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

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Seedlings lacking the PTM protein do not show a *genomes uncoupled (gun)* mutant phenotype[1]

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Summary sentence: The *ptm* mutant of Arabidopsis does not show a *genomes uncoupled* mutant phenotype and PTM is therefore unlikely to function in chloroplast-to-nucleus signalling as previously reported.

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Chloroplast development requires communication between the nucleus and the developing chloroplast to ensure that this process is optimised (Jarvis and López-Juez, 2013; Chan et al., 2016). This is especially true during de-etiolation as mis-regulation of chloroplast development can lead to seedling death from photo-oxidative damage. Retrograde signalling from the developing chloroplast (plastid) to the nucleus, which is termed biogenic signalling (Pogson et al., 2008), can be revealed using either the bleaching herbicide Norflurazon (NF), an inhibitor of carotenoid synthesis, or the plastid translation inhibitor, lincomycin (Lin) to damage the plastid. Under these conditions there is a strong down regulation of hundreds of nuclear genes (Koussevitzky et al., 2007; Aluru et al., 2009; Page et al., 2016). Despite decades of research, the biogenic retrograde signalling pathway is still very poorly understood. What we do know has mostly come from an innovative screen by the group of Joanne Chory in which *genomes uncoupled* (*gun*) mutants were identified that retained nuclear gene expression of chloroplast-related genes after NF treatment (Susek et al., 1993). This screen now defines the *gun* phenotype: increased expression, compared to wild-type (WT), of nuclear genes following chloroplast damage. In total six original *gun* mutants have been described. GUN1 is a pentatricopeptide repeat protein with a still unknown function (Koussevitzky et al, 2007). The other GUNs are all related to the tetrapyrrole pathway (Mochizuki et al, 2001; Larkin et al, 2004; Woodson et al., 2011). Further analysis of these mutants has supported the idea that tetrapyrroles are important for plastid signalling (Vinti et al., 2000; Strand et al., 2003; Moulin et al., 2008; Mochizuki et al., 2008; Voigt et al., 2010) and our current understanding is that the synthesis of heme by ferrochelatase 1 results in a positive signal that promotes expression of nuclear-encoded chloroplast genes (Woodson et al., 2011; Terry and Smith, 2013).

Additional mutants identified through screens for a *gun* phenotype are the blue-light photoreceptor mutant *cry1* (Ruckle et al., 2007) and the *coe1* mutant lacking a functional mitochondrial transcription termination factor 4 (Sun et al., 2015). A number of *happy on norflurazon* (*hon*) mutants were also identified by screening seedlings grown on NF under lower light intensities (Saini et al., 2011). This identified one *hon* mutation in the ClpR4 subunit of the chloroplast-localized Clp protease complex (Saini et al., 2011). Other mutants with a *gun* phenotype have been identified *via* informed approaches to test potential signalling components. These include the transcription factor mutants *abi4* (Koussevitzky et al, 2007), *hy5* (Ruckle et al., 2007) and *glk1glk2* (Waters et al., 2009). Interestingly, *GOLDEN2-LIKE* (*GLK*) overexpressing plants (Leister and Kleine, 2016) have also been reported to show *gun* phenotypes, perhaps reflecting the complex relationship between the

anterograde signals by which the nucleus controls chloroplast development and retrograde signalling (Martin et al., 2016).

In 2011, Sun et al identified a PHD transcription factor associated with the chloroplast envelope, called PTM, which they proposed mediates chloroplast signals to the nucleus through cleavage in response to changes in plastid status. Accumulation of the N-terminus of the protein in the nucleus would then inhibit nuclear gene expression. Consistent with this, they reported that the *ptm* mutant has a *gun* phenotype with elevated expression compared to WT of *Lhcb* on both NF and Lin. This was a significant result for the field as it defined a mechanism for plastid signalling, and is unsurprisingly included in numerous models for this pathway (*e.g*. Chan et al, 2016; Bobik and Burch-Smith, 2015; Terry and Smith, 2013; Barajas-López et al, 2013). Subsequent studies from the same group have suggested that PTM functions in retrograde signalling from the chloroplast to regulate flowering under high light (Feng et al, 2016) and in the integration of light and chloroplast retrograde signalling during de-etiolation (Xu et al, 2016). However, the demonstration that PTM shows a *gun* phenotype and is involved in retrograde signalling has yet to be supported by additional experimental data from other groups.

Given the potential importance of PTM for our understanding of plastid signalling we have further examined the role of PTM in responses to NF and Lin in two different laboratories. For the experiments at Southampton, it was necessary for us to isolate the same insertional *ptm* mutant allele described in Sun et al (2011) from the SALK collection because this was no longer available from the authors. Isolation of the *ptm* mutant for this study, which we name here as *ptm-1*, is described in Figure S1. Analysis of gene expression after NF treatment was then performed. As shown in Figure 1A, 5 µM NF treatment using the experimental conditions (1% sucrose, 25 $\mu mol.m^{-2}.s^{-1}$ white light (WL) for 7 d) of Woodson et al (2011) resulted in no change in gene expression for a suite of five photosynthesis-related genes (including *LHCB2.1* used by Sun et al (2011) for their real-time PCR experiments) in *ptm-1* compared to WT seedlings, whereas there was clear rescue of gene expression in the control *gun5* and *gun6* mutants. Next we repeated the experiment under identical conditions (2% sucrose, 4d dark followed by 3d 120 $\mu mol.m^{-2}.s^{-1}$ WL) to those reported in Sun et al (2011). Under these conditions we also saw rescue of gene expression in *gun5* and *gun6*, but not in *ptm-1* (Figure 1B). These studies were performed using *ADF2* as a reference gene. To confirm that the lack of a *gun* phenotype in *ptm1* was not related to the choice of reference gene, we also normalised the data using *YLS8*, which gave essentially identical results (Figure S2). Finally, we examined expression under conditions we have

previously described (McCormac and Terry, 2004). With 3d dark followed by 3d 120 µmol.m^{-2}.s^{-1} WL we also saw no *gun* phenotype for *ptm-1* either in the presence or absence of sucrose (Figure S3). Only under one particular set of conditions did we see any indication of a rescue of gene expression in *ptm-1* after NF treatment. Under these conditions (1% sucrose, 2d dark followed by 3d 100 µmol.m^{-2}.s^{-1} WL with a lower NF concentration of 1 µM) we saw a very small, but statistically significant increase for *LHCB2.1* and *HEMA1*, but not for the other three genes tested (Figure S4). Given that under these conditions *gun1-1* rescue was complete for both genes (>300% for *HEMA1*) we do not believe this one exception supports a role for PTM in the plastid signalling pathway exposed by NF treatment.

The *ptm-1* mutant was also reported to result in elevated gene expression compared to WT seedlings when grown on Lin (Sun et al., 2011). We therefore also tested *ptm-1* under these conditions. As shown in Figure 2, *ptm-1* failed to result in elevated gene expression on Lin while *gun1-1* (Koussevitzky et al., 2007) and *gun1-103* (see methods) control seedlings, both showed strong rescue of gene expression (Figure 2). This was true whether seedlings were grown in the dark (Figure 2A) or in the light (Figure 2B), and was independent of the reference gene used (Figure S5).

