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Sigma factor-mediated plastid retrograde signals control nuclear gene expression

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SUMMARY

Retrograde signalling from plastids to the nucleus is necessary to regulate the organelle's proteome during the establishment of photoautotrophy and fluctuating environmental conditions. Studies that used inhibitors of chloroplast biogenesis have revealed that hundreds of nuclear genes are regulated by retrograde signals emitted from plastids. Plastid gene expression is the source of at least one of these signals, but the number of signals and their mechanisms used to regulate

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Additional Supporting Information may be found in the online version of this article.

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nuclear gene expression are unknown. To further examine the effects of plastid gene expression on nuclear gene expression, we analyzed Arabidopsis mutants that were defective in each of the six *sigma factor* (*SIG*) genes that encode proteins utilized by plastid-encoded RNA polymerase to transcribe specific sets of plastid genes. We showed that SIG2 and SIG6 have partially redundant roles in plastid transcription and retrograde signalling to control nuclear gene expression. The loss of GUN1 (a plastid-localized pentatricopeptide repeat protein) is able to restore nuclear (but not plastid) gene expression in both *sig2* and *sig6*, whereas an increase in heme synthesis is able to restore nuclear gene expression in *sig2* mutants only. These results demonstrate that sigma factor function is the source of at least two retrograde signals to the nucleus; one likely to involve the transcription of $tRNA^{Glu}$. A microarray analysis showed that these two signals accounted for at least one subset of the nuclear genes that are regulated by the plastid biogenesis inhibitors norflurazon and lincomycin. Together these data suggest that such inhibitors can induce retrograde signalling by affecting transcription in the plastid.

Keywords

chloroplast; retrograde signalling; transcription; tetrapyrroles; sigma factors; photosynthesis

INTRODUCTION

Over 95% of the ~3000 proteins in the plastid are encoded in the nuclear genome, translated in the cytoplasm, and then imported into the organelle (Martin *et al.*, 2002; Barbrook *et al.*, 2006). Communication between the separate compartments is therefore necessary to regulate the stoichiometry of multi-subunit complexes encoded by both genomes, the proteome of the plastid, and to ensure that the organelle can meet the metabolic and energy demands of the cell (Woodson and Chory, 2008). Many plastid functions (e.g. DNA replication, division, and most gene regulation) are regulated by nuclear factors; this control is referred to as anterograde control or signalling. Conversely, the functional and developmental state of the plastid (e.g. undeveloped, stressed) allows the organelle to send retrograde signals to the nucleus to regulate gene expression. H_2O_2 (Laloi *et al.*, 2006), singlet oxygen (Wagner *et al.*, 2004), the plastid redox state (Pfannschmidt *et al.*, 2003), and drought stress (Estavillo *et al.*, 2011) act as ‘operational’ retrograde signals from mature photosynthesizing plastids that regulate nuclear genes to alter photosystem stoichiometry or to induce stress responses (Pogson *et al.*, 2008).

At the same time, ‘developmental’ signals from plastids also regulate early chloroplast biogenesis as a seedling shifts from a heterotrophic to a photoautotrophic lifestyle. Such knowledge has been derived primarily from studies that used herbicide drugs that inhibit organelle function and development, e.g. norflurazon (NF), an inhibitor of phytoene desaturase and carotenoid biosynthesis that leads to photobleaching, or lincomycin (Linc), an inhibitor of 70S ribosomes and plastid translation (Nott *et al.*, 2006). From these studies it was gleaned that hundreds of Photosynthetic Associated Nuclear Genes (PhANGs) are sensitive to the functional state of the plastid and, if plastid gene expression and/or plastid tetrapyrrole biosynthesis are attenuated, PhANGs are down-regulated as a response.

Consequently, plastid RNA transcripts, proteins, and tetrapyrroles have all been posited to be potential signalling molecules (Fernandez and Strand, 2008; Pogson *et al.*, 2008).

Specific and unbiased genetic screens in *Arabidopsis thaliana* have identified six *genomes uncoupled* (*gun*) mutants that still express PhANGs even when photobleached by NF (Susek *et al.*, 1993). *GUN2-6* encode for proteins clustered around the heme/chlorophyll branch point of the tetrapyrrole biosynthetic pathway (Mochizuki *et al.*, 2001; Larkin *et al.*, 2003). The one gain-of-function mutant, *gun6*, has an increase of ferrochelatase I (FC1) activity leading to the hypothesis that increasing FC1-produced heme in undeveloped chloroplasts was a positive signal to regulate PhANG expression (Woodson *et al.*, 2011).

gun1 mutants were isolated from genetic screens that used both NF and Linc, and led to a model in which GUN1 is required for multiple-stress regulated pathways (Koussevitzky *et al.*, 2007). Although the precise biochemical activity of GUN1 is not known, it is a chloroplast-localized pentatricopeptide repeat (PPR)-containing protein, which is believed to be one of the specific RNA binding factors that post-transcriptionally regulate gene expression by altering editing, splicing, stability, or translation (Delannoy *et al.*, 2007). Therefore, a plausible model for GUN1 is that it regulates one or a small set of chloroplast RNAs (or protein product(s)) that is necessary for retrograde signalling.

Although clearly involved in retrograde signalling, the intertwined nature of transcription, translation, and tetrapyrrole synthesis within plastids has made it difficult to determine the exact sources of these signals. For instance, because much transcription in chloroplasts is performed by plastid-encoded RNA polymerase (PEP), inhibition of translation by Linc will also reduce most RNA transcripts within the plastid (Gray *et al.*, 2003). Also, PEP-transcribed $tRNA^{Glu}$ is the starting precursor for all tetrapyrroles within the plastid (Schon *et al.*, 1986). Therefore, tetrapyrrole metabolism is affected directly by inhibition of either transcription or translation. Because drug inhibitors used in these studies are likely to affect all of these processes, their usefulness in the study of retrograde signalling has been criticized (Pfannschmidt, 2010).

To analyze further the role that gene expression plays in plastid signalling, here we monitored retrograde signalling when chloroplast transcription had been specifically perturbed without inhibitors. To this end, we analyzed Arabidopsis mutants that were defective in each of the six *sigma factor* (*SIG*) genes that encode proteins utilized by PEP to transcribe specific sets of plastid genes. These studies allowed us to demonstrate that: (i) SIG2 and SIG6 are the source of two plastid retrograde signals—one is transcription of plastid $tRNA^{Glu}$ by SIG2 to increase tetrapyrrole synthesis and promote PhANG expression in the nucleus, (ii) these two signals affect many of the same genes as do drug inhibitor treatments, which suggested that a common signal is shared, and (iii) GUN1 and tetrapyrroles are important for genome co-ordination under physiologically relevant conditions.

