genome uncoupled1 Mutants Are Hypersensitive to Norflurazon and Lincomycin[1][OPEN]

Xiaobo Zhao,[a,b] Jianyan Huang,[a,b] and Joanne Chory[a,b,2,3]

[a]Plant Biology Laboratory, Salk Institute for Biological Studies, La Jolla, California 92037
[b]Howard Hughes Medical Institute, Salk Institute for Biological Studies, La Jolla, California 92037

ORCID ID: 0000-0002-3664-8525 (J.C.)

Chloroplast development and function relies on the coordinated regulation of chloroplast and nuclear gene expression. Generally, the nucleus is in charge; however, chloroplasts also can signal back to the nucleus via retrograde signaling pathways (Koussevitzky et al., 2007; Woodson and Chory, 2008; Chi et al., 2013; Kim and Apel, 2013; Chan et al., 2016; de Souza et al., 2017). Retrograde signaling pathways are activated when chloroplasts are stressed in high light (Woodson et al., 2015; Chan et al., 2016), heat (Sun and Guo, 2016), or during photosynthesis, which generates reactive oxygen species (Gollan et al., 2015; Chan et al., 2016). Retrograde signaling pathways also play an important role during early leaf development when chloroplasts are establishing a photoautotrophic physiology (Chan et al., 2016; Hernández-Verdeja and Strand, 2018).

In an effort to identify components of retrograde signaling, pharmacological studies were conducted using the photosensitizing herbicide norflurazon (NF), a noncompetitive inhibitor of phytoene desaturase, and lincomycin (Linc), a chloroplast-specific protein synthesis inhibitor. Blocking photosynthesis with NF results in the down-regulation of hundreds of nuclear genes mostly for chloroplast-destined proteins, for example, *LHCB* (light-harvesting chlorophyll *a*/*b*-binding protein) and *RBCS* (Rubisco small subunit) genes. This observation was used in genetic screens (conducted mostly in our lab) in Arabidopsis (*Arabidopsis thaliana*), resulting in the discovery of six *GENOMES UNCOUPLED* (*GUN*) loci. GUN2 to GUN6 are enzymes involved in tetrapyrrole biosynthesis or metabolism (Susek et al., 1993; Mochizuki et al., 2001; Larkin et al., 2003; Woodson et al., 2011). By contrast, GUN1 is a chloroplast-localized pentatricopeptide repeat protein with a C-terminal small MutS-related domain. GUN1 appears to play a role in multiple stress-related retrograde signaling pathways (Koussevitzky et al., 2007). Despite numerous studies of *GUN1* (Ruckle and Larkin, 2009; Cottage et al., 2010; Tadini et al., 2016; Paieri et al., 2018), its exact biochemical mechanism as well as its precise role in retrograde signaling remain enigmatic. A major problem is that *gun1* mutants are difficult to study because they have no visible phenotype that distinguishes them from the wild type under normal growth conditions. Rather, *gun1* mutants must be stably transformed with reporter genes and grown on NF or Linc to be identified (Susek et al., 1993; Koussevitzky et al., 2007). In view of the important role of GUN1 in retrograde signaling, we have been searching for a visible phenotype associated with *gun1* mutants. Here, we report that *gun1* mutants have a visible hypersensitive phenotype when grown on NF or Linc.

Previously, we screened *gun* mutants on medium containing 5 µM NF or 220 µg/mL Linc (with 1% or 2% Sucrose). When grown on 5 µM NF or 220 µg/mL Linc, *gun1* mutant and wild-type seedlings are bleached and look almost identical, except that *gun1* mutants accumulate less anthocyanin than the wild type (Cottage et al., 2010). However, due to the interplay between sucrose and retrograde signaling pathways (Cottage et al., 2010), we now exclude sucrose from the NF or Linc treatment (Supplemental Methods), and this does not affect the detection of the *gun* phenotype (higher nuclear gene expression levels in mutants, compared with the wild type, following chloroplast damage; see results below).