To verify further whether we could detect a *gun* mutant phenotype for *ptm* mutants, we also performed experiments in parallel in Kyoto. For this set of experiments two *ptm* alleles were used, the original *ptm* mutant (*ptm-1* OL) was obtained from Lixin Zhang (CAS, Beijing; Sun et al., 2011) and independently from the SALK collection (*ptm-1*) and, in addition, a second *ptm* allele, *ptm-2*, was also identified from the SALK collection (Fig S1). As shown in Figure 3 none of the *ptm* mutants showed an elevation of *LHCB1.2* (although the primer set used is also likely to detect *LHCB1.1* and *LHCB1.3)* or *LHCB2.1* expression after NF or Lin treatment compared to WT, while a strong increase was observed in the *gun1-1* control.

In conclusion, rigorous testing of the phenotype of *ptm* mutants on NF and Lin shows that the *ptm* mutant does not show elevated expression of photosynthetic genes compared to WT. This was true whether using the conditions described in the original publication or other conditions used routinely to test plastid signalling responses. One possible difference between our study and that of Sun et al (2011) is that they used RNA gel blot analysis for most of their experiments. The probe used should preferentially detect *LHCB1.1*, but might also be expected to detect *LHCB1.2* and *LHCB1.3,* and possibly other *LHCB* genes. In our experiments we have tested both *LHCB1.1* and *LHCB1.2,* so it remains possible that changes in another *LHCB* gene could account for the observed phenotype in

the original paper (Sun et al., 2011). However, Sun et al (2011) also reported the same gene expression phenotype for *ptm* using real-time PCR and a primer pair that most closely matches *LHCB2.1,* and we did not detect an increase in expression for this gene in our experiments (with one exception). We therefore believe it is unlikely that differences in detection methods or genes tested can account for the observed differences in phenotype. Moreover, if PTM is to be considered an important player in plastid signalling, the *gun* phenotype of *ptm* should be robust enough to withstand this level of scrutiny. We have not tested other results reported by Sun et al (2011). However, we note that the 3-fold elevation of expression of *PTM* on NF measured using *PTM:GUS* was not apparent in our experiments (Figures 1 and S3) and the reduction in *PTM* expression in *gun1* after NF and Lin treatment was also not observed (Figure 3). In fact *PTM* expression was moderately (but significantly) elevated in *gun1-1* in our study (Figure 3). Whether our result has implications for other PTM signalling roles (Feng et al., 2016; Xu et al., 2016) is currently unknown, but should be the subject of further scrutiny.

The signalling pathway by which the status of the developing chloroplast is relayed to the nucleus is one of the few remaining plant signalling pathways that we know of, but for which we have little idea of the signalling components involved. We believe this study resolves one of the major discrepancies in plastid signalling research by eliminating a major role for PTM, and paves the way for more focussed studies that build on recent progress on the role of tetrapyrroles and chloroplast protein homeostasis in plastid retrograde signalling (Woodson et al., 2011; Murata et al., 2015; Ibata et al., 2016; Tadini et al., 2016).

Supplemental data

Supplemental Figure S1. Characterisation of the *ptm* T-DNA insertion mutants

Supplemental Figure S2. The phenotype of *ptm-1* after NF treatment using the Sun et al (2011) method normalised to *YLS8*

Supplemental Figure S3. The phenotype of *ptm-1* after NF treatment using the McCormac & Terry (2004) method in the presence and absence of sucrose

Supplemental Figure S4. The phenotype of *ptm-1* after NF treatment using a modification of the McCormac & Terry (2004) method in the presence of sucrose

Supplemental Figure S5. The phenotype of *ptm-1* after Lin treatment normalised to *YLS8*

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Figure Legends

Figure 1. **The *ptm-1* mutant does not show a *gun* phenotype on Norflurazon (NF).** Seedlings were grown on half-strength Linsmaier and Skoog medium (A) supplemented with 1% sucrose and 0.8% agar (pH 5.7) with (dark grey bars) or without (light grey bars) 5 µM NF under continuous low white light (25 µmol.m^{-2}.s^{-1}) for 7 d, or (B) supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 µM NF under the following conditions: an initial 2 h WL treatment (120 µmol.m^{-2}.s^{-1}) to stimulate germination, 4 d dark, 3 d WLc (120 µmol m^{-2} s^{-1}). For (A) and (B), *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).

Figure 2. The *ptm* mutant does not show a *gun* phenotype on lincomycin (Lin). Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin in dark for 5 d (A), or (B) on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin under the following conditions: 2 d dark, 3 d WL (100 µmol.m^{-2}.s^{-1}). For (A) and (B), the *genomes uncoupled, gun1-1* and *gun1-103* mutants were included as positive controls (known to rescue gene expression on Lin). Expression is relative to WT -Lin and normalised to *ACTIN2* (*ACT2*, At3g18780) used in Sun et al. (2011). Data shown are 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 ($p<0.05$).

Figure 3. **A second *ptm* mutant allele does not show a *gun* phenotype on Norflurazon (NF) or lincomycin (Lin).** Seedlings were grown on Murashige and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8), and either (A) 2.5 µM NF or (B) 560 µM Lin. All seedlings were grown under continuous white light (WLc, 100 $\mu mol.m^{-2}.s^{-1}$) for 4 d at 23 °C. Three *ptm* mutant lines were tested: *ptm-1* (OL) is the original line as used in Sun *et al*., 2011; *ptm-1* is the same insertion line as *ptm-1* (OL), Salk_013123, but obtained independently from the stock centre; *ptm-2* is a second insertion line, Salk_073799. The *genomes uncoupled 1-1* (*gun1-1*) mutant was included as a positive control (known to rescue nuclear gene expression on NF and Lin). Expression was determined with qRT-PCR and is relative to WT +NF and normalised to *TUBULIN BETA CHAIN 2* (*TUB2*, At5g62690). Data shown are the means +SEM of five independent biological replicates. Asterisks denote a significant difference *vs*. WT +NF, Student's *t*-test ($p<0.05$).

