Letter

Plastid-to-Nucleus Retrograde Signalling during Chloroplast Biogenesis Does Not Require ABI4[1][OPEN]

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Chloroplast-to-nucleus retrograde signaling pathways function during chloroplast development to enable coordination of the nuclear and chloroplast genomes for the assembly of the photosynthetic apparatus (Chan et al., 2016). This coordination is extremely important for seedling survival, as misregulation of photosynthetic development can lead to severe photooxidative damage and seedling lethality. The pathways mediating chloroplast-to-nucleus retrograde signaling during chloroplast development, termed biogenic signaling, are still poorly understood, but the transcription factor ABSCISIC ACID-INSENSITIVE4 (ABI4) has been proposed as an important downstream component (Koussevitzky et al., 2007) and features prominently in all published models (Chan et al., 2016; de Souza et al., 2017; Brunkard and Burch-Smith, 2018; Hernández-Verdeja and Strand, 2018). However, we had observed that chloroplast-to-nucleus retrograde signaling was not affected in *abi4* mutants. Given the prevalence of ABI4 in retrograde signaling models, we have now systematically assessed the phenotype of *abi4* mutants in an attempt to clarify the role of ABI4 in this signaling pathway. Here, we have analyzed the expression of eight retrograde-regulated nuclear genes following treatments with norflurazon (NF) and lincomycin (Lin), which block chloroplast development, in multiple *abi4* alleles and in four different laboratories. Our analyses show no consistent effect of *abi4* mutations on the retrograde response and do not support a role for ABI4 in this pathway. Therefore, we propose that ABI4 be omitted from future models of biogenic chloroplast-to-nucleus retrograde signaling.

Biogenic chloroplast-to-nucleus retrograde signaling pathways have been demonstrated using mutants that lack normal chloroplast development or the application of treatments such as the carotenoid synthesis inhibitor NF or the plastid translation inhibitor Lin. Both chemical treatments lead to chloroplast damage and a photobleached phenotype and result in a severe reduction in the expression of most photosynthesis-related nuclear genes (Koussevitzky et al., 2007; Woodson et al., 2013). The signaling pathway mediating this response remains unknown, but clues have come from the isolation of mutants that show less inhibition of nuclear gene expression after chloroplast damage. These mutants, termed *genomes uncoupled* or *gun* mutants, were identified originally as having elevated expression of the nuclear gene *LIGHT HARVESTING CHLOROPHYLL A/B BINDING PROTEIN1.2* (*LHCB1.2*) after NF treatment, a response that has become known as a *gun* phenotype. The original screens resulted in six loci that are important in retrograde signaling: five of these encode components of the tetrapyrrole biosynthesis pathway and rescue expression on NF (Mochizuki et al., 2001; Larkin et al., 2003; Woodson et al., 2011), while the sixth, *gun1*, lacks a pentatricopeptide repeat protein and can rescue expression on both NF and Lin (Koussevitzky et al., 2007). Based on these discoveries, the current model for chloroplast-to-nucleus retrograde signaling during chloroplast biogenesis is that signals from different sources, including tetrapyrrole biosynthesis, are integrated by GUN1 and relayed to the nucleus (Chan et al., 2016).

ABI4 was first identified in a screen for mutants that could germinate in the presence of abscisic acid