RESULTS

Plastid transcription is necessary for full *LHCB* expression in Arabidopsis

Block of plastid transcription in barley, wheat, or mustard leads to repression of PhANGs (Lukens *et al.*, 1987; Rapp and Mullet, 1991; Pfannschmidt and Link, 1997). To test if plastid transcription is important for regulation of PhANGs in Arabidopsis, we monitored the expression of PhANGs in seedlings that had been grown on media that contained the drug rifampicin, which selectively inhibits PEP activity. To this end, we used the genetic construct (6–3) that contained the canonical PhANG *LHCB1.2* (*Light Harvesting Chlorophyll Binding protein*) minimal promoter driving the *LUC* gene (Koussevitzky *et al.*, 2007). In agreement with previous studies, rifampicin treatment (50 μg ml^{-1}) reduced *LHCB* expression in 2-day-old seedlings in a lightindependent manner (Figure 1a). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis confirmed that transcript levels of *LHCB1.2*, the PhANG *Carbonic anhydrase 1* (*CA1*), and the PEP-dependent plastid genes *psbA* and *psbD* were reduced. Conversely, control genes [nuclear genes encoding plastid-localized proteins that are not regulated by plastid retrograde signals (Table S2) (Koussevitzky *et al.*, 2007)] and the NEP-dependent plastid genes were unchanged or elevated (Figure S1). Together these results suggested that transcription of some plastid genes is necessary for the full expression of *LHCB* and *CA1* in Arabidopsis.

SIG2 and *SIG6* are necessary for full PhANG expression in seedlings

In land plants, prokaryotic-derived nuclear-encoded sigma factors are involved in PEP promoter recognition and are important for the transcription of certain sets of PEP-transcribed plastid genes (Lysenko, 2007; Schweer *et al.*, 2010). The Arabidopsis nuclear genome encodes six sigma factors with partially redundant functions in plastid transcription. Because the previous experiment showed that PEP activity is required for full *LHCB* expression, we surmised that a certain sigma factor(s) might also be required. To investigate this possibility, we obtained T-DNA lines and disrupted each of the six *SIG* genes (Table S1). Quantitative real-time PCR (qPCR) analysis showed that, except for *sig1*, each of the T-DNAs severely reduced the level of steady-state RNA transcript (Figure S2a). Except for the *sig2* T-DNA, all the insertions were in exons, a finding that suggested that even the low levels of transcripts are not expected to encode functional proteins.

In agreement with previous reports, mutations that affected *SIG2* (Shirano *et al.*, 2000) or *SIG6* (Ishizaki *et al.*, 2005) resulted in pale seedlings that accumulated significantly less chlorophyll (Figures 1b and S2f). Mutants that lacked the other four sigma factors had a wild-type (wt)- like physical appearance as has been reported for *sig1* (Lai *et al.*, 2011), *sig3* (Zghidi *et al.*, 2007), *sig4* (Favory *et al.*, 2005), and *sig5* (Nagashima *et al.*, 2004b). Several plastid transcripts failed to accumulate to wt levels in these mutants. As has been reported previously, *sig2* had lower levels of *psaJ*, *psbA*, and *psbD* (Hanaoka *et al.*, 2003; Privat *et al.*, 2003; Nagashima *et al.*, 2004a), *sig3* had reduced levels of *psbN* (Zghidi *et al.*, 2007), *sig4* had reduced levels of *ndhF* (Favory *et al.*, 2005), and *sig6* had lower transcript levels of all the PEP-dependent genes that we tested (Ishizaki *et al.*, 2005; Loschelder *et al.*, 2006) (Figure 1c). In addition, we observed that *sig2*, *sig3*, and *sig4* mutants failed to accumulate normal levels of additional PEP-transcribed transcripts (*psbB* and *psbN* in *sig2*, *psaJ* in *sig3*,

psaJ and *rbcL* in *sig4*); the *sig2* mutant had the most severe defect. *sig1* and *sig5* seedlings had essentially wt levels of plastid transcripts. Only PEP-transcribed genes appeared to be affected as nuclear-encoded polymerase (NEP) transcribed transcripts (*clpP*, *rpoB*, *rpoC*) were either at wt levels or even elevated in *sig2* and *sig6* lines (Figure S2b).

Next, we tested if the reduced plastid transcripts levels led to reduced PhANG expression in any of the *sig* mutants, i.e. is there retrograde signalling? Although the PhANG *RBCS2B* (RuBisCO small subunit 2B) was slightly reduced in *sig3* and *sig4*, the most drastic effects were observed in *sig2* and *sig6* (Figure 1d). In these two lines, the PhANGs *LHCB1.2*, *LHCB2.2*, *CA1*, *RBCS2B* and *Plastocyanin* (*PC*) levels were reduced compared with wt, whereas levels of control gene transcripts were unchanged or elevated (Figure S2c). This phenotype was not light dependent as etiolated *sig2* and *sig6* seedlings also had reduced levels of PhANG and plastid transcripts (Figures 2a and S3a). In the *sig2* and *sig6* mutants, the observed defects in chlorophyll synthesis (Figure S2f,g), plastid (Figure S2d) and PhANG transcript levels (Figure S2e) were complemented by reintroduction of a wt copy of either *SIG2* or *SIG6*, and confirmed causality of the T-DNA insertions. As predicted, both the overexpressed SIG2-YFP-HA and SIG6-YFP-HA appear to be localized only to chloroplasts (Figure S2h).

SIG2 and *SIG6* are partially redundant for genome co-ordination

Plants that lack SIG2 or SIG6 are virescent (leaves emerge yellow or pale green and then eventually turn green (Hanaoka *et al.*, 2003; Ishizaki *et al.*, 2005)). We therefore performed a time course analysis of seedlings grown from 2 to 10 days. Although there were individual differences between both the genes and the genotypes, the general trend (with one exception, *psaJ*) was that both plastid and PhANG transcripts were lowest at day 2 and accumulated to or near to wt levels by day 10 in the *sig2* and *sig6* mutants (Figure S4a). In agreement with the transcript data, three plastid-encoded proteins accumulated more slowly in the *sig2* or *sig6* mutants compared with wt (Figure S4b).

To assess whether there was genetic redundancy between *SIG2* and *SIG6*, we generated a *sig2/sig6* double mutant. The *sig2*/*sig6* mutant was severely pale (Figure 3a) even after 5 weeks of growth on medium supplemented with 1% sucrose (Figure S5b). Although the phenotype of the *sig2* or *sig6* single mutant was similar to wt after 1 week, plastid PEP (but not NEP) dependent transcripts and PhANG transcripts failed to accumulate in *sig2/sig6* (Figure 3b,c). A small reduction in the level of control gene transcripts suggested that the double mutant had a broader effect on nuclear genes encoding plastid proteins.

GUN1 is required in the plastid transcription signalling pathway

To test if GUN1, which is involved in multiple retrograde signalling pathways (Koussevitzky *et al.*, 2007), was also involved in the retrograde signalling observed in the *sig2* and *sig6* mutants, we generated *sig2/gun1* and *sig6/gun1* double mutants. The *gun1* mutation partially restored the expression of several PhANGs in both *sig2* and *sig6* mutants, and this effect was light independent (Figure 2a, b) and confirmed that GUN1 acts in a light-independent signalling pathway (Ruckle *et al.*, 2007). The restoration of nuclear gene expression by *gun1* could not be explained by a concurrent increase in plastid transcripts

(Figure S3a, b) or protein (Figure 2c). The *gun1* mutation had little effect on plastid transcripts and proteins in the *sig2* mutant, but further reduced their accumulation in the *sig6* mutant. At the visual level, the *sig2/gun1* mutant was similar in appearance to *sig2*, but *gun1/sig6* was noticeable paler and sicker than *sig6* after 7 days (Figure S3c).