Supplemental Fig. S1. **Characterisation of the *ptm* T-DNA insertion mutants.** (A) *PTM* gene structure, with black boxes representing exons. The approximate location of the Salk_013123 (*ptm-1*) and Salk_073799 (*ptm-2*) T-DNA inserts, genotyping primers (LB, LP, RP) and qRT-PCR primers (qF1, qR1, qF2, qR2) are indicated. The *ptm-2* mutant has tandem T-DNA insertions with a 24 bp deletion, in which the LB primer binding site is located at each end of the tandem insertion. Precise T-DNA insertion sites in (B) *ptm-1* and (C) *ptm-2* as revealed by sequencing. For (B) and (C) amino acid single letter codes are given above DNA sequences, with the T-DNA sequences underlined in black. Sequence is given from the LP and RP sides of the *ptm-2* T-DNA insertion in (C), to demonstrate the site of the 24 bp deletion (underlined in red in the WT sequence). (D) PCR genotyping of *ptm-1* and *ptm-2* mutants. Primers shown in (A) were used to amplify the following: *ptm-1* - WT band (LP1 + RP1, predicted size 1,098 bp) and mutant band (LB + RP1, predicted size 687 bp); *ptm-2* - WT band (LP2 + RP2, predicted size 1,142 bp) and two mutant bands (LB + RP2, predicted size 661 bp, and LB + LP2, predicted size 904 bp). MW = molecular weight marker. (E) Expression of *PTM* in WT and *ptm-1* seedlings as determined by qRT-PCR. This analysis was repeated under the conditions used in this study: the growth conditions in McCormac & Terry, 2004 (white bars), Sun *et al*., 2011 (grey bars) and Woodson *et al*., 2011 (black bars), all in the absence of NF. Expression is relative to WT for each condition and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data represent the mean + SEM of three independent biological replicates, asterisks indicate a significant difference *vs*. WT ($p < 0.05$, Student's *t*-test).

Supplemental Fig. S2. **Normalisation of expression data to a different reference gene does not reveal a *gun* phenotype for *ptm-1*.** Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 µM NF under the following conditions: an initial 2 h WL treatment (120 µmol.m^{-2}.s^{-1}) to stimulate germination, 4 d dark, 3 d WLc (120 µmol m^{-2} s^{-1}). *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *YELLOW LEAF SPECIFIC GENE 8* (*YLS8*, At5g08290). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).

Supplemental Fig. S3. **Growth under a third set of conditions fails to find a *gun* phenotype in *ptm-1*.** Seedlings were sown onto half-strength Murashige and Skoog medium supplemented with 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 µM NF, and either in the presence (A) or absence (B) of 1% sucrose. For (A) and (B), seedlings were grown under the following conditions: an initial 2 h WL treatment (120 µmol.m^{-2}.s^{-1}) to stimulate germination, 3 d dark, 3 d WLc (120 µmol.m^{-2}.s^{-1}). *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).

Supplemental Figure S4. The *ptm* mutant shows a very weak *gun* phenotype for some genes under low (1 µM) Norflurazon (NF). Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 1 µM NF under the following conditions: 2 d dark, 3 d WLc (100 µmol.m^{-2}.s^{-1}). The *genomes uncoupled 1* (*gun1-1*) mutant was included as positive control (known to rescue gene expression on NF). Expression is relative to WT -NF and normalised to *YELLOW LEAF SPECIFIC GENE 8 (YLS8,* At5g08290). Data shown are means

+SEM of three independent biological replicates. Asterisks denote a significant difference vs. WT for the same treatment (-NF or + NF), Student's t-test ($p<0.05$).

Supplemental Figure S5. The *ptm* mutant does not show a *gun* phenotype on lincomycin (Lin). Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin in dark for 5 d (A), or (B) on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM LIN under the following conditions: 2 d dark, 3 d Wlc (100 $\mu mol.m^{-2}.s^{-1}$). For (A) and (B), two alleles of *genomes uncoupled 1* (*gun1-1* and *gun1-103*) mutants were included as positive control (known to rescue gene expression on Lin). Expression is relative to WT -Lin and normalised to *YELLOW LEAF SPECIFIC GENE 8 (YLS8,* At5g08290). Data shown are 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 ($p<0.05$).

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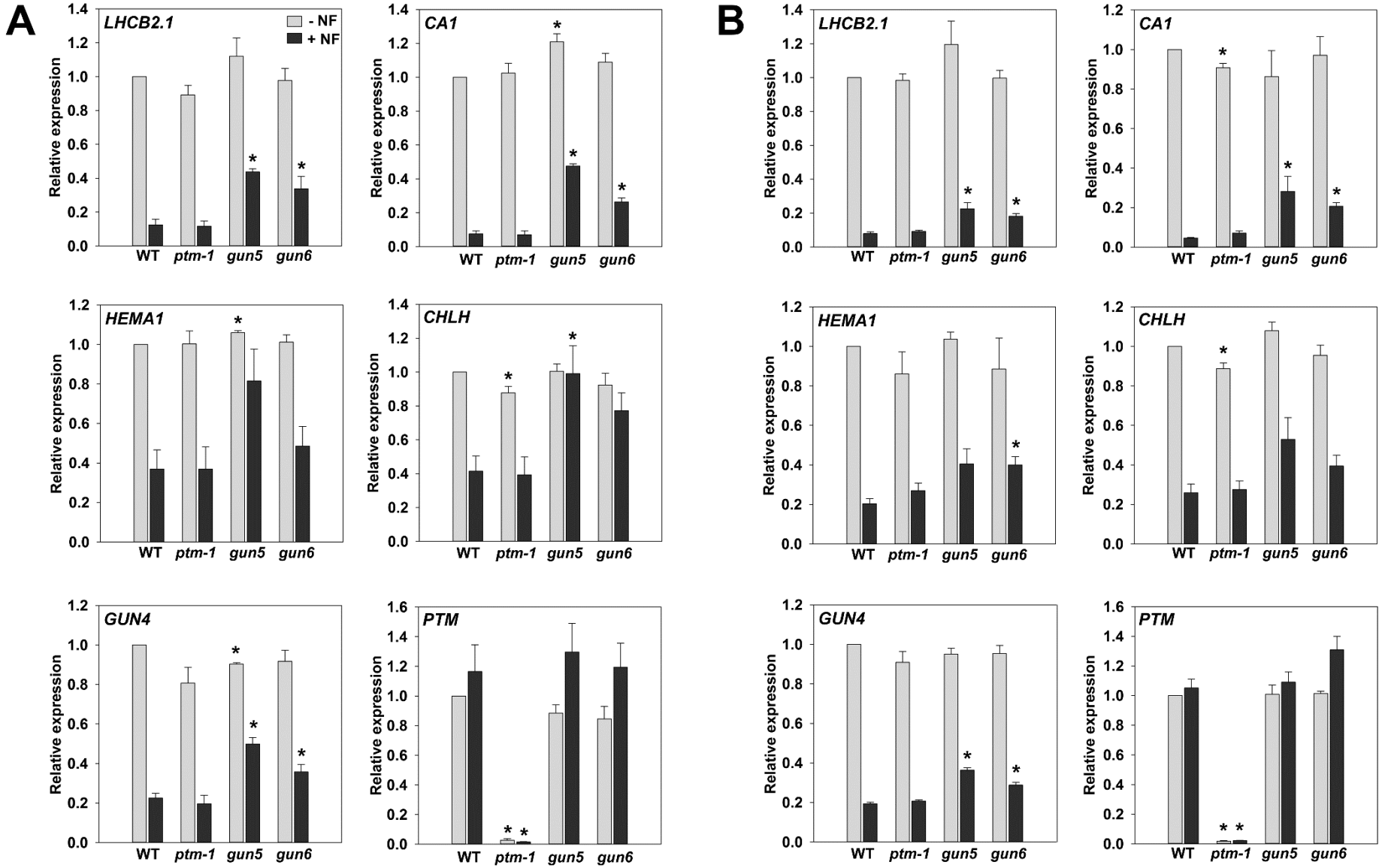


Figure 1. The *ptm-1* mutant does not show a *gun* phenotype on Norflurazon (NF). Seedlings were grown on half-strength Linsmaier and Skoog medium (A) supplemented with 1% sucrose and 0.8% agar (pH 5.7) with (dark grey bars) or without (light grey bars) 5 µM NF under continuous low white light (LWLc, 25 µmol m^{-2} s^{-1}) for 7 d, or (B) supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 µM NF under the following conditions: an initial 2 h WL treatment (120 µmol m^{-2} s^{-1}) to stimulate germination, 4 d dark, 3 d WLc (120 µmol m^{-2} s^{-1}). For (A) and (B), *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).