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(Finkelstein, 1994) and subsequently was shown to be related to a family of transcription factors containing an APETALA2 (AP2) domain, one of 147 AP2/ethylene response element-binding proteins in the Arabidopsis (*Arabidopsis thaliana*) genome (Nakano et al., 2006). ABI4 has been implicated in many growth and developmental responses in plants, with *abi4* mutants also being identified independently in screens for sugar signaling mutants (León et al., 2013). These roles include signaling from the mitochondria to regulate *ALTERNATIVE OXIDASE1a* (Giraud et al., 2009) and chloroplast-to-nucleus retrograde signaling during chloroplast development (Koussevitzky et al., 2007). A role for ABI4 in chloroplast-to-nucleus signaling was first proposed by Nott et al. (2006) based on the reduced inhibition of a heterologous *RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN* (*RBCS*)-*GUS* reporter in an *abi4* mutant background after NF treatment (Acevedo-Hernández et al., 2005; although no effect of *abi4* was seen for an NF-responsive minimal *CMA5* promoter construct) and their own data, later published as Koussevitzky et al. (2007), showing that *abi4* also rescued *LHCB* expression after Lin treatment. From that point, ABI4 became established as a signaling intermediate in biogenic retrograde signaling and is included routinely in all published models. Despite this, the evidence for a role for ABI4 in chloroplast-to-nucleus signaling is not undisputed. Although some recent studies support a role for ABI4 (Sun et al., 2011; Zhang et al., 2013; Guo et al., 2016), others have not observed a *gun* phenotype on NF or Lin when looking at the expression of *CARBONIC ANHYDRASE1* (*CA1*; Cottage and Gray, 2011), *LHCB1.1* (Kerchev et al., 2011), or *GOLDEN2-LIKE1* (*GLK1*; Martín et al., 2016). An *abi4* mutant also was unable to rescue the loss of nuclear gene expression in the *ppi2* mutant, in contrast to *gun1* (Kakizaki et al., 2009). We also independently observed that *abi4* mutants did not show a *gun* phenotype in our assays. Therefore, to try and resolve the question of whether ABI4 is required for biogenic retrograde signaling, we systematically assessed the phenotypes of four different *abi4* alleles across four different research laboratories in three locations (Southampton, United Kingdom; Kyoto, Japan; and Munich, Germany).

The four different alleles of *abi4* used in this study were the *abi4-102* allele used by Koussevitzky et al. (2007), the *abi4-1* allele used by Sun et al. (2011), and two alleles that have not been characterized previously in terms of retrograde signaling, *abi4-2* and *abi4-4* (Supplemental Fig. S1; Supplemental Table S1; Supplemental Methods). Previous studies supporting a role for ABI4 based their conclusions on changes in *LHCB* expression measured by RNA gel blotting (Koussevitzky et al., 2007) or reverse transcription quantitative PCR (RT-qPCR) experiments with *LHCB2.1* (Sun et al. [2011] used a primer pair that most closely matched this gene) or *LHCB1.2* (Guo et al., 2016) in the presence of Suc. Therefore, we included both of these genes in our analysis, which also was performed in the presence of Suc (for a summary of the conditions used in this study, see Supplemental Table S2). As shown in Figure 1A, the expression of *LHCB1.2*, *LHCB2.1*, and three additional chlorophyll synthesis genes, *HEMA1*, *CHLH*, and *GUN4*, which show a strong dependence on GUN-mediated retrograde signaling (Moulin et al., 2008; Page et al., 2017), was strongly down-regulated in the presence of NF in wild-type seedlings with no increase in expression observed in any of the four *abi4* alleles tested. In contrast, the *gun1-103* mutant showed a strong rescue of nuclear gene expression in all cases. In parallel experiments performed in Kyoto, which included two additional NF down-regulated genes, *RBCS1A* and *GLK1*, and *gun1-102* as a control, identical results were observed, although a small but statistically significant increase was seen for *LHCB1.2* in the *abi4-102* mutant only (Fig. 1B). Similar experiments using Lin to inhibit nuclear gene expression also showed essentially the same results, except that a very small, but significant, *gun* phenotype was observed in *abi4-2* for *LHCB2.1*, *CHLH*, and *GUN4* and in *abi4-102* for *HEMA1* and *CHLH* in the experiments performed in Southampton (Fig. 2A). This was under conditions in which *gun1-103* rescued expression almost completely (Fig. 2A). However, no *gun* phenotype was observed in the experiments performed in Kyoto, including for *LHCB2.1* in *abi4-2* (Fig. 2B). To confirm that the lack of a *gun* phenotype was not due to the choice of reference gene, we replotted the data in Figure 2A using *ACTIN2* (Sun et al., 2011) instead of *YLS8*. This made no difference to the conclusion, with a small, but significant, response seen only for the *LHCB2.1* gene in *abi4-2* (Supplemental Fig. S2A).