We considered the possibility that GUN1 affects the abundance of a specific transcript or small set of transcripts not included in our qPCR analysis. We, therefore, analyzed plastid transcripts in these mutants by strandspecific RNA sequencing. Because the *sig2* mutation primarily affects tRNA transcription (Kanamaru *et al.*, 2001) and such an analysis is not practical for monitoring small structured RNAs, we tested wt, *gun1*, *sig6* and *sig6*/*gun1* plants. Of the 72 plastid genes that we could reliably monitor, the *sig6* mutation reduced steady-state transcript levels of most of the PEP-dependent genes (Figures 4 and S6a). This analysis showed that there was a strong correlation to the expression changes of plastid genes that we monitored by qRT-PCR (Figure S6c). The *gun1* allele alone had no significant effect on any plastid transcript and the addition of *gun1* did not restore expression of any single plastid transcript in *sig6* mutants (Figure S6b). Instead, *gun1* addition led to a global reduction of all 72 plastid transcripts, both PEP and NEP-dependent, in this background. Although our analysis could not account for the effect of small or structured RNA molecules (tRNAs, rRNAs, etc.) we concluded that the *gun1* mutation does not restore PhANG expression in the *sig6* mutant by globally restoring plastid transcript levels or recoupling genome expression.

Tetrapyrroles are involved in SIG2 dependent genome co-ordination

Our published genetic studies have previously linked plastid tetrapyrrole synthesis to retrograde signalling to the nucleus (Mochizuki *et al.*, 2001; Larkin *et al.*, 2003; Woodson *et al.*, 2011). The starting substrate for all tetrapyrroles is glutamyl-tRNA that is enzymatically produced from glutamate and the PEP-transcribed $tRNA^{Glu}$. We, therefore, investigated the possibility that perturbations in the tetrapyrrole pathway may be responsible for lowered PhANG expression in *sig2* and *sig6*. Indeed, both $tRNA^{Glu}$ and glutamyl- tRNA levels were decreased in *sig6* and especially in *sig2* (Figures 5a and S7). This decrease of the tetrapyrrole starting substrate is likely to be responsible for the reduced levels of the other tetrapyrroles we measured. The rate-limiting early intermediate δ-aminolevulinic acid (ALA) (Figure 5c), the dark-accumulating late intermediate protochlorophyllide (Pchlide) (Figure 5b), and chlorophyll (Figure S2f) were reduced in both *sig2* and *sig6*. Interestingly, total non-covalently attached heme levels (Figure 5d) were decreased in *sig2*, but not in *sig6* mutants, suggesting that these sigma factors may contribute differently to the tetrapyrrole pathway(s).

Next, we tested if the *gun1* mutation affected tetrapyrrole metabolism in either *sigma* mutant. Indeed, *gun1* increased ALA, heme, and Chl levels in the *sig2* (but not the *sig6*) mutant (Figures 5c,d and S2f). Pchlide levels, however, were not elevated significantly (*P*-value = 0.18; Figure 5b). The ability of *gun1* to increase tetrapyrrole synthesis in *sig2*, however, was not achieved by an increase in total $tRNA^{Glu}$ transcripts or glutamyl-tRNA levels (Figures 5a and S7).

If reduced PhANG expression in the *sig2* mutant was due to decreased tetrapyrrole synthesis, as suggested by the previous experiments, then exogenous feeding of the precursor ALA should be able to restore PhANG expression. Indeed, when low levels of ALA (32–64 nM) were added to the medium, *LHCB* expression increased in etiolated *sig2* seedlings (Figure 6b). A similar trend was not observed in *sig6* mutants, in which *LHCB* was unresponsive to low levels of ALA. At higher levels of ALA (>250 nM) *LHCB* expression was repressed in all genotypes. This finding may be due to the increase of toxic tetrapyrrole intermediates, which led to photobleaching after light exposure (Figure S8d) and are known to affect chloroplast DNA levels even in the dark (Vinti *et al.*, 2000).

As there appeared to be a correlation between tetrapyrrole levels in PhANG expression in the *sig2* mutant, we next tested the genetic interaction of the *sig* mutants with *gun5* (a weak allele of *chlH* encoding the H subunit of the Mg-chelatase, the first enzyme of the Mg branch of the tetrapyrrole pathway). Analysis of the double mutants demonstrated that the *gun5* mutation partially restored PhANG expression in etiolated *sig2*, but not *sig6* mutants (Figure 2a). Plastid transcript levels, on the other hand, did not change significantly (Figure S3a). To observe the effect of *gun5* on tetrapyrrole synthesis we measured the end products of the branched tetrapyrrole pathway. As expected, combining *gun5* with either *sig* mutant reduced Chl (Figure S2f) and Pchlide (Figure 5b) levels further due to blocking the Mg branch of the tetrapyrrole pathway. Heme levels, however, were increased significantly (*P*-value < 0.01) in the *sig2* (but not the *sig6*) mutant by the addition of *gun5* (Figure 5d). Together these data suggested that by diverting flow of the tetrapyrrole pathway towards heme synthesis, the *gun5* mutation was able to partially restore PhANG expression in the *sig2* mutant.

FC1-produced heme has previously been hypothesized to be a signalling molecule from developing chloroplasts that have been photobleached with NF (Woodson *et al.*, 2011). To test if a similar situation exists in *sig2* mutants, we constructed transgenic lines that overexpressed either *FC1* or *FC2*. As during a norflurazon treatment, *FC1* overexpression increased PhANG expression in a *sig2* mutant without affecting plastid gene expression (Figure 6a, S8a). *FC2* overexpression, however, was unable to affect PhANG expression even though comparable levels of transcript and protein were synthesized (Figure S8b,c).

Plastid transcription plays a role in *GUN* signalling in herbicide-treated plants

The above results suggested that the effects of the *sig2* and *sig6* mutations on retrograde signalling are similar to those of herbicides and plastid biogenesis inhibitors such as NF and Linc, which also reduce plastid transcript levels (Gray *et al.*, 2003). This finding led us to test if the sigma factors were playing a role in regulation of PhANGs in NF-treated seedlings. Indeed, photobleaching wt seedlings with NF reduced transcript levels of all six *SIG* genes (Figure 7a), which may have contributed to a reduction of plastid transcript levels (Figure S9). In addition, overexpression of either *SIG2* or *SIG6* was able to partially restore PhANG expression in these photobleached seedlings (Figure 7b).

To determine the extent to which SIG-mediated signals overlap with each other and with drug inhibitor-induced signals, we performed a microarray analysis of wt, *sig2*, *sig6*, wt treated with NF, and wt treated with Linc. Compared with untreated wt seedlings, 1638

nuclear genes were differentially expressed at least twofold in at least one group. A cluster analysis of these genes showed that while the drug treatments had a broader affect on nuclear gene expression, there is significant correlation between the groups (Figure 7c). One major cluster of 714 down-regulated genes was enriched significantly for plastid (353 genes), and photosynthesis-related genes [photosynthesis (81 genes), photosystem (42 genes), thylakoid (68 genes), and tetrapyrrole metabolic process (20 genes)]. A comparison of genes that were differentially expressed in each group (cutoff = twofold change in expression) revealed that there were significant overlaps between the *sigma* mutants and drug treatments (Table S5 and Figure S10e,f). For instance, 76% of the Linc group genes were shared with the NF group and 49% of the *sig2* group genes were shared with the *sig6* group. Furthermore, 43% and 60% of genes up- and down-regulated, respectively, in *sig2* were similarly affected by at least one of the drug treatments and 55% and 96% of genes up- and down-regulated, respectively, in *sig6* were similarly affected by at least one of the drug treatments. Together these results suggest that at least part of the NF and Linc responses are due to a decrease in SIG function and/or plastid transcription.

