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Short title: *abi4* is not a *gun* mutant

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Plastid-to-nucleus retrograde signalling during chloroplast biogenesis does not require ABI4[1]

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Summary sentence: Multiple *abi4* alleles fail to show a deficiency in chloroplast-to-nucleus retrograde signalling indicating that, contrary to contemporary models, ABI4 is not a component of this signalling pathway.

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Chloroplast-to-nucleus retrograde signalling pathways function during chloroplast development to enable co-ordination of the nuclear and chloroplast genomes for the assembly of the photosynthetic apparatus (Chan et al., 2016). This co-ordination is extremely important for seedling survival as mis-regulation of photosynthetic development can lead to severe photo-oxidative damage and seedling lethality. The pathways mediating chloroplast-to-nucleus retrograde signalling during chloroplast development, termed biogenic signalling, are still poorly understood, but the transcription factor ABSCISIC ACID-INSENSITIVE 4 (ABI4) has been proposed as an important downstream component (Koussevitzky et al., 2007) and features prominently in all published models (for recent examples see Brunkard and Burch-Smith, 2018; Chan et al., 2016; Hernández-Verdeja and Strand, 2018; de Souza et al., 2017). However, we had observed that chloroplast-to-nucleus retrograde signalling was not affected in *abi4* mutants. Given the prevalence of ABI4 in retrograde signalling models, we have now systematically assessed the phenotype of *abi4* mutants in an attempt to clarify the role of ABI4 in this signalling pathway. Here, we have analysed 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. We therefore propose that ABI4 is omitted from future models of biogenic chloroplast-to-nucleus retrograde signalling.

Biogenic chloroplast-to-nucleus retrograde signalling 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 signalling 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 originally identified as having elevated expression of the nuclear gene *LIGHT HARVESTING CHLOROPHYLL A/B BINDING PROTEIN 1.2* (*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 signalling: five of these encode components of the tetrapyrrole biosynthesis pathway and rescue expression on NF (Mochizuki et al., 2001; Larkin et al., 2004; Woodson et al., 2011), while the sixth, *genomes*

uncoupled 1 (*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 signalling 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 (ABA) (Finkelstein, 1994) and was subsequently shown to be related to a family of transcription factors containing an Apetala 2 (AP2) domain, one of 147 AP2/ ethylene response element binding proteins (EREBPs) 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 independently identified in screens for sugar signalling mutants (León et al., 2013). These roles include signalling from the mitochondria to regulate *ALTERNATIVE OXIDASE1a* (Giraud et al., 2009) and chloroplast-to-nucleus retrograde signalling during chloroplast development (Koussevitzky et al., 2007). A role for ABI4 in chloroplast-to-nucleus signalling 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; but no effect of *abi4* was seen for an NF-responsive minimal *CMA5* promoter construct) and their own data, later published in Koussevitzky et al. (2007), showing that *abi4* also rescued *LHCB* expression after Lin treatment. From this point, ABI4 has become established as a signalling intermediate in biogenic retrograde signalling and is routinely included in all published models. Despite this, the evidence for a role for ABI4 in chloroplast-to-nucleus signalling 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 expression of *CARBONIC ANHYDRASE 1* (*CA1*; Cottage and Gray 2011), *LHCB1.1* (Kerchev et al., 2011) or *GOLDEN2-LIKE 1* (*GLK1*; Martin et al., 2016). An *abi4* mutant was also unable to rescue the loss of nuclear gene expression in the *ppi2* mutant, in contrast to *gun1* (Kakizaki et al., 2009). We had 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 signalling, we have systematically assessed the phenotype of four different *abi4* alleles across four different research laboratories in three locations (Southampton, Kyoto and Munich).

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 previously been characterized in terms of retrograde signalling, *abi4-2* and *abi4-4* (Supplemental Fig. S1; see also Supplemental Materials and Methods). Previous studies supporting a role for ABI4 have based their conclusions on changes in *LHCB* expression measured by RNA gel blotting (Koussevitzky et al. 2007), or reverse transcription quantitative PCR experiments (RT-qPCR) 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 sucrose. We therefore included both of these genes in our analysis, which was also performed in the presence of sucrose (see Supplemental Table S1 for a summary of the conditions used in this study). As shown in Figure 1A, expression of *LHCB2.1, LHCB1.2* and three additional chlorophyll synthesis genes, *HEMA1*, *CHLH* and *GUN4*, which show a strong dependence on GUN-mediated retrograde signalling (Moulin et al., 2008; Page et al., 2017), were strongly down-regulated in the presence of NF in wild-type (WT) 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 downregulated 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 a strong response (Fig.