In the final set of experiments to test for a *gun* phenotype in *abi4*, which were performed in Munich, analysis was conducted using both RNA gel-blot analysis, as used in the original Koussevitzky et al. (2007) study, and RT-qPCR (Fig. 3). RNA gel-blot analyses of *LHCB1.2*, *LHCB2.1*, and *CA1* showed no evidence for elevated gene expression after NF treatment in three *abi4* alleles, while three *gun1* alleles all showed strong responses (Fig. 3A). Similar results were observed after a shorter 6-d treatment with NF and WLc (Supplemental Fig. S2B). After Lin treatment, a very small increase in expression was observed for *LHCB1.2* and *LHCB2.1*, but not for *CA1*, and only in *abi4-102*, not in *abi4-1* or *abi4-2* (Fig. 3B). Since Koussevitzky et al. (2007) used the *abi4-102* allele, this result may account for their observations, but with the absence of a phenotype in the other *abi4* alleles tested, it cannot be interpreted as supporting a role for ABI4. Finally, analysis of *abi4-1* (used by Sun et al., 2011) did not show a *gun* phenotype for either *LHCB1.2* or *LHCB2.1* after NF or Lin treatment under conditions in which the positive controls *gun1-103* and a *GLK1*-overexpressing line (Leister and Kleine, 2016; Martín et al., 2016) both resulted in a strong rescue of gene expression (Fig. 3, C and D). This result was not dependent on the reference gene used (Supplemental Fig. S2, C and D). Interestingly, simultaneous analysis of