Given the use of NF in our mutant screening, one would expect to uncover mutants that were resistant to NF, for example, mutants that could metabolize NF or ones that could not uptake NF. In addition, one might imagine that indirect effects from mutations that affect chloroplast metabolism/physiology could confound the screen. We did not find such mutants in our original screen. However, when we lowered the concentration of NF to 20 nM, we surprisingly found that a *GUN1* null mutant, *gun1-9*, displayed a visible pale yellow or white and smaller cotyledon phenotype compared with the wild type (Fig. 1A), which indicated that *gun1-9* was actually hypersensitive to NF.

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[2]Senior author.

[3]Author for contact: chory@salk.edu.

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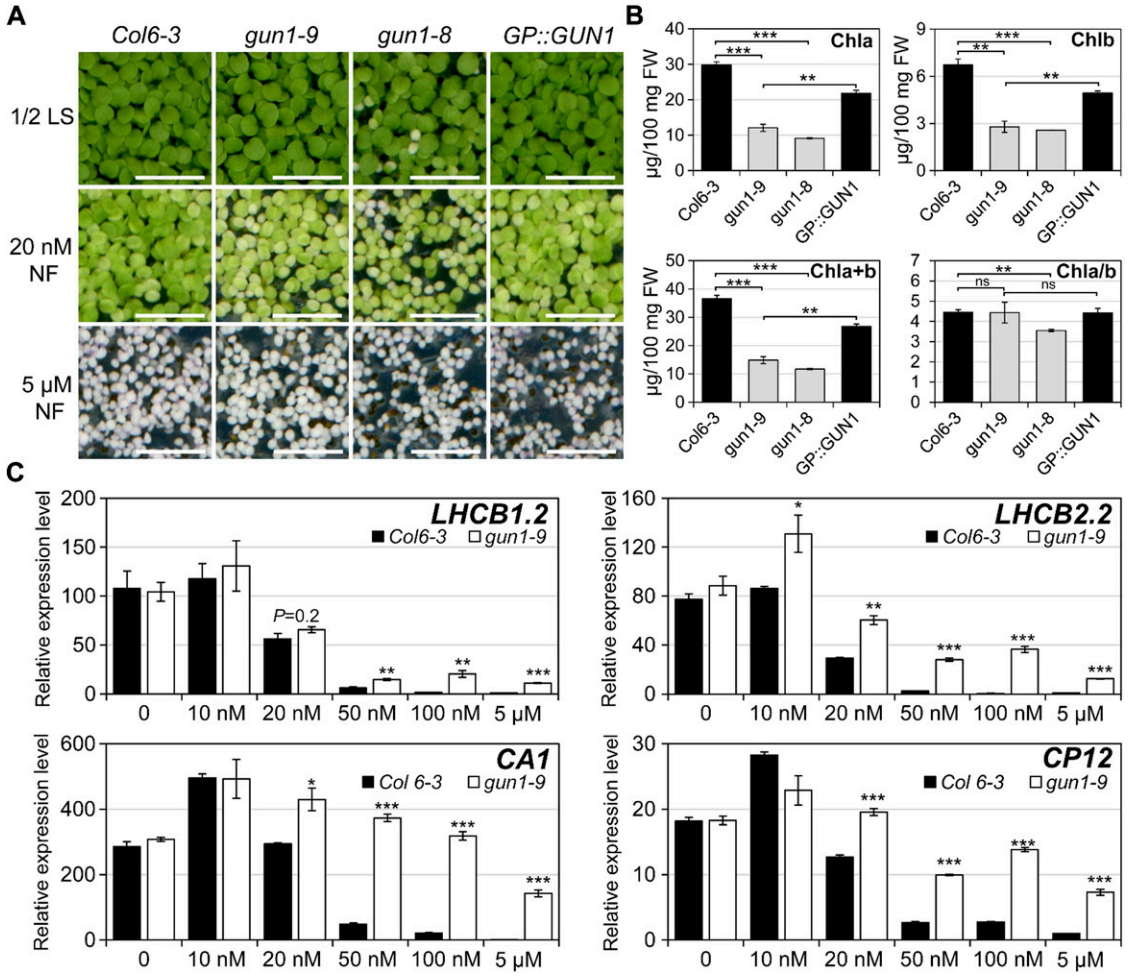