DISCUSSION

SIG2 and SIG6 coordinate plastid and nuclear gene expression in developing seedlings

In this study we aimed to determine the role of plastid transcription in retrograde signalling. To this end, we analyzed mutants that were defective in all six *SIG* genes and hypothesized that each mutant, defective in transcription of a certain set of plastid genes, may exhibit retrograde signalling without additional stresses. Although most of the *sig* mutants were defective in the expression of at least one plastid gene that we monitored, *sig2* and *sig6* mutants were clearly the most affected and the only mutants to have pale phenotypes (Figure 1b–d). Two other mutants, *sig1* and *sig5* appeared to be essentially wt in both appearance and plastid transcript accumulation even though SIG1 and SIG5 are normally present in the conditions that we tested (Demarsy *et al.*, 2006). Previous reports, however, have shown that these two proteins may have more specialized functions: SIG1 being involved in WRKY-mediated pathogen response (Lai *et al.*, 2011) and redox control of *psaA* expression (Shimizu *et al.*, 2010) and SIG5 being a stress-induced sigma factor that directs transcription of *psbD* at a blue light-responsive promoter (Nagashima *et al.*, 2004b).

Analysis of nuclear gene expression in the *sig* mutants revealed that both *sig2* and *sig6* had a significant reduction of PhANG transcripts, a finding that suggested that the reduced transcription of a certain plastid transcript or set of transcripts was causing a retrograde response. The double mutant, *sig2*/*sig6*, had a much more severe reduction of both plastid and PhANG transcripts even after 7 days, at which time the single mutants would have recovered (Figure 3a–c). Such a phenotype supports the hypothesis that the sigma factors are partially redundant and utilize the same or overlapping promoters for some genes (Lysenko, 2007). Also, the increased accumulation of SIG6 has been proposed to partially complement the SIG2 deficiency (Privat *et al.*, 2003; Nagashima *et al.*, 2004a; Ishizaki *et al.*, 2005). Alternatively, the severe phenotype of the double mutant may be due to the additive affect of a larger number of transcripts that do not accumulate properly. Given that SIG2 and SIG6 contributed to retrograde signalling differently (see below), such an explanation seems to be

responsible at least partly. In any case, these data suggest that the roles of SIG2 and SIG6 in plastid biogenesis and retrograde signalling cannot be replaced by the other endogenously expressed sigma factors. SIG3 and SIG4 may also contribute to retrograde signalling to some extent as their respective mutations caused a mild reduction in some PhANG transcripts (*RBS2B*, *CA1*). These two sigma factors are perhaps the least well characterized of the six in Arabidopsis (Lysenko, 2007), and a more detailed analysis (e.g. combination mutants) would be required to determine their role in retrograde signalling.

SIG2 retrograde signalling involves tetrapyrrole synthesis

SIG2 has a specialized role in transcription of tRNAs in the plastid, including $tRNA^{Glu}$, the starting substrate for all tetrapyrroles in the cell (Kanamaru *et al.*, 2001). As a result, ALA, Chl, Pchlide, and heme all accumulate to lower levels in *sig2* mutants (Figures 5b–d and S2f). Such a defect in transcription is relevant considering that tetrapyrroles are candidate retrograde signalling molecules in yeast (Zhang and Hach, 1999; heme), green algae (Vasileuskaya *et al.*, 2005; heme, Mg-Protoporphyrin IX), red algae (Kobayashi *et al.*, 2009; Mg-Protoporphyrin IX), and humans (Yin *et al.*, 2007; heme). In Arabidopsis, analysis of the *gun* mutants revealed that PhANGs are regulated by increased heme synthesis by FC1 in NF-photobleached seedlings (Woodson *et al.*, 2011). This situation led us to speculate if the decrease of $tRNA^{Glu}$ transcripts and tetrapyrrole synthesis in *sig2* mutants was responsible for the decreased PhANG expression.

ALA feeding (Figure 6b), addition of the *gun5* allele (Figure 2a), or overexpression of *FC1* (Figure 6a) increased PhANG expression in the *sig2* mutant, a finding that suggested that increasing heme synthesis can overcome the *sig2* retrograde phenotype. Although ALA feeding or FC1 overexpression would increase heme levels directly, the *gun5* mutation may be able to increase heme levels by blocking the Mg branch of the tetrapyrrole pathway and by redirecting flow towards heme synthesis. Indeed, *gun5* did increase steady-state heme levels in the *sig2* mutant (Figure 5d). Somewhat curiously, however, the *gun5* mutation only increased PhANG transcripts in etiolated *sig2* seedlings. This situation may be due to HY5-mediated light signalling masking the effect of *gun5* and increased heme synthesis (Ruckle *et al.*, 2007). Alternatively, the increased cellular demand for metabolic heme in de-etiolated seedlings may reduce the pool that can act as a signal. Interestingly, overexpression of *FC2* was unable to affect PhANG expression in *sig2* mutants, a finding that was consistent with the hypothesis that only FC1-produced heme is able to induce PhANG expression in undeveloped seedlings (Woodson *et al.*, 2011).

sig6 mutants also have decreased levels of $tRNA^{Glu}$, ALA, Pchlide and Chl. Heme levels, however, appear to be unaffected (Figures 5a–d and S2f). This factor may be due to the less severe reduction in both $tRNA^{Glu}$ and ALA compared with the *sig2* mutant. Alternatively, the $tRNA^{Glu}$ supplied by SIG2 and SIG6 may contribute to different pools of ALA used for heme and Chl synthesis (Czarnecki *et al.*, 2011). Various attempts to further increase heme levels (ALA feeding, *gun5* mutation) did not increase PhANG expression in *sig6* mutants as they did in *sig2* mutants (Figures 2a and 6b). Together these results indicated that the retrograde signals mediated by SIG2 and SIG6 are distinct and that SIG2 signals to the nucleus by increasing tetrapyrrole synthesis through $tRNA^{Glu}$ transcription.

SIG2- and SIG6-mediated retrograde signalling requires GUN1

The plastid-localized PPR protein GUN1 is necessary for the repression of PhANGs during a variety of stresses and conditions, leading to a model in which GUN1 integrates various chloroplast signals (Koussevitzky *et al.*, 2007). Consistent with such a role, *GUN1* is necessary for both the SIG2 and SIG6 retrograde signals to regulate PhANG expression (Figure 2a). Although PhANG expression was partially restored by the *gun1* mutation in *sig2* and *sig6*, plastid transcript levels (Figure S3a), plastid protein levels (Figure 2c), and the pale appearances (Figure S3c) of these mutants were not restored. This situation is particularly apparent in the *sig6* mutant, in which the addition of *gun1* resulted in a decrease in both plastid transcript and proteins levels. Of the 72 plastid genes that we could reliably monitor by strand-specific RNA-sequencing, steady-state levels of all 72 were decreased to some extent (Figures 4 and S6b). Presumably this situation led to the sick and pale visual phenotype of *sig6/ gun1* mutants. Together, these data indicate that the *gun1* mutation does not restore SIG2 and SIG6 function and that GUN1 either (i) affects a smaller or structured RNA(s) not included in our analysis, (ii) affects translation of a specific RNA(s) like some PPR proteins (Schmitz-Linneweber and Small, 2008), or (iii) affects a plastid process other than RNA metabolism.