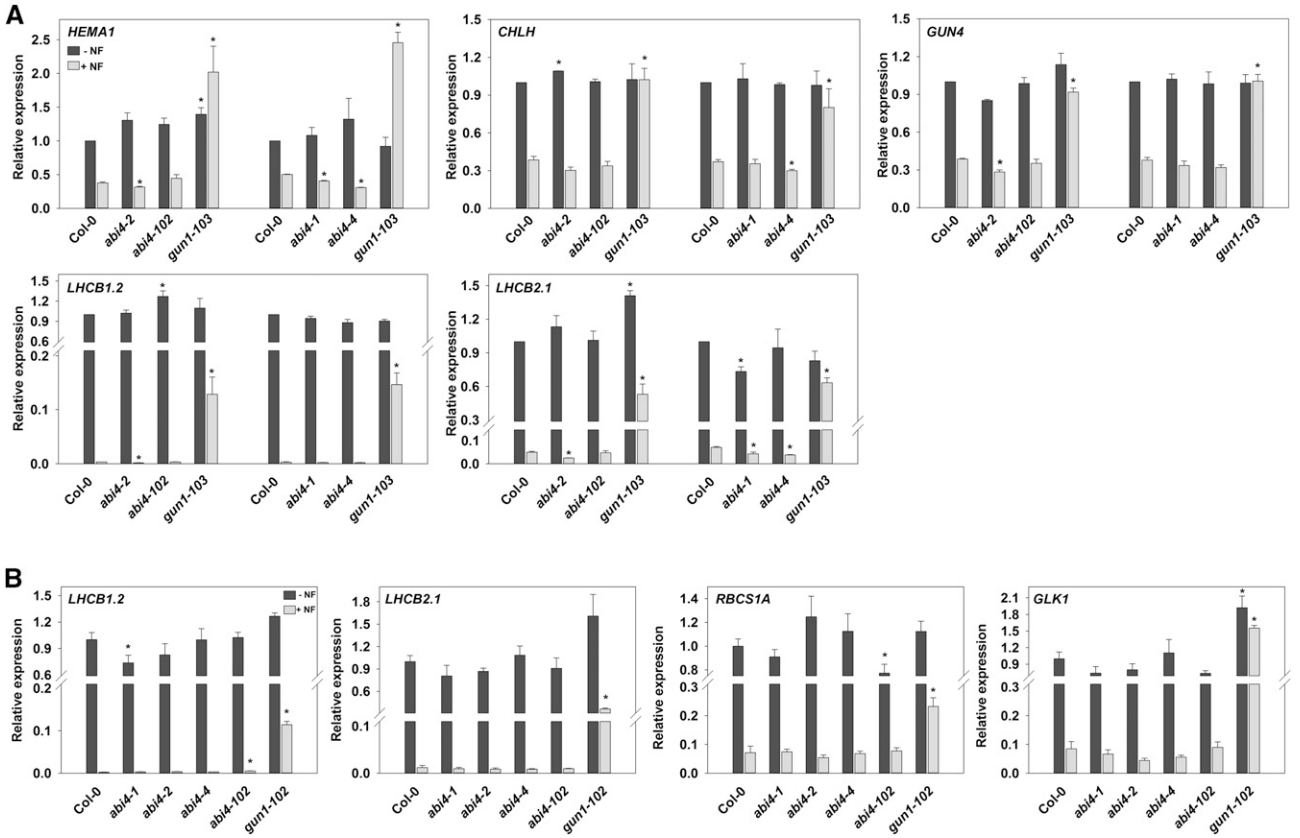

Figure 1. *abi4* mutants do not show a *gun* phenotype on NF. A, Seedlings were grown on one-half-strength Murashige and Skoog medium supplemented with 1% (w/v) Suc and 1% (w/v) agar (pH 5.8) with (light gray bars) or without (dark gray bars) 1 μM NF for 2 d of dark followed by 3 d of continuous white light (WLc; 100 μmol m^{-2} s^{-1}). B, Seedlings were grown on Murashige and Skoog medium supplemented with 2% (w/v) Suc and 0.8% (w/v) agar (pH 5.8) with (light gray bars) or without (dark gray bars) 2.5 μM NF and grown for 4 d in WLc (100 μmol m^{-2} s^{-1}). Expression was determined by RT-qPCR and is relative to wild-type Columbia-0 (Col-0) −NF and normalized to *YELLOW LEAF SPECIFIC GENE8* (*YLS8*, At5g08290; A) or to *TUBULIN BETA-CHAIN2* (*TUB2*, At5g62690; B). Data shown are means + SE of three independent biological replicates. Asterisks denote significant differences versus the wild type for the same treatment (−NF or +NF) by Student's *t* test ($P < 0.05$).

expression in the *ptm1* mutant (Supplemental Fig. S2, E and F) confirmed that a third laboratory failed to see a *gun* phenotype for this mutant, consistent with our previous study (Page et al., 2017).

In the original study by Koussevitzky et al. (2007), it was reported that there was significant overlap of *gun1*- and *abi4*-regulated genes (approximately 50% of derepressed or repressed genes) following transcriptome analysis, and this finding was used to support the hypothesis that they act in the same retrograde pathway. Here, we reanalyzed this data set and compared the response to Lin in *abi4-102* and *gun1-1*. As shown in Supplemental Figure S3, the response in *abi4-102* clustered with the wild type after Lin treatment in contrast to *gun1-1*, but it did show some difference from the wild type in control conditions (Supplemental Fig. S3, A and B). Expression analysis after Lin treatment correlated well between the wild type and *abi4-102* but not between the wild type and *gun1-1* overall (Supplemental Fig. S3C), and this could be seen clearly when changes in the expression of individual photosynthesis (Supplemental Fig. S3D) and tetrapyrrole biosynthesis (Supplemental Fig. S3E) genes were analyzed. Similar conclusions were drawn from this data set by Martín et al. (2016) in the context of PHYTOCHROME INTERACTING FACTOR-regulated genes. Therefore, these results do not support a role for ABI4 in the same retrograde pathway as GUN1.

One of the observations that supported a prominent role for ABI4 in chloroplast-to-nucleus retrograde signaling was that *ABI4* gene expression was strongly up-regulated on NF and Lin and that this response was completely absent in the *gun1* mutant (Sun et al., 2011). This followed on from initial observations that *ABI4* expression was reduced in *gun1* in the presence and absence of Lin (Koussevitzky et al., 2007). We tested this response in our assays and observed very different results. In this case, treatment with NF or Lin resulted in a 34- or 6-fold increase in *ABI4* expression in wild-type seedlings, respectively, and expression was even more strongly up-regulated in the three different *gun1* alleles

