-GUN1-PS complex purified by gel filtration. (*D*) Absorbance difference spectra of hemin-GUN1-PS minus hemin solution at different hemin concentrations. (*E*) Change in absorbance of the Soret peak plotted against hemin concentration was used to determine the dissociation constant (K_D) of the heme-GUN1-PS complex assuming a 1-site binding model. (*F*) Absorption spectra of Proto IX and Proto IX-GUN1-PS complexes. (*G*) Absorbance difference spectra of Proto IX-GUN1-PS minus hemin solution at different hemin concentrations. (*H*) Change in absorbance of the Soret peak plotted against Proto IX concentration was used to determine the dissociation constant (K_D) of the Proto IX-GUN1-PS complex assuming a 1-site binding model. (*I*) Binding of FLAG-tagged GUN1 isolated from *Arabidopsis* lines A3022 and A3026 (overexpressed in a *gun1* mutant background) to hemin beads. The GUN5 protein (expressed in the *cch* mutant background) is shown as a control.

5-fold reduction in K_M (Fig. 2). It is likely that GUN1 stimulates FC1 activity through enhancing substrate affinity in a similar way to GUN4 enhancement of Mg-chelatase activity (10). Indeed, like GUN4, we have confirmed that GUN1-PS can also bind to Proto IX (Fig. 3 *F*–*H*). As interaction of GUN1 and CHLD is proposed (24) and GUN1-PS can bind to MgProto (*SI Appendix*, Fig. S5), the possibility that GUN1 also stimulates Mg-chelatase cannot be ruled out. However, it should be noted that GUN1 product binding is more than 10-fold weaker than GUN4 (K_D value for Mg-deuteroprotoporphyrin binding to GUN4 is 0.26 μM) (10). Therefore, it is unlikely that GUN1 can also regulate Mg-chelatase activity. The control protein used for this assay, BSA, also binds heme (42), and thus enhancement of FC1 activity by GUN1 appears to be quite specific and not simply a function of product binding. A stimulation of FC1 activity by GUN1 is surprising, given that GUN1ox lines showed reduced heme levels, and it is probable that GUN1-dependent FC1 stimulation does not reflect the total heme content. As functional expression of recombinant *Arabidopsis* FC2 protein has not been successful, we could not test whether GUN1 could also promote FC2 activity, but given that the interaction of GUN1 is reported to be specific for FC1 (24) this is not likely.

To understand why GUN1 restricts tetrapyrrole synthesis, but promotes FC1 activity, will take more detailed analysis of heme metabolism in young seedlings. However, one possible explanation is that GUN1 enhances FC1 activity to ensure a supply of heme to cellular locations outside of the plastids under conditions in which tetrapyrrole synthesis is maintained at a low level, i.e., before transcriptional up-regulation of the tetrapyrrole pathway by light (43). Once seedlings are exposed to light, the flow through the tetrapyrrole pathway is greatly increased, which would negate the need for GUN1 to promote FC1 activity. Degradation of GUN1 would then redress the balance between FC1 and FC2 to favor FC2 activity required to synthesize photosynthetic hemes (44). As reported previously by us and other groups it is apparent that endogenous levels of total heme do not correlate with a *gun* phenotype (21, 45). This is perhaps not surprising as only the activity of FC1 results in a *gun* phenotype. The heme produced specifically by FC1 cannot be determined by current methods, but by looking in etiolated seedlings, we have conditions in which a bigger proportion of the heme pool is likely to be derived from FC1. One other point to note is that in the experiments described by Espinas et al. (45) seedlings were not fed ALA. Feeding ALA bypasses the regulatory ALA synthesis step and exacerbates any