3A). Similar results were observed after a shorter 6 d treatment with NF and continuous white light (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 *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, 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; Martin 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 expression in the *ptm1* mutant (Supplemental Fig. S2, E and F) confirmed that a third laboratory has 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 de-repressed 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 have re-analysed 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 WT after Lin treatment in contrast to *gun1-1*, but did show some difference from WT in control conditions (Supplemental Fig. S3, A and B). Expression analysis after Lin treatment correlated well between WT and *abi4-102*, but not between WT and *gun1-1* overall (Supplemental Fig. S3C), and this could be clearly seen when changes in expression of individual photosynthesis (Supplemental Fig. S3D) and tetrapyrrole biosynthesis (Supplemental Fig. S3E) genes were analysed. Similar conclusions were drawn from this data set by Martin et al (2016) in the context of PHYTOCHROME INTERACTING FACTOR (PIF)-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 signalling 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-fold and 6-fold increase in *ABI4* expression in WT seedlings, respectively, and expression was even more strongly upregulated in the three different *gun1* alleles tested (Supplemental Fig. S4A). An increase in *ABI4* expression was also observed in *gun1* mutants when analysed by RNA 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 regulation of *ABI4* via a GUN1-mediated retrograde signalling pathway.

Recent models for biogenic chloroplast-to-nucleus retrograde signalling 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 signalling has not supported such a model (Page et al., 2017; and this study). Here we have re-evaluated the role of ABI4 in biogenic retrograde signalling using the same basic experimental conditions, such as the presence of sucrose and developmental age of the seedlings and by testing the same genes. If ABI4 has a major role in this signalling pathway (and previous studies have shown the response to be almost as strong as *gun1*; e.g. 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 that we observe 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 signalling. As noted earlier, other studies have also reported a lack of a *gun* phenotype for *abi4* mutants (Cottage and Gray 2011; Kerchev et al., 2011; Martin et al., 2016) and our results can be considered to be in agreement with these. We therefore recommend that ABI4 should be omitted from future models of chloroplast-to-nucleus retrograde signalling. There have been some significant recent developments in our understanding of the importance of tetrapyrroles and chloroplast protein homeostasis in plastid retrograde signalling (Woodson 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

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 norflurazon (NF) and lincomycin (Lin) treatments.

Supplemental Figure S3. Re-analysis of microarray data from Koussevitzky et al. (2007).

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

Supplemental Table S1. Summary of treatment conditions used in this study.

Supplemental Table S2. Primers used in this study.

Supplemental Methods. Supplemental materials and methods.

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Figure Legends

Figure 1. ***abi4*** **mutants do not show a** ***gun*** **phenotype on Norflurazon (NF).** A, Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 1% (w/v) sucrose and 1% (w/v) agar (pH 5.8), with (light grey bars) or without (dark grey bars) 1 µM NF for 2 d dark followed by 3 d continuous white light (WLc; 100 $\mu mol.m^{-2}.s^{-1}$). B, Seedlings were grown on Murashige and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8), with (light grey bars) or without (dark grey bars) 2.5 µM NF and grown for 4 d in WLc (100 $\mu mol.m^{-2}.s^{-1}$). Expression was determined by RT-qPCR and is relative to WT (Col-0) -NF and normalized to *YELLOW LEAF SPECIFIC GENE 8* (*YLS8*, At5g08290; A), or to *TUBULIN BETACHAIN 2* (*TUB2*, At5g62690; B). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT (Col-0) for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).

Figure 2. ***abi4*** **mutants do not show a** ***gun*** **phenotype on lincomycin (Lin).** A, Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8) with (light grey bars) or without (dark grey bars) 0.5 mM Lin for 5 d dark after an initial 2 h WL treatment (120 $\mu mol.m^{-2}.s^{-1}$). B, Seedlings were grown on Murashige and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH

239 5.8), with or without 450 µM Lin for 4 d in continuous WL (100 µmol.m^{-2}.s^{-1}). Expression was
240 determined by RT-qPCR and is relative to WT (Col-0) -Lin and normalized to *YELLOW LEAF*
241 *SPECIFIC GENE 8* (*YLS8*, At5g08290; A), or to *TUBULIN BETACHAIN 2* (*TUB2*, At5g62690; B).
242 The expression values for the control condition -Lin in (B) are the same as shown in Figure 1B
243 (- NF; dark grey bars). Data shown are the means +SEM of three independent biological
244 replicates. Asterisks denote a significant difference *vs*. WT (Col-0) for the same treatment (-
245 Lin or +Lin), Student's *t*-test (p<0.05).