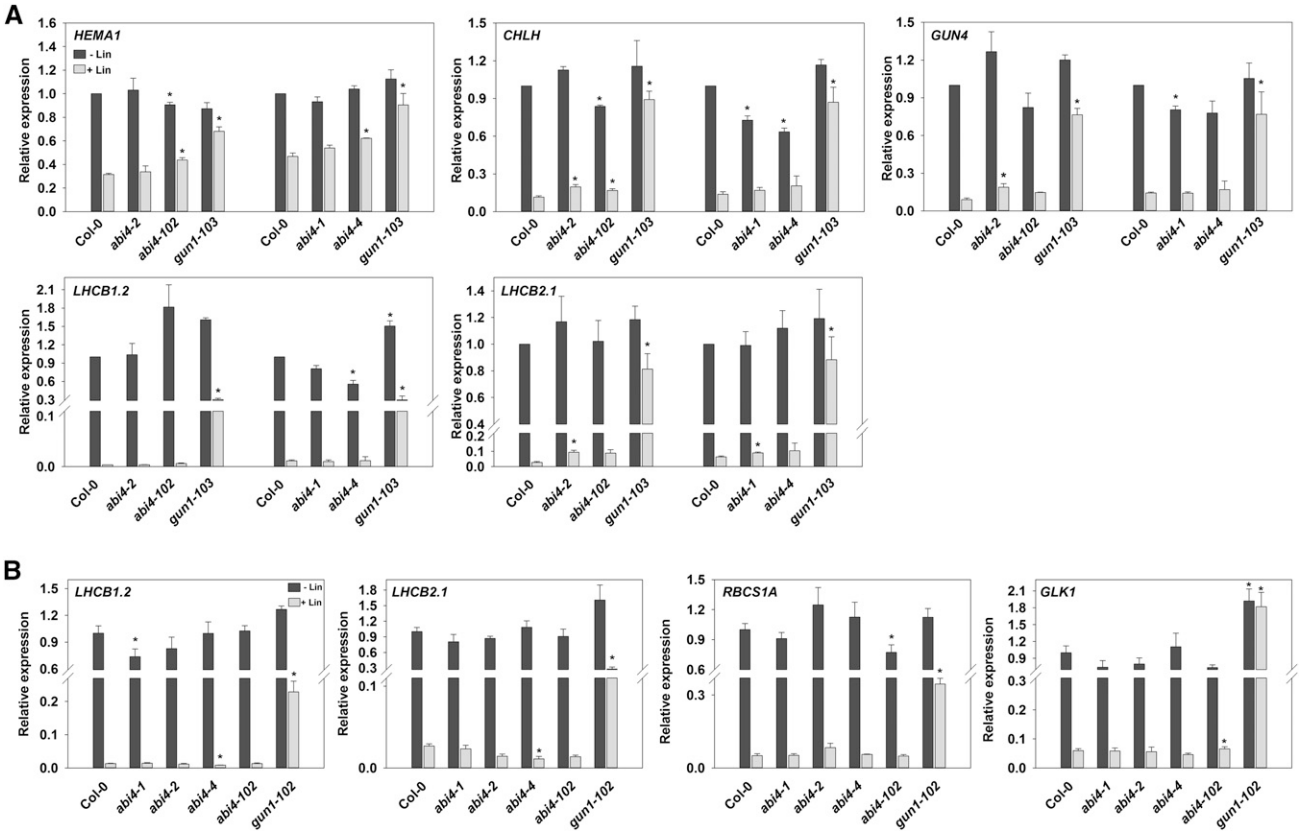

Figure 2. *abi4* mutants do not show a *gun* phenotype on Lin. A, Seedlings were grown on one-half-strength Linsmaier and Skoog medium supplemented with 2% (w/v) Suc and 0.8% (w/v) agar (pH 5.8) with (light gray bars) or without (dark gray bars) 0.5 mM Lin for 5 d of dark after an initial 2 h of WLc treatment (120 μmol m^{-2} s^{-1}). B, Seedlings were grown on Murashige and Skoog medium supplemented with 2% (w/v) Suc and 0.8% (w/v) agar (pH 5.8) with or without 450 μM Lin for 4 d in WLc (100 μmol m^{-2} s^{-1}). Expression was determined by RT-qPCR and is relative to wild-type Columbia-0 (Col-0) −Lin and normalized to *YLS8* (At5g08290; A) or to *TUB2* (At5g62690; B). The expression values for the control condition −Lin in B are the same as those shown in Figure 1B (−NF; dark gray bars). Data shown are means + SE of three independent biological replicates. Asterisks denote significant differences versus the wild type for the same treatment (−Lin or +Lin) by Student's *t* test ($P < 0.05$).