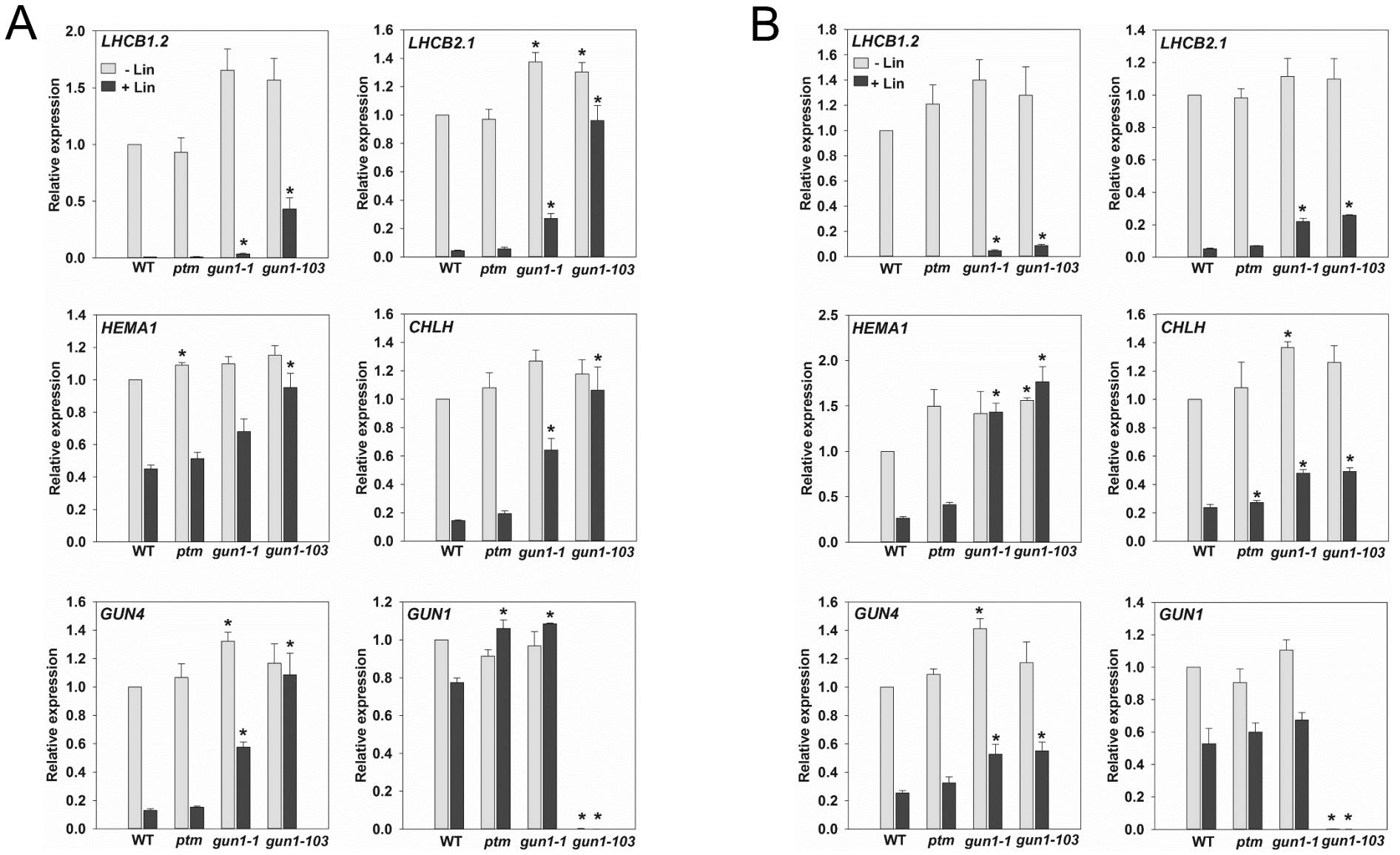


Figure 2. The *ptm* mutant does not show a *gun* phenotype on lincomycin (Lin). Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin in dark for 5 d (A), or (B) on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin under the following conditions: 2 d dark, 3 d Wlc (100 µmol $m^{-2}s^{-1}$). For (A) and (B), two alleles of *genomes uncoupled 1* (*gun1-1 and gun1-103*) mutants were included as positive control (known to rescue gene expression on Lin). Expression is relative to WT -Lin and normalised to *ACTIN2 (ACT2,* At3g18780*)* used in Sun et al (2011). Data shown are 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 ($p<0.05$).