246

247 **Figure 3**. ***abi4* mutants do not show a *gun* phenotype after Norflurazon (NF) or lincomycin**
248 **(Lin) treatment.** A and B, Expression of photosynthetic genes after NF and Lin treatments
249 determined by RNA blot analysis. Seedlings were grown on half-strength Murashige and
250 Skoog medium supplemented with 1% (w/v) sucrose and 1% (w/v) agar (pH 5.8), with or
251 without 5 µM NF (A) or 0.5 mM Lin (B). For NF treatments (A) seedlings were grown 4 d dark
252 and 3 d continuous white light (WLc; 100 µmol.m^{-2}.s^{-1}), and for Lin treatments (B) seedlings
253 were grown for 6 d in WLc (100 µmol.m^{-2}.s^{-1}). Five micrograms of total RNA was loaded per
254 sample with methylene blue staining of rRNA as a loading and RNA transfer control. One of
255 three independent experiments is shown with values indicating the mean ± SEM of
256 densitometric scans from all three experiments. C and D, Expression of photosynthetic genes
257 after NF and Lin treatments determined by RT-qPCR. Seedlings were grown on half-strength
258 Murashige and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH
259 5.8), with or without 5 µM NF (C) or 0.5 mM Lin (D) under the same growth conditions as for
260 A and B. Expression is relative to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000) and
261 data shown are the means +SEM of three independent biological replicates. Asterisks denote
262 a significant difference *vs*. WT (Col-0) for the same treatment (NF or Lin), Student's *t*-test
263 (p<0.05).

264

265 **Supplemental Figure S1**. **Characterization of the four *abi4* mutant alleles used in this study.**
266 A, Diagram depicting the *abi4-1*, *abi4-2*, *abi4-4* and *abi4-102* mutation sites in the *ABI4* gene.
267 Black box represents the exon and grey box the 3' UTR region. B, The precise location of a
268 single base deletion in *abi4-1*, a T-DNA insert in SALK_080095 (*abi4-2*), and C/T and G/A
269 nucleotide substitutions in *abi4-4* and *abi4-102*, respectively. These were confirmed by
270 sequencing. Amino acid single letter codes are given in grey above each fragment of the
271 wild-type *ABI4* DNA sequence and amino acid changes resulting from different mutations
272 are given in red below the sequence. The T-DNA sequence in *abi4-2* is marked in grey italics.

Numbers on the left indicate nucleotide position from the start of the exon. C, Confirmation of the T-DNA insertion in the *abi4-2* mutant by PCR. Primers (see Supplemental Table S2) were used to amplify the *ABI4* WT band (LP2 + RP2, predicted size 1,061 bp) and the mutant band (LB + RP2, predicted size 774 bp); MW = molecular weight marker. D, Sensitivity of *abi4-1*, *abi4-2*, *abi4-4* and *abi4-102* mutant alleles to abscisic acid (ABA). Fifty seeds of each genotype were sown on half-strength Murashige and Skoog medium supplemented with 1% (w/v) agar (pH 5.6) with 5 µM ABA and without sucrose, and grown for 10 d in WLc (100 $\mu mol.m^{-2}.s^{-1}$). Representative photographs are shown (scale bar represents 5 mm) with percentage seeds germinated given below (± S.D.; n=3).