These data are consistent with the model that GUN1 acts downstream of plastid stresses and developmental defects (Koussevitzky *et al.*, 2007). However, as *gun1* is able to increase steady-state heme levels in *sig2* mutants (Figure 5d), it is tempting to speculate that GUN1 may also be affecting $tRNA^{Glu}$ transcripts (Figure 5a). Northern blot analysis demonstrated that *gun1* does not increase the steady-state levels of this transcript. GUN1, however, may be involved in regulating the fate of the transcript (i.e. translation versus tetrapyrrole synthesis) as there is only one $tRNA^{Glu}$ gene in the plastid genome. Alternatively, as a recent report has shown that separate pools of ALA exist for heme and Chl biosynthesis (Czarnecki *et al.*, 2011), GUN1 may help dictate the pool of ALA to which $tRNA^{Glu}$ transcripts contribute. This approach is especially interesting, considering that *gun1* is able to increase heme but not Pchlide levels in *sig2*. Our laboratory is currently investigating these possibilities.

Recent research from other laboratories has suggested that the regulation of PEP and sigma factors is quite complex. The PPR protein Delayed Greening 1 (DG1) interacts with SIG6 and is required for the expression of many PEP-dependent genes in seedlings (Chi *et al.*, 2010). The possibility that DG1 regulates retrograde signals such as GUN1 has not been investigated. Phosphorylation studies were carried out with SIG1 and SIG6 that demonstrated that such post-translational modifications can change their affinity to different plastid promoters. In the case of SIG1, phosphorylation in regulated by the redox state of plastoquinone (Schweer *et al.*, 2010; Shimizu *et al.*, 2010; Turkeri *et al.*, 2012). How this additional level of sigma regulation will also affect retrograde signalling should be a topic of subsequent studies. Furthermore, the redox state of photosynthetic electron transport chain has a more general affect on PEP activity in photosynthesizing chloroplasts (Arsova *et al.*, 2010; Kindgren *et al.*, 2012). Under high light intensities, PEP activity is the source of a signal to the nucleus to regulate *LHCB*, but this signalling does not appear to involve GUN1 or tetrapyrroles. Lastly, a role for SIG6 in retrograde signalling during singlet oxygen stress

has been reported (Coll *et al.*, 2009). A genetic screen uncovered a *sig6* mutant (*soldat8*) that was able to survive the high levels of singlet oxygen in the Arabidopsis *flu* mutant that has uncontrolled tetrapyrrole synthesis. Together these studies demonstrate that the effect of plastid transcription on plastid-nuclear communication is quite complex, involves multiple signals, and needs further examination.

sigma mutants validate drug inhibitor studies

Our studies strongly suggested that the retrograde signals in the *sig2* and *sig6* mutants are similar to those induced by drug inhibitors. Similar to NF-photobleached seedlings, the *gun5* mutation (Figure 2a), *FC1* overexpression (Figure 6a), and ALA feeding (Figure 6b) restored PhANG expression in *sig2* mutants and the *gun1* mutation restored PhANG expression in both *sig2* and *sig6* mutants (Figure 2a,b; Koussevitzky *et al.*, 2007; Mochizuki *et al.*, 2001; Woodson *et al.*, 2011). *SIG2* and *SIG6* overexpression increased PhANG expression in NF-treated seedlings (Figure 7b). Lastly, a microarray analysis demonstrated that within the broad effect that NF and Linc have on nuclear genes expression, there is a core response shared with the *sigma* mutants. This relative weakness of the SIG2 and SIG6 signals may be due to their partial redundancy and that the *sig2* and *sig6* mutants still have photosynthesizing chloroplasts. Taken together, however, these data suggest that at least part of the NF and Linc responses are due to a decrease in SIG function and/or plastid transcription.

Although pharmacology has played an important role in pathway analysis in metazoans (Liu and Butow, 2006; Su, 2006; Conradt, 2009) and genetic screens that used chloroplast inhibitors have identified specific processes involved in retrograde signalling (Nott *et al.*, 2006), these studies have recently been under scrutiny (Pfannschmidt, 2010). Therefore, the ability of the *sig* mutants to phenocopy the ‘GUN signal’ is significant. It validates previous studies by demonstrating NF (and likely Linc) can elicit specific plastid signals by decreasing plastid transcription. We provide additional evidence that these signals are not dependent on light (Ruckle *et al.*, 2007) or that these signals are due to reactive oxygen species (ROS) or severe plastid/cell damage that has been speculated by some groups (Mochizuki *et al.*, 2008; Saini *et al.*, 2011). At the same time, the use of *sig* mutants offers an alternative system to monitor retrograde signalling when only some plastid genes are affected. The ability to study these signals independent of light is also an advantage as light signalling networks are known to complicate the analysis of retrograde signalling mechanisms (Ruckle *et al.*, 2007). New genetic screens and in-depth analyses that used these mutants could provide clues as to the mechanisms by which signals are transmitted to the nucleus.

CONCLUSIONS

As seeds germinate, the nucleus must provide sigma factors to interact with the PEP core enzyme to start transcription in the developing chloroplast. The work presented here demonstrates that the successful PEP-mediated expression of $tRNA^{Glu}$ by SIG2 and other photosynthesis genes by SIG6 in the plastid are required to establish a communication pathway with the nucleus to regulate chloroplast development. This signalling network is

independent of light and requires GUN1. The ability to elicit specific retrograde signals with *sigma* mutations offers a novel way to elucidate the mechanisms by which plastids communicate with the nucleus.

EXPERIMENTAL PROCEDURES

Biological material, growth conditions, and treatments

The Arabidopsis transgenic line 6-3 (Col-0/6-3) (Koussevitzky *et al.*, 2007) in the *Columbia* background was used as the wt and parent line for transgenic constructs. This line contains the *LUC* (Luciferase) marker under the control of a minimal *LHCB1.2* promoter. *gun1-9*, *gun5-1*, *sig1-1*, *sig3-2*, and *sig6-1* were described previously (Ishizaki *et al.*, 2005; Koussevitzky *et al.*, 2007; Lai *et al.*, 2011; Susek *et al.*, 1993; Zghidi *et al.*, 2007; Methods S1). The T-DNA insertion lines (Alonso *et al.*, 2003) *sig1-1* (SALK_147985), *sig2-2* (SALK_045706), *sig3-2* (SALK_009166), *sig4- 2* (SALK_078760), *sig5-3* (SALK_141383) and *sig6-1* (SAIL_893_C09) were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/). Double mutants were obtained by crossing mutant lines and all genotypes were confirmed by PCR-based markers. Primer sequences for PCR are listed in Methods S1.

Seeds were surface sterilized using chlorine gas for 16 h and plated on Linsmaier and Skoog medium pH 5.7 (Caisson Laboratories, North Logan, UT, USA) with 0.6% micropropagation type-1 agar powder (Caisson Laboratories). After a 4–6 day stratification in the dark at 4°C, plates were moved to constant light conditions of 25 μmol m^{-2} s^{-1}. To grow etiolated seedlings, plates were wrapped in aluminum foil after a 2-h exposure to light. These dark-grown seedlings were harvested under dim green light. For plastid transcription inhibition studies, rifampicin powder (Sigma) [(final) 50 μg ml^{-1}] was added directly to cool medium before solidification. For photobleaching experiments, medium was supplemented with 5 μM NF (Supelco, Bellafonte, PA, USA) or 220 μg ml^{-1} Linc (Sigma). For experiments with the seedling lethal *sig2*/*sig6* mutant, medium was supplemented with 1% sucrose (w/v). To minimize differences in seed quality for a given experiment, all seeds from all genotypes were harvested from plants grown concurrently in the same growth chambers.