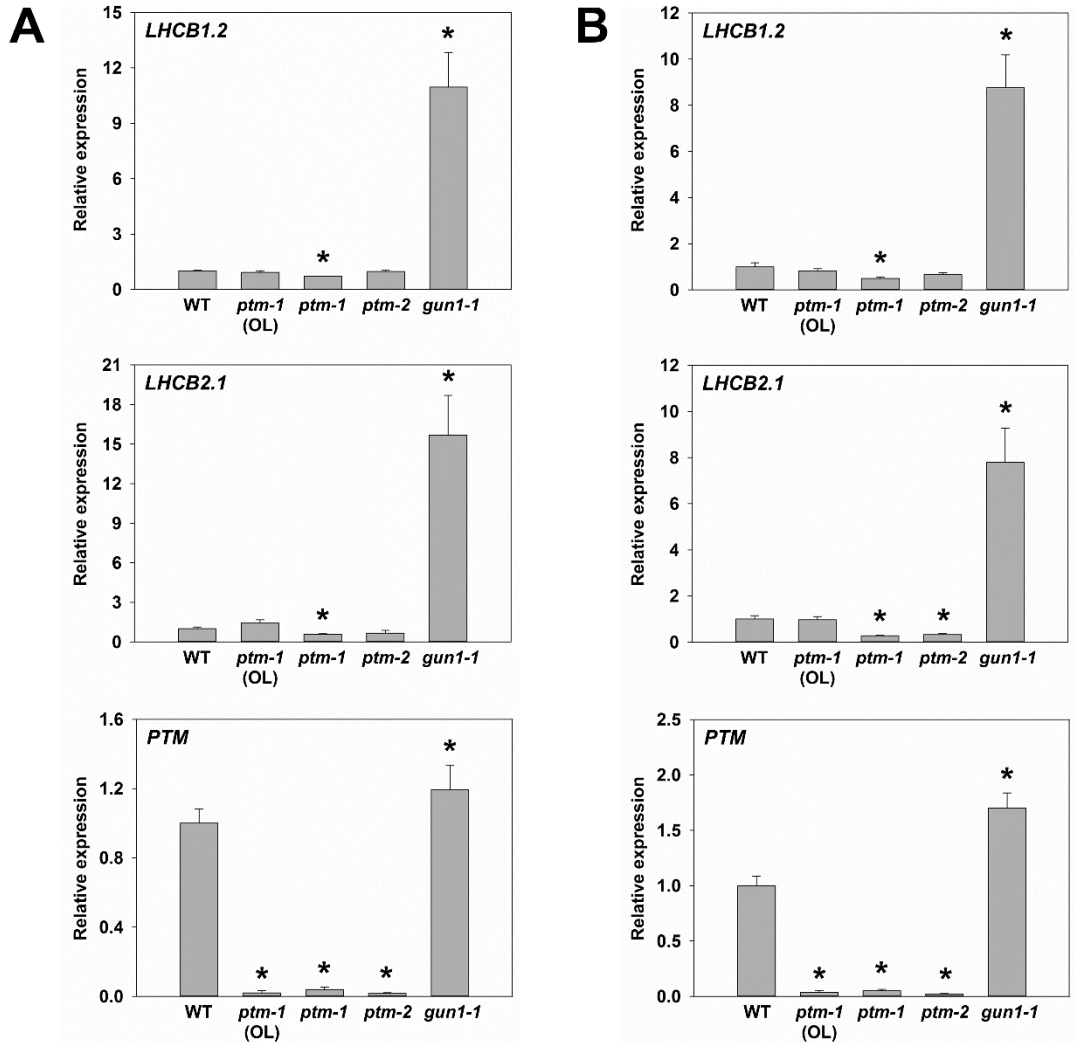
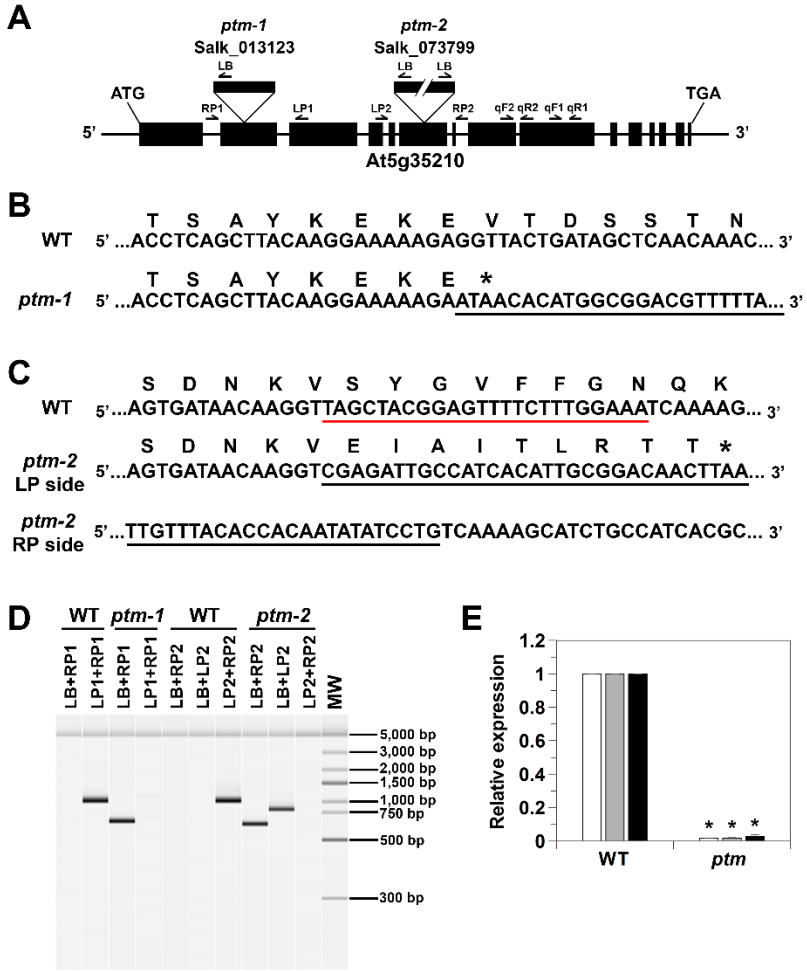
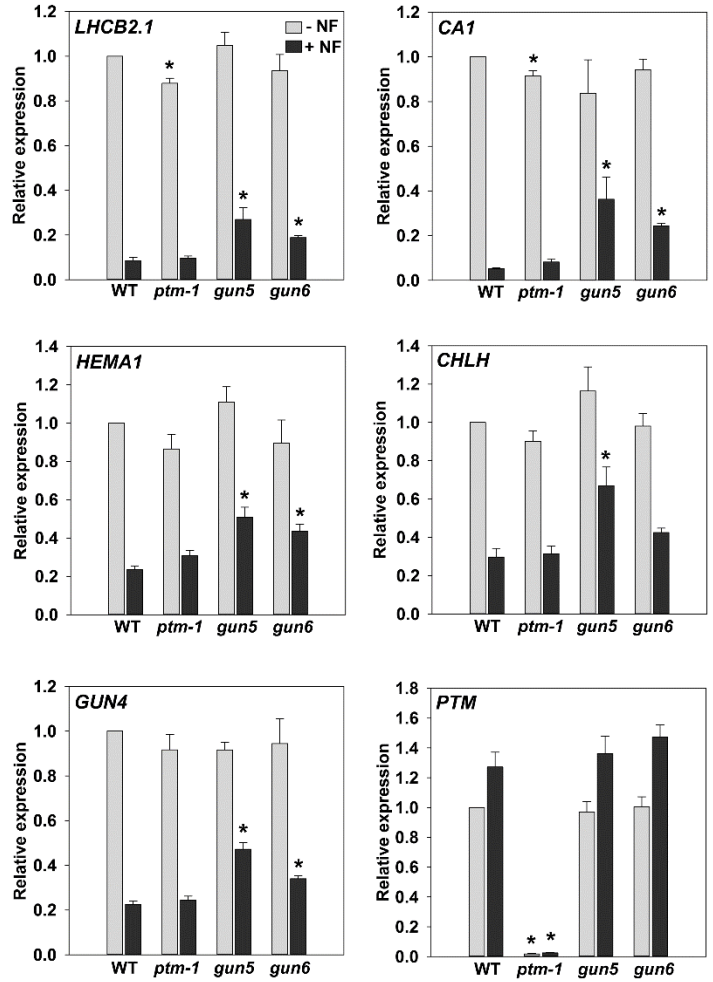