Supplemental Figure S2. **Additional analyses of retrograde regulation of photosynthetic gene expression after norflurazon (NF) and lincomycin (Lin) treatments.** A, The data shown in Figure 2A were re-plotted with the reference gene *ACTIN2* (*ACT2*, At3g18780) used by Sun et al. (2011). Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8) with (light grey bars) or without (dark grey bars) 0.5 mM Lin for 5 d dark after an initial 2 h WLc treatment (120 $\mu mol.m^{-2}.s^{-1}$). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-Lin or +Lin), Student's *t*-test ($p<0.05$). B, RNA gel blot analysis of photosynthetic gene expression after NF treatment. Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 1% (w/v) sucrose and 1% (w/v) agar (pH 5.8), with or without 5 µM NF, for 6 d in WLc (100 $\mu mol.m^{-2}.s^{-1}$). Five micrograms of total RNA was loaded per sample with methylene blue staining of rRNA as a loading and RNA transfer control. One of two independent experiments is shown with values indicating the mean ± SEM of densitometric scans from both experiments. C and D, The data shown in Figure 3, C and D were re-plotted with the reference gene *ACT2*. Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8), with or without 5 µM NF (C) or 0.5 mM Lin (D). For NF treatments (C) seedlings were grown 4 d in the dark and 3 d under WLc (100 $\mu mol.m^{-2}.s^{-1}$), and for Lin treatments (D) seedlings were grown for 6 d in WLc (100 $\mu mol.m^{-2}.s^{-1}$). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference vs. WT for the same treatment (NF or Lin), Student's t-test ($p<0.05$). E and F, The retrograde response of the *ptm1* mutant (Munich lab). Seedlings were grown as for C and D, respectively, and expression is shown relative to *ACT2*. Col-0 and *gun1-103* data are the same as in Figure 3, C and D and

Supplemental Figure S2, B and C. Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference vs. WT for the same treatment (NF or Lin), Student's *t*-test (p<0.05).

Supplemental Figure S3. **Re-analysis of microarray data from Koussevitzky et al. (2007).**

A, Hierarchical clustering dendrogram of the control (MS) and Lin treated (Lin) microarray datasets (GSE5770) using MAS5 normalised expression values in wild type (Col-0), *abi4-102* and *gun1-1* from two biological replicates. Data have been clustered according to the complete-linkage method. B, Principal components analysis on the whole transcriptome expression data set using samples as features. C, Log_2-fold change in expression in Col-0 on Lin correlated positively with log_2-fold change in expression caused by Lin in the *abi4-102* mutant, but not in the *gun1-1* mutant. Pearson's R is calculated for all genes (grey) and the subset of genes down-regulated at least 2-fold in Col-0 after Lin treatment (purple). D and E, Heat maps of the log_2 fold-change in expression of selected photosynthetic (D) and tetrapyrrole (E) genes after Lin treatment in Col-0, *gun1-1* and *abi4-102*. Selected transcriptomes are shown from 5-d-old seedlings grown on ½ MS supplemented with or without 0.5 mM Lin in constant white light. Down-regulated and up-regulated genes are shown in purple and green, respectively.

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

Expression of *ABI4* in WT (Col-0) and *gun1* mutants after treatment with norflurazon (NF) and lincomycin (Lin). A, Seedlings were grown on Murashige and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8), with or without 2.5 µM NF or 560 µM Lin for 4 d in continuous WL (100 $\mu mol.m^{-2}.s^{-1}$). Expression of *ABI4* was determined by RT-qPCR and is relative to control WT (Col-0) grown without NF or Lin and normalised to *TUBULIN BETA CHAIN 2* (*TUB2*, At5g62690). Data shown are the means +SEM of three independent biological replicates. B, RNA gel blot analysis of *ABI4* expression after NF and Lin treatment. Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 1% (w/v) sucrose and 1% (w/v) agar (pH 5.8), with or without 5 µM NF or 0.5 mM Lin. For NF treatments, seedlings were grown for 4 d in the dark and 3 d under WLc (100 $\mu mol.m^{-2}.s^{-1}$) and for Lin treatments, seedlings were grown for 6 d in WLc (100 $\mu mol.m^{-2}.s^{-1}$). Five micrograms of total RNA was loaded per sample with methylene blue staining of

rRNA as a loading and RNA transfer control. One of three independent experiments is shown with values indicating the mean ± SEM of densitometric scans from all three experiments.