Figure 1. *gun1* mutants are hypersensitive to NF. A, The phenotypes of wild-type *Col6-3*, *gun1* mutants, and *gun1-9* complemented line (*GP*::*GUN1*) grown on 20 nM NF with the following growth condition: 24 h light (100 μmol.m^{-2}.s^{-1}) at 22°C for 6 d. 1/2 LS, No NF. The concentrations of NF are indicated on left. Commonly used concentration of NF is 5 μM. Scale bar = 0.5 cm. B, The chlorophyll content differences among wild-type *Col6-3*, *gun1* mutants, and *gun1-9* complemented line under 20 nM NF (24 h, 100 μmol.m^{-2}.s^{-1} light at 22°C for 6 d). The *y* axis is the chlorophyll levels (μg per 100 mg fresh weight). Chla, Chlorophyll *a*; Chlb, chlorophyll *b*; Chla+b, total chlorophyll; Chla/b, chlorophyll *a*/*b* ratio. Data are mean ± standard error of the mean (SEM) (three biological replicates). $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant (two-tailed Student's *t* test). C, RT-qPCR analysis of multiple *gun* phenotype marker gene expression profile in wild-type *Col6-3* and *gun1-9* mutant under different concentrations of NF with the following growth condition: 24 h light (100 μmol.m^{-2}.s^{-1} at 22°C for 6 d. The *x* axis is the sample with different concentrations of NF. The *y* axis represents the relative expression level, and the expression level of each gene in *Col6-3* under 5 μM NF is set to 1. Data are mean ± SEM (three biological replicates). Asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed Student's *t* test) indicate the significant difference in *gun1-9* versus wild-type *Col6-3* for the same treatment.

Similar results were observed for another *gun1* null mutant, *gun1-8* (Fig. 1A). Transformation of *gun1-9* with a GFP-tagged GUN1 expressed from *GUN1*'s promoter (*GP*::*GUN1*; Koussevitzky et al., 2007) rescued *gun1-9*'s hypersensitive phenotype (Fig. 1A). These observations confirmed the hypersensitive phenotype of *gun1* mutants was caused by the loss of function of *GUN1*. To quantify this hypersensitive phenotype, we measured chlorophyll content in the wild type, *gun1* mutants, and *GP*::*GUN1* line (Supplemental Methods). When grown on 20 nM NF, *gun1* mutants had significantly lower levels of chlorophyll *a*, chlorophyll *b*, and total chlorophyll compared with the wild type (Fig. 1B). Thus, the chlorophyll content profile also revealed the hypersensitive phenotype of *gun1* mutants. Although the *gun1-9* complemented line did not restore the chlorophyll content fully to the wild-type level, it rescued approximately 73% of *gun1-9*'s phenotype (Fig. 1B). GUN1 is a short-lived protein of very low abundance, accumulating to detectable levels only under