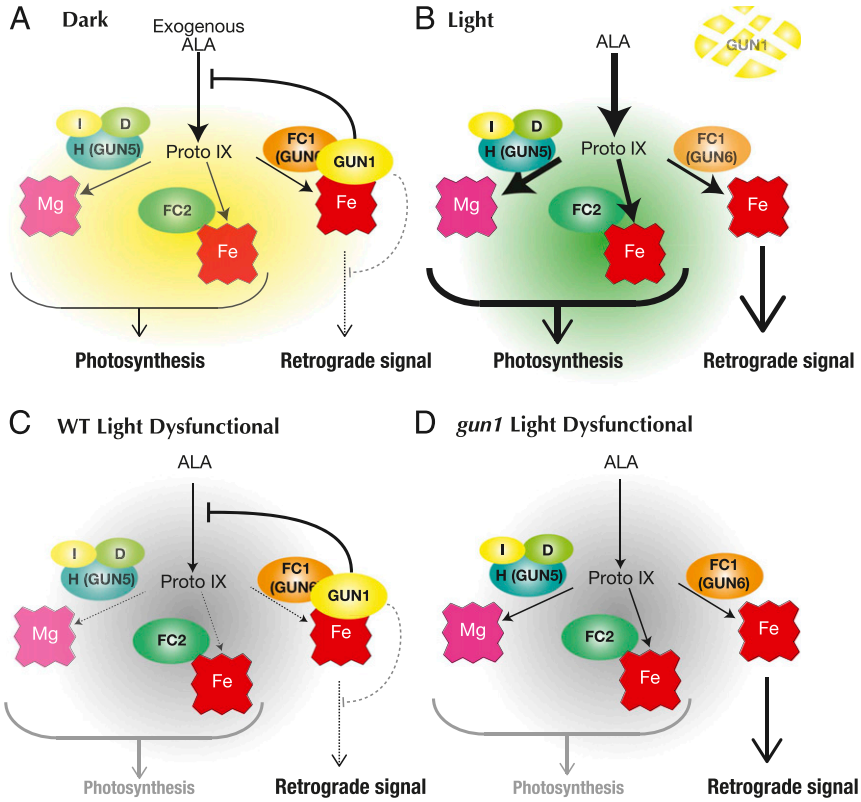

Fig. 4. Model for GUN1 function in tetrapyrrole metabolism. (*A*) In the dark GUN1 represses flow through the tetrapyrrole pathway (as observed under conditions in which flow is artificially increased via feeding of 5-aminolevulinic acid [ALA]), but promotes FC1 activity to ensure a sufficient supply of heme to cellular locations outside of the plastids. It may also prevent release of the retrograde signal through heme binding. (*B*) In the light GUN1 is degraded by the ClpC protease promoting total tetrapyrrole synthesis required for chloroplast development. Under these conditions, FC1 activity is no longer promoted, but the increased tetrapyrrole flux ensures a sufficient supply of substrate to FC1 to supply heme to cellular locations outside of the plastids. The absence of GUN1 also permits release of the retrograde signal. (*C*) After inhibition of chloroplast function (for example with norflurazon that causes an inhibition of tetrapyrrole synthesis) the presence of GUN1 prevents a retrograde signal. (*D*) Under these dysfunctional conditions the absence of GUN1 promotes tetrapyrrole synthesis and retrograde signal release. I, CHLI; D, CHLD; H/GUN5, CHLH; and FC, ferrochelatase.

differences between WT and *gun1*. Only by doing this can we observe the effects of GUN1 on heme metabolism using currently available analytical techniques.

The observations presented here demonstrate that GUN1 alters tetrapyrrole metabolism (Fig. 4) and therefore that all described *gun* mutations affect this pathway. This observation therefore supports a model in which tetrapyrroles are mediators of a single biogenic retrograde signal during deetiolation and that GUN1 and GUN2-6 function in the same pathway to regulate nuclear gene expression. Previous studies have failed to define the genetic relationship between the 2 groups of mutants. However, the enhanced phenotype of *gun1gun4* or *gun1gun5* double mutants compared with the single mutants (9, 46) was proposed to be the result of using the weaker *gun1-1* allele with double mutants containing the stronger *gun1-9* allele not showing an additive phenotype, consistent with a strong overlap of the *gun1* and *gun5* transcriptomes (5). It was also reported that *HEMA1* and *LHCB* expression showed a different level of dependence on GUN1 and GUN5 (41). This might be explained by the fact that while both *gun1* and *gun5* might promote heme synthesis these mutations have opposite effects of Mg-porphyrin synthesis. Over accumulation of Mg-porphyrins results in singlet oxygen synthesis that is associated with down-regulation of nuclear gene expression and a loss of the ability to green (14, 47, 48). The overaccumulation of Pchlide in *gun1* could also be associated with inhibition of gene expression and greening under some conditions (41), which could explain some of the complex genetic interactions observed and the deetiolation phenotype of *gun1* mutants (49). In addition, the *gun1* mutant exhibits a *gun* phenotype when treated with either NF or Lin, while the tetrapyrrole mutants (*gun2-6*) only show a *gun* phenotype on NF. The enhancement of nuclear gene expression in *gun1* also after inhibition of translation might be explained by the interaction of GUN1 with proteins involved in plastid protein homeostasis (23–26) where it could provide a link between protein and tetrapyrrole synthesis pathways.

The prevailing model is that FC1-dependent heme synthesis is required to generate a positive signal that is inhibited by treatments affecting chloroplast function such as NF and Lin (22, 39). Our observations on the restriction of heme synthesis by GUN1 in seedlings are consistent with this model as the *gun1* mutation would be expected to result in more flow through FC1 after such a treatment (Fig. 4). However, the demonstration that GUN1 enhances FC1 activity does not appear to support it. One scenario that could reconcile these two observations is if the restriction of tetrapyrrole synthesis by GUN1 was a more significant effect than enhancement of FC1, such that overall there was still less FC1-dependent heme in the presence of GUN1 than in its absence. Our own data showing that heme levels were reduced overall in GUN1ox lines do support such an interpretation. Alternatively, or perhaps in addition to its role inhibiting tetrapyrrole synthesis, it is possible that binding of FC1-synthesized heme by GUN1 blocks release or propagation of the retrograde signal (Fig. 4). In this case, GUN1 degradation in the light would ensure an increased signal to promote further

chloroplast protein synthesis for continued development and the supply of new chloroplasts.

GUN1 has been reported to be involved in many processes in the chloroplast from gene editing to protein import and synthesis (24, 25, 27–29), and defects in all these processes affect nuclear gene expression. Here we show a link between GUN1 function and tetrapyrrole synthesis that offers the potential of a unifying hypothesis for biogenic retrograde signaling. Future work will focus on understanding the relationship between these different chloroplast processes and the tetrapyrrole synthesis pathway and the role of GUN1 in this relationship.

Materials and Methods

Plant material and growth conditions, Pchlide measurements, heme detection by chemiluminescence, heme-binding assays, ferrochelatase assay, absorbance spectroscopy, circular dichroism spectrometry, RNA extraction and gene expression analysis by reverse transcription quantitative PCR (RT-qPCR), construction of GUN1-FLAG lines, and plasmid constructions and expression of recombinant proteins are described in *SI Appendix, SI Materials and Methods*.

Data Availability. Enzymology data are summarized according to the guidelines of the STRENDA commission.

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