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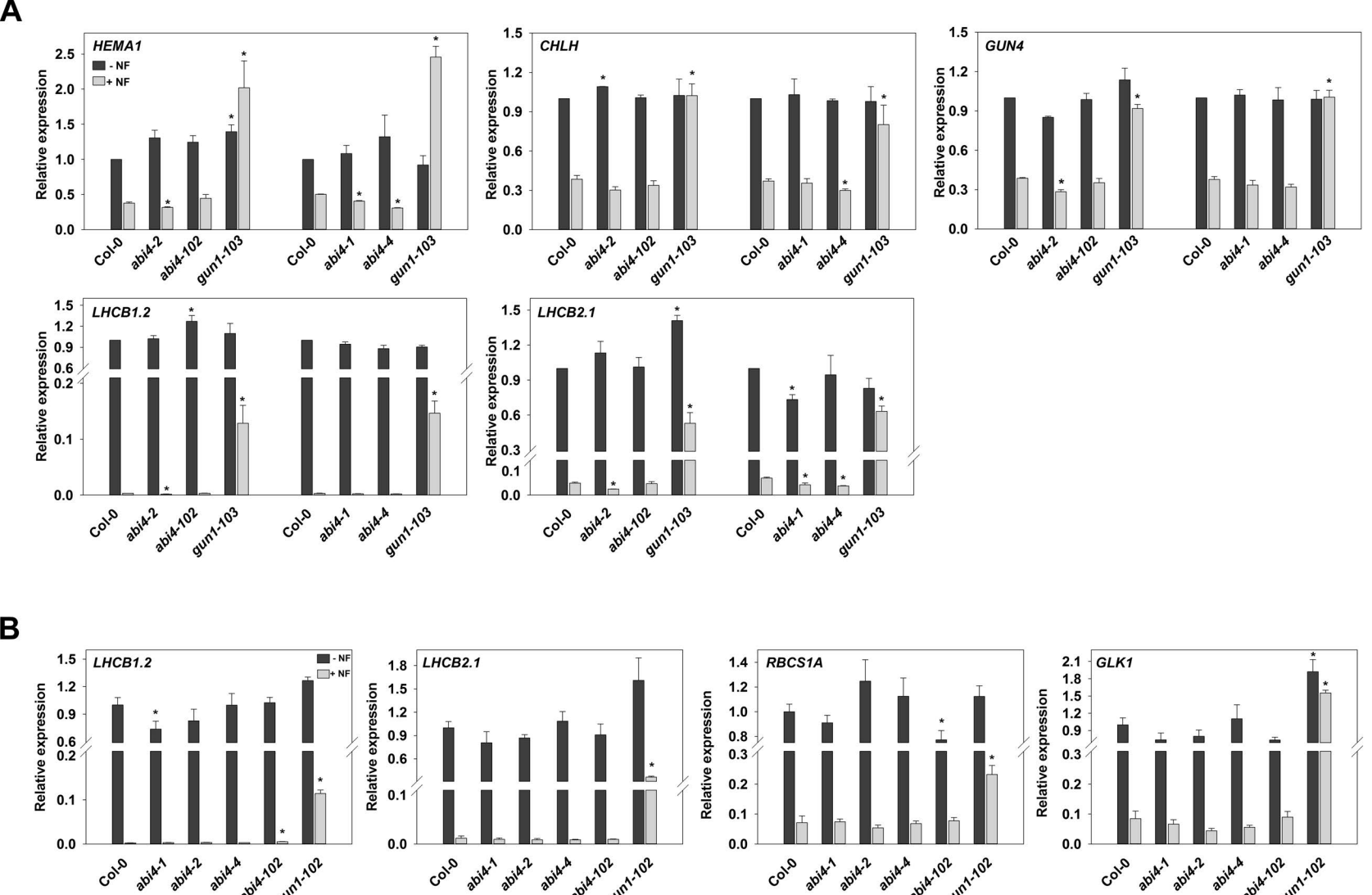

Figure 1. *abi4* mutants do not show a *gun* phenotype on Norflurazon (NF). A, Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 1% (w/v) sucrose and 1% (w/v) agar (pH 5.8), with (light grey bars) or without (dark grey bars) 1 µM NF for 2 d dark followed by 3 d continuous white light (WLc; 100 µmol.m^{-2}.s^{-1}). B, Seedlings were grown on Murashige and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8), with (light grey bars) or without (dark grey 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 WT (Col-0) -NF and normalized to *YELLOW LEAF SPECIFIC GENE 8* (*YLS8*, At5g08290; A) or to *TUBULIN BETA CHAIN 2* (*TUB2*, At5g62690; B). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT (Col-0) for the same treatment (-NF or +NF), Student's *t*-test (p<0.05).