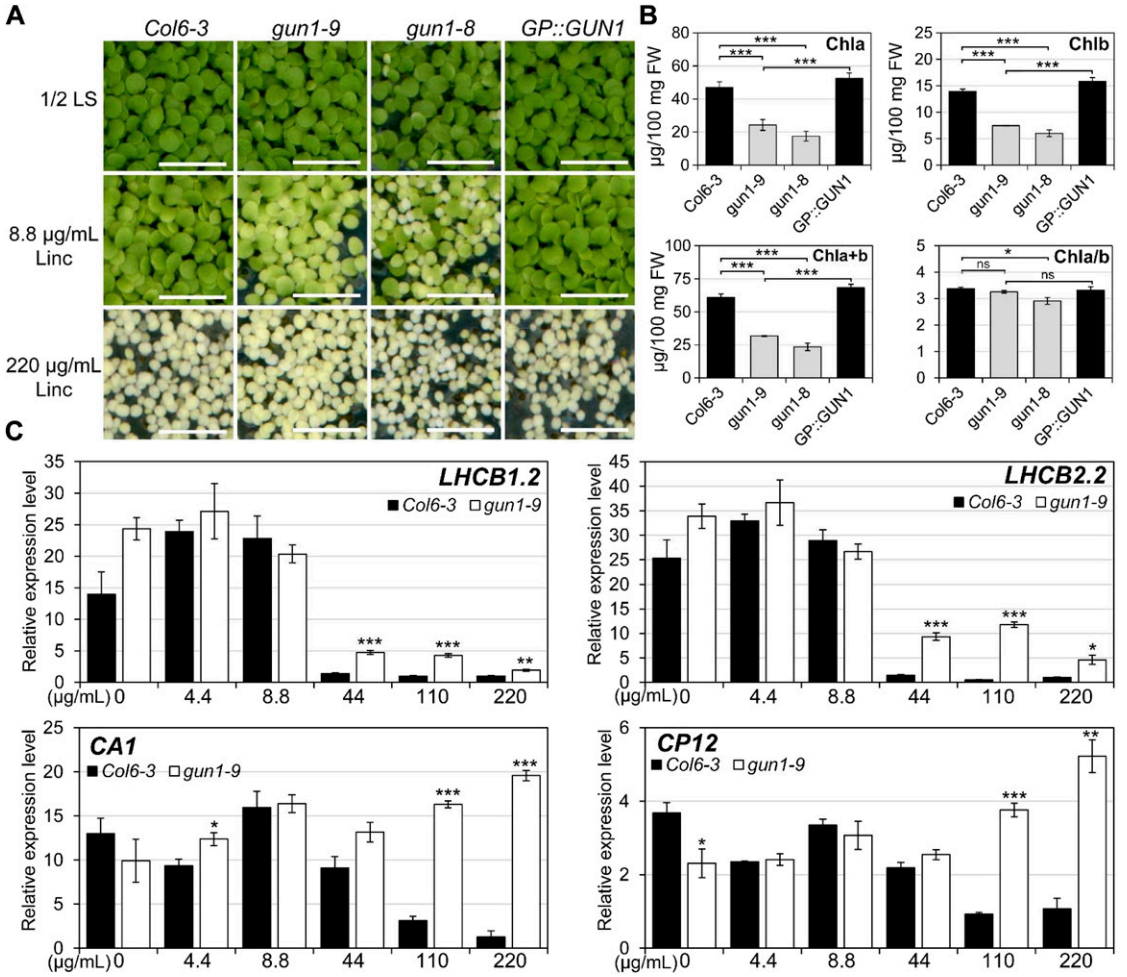

Figure 2. *gun1* mutants are hypersensitive to Linc. A, The phenotypes of wild-type *Col6-3*, *gun1* mutants, and *gun1-9* complemented line (*GP*::*GUN1*) grown on 8.8 μg/mL Linc with the following growth condition: 24 h light (100 μmol.m^{-2}.s^{-1}) at 22°C for 6 d. 1/2 LS, No Linc. The concentrations of Linc are indicated on left. The commonly used concentration of Linc is 220 μg/mL. Scale bar = 0.5 cm. B, The chlorophyll content difference among wild-type *Col6-3*, *gun1* mutants, and *gun1-9* complemented line under 8.8 μg/mL Linc (24 h, 100 μmol.m^{-2}.s^{-1} light at 22°C for 6 d). The *y* axis is the chlorophyll levels (μg per 100 mg fresh weight). Chla, Chlorophyll *a*; Chlb, chlorophyll *b*; Chla+b, total chlorophyll; Chla/b, chlorophyll *a*/*b* ratio. Data are mean ± SEM (three biological replicates). *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant (two-tailed Student's *t* test). C, RT-qPCR analysis of multiple *gun* phenotype marker gene expression profile in wild-type *Col6-3* and *gun1-9* mutant under different concentrations of Linc with the following growth condition: 24 h light (100 μmol.m^{-2}.s^{-1}) at 22°C for 6 d. The *x* axis is the sample with different concentrations (μg/mL) of Linc. The *y* axis represents the relative expression level, and the expression level of each gene in *Col6-3* under 220 μg/mL Linc is set to 1. Data are mean ± SEM (three biological replicates). Asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student's *t* test) indicate the significant difference in *gun1-9* versus wild-type *Col6-3* for the same treatment.

stress that involves retrograde signaling like NF and Linc treatment or during chloroplast biogenesis (Wu et al., 2018). Although *GUN1* was driven by its own promoter in the *GP*::*GUN1* line, *GUN1* expression was transgenic. It is possible that the GUN1 levels in *GP*::*GUN1* were different from the levels of native GUN1 in the wild type, especially under NF treatment. Thus, the *GP*::*GUN1* line only partially complemented the *gun1-9* phenotype of the NF-treated seedlings. Unfortunately, we cannot test this because despite multiple attempts, we have not been able to obtain an antibody that works for native GUN1.