For cloning purposes, *E. coli* and *Agrobacterium tumefaciens* strains were grown in liquid Miller nutrient broth or solid medium that contained 1.5% agar (w/v). Cells were grown at 37°C (*E. coli*) or 28°C (*A. tumefaciens*) with the appropriate antibiotics and liquid medium was shaken at 225 rpm.

RNA extraction, real-time quantitative PCR, and northern blotting

Total RNA was extracted from whole seedlings using the Spectrum Plant Total RNA kit (Sigma-Aldrich, http://www.sigmaaldrich.com) and cDNA was synthesized using the Maxima first strand cDNA synthesis kit (Fermentas, http://www.fermentas.com) following the manufacturer's instructions. Real-time PCR was performed using a C1000 Thermal Cycler and CFX384 R-T System detection instrument (Biorad, http://www.bio-rad.com). The following standard thermal profile was used for all PCRs: 95°C for 3 min, 40 cycles of

95°C for 10 s and 60°C for 30 s. Expression levels for all genes were normalized using *Actin2* and *18S* rRNA as standards. The primers used for qPCR are listed in Methods S1.

Northern blot analysis of $tRNA^{Glu}$ and glutamyl-tRNA were performed as follows: for $tRNA^{Glu}$ (deacetylated form), total RNA was isolated from whole seedlings using the *mir*VanaTM miRNA Isolation Kit (Ambion, http://www.invitrogen.com/site/us/en/home/brands/ambion.html). Then, 0.5 μg of total RNA was separated on denaturing 6% urea polyacrylamide gel electrophoresis (PAGE) and transferred to a nylon membrane. Hybridization was carried out following standard procedures previously described (Chory *et al.*, 1991). $tRNA^{Glu}$ DNA probes were generated from a plasmid that contained the *trnE* genomic DNA. The primers used for probe amplification are listed in Methods S1. For the aminoacylated form of $tRNA^{Glu}$ (glutamyl-tRNA), total RNA isolation was carried out under acidic conditions using the Trizol reagent (Invitrogen http://www.invitrogen.com). Then, 0.5 μg of total RNA was separated by 6.5% acid urea PAGE and transferred as previously described (Kohrer and Rajbhandary, 2008). Membrane was subjected to northern blot hybridization as described above.

RNA-Seq library construction and analysis

Total RNA was isolated from 2-day-old seedlings as described above. For each sample, 10 μg of RNA was depleted of rRNAs following the manufacturer's protocol included in the Ribominus kit from Life Technologies, http://www.lifetechnologies.com/uk/en/home.html. 50 ng of rRNA-depleted samples were used for strand-specific library construction according to the 'Directional mRNA-Seq Library Prep Pre-Release' protocol (Illumina, CA, USA). Libraries were sequenced on an Illumina GAIIx instrument. Sequencing data were aligned to the Arabidopsis TAIR10 reference genome using TOPHAT (version 1.3.3)/ BOWTIE (version 0.12.5) using default commands with the exception of the –segment-length 20 and the –library-type fr-second strand flags (Langmead *et al.*, 2009; Trapnell *et al.*, 2009). Cufflinks software was used for quantification of transcript abundance (Trapnell *et al.*, 2010).

Microarray expression and cluster analysis

Total RNA was extracted from 2-day-old seedlings from three separate biological experiments. The Affymetrix GeneChip Arabidopsis ATH1 Genome Array (Affymetrix, Santa Clara, CA, USA) was used to analyze the expression of ~24 000 genes according to the manufacturer's instructions. Laboratory procedures and data analysis are described in detail in Methods S1.

Luciferase activity measurements

Single 2-day-old seedlings were placed in a microplate well that contained 100 μl Linmaier and Skoog medium pH 5.7. Next, 100 μl of water that contained 2 mM D–luciferin and 0.1% Tween 20 were added followed by a 1 h incubation at room temperature. Luminescence was measured with a Promega (http://www.promega.com/) Glomax 96 microplate luminometer.

Chlorophyll measurements

Two-day-old seedlings were frozen in liquid N_2 and homogenized. Chlorophyll was extracted twice in ice-cold 80% acetone and cell debris was removed by centrifugation at 10 000 ***g*** for 20 min at 4°C. Protein levels were determined by Bradford assay after solubilizing the dried pellet in 0.1 N NaOH. Chlorophyll was measured spectrophotometrically and levels were calculated according to Hendry and Price (1993).

Heme measurements

Two-day-old seedlings were frozen in liquid N_2 and homogenized. Total non-covalently bound heme was extracted twice using acetone with 2% HCL (v:v) and measured by the chemiluminescence-based method described by Masuda and Takahashi (2006) using a Promega Glomax 96 microplate luminometer that measures reconstituted activity from apo-horseradish peroxidase (Biozyme, Blaenovan, Wales). Protein levels were determined by Bradford assay after solubilizing the dried pellet in 0.1 N NaOH.

Pchlide measurements

Pchlide was extracted and quantified as previously described (Shin *et al.*, 2009). Pchlide levels were determined by measuring fluorescence in a Tecan Safire^2 fluorometer.

ALA measurements

ALA accumulation was measured as described previously (Beator and Kloppstech, 1993) with some modifications. Two-day-old Arabidopsis seedlings were transferred to liquid Linsmaier and Skoog (LS) medium that contained 1.3 mM levulinic acid (Sigma) and incubated for 16 h. Tissue was frozen in liquid nitrogen, homogenized in 1 N trichloroacetic acid (TCA), 1% sodium dodecyl sulphate (SDS) and centrifuged at 65 000 ***g*** for 10 min at 4°C. The resulting protein pellet was washed twice with acetone, resuspended in 0.1 N NaOH, centrifuged at 65 000 ***g*** for 10 min at 4°C, and the protein concentration of the supernatant was determined using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). The cleared extract was neutralized with an equal volume of 0.5 M NaH_2PO_4, pH 7.5 and condensed with 50 μl of ethylacetoacetate (Sigma) by boiling for 10 min. The cooled extract was treated with an equal volume of modified Ehrlich's reagent (0.2 g of *p*-dimethylaminobenzaldehyde (Sigma), 8.4 ml of acetic acid, 1.6 ml of 70% perchloric acid), centrifuged for 5 min and assayed spectrophotometrically at 553 nm using a Tecan Saphire plate reader. The amount of porphobilinogen formed from ALA was determined using a coefficient of extinction of 7.45×10^4 M^{-1} cm^{-1} (Harel and Klein, 1972) and normalized to protein content.

Confocal imaging

Confocal microscopy was performed with a Leica SP/2 inverted microscope. Image analysis was performed with the Leica SP/2 software package and the ImageJ bundle provided by the Wright Cell Imaging facility.

Protein extraction and western blot analysis

Protein extraction and western blot analysis were performed using standard laboratory procedures and described in detail in Methods S1.