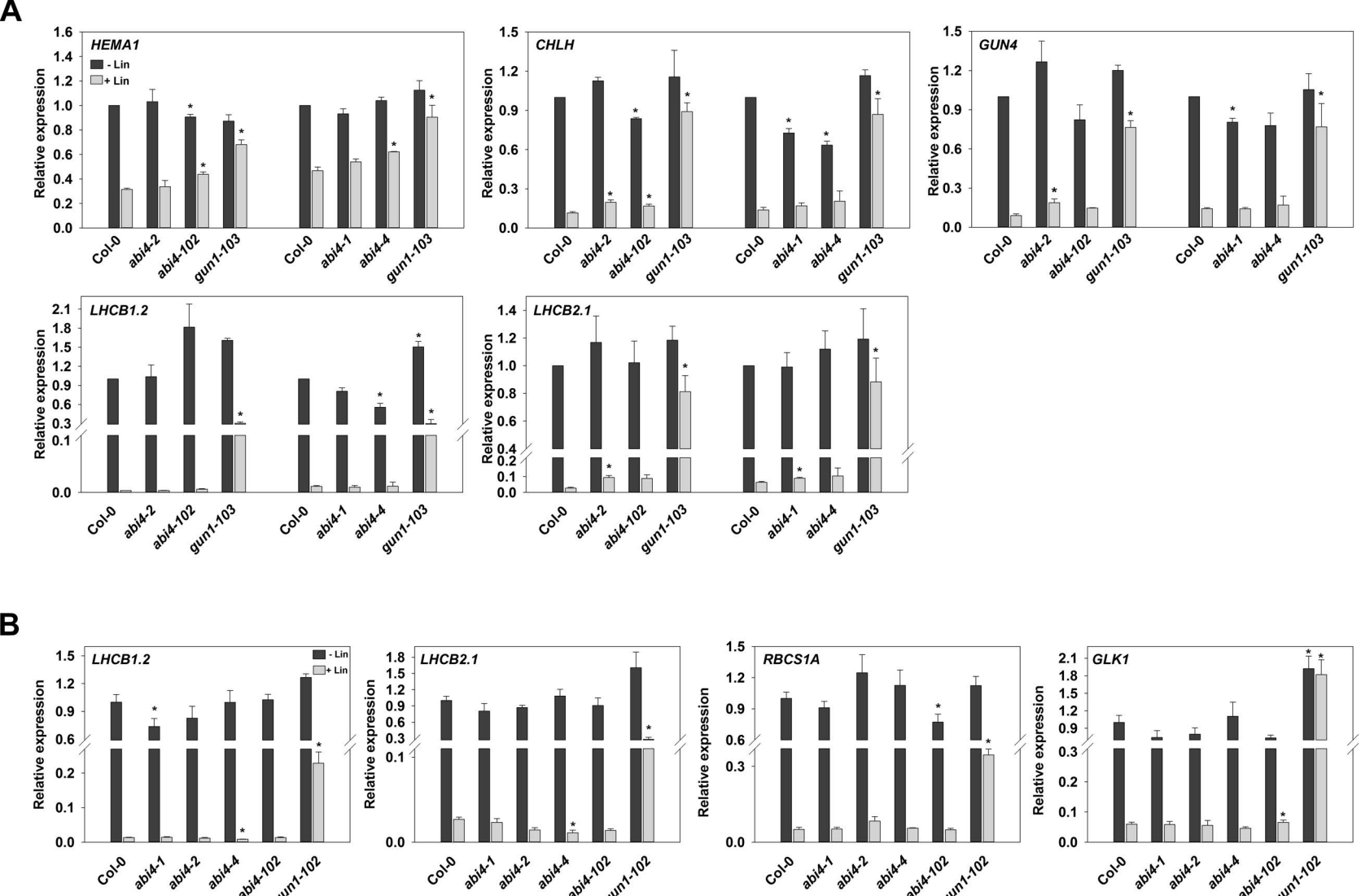

Figure 2. *abi4* mutants do not show a *gun* phenotype on lincomycin (Lin). A, Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8) with (light grey bars) or without (dark grey bars) 0.5 mM Lin for 5 d dark after an initial 2 h WL treatment (120 µmol.m^{-2}.s^{-1}). B, Seedlings were grown on Murashige and Skoog medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8), with or without 450 µM Lin for 4 d in continuous WL (100 µmol.m^{-2}.s^{-1}). Expression was determined by RT-qPCR and is relative to WT (Col-0) -Lin and normalized to *YELLOW LEAF SPECIFIC GENE 8* (*YLS8*, At5g08290; A), or to *TUBULIN BETA CHAIN 2* (*TUB2*, At5g62690; B). The expression values for the control condition -Lin in (B) are the same as shown in Figure 1B (- NF, dark grey bars). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT (Col-0) for the same treatment (-Lin or +Lin), Student's *t*-test (p<0.05).