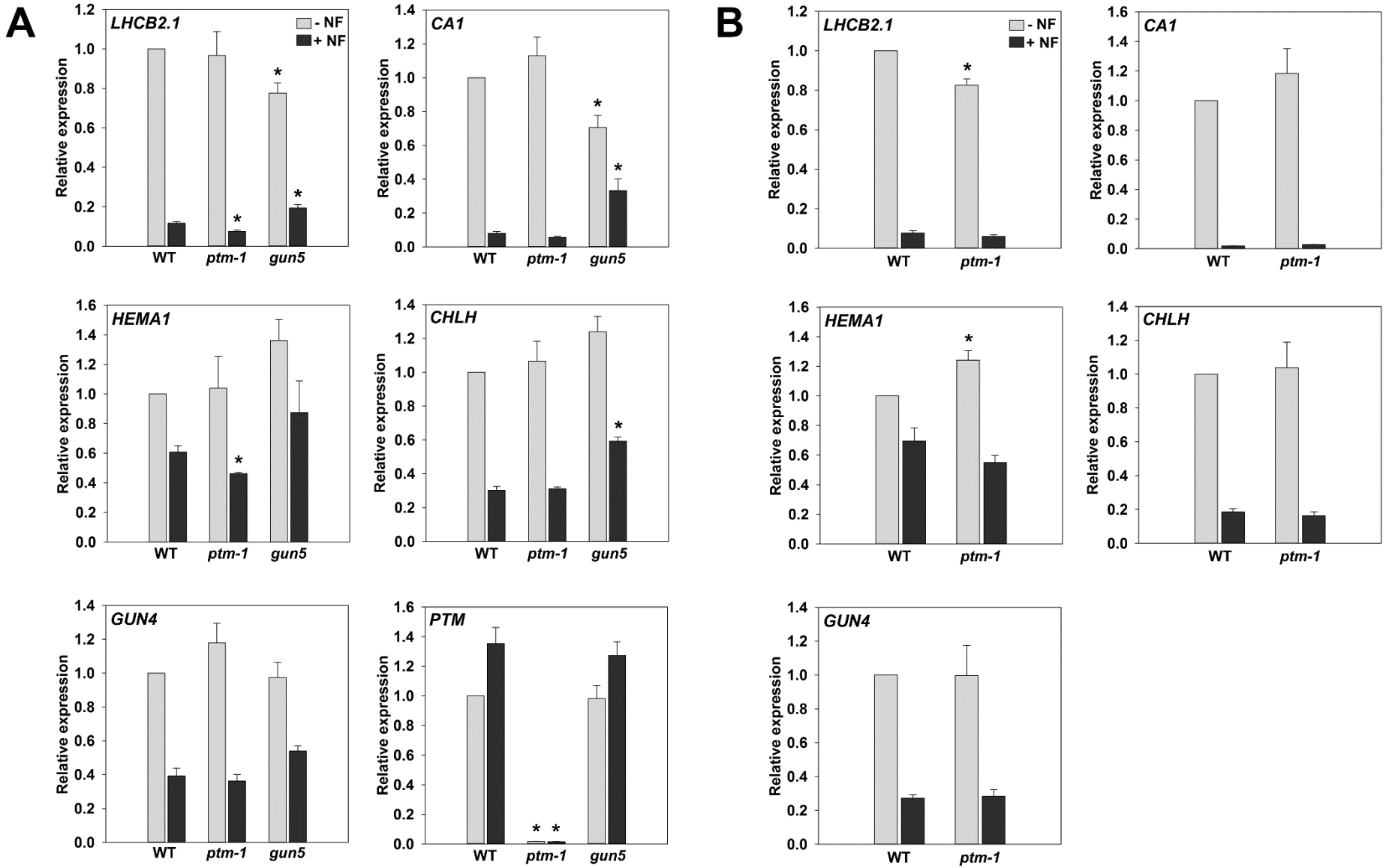
Figure 3. A second *ptm* mutant allele does not show a *gun* phenotype on Norflurazon (NF) or lincomycin (Lin). Seedlings were grown on Murashige and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8), and either (A) 2.5 µM NF or (B) 560 µM Lin. All seedlings were grown under continuous white light (WLc, 100 µmol m^{-2} s^{-1}) for 4 d at 23 °C. Three *ptm* mutant lines were tested: *ptm-1* (OL) is the original line as used in Sun *et al*., 2011; *ptm-1* is the same insertion line as *ptm-1* (OL), Salk_013123, but obtained independently from the stock centre; *ptm-2* is a second insertion line, Salk_073799. The *genomes uncoupled 1-1* (*gun1-1*) mutant was included as a positive control (known to rescue nuclear gene expression on NF and Lin). Expression was determined with qRT-PCR and is relative to WT +NF and normalised to *TUBULIN BETA CHAIN 2* (*TUB2*, At5g62690). Data shown are the means +SEM of five independent biological replicates. Asterisks denote a significant difference *vs*. WT +NF, Student's *t*-test ($p<0.05$).