To examine the relationship between the hypersensitive phenotype and the molecular *gun* phenotype of *gun1* mutants, we set up a detailed dose response assay with 0, 10 nM, 20 nM, 50 nM, 100 nM, and 5 μM NF (Supplemental Fig. S1A) and examined the expression changes of multiple *gun* phenotype marker genes (RNAs of which accumulated in *gun* mutants in the presence of NF; Woodson et al., 2013) (Supplemental

Methods). The results indicated that when grown on 20 nM NF, *LHCB2.2*, *CA1* (carbonic anhydrase 1), and *CP12* (chloroplast protein 12) showed significantly higher expression levels in *gun1-9* compared with the wild type (Fig. 1C), demonstrating that retrograde signaling pathways may have already been activated. However, the NF-sensitive differences between *gun1* mutants and the wild type could not simply be explained by the expression level changes of these marker genes. A possible explanation for this might be that under NF, at the genome-wide scale, a portion of nuclear genes had higher expression levels in *gun1* mutants while another portion of nuclear genes had lower expression levels in *gun1* mutants compared with the wild type (Koussevitzky et al., 2007). Another possible reason is that GUN1 also may play a role in chloroplast development, and the hypersensitive phenotype is caused by the loss of function of *GUN1* in both chloroplast development and retrograde signaling.

As *gun1* mutants also exhibit a *gun* phenotype in response to Linc (Koussevitzky et al., 2007), we carried out a dose response assay of Linc (4.4 µg/mL, 8.8 µg/mL, 44 µg/mL, 110 µg/mL, and 220 µg/mL) as well. The assay again showed that under 8.8 µg/mL Linc, more *gun1* mutant seedlings were pale yellow or white with smaller cotyledons compared with the wild type while *gun1-9* complemented plants resembled the wild type (Fig. 2A; Supplemental Fig. S1B). Chlorophyll content profiles under 8.8 µg/mL Linc also showed that *gun1* mutants had significantly lower chlorophyll levels compared with the wild type and the *gun1-9* complemented plants restored to wild-type levels (Fig. 2B). These results suggest that *gun1* mutants also are hypersensitive to Linc. However, we examined the expression of marker genes and found that 8.8 µg/mL Linc did not affect marker gene expression in *gun1-9*. The concentration of Linc needed to increase to 44 µg/mL to activate retrograde signaling (Fig. 2C). This indicated that under low concentrations of Linc (e.g. 8.8 µg/mL), the chloroplast development in *gun1* mutants was affected but the concentration of Linc was not high enough to activate GUN1-related retrograde signaling pathways. This further suggests GUN1's role both in chloroplast development and retrograde signaling under stress conditions.

We conclude that seedlings lacking *GUN1* in fact present a hypersensitive phenotype to both NF and Linc treatments. This finding suggests that NF treatment may cause the accumulation of a "toxic" intermediate in the plant, which in turn down-regulates nuclear gene expression. The NF and Linc hypersensitive phenotype of *gun1* mutants shows that GUN1 may play a role in early development of chloroplasts as well as in retrograde signaling. In addition, these observations suggest conditions for conducting sensitized genetic screens for new *gun* mutants. Our discovery of a visible phenotype for *gun1* mutants will help clarify and define the role of GUN1 in chloroplast development and retrograde signaling.

Accession Numbers

The accession numbers for the genes mentioned in this article are as follows: *GUN1*, AT2G31400; *LHCB1.2*, AT1G29910; *LHCB2.2*, AT2G05070; *CA1*, AT3G01500; and *CP12*, AT3G62410.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. The seedling phenotype of *Col6-3* wild type and *gun1-9* mutant under different concentrations of NF or Linc treatment.

Supplemental Table S1. Primers used in RT-qPCR analysis.

Supplemental Methods. Supplemental methods.

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