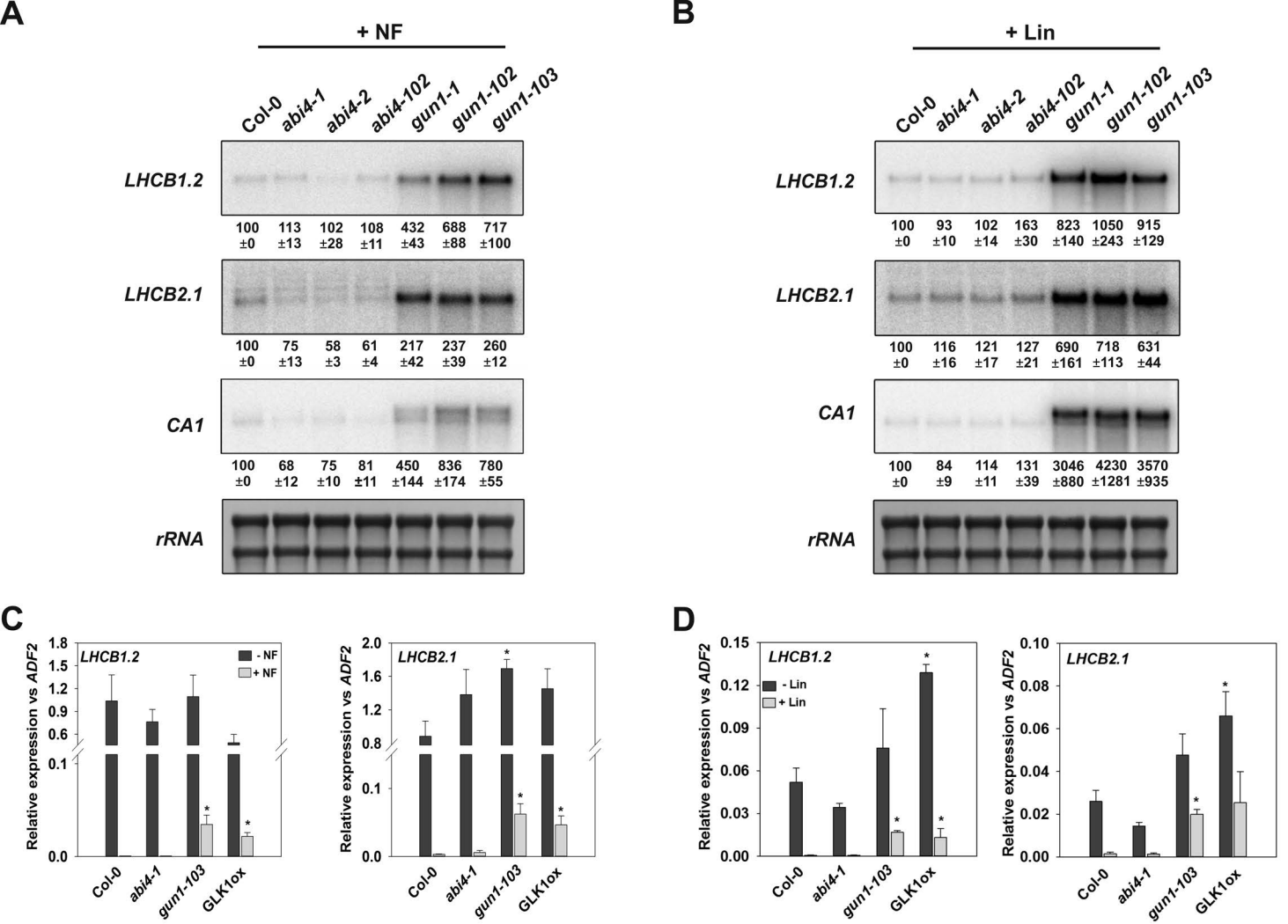

Figure 3. *abi4* mutants do not show a *gun* phenotype after Norflurazon (NF) or lincomycin (Lin) treatment. A and B, Expression of photosynthetic genes after NF and Lin treatments determined by RNA blot analysis. Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 1% (w/v) sucrose 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 4 d dark and 3 d continuous white light (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. One of three independent experiments is shown with values indicating the mean ± SEM 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 half-strength Murashige and Skoog medium supplemented with 2% (w/v) sucrose 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 DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000) and data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT (Col-0) for the same treatment (NF or Lin), Student's *t*-test (p<0.05).

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