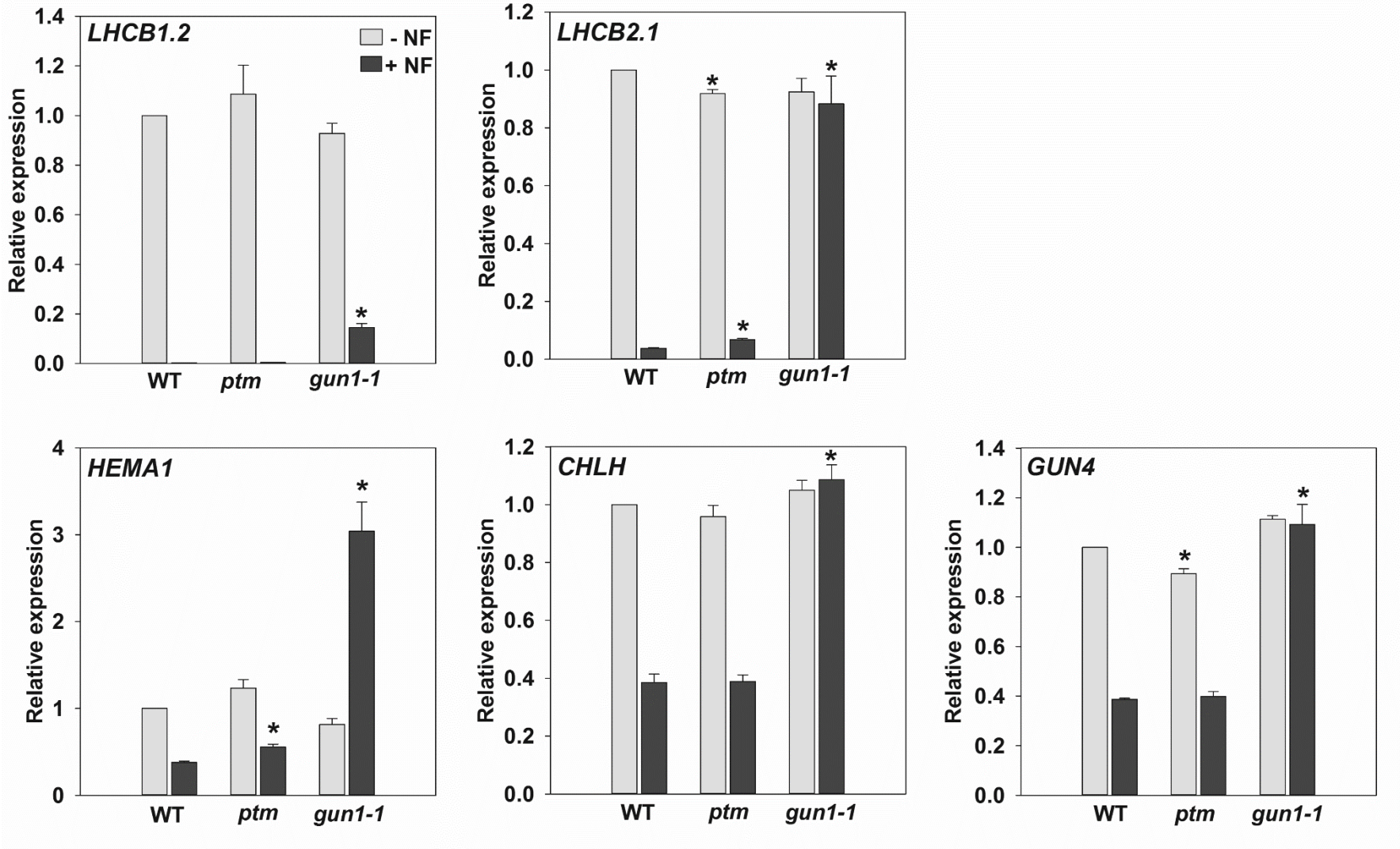
Supplemental Fig. S1. Characterisation of the *ptm* T-DNA insertion mutants. (A) *PTM* gene structure, with black boxes representing exons. The approximate location of the Salk_013123 (*ptm-1*) and Salk_073799 (*ptm-2*) T-DNA inserts, genotyping primers (LB, LP, RP) and qRT-PCR primers (qF, qR) are indicated. The *ptm-2* mutant has tandem T-DNA insertions with a 24 bp deletion, in which the LB primer binding site is located at each end of the tandem insertion. Precise T-DNA insertion sites in (B) *ptm-1* and (C) *ptm-2* as revealed by sequencing. For (B) and (C) amino acid single letter codes are given above DNA sequences, with the T-DNA sequences underlined in black. Sequence is given from the LP and RP sides of the *ptm-2* T-DNA insertion in (C), to demonstrate the site of the 24 bp deletion (underlined in red in the WT sequence). (D) PCR genotyping of *ptm-1* and *ptm*-2 mutants. Primers shown in (A) were used to amplify the following: *ptm-1* - WT band (LP1 + RP1, predicted size 1,098 bp) and mutant band (LB + RP1, predicted size 687 bp); *ptm-2* - WT band (LP2 + RP2, predicted size 1,142 bp) and two mutant bands (LB + RP2, predicted size 661 bp, and LB + LP2, predicted size 904 bp). MW = molecular weight marker. (E) Expression of *PTM* in WT and *ptm-1* seedlings as determined by qRT-PCR. This analysis was repeated under the three conditions used in this study: growth condition 1 (GC1, white bars), GC2 (grey bars) and GC3 (black bars), all in the absence of NF. Expression is relative to WT for each GC and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data represent the mean + SEM of three independent biological replicates, asterisks indicate a significant difference *vs.* WT ($p < 0.05$, Student's *t*-test).



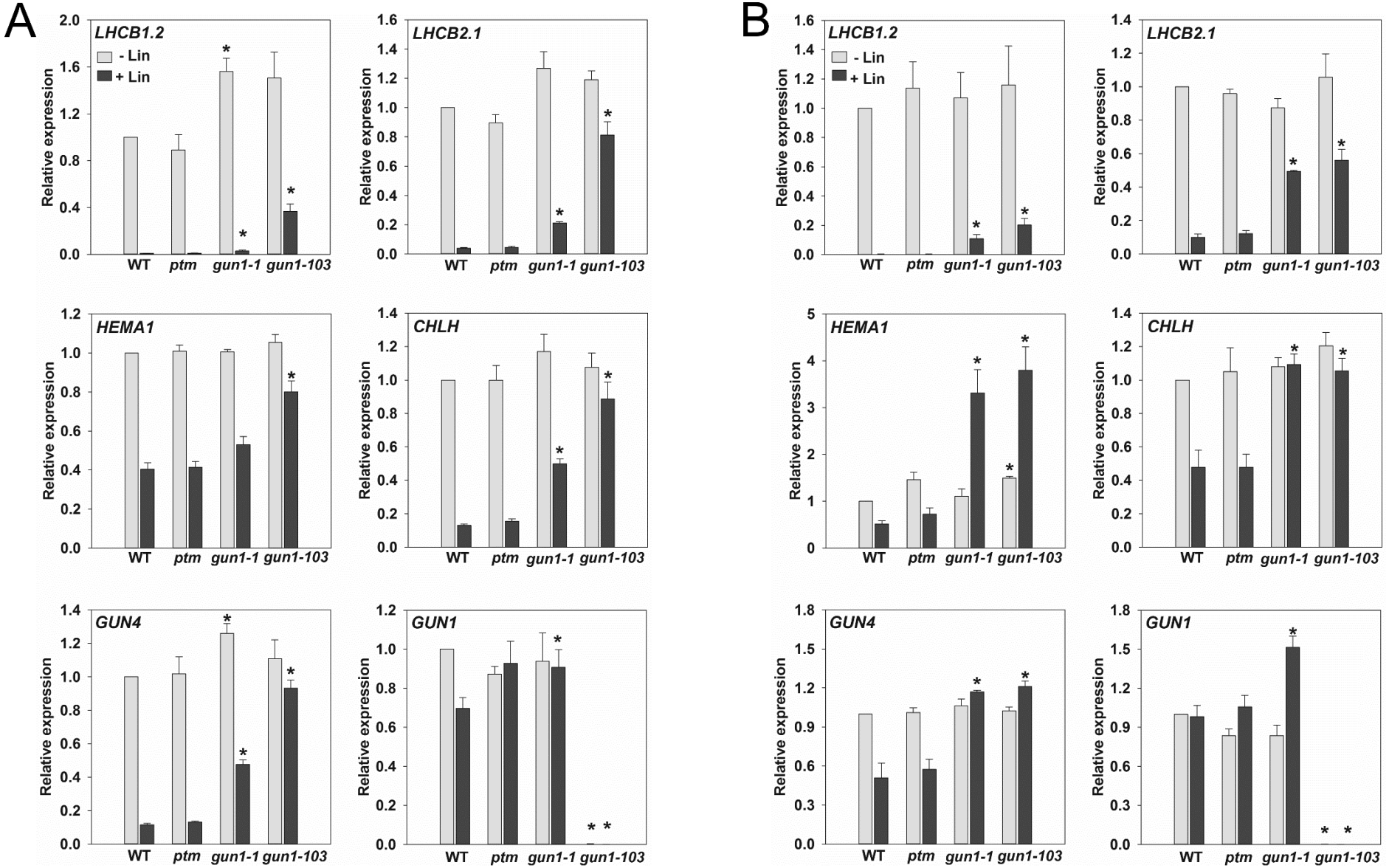
Supplemental Fig. S2. Normalisation of expression data to a different reference gene does not reveal a *gun* phenotype for *ptm-1*. Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 µM NF under the following conditions: an initial 2 h WL treatment (120 µmol m^{-2} s^{-1}) to stimulate germination, 4 d dark, 3 d WLc (120 µmol m^{-2} s^{-1}). *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *YELLOW LEAF SPECIFIC GENE 8* (*YLS8*, At5g08290). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).