Construction of overexpression plasmids

Plasmids and plant lines used in this study are listed in Methods S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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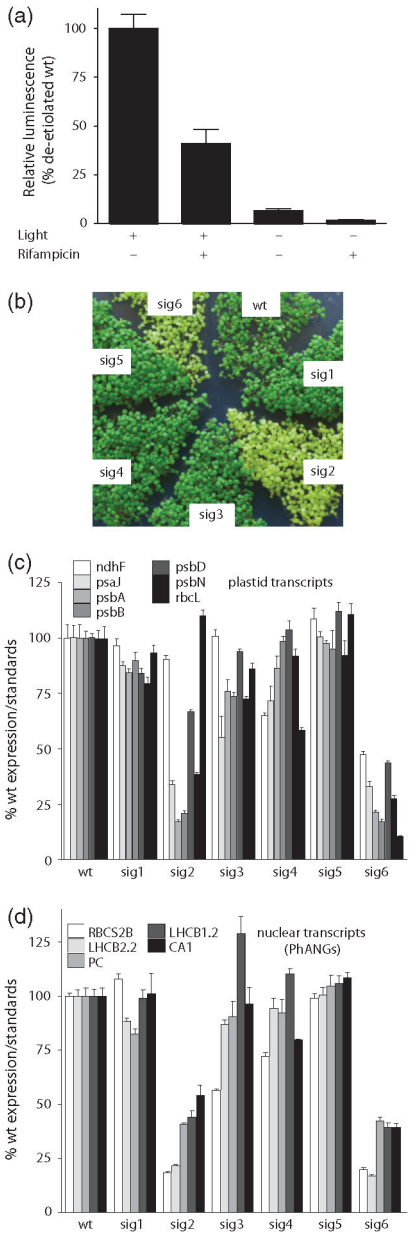

Figure 1.

SIG2 and SIG6-mediated plastid transcription is required for PhANG expression. (a) The average luciferase activity of 2-day-old etiolated (− light) and de-etiolated (+ light) seedlings that harbour the *Luc* gene under the control of a minimal *LHCB1.2* promoter (6–3) and that have been germinated in the presence or absence of 50 μg ml^{-1} rifampicin is shown. Data shown are the mean ± standard error of the mean (SEM) of 16 individual seedlings. (b) Image of 3-day-old *sigma* mutant seedlings. Quantitative polymerase chain reaction (qPCR) analysis of steady-state (c) plastid and (d) PhANG mRNA transcript levels of 2-day-old seedlings. Data shown are the mean ± SEM of triplicate reactions of a representative experiment.

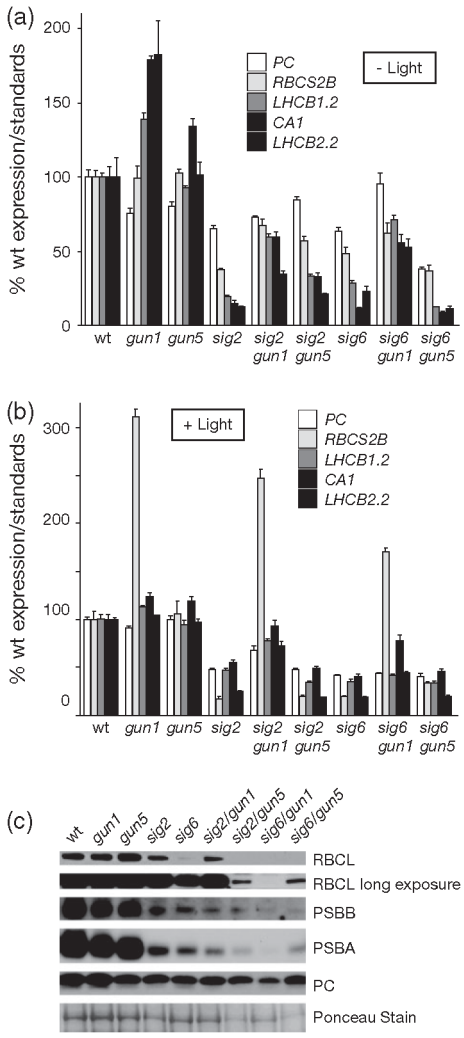

Figure 2. Genetic interactions between *sigma* and *gun* mutants

Quantitative polymerase chain reaction (qPCR) analysis of steady-state PhANG mRNA transcript levels of 2-day-old (a) etiolated and (b) de-etiolated seedlings. Data shown are the mean ± standard error of the mean (SEM) of triplicate reactions of a representative experiment. (c) Western blot analysis of plastid proteins. 20 μg of total protein was fractioned by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted. Filters were probed with antibodies that were specific for the proteins indicated to the right.

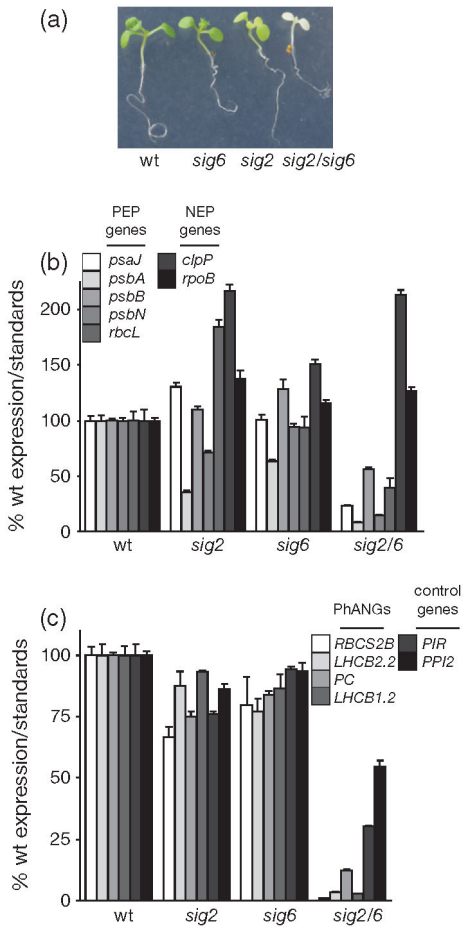

Figure 3. SIG2 and SIG6 are partially redundant for genome coordination

(a) Image of 7-day-old *sigma* mutant seedlings that were grown on medium supplemented with 1% sucrose. Quantitative polymerase chain reaction (qPCR) analysis of steady-state (b) plastid and (c) nuclear mRNA transcript levels of 7-day-old seedlings. Data shown are the mean ± standard error of the mean (SEM) of triplicate reactions of a representative experiment. The *sig2*/*sig6* was propagated as heterozygous for the *sig6* allele.

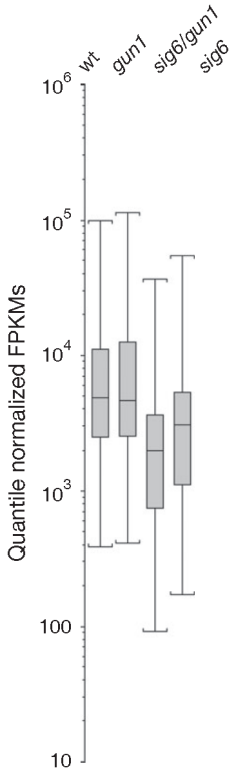

Figure 4. The *gun1* mutation further reduces plastid transcripts in *sig6* mutants

Analysis of steady-state plastid transcripts from 2-day-old seedlings as determined by strand-specific RNA-Seq analysis. Box-plot representation of plastid gene expression levels (Quantile Normalized Fragments Per Kilobase of exon model per Million mapped fragments (FPKMs) reveals that the plastid transcriptome is globally reduced in *sig6* mutants and that this reduction is further enhanced in *sig6/gun1* mutants.