tested (Supplemental Fig. S4A). An increase in *ABI4* expression also was observed in *gun1* mutants when analyzed by RNA gel-blot analysis (Supplemental Fig. S4B). Thus, although the induction of *ABI4* expression under these stress conditions was confirmed in this study, the response in *gun1* was opposite to that reported previously and not consistent with the regulation of *ABI4* via a GUN1-mediated retrograde signaling pathway.

Recent models for biogenic chloroplast-to-nucleus retrograde signaling have ABI4 acting downstream of GUN1 in a PTM-dependent pathway. While the strong gene expression phenotype of different *gun1* mutant alleles has been verified in many studies, including this one, further analysis of the role of PTM in retrograde signaling has not supported such a model (Page et al., 2017; this study). Here, we have reevaluated the role of ABI4 in biogenic retrograde signaling using the same basic experimental conditions, such as the presence of Suc and developmental age of the seedlings, and by testing the same genes. If ABI4 has a major role in this signaling pathway (and previous studies have shown the response to be almost as strong as that of *gun1*; Sun et al., 2011), then we would expect to see some response under the conditions tested across the three different locations in which our experiments were conducted. The results presented here show no consistent or strong *gun* phenotype for multiple *abi4* alleles across multiple laboratories and, therefore, do not support a role for ABI4 in biogenic retrograde signaling. As noted earlier, other studies also have reported a lack of a *gun* phenotype for *abi4* mutants (Cottage and Gray, 2011; Kerchev et al., 2011; Martín et al., 2016), and our results can be considered to be in agreement with these. Therefore, we recommend that ABI4 should be omitted from future models of chloroplast-to-nucleus retrograde signaling. There have been some significant recent developments in our understanding of the importance of tetrapyrroles and chloroplast protein homeostasis in plastid retrograde signaling (Woodson