Supplemental Fig. S3. Growth under a third set of conditions fails to find a *gun* phenotype in *ptm-1*. Seedlings were sown onto half-strength Murashige and Skoog medium supplemented with 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 5 μM NF, and either in the presence (A) or absence (B) of 1% sucrose. For (A) and (B), seedlings were grown under the following conditions: an initial 2 h WL treatment (120 μmol m^{-2} s^{-1}) to stimulate germination, 3 d dark, 3 d WLc (120 μmol m^{-2} s^{-1}). *genomes uncoupled 5* (*gun5*) and *gun6* mutants were included as positive controls (known to rescue nuclear gene expression on NF). Expression was determined with qRT-PCR and is relative to WT -NF and normalised to *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000). Data shown are the means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs*. WT for the same treatment (-NF or +NF), Student's *t*-test ($p<0.05$).



Supplemental Figure S4. The *ptm* mutant does not show a clear *gun* phenotype on Norflurazon (NF). Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 1 µM NF under the following conditions: 2 d dark, 3 d WLc (100 µmol $m^{-2}s^{-1}$). The *genomes uncoupled 1* (*gun1-1*) mutants was included as positive control (known to rescue gene expression on NF). Expression is relative to WT -NF and normalised to *YELLOW LEAF SPECIFIC GENE 8 (YLS8,* At5g08290*)*. Data shown are means +SEM of three independent biological replicates. Asterisks denote a significant difference *vs.* WT for the same treatment (-NF or + NF), Student's *t*-test ($p<0.05$).



Supplemental Figure S5. The *ptm* mutant does not show a *gun* phenotype on lincomycin (Lin). Seedlings were grown on half-strength Linsmaier and Skoog medium supplemented with 2% sucrose and 0.8% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin in dark for 5 d (A), or (B) on half-strength Murashige and Skoog medium supplemented with 1% sucrose and 1% agar (pH 5.8) with (dark grey bars) or without (light grey bars) 0.5 mM Lin under the following conditions: 2 d dark, 3 d Wlc (100 µmol $m^{-2}s^{-1}$). For (A) and (B), two alleles of *genomes uncoupled 1* (*gun1-1 and gun1-103*) mutants were included as positive control (known to rescue gene expression on Lin). Expression is relative to WT -Lin and normalised to *YELLOW LEAF SPECIFIC GENE 8 (YLS8,* At5g08290*)*. Data shown are 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 ($p<0.05$).

Supplemental Table S1. Primers used in this study.

Primer Name	Gene name (AGI)	Primer sequence (5' > 3')	Amplicon length (bp)	Notes
LP1	*PTM* (At5g35210)	TTGACAACTGCATCTCCATTG	1,098	PCR genotyping *ptm-1* (WT band)
RP1		CTAGCAGATTTGGTCATTGGG		
LP2	*PTM* (At5g35210)	TACACTTGGGGTTCCACAGAG	1,142	PCR genotyping *ptm-2* (WT band)
RP2		TTTTACCATGGCAAGAACTGC		
LP	*GUN1* (AT2G31400)	ATGTTTAGTAGCCACGCATGG	1,110	PCR genotyping *gun1-103* (WT band)
RP		TTGATCGATGGGTACTCGAAG		
LB	Salk_013123	ATTTTGCCGATTTCGGAAC	687 (with RP1)	T-DNA primer for PCR genotyping *ptm* mutants (mutant bands)
	Salk_073799 (RP side)		661 (with RP2)	
	Salk_073799 (LP side)		904 (with LP2)	
LB	SAIL_742_A11	GTGTACCAAACAACGCTTTACAGC	760 (with RP)	T-DNA primer for PCR genotyping *gun1* mutant (mutant band)
qF1	*PTM* (At5g35210)	TCCTCTCCTGGTATCAGTTTCC	181	qRT-PCR primer for *PTM* (Southampton)
qR1		CTCTGCCTCGGGTTTCACA		
qF2	*PTM* (At5g35210)	GTATAATCCTGGGTTAACGTATATTCACTG	103	qRT-PCR primer for *PTM* (Kyoto)
qR2		CCCAACAACTTCAGGAATTTGTGAATC		
Lhcb1.2_F	*LHCB1.2* (At1g29910)	GTGTGACAATGAGGAAGACTGTTGCC	381	qRT-PCR primer for *LHCB1.2* (Kyoto)
Lhcb1.2_R		AAATGCTCTGAGCGTGGACCAAGCTA		
Lhcb1.2_F	*LHCB1.2* (At1g29910)	GAGTGAGAGACATGAGGAGAAAG	60	qRT-PCR primer for *LHCB1.2* (Southampton)
Lhcb1.2_R		ACATCTGAAAGTCTCAAACCATC		
Lhcb2.1_F	*LHCB2.1* (At2g05100)	GTGACCATGCGTCGTACCGTC	246	qRT-PCR primer for *LHCB2.1* (Kyoto)
Lhcb2.1_R		CTCAGGGAATGTGCATCCGAG		
Lhcb2.1_F	*LHCB2.1* (At2g05100)	CTCCGCAAGGTTGGTGTATC	142	qRT-PCR primer for *LHCB2.1* (Southampton)
Lhcb2.1_R		CGGTTAGGTAGGACGGTGTAT		
CA1_F	*CA1* (At3g01500)	GCTTCTTTCTCACTTCACTTTCTC	189	qRT-PCR primer for *CA1* (Southampton)
CA1_R		CAATGATAGGAGCAGGAGCG		
HEMA1_F	*HEMA1* (At1g58290)	GCTTCTTCTGATTCTGCGTC	128	qRT-PCR primer for *HEMA1* (Southampton)
HEMA1_R		GCTGTGTGAATACTAAGTCCAATC		
CHLH_F	*CHLH* (At5g13630)	CATTGCTGACACTACAACTGC	145	qRT-PCR primer for *CHLH* (Southampton)
CHLH_R		CTTCTCTATCTCACGAACTCCTTC		
GUN1_F	*GUN1* (AT2g31400)	GCTACTAAACATACGCTCCATTG	115	qRT-PCR primer for *GUN1* (Southampton)
GUN1_F		TCGTCTTAGTGCTCCGTCTC		
GUN4_F	*GUN4* (At3g59400)	CAATCTCACTTCGGACCAAC	