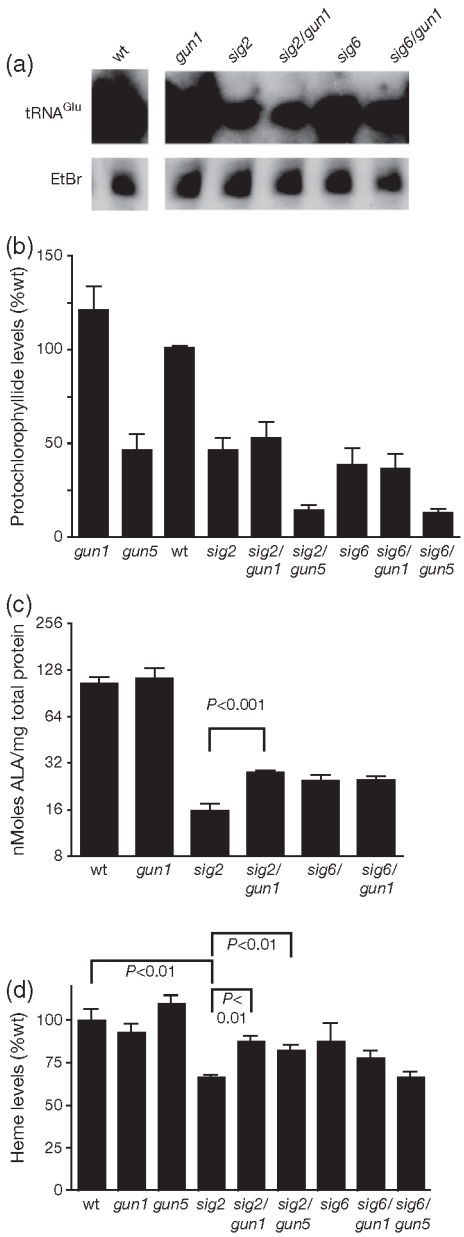

Figure 5. Analysis of tRNA^{Glu} and tetrapyrrole levels in *sig2* and *sig6* mutants

(a) Northern blot analysis of tRNA^{Glu}; (b) steady-state levels of Pchlide; (c) ALA biosynthetic capacity; and (d) steady-state levels of non-covalently bound heme in 2-day-old seedlings. Error bars indicate the mean ± standard error of the mean (SEM) of two independent experiments with biological duplicates.

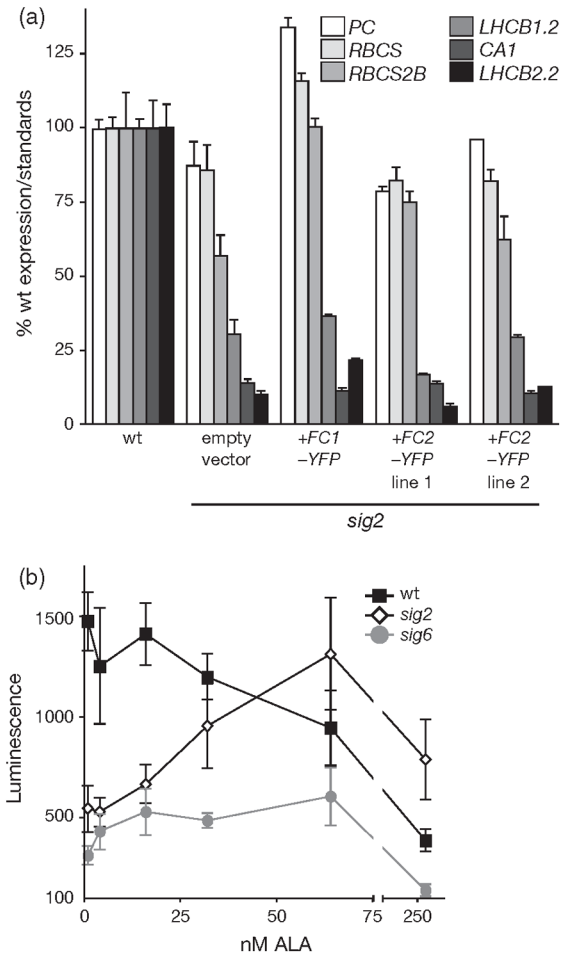

Figure 6. SIG2-mediated signals involve tetrapyrrole synthesis

(a) Quantitative polymerase chain reaction (qPCR) analysis of steady-state PhANG mRNA transcript levels of 2-day-old seedlings. Data shown are the mean ± standard error of the mean (SEM) of triplicate reactions of a representative experiment.

(b) *LHCB1.2* promoter-driven luciferase activity in 2-day-old etiolated seedlings grown in the presence of the indicated the amount of δ-aminolevulinic acid (ALA). Mean ± standard error of the mean (SEM) of eight individual seedlings is shown.

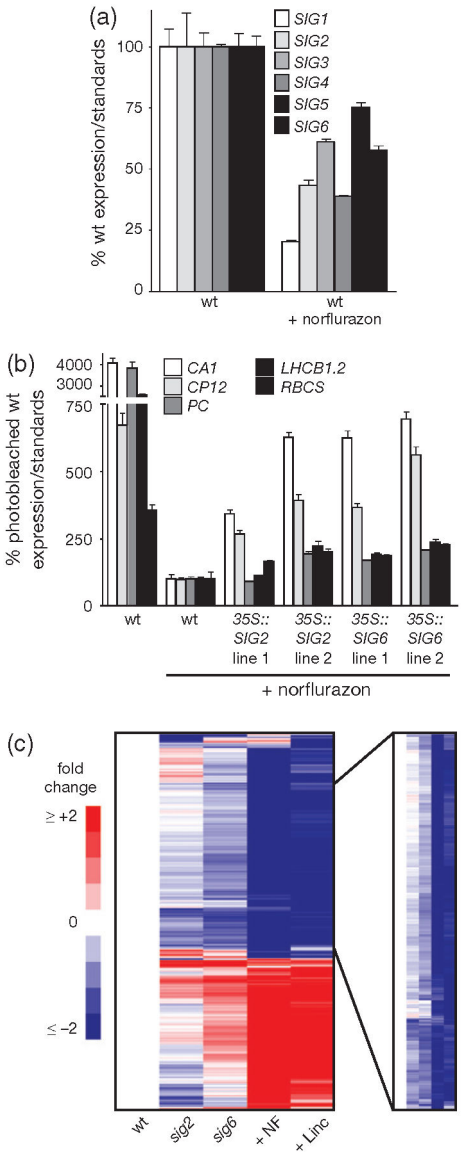

Figure 7. Relationship between SIG-mediated and herbicide-inhibitor signals

Quantitative polymerase chain reaction (qPCR) analysis of steady-state (a) *Sigma* and (b) PhANG mRNA transcript levels of 2-day-old seedlings. Data shown are the mean ± standard error of the mean (SEM) of triplicate reactions of a representative experiment. (c) A heat-map based on a microarray analysis depicts 1638 genes differentially (≥twofold) expressed in the *sigma* mutants (*sig2* or *sig6*) or by herbicide-inhibitor treatment (NF or Linc). Average values were calculated using untreated wild type (wt) as a reference. To the right is a zoomed image of one cluster of 714 similarly expressed genes that was subjected to gene ontology analysis, 353 of which encode plastid proteins.