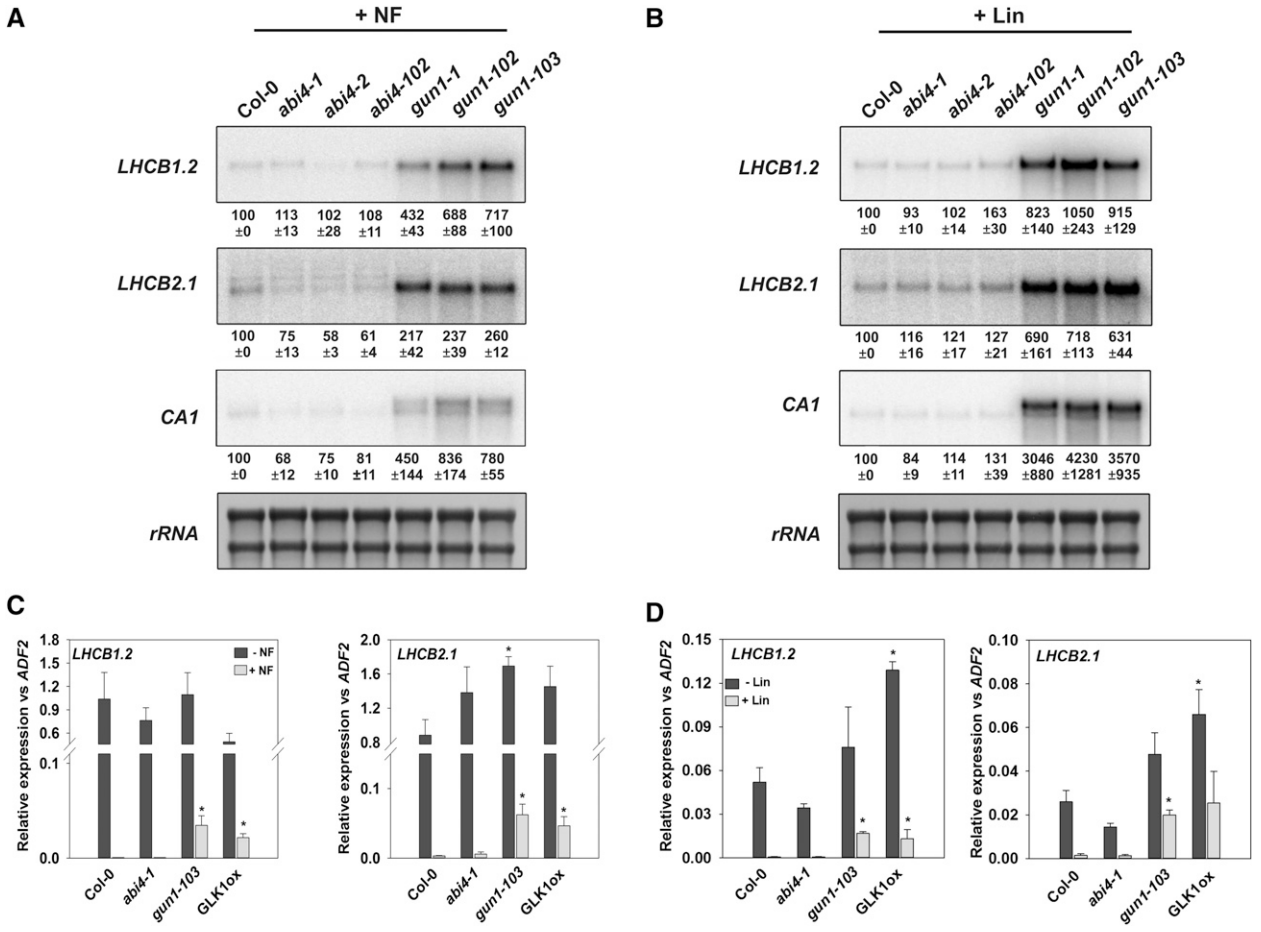

Figure 3. *abi4* mutants do not show a *gun* phenotype after NF or Lin treatment. A and B, Expression of photosynthetic genes after NF and Lin treatments determined by RNA gel-blot analysis. Seedlings were grown on one-half-strength Murashige and Skoog medium supplemented with 1% (w/v) Suc and 1% (w/v) agar (pH 5.8) with or without 5 μM NF (A) or 0.5 mM Lin (B). For NF treatments (A), seedlings were grown for 4 d of dark and 3 d in WLc (100 μmol m^{-2} s^{-1}), and for Lin treatments (B), seedlings were grown for 6 d in WLc (100 μmol m^{-2} s^{-1}). Five micrograms of total RNA was loaded per sample with Methylene Blue staining of rRNA as a loading and RNA-transfer control. Results from one of three independent experiments are shown, with values indicating means ± SE of densitometric scans from all three experiments. C and D, Expression of photosynthetic genes after NF and Lin treatments determined by RT-qPCR. Seedlings were grown on one-half-strength Murashige and Skoog medium supplemented with 2% (w/v) Suc and 0.8% (w/v) agar (pH 5.8) with or without 5 μM NF (C) or 0.5 mM Lin (D) under the same growth conditions as for A and B. Expression is relative to *ACTIN DEPOLYMERIZING FACTOR2* (*ADF2*, At3g46000), and data shown are means + SE of three independent biological replicates. Asterisks denote significant differences versus wild-type Columbia-0 (Col-0) for the same treatment (NF or Lin) by Student's *t* test ($P < 0.05$).

et al., 2011; Tadini et al., 2016; Paieri et al., 2018; Wu et al., 2018), and attention can now focus on these areas of research.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Characterization of the four *abi4* mutant alleles used in this study.

Supplemental Figure S2. Additional analyses of retrograde regulation of photosynthetic gene expression after NF and Lin treatments.

Supplemental Figure S3. Reanalysis of microarray data from Koussevitzky et al. (2007).

Supplemental Figure S4. Regulation of *ABI4* gene expression by retrograde signaling.

Supplemental Table S1. Primers used in this study.

Supplemental Table S2. Summary of treatment conditions used in this study.

Supplemental Methods. Supplemental materials and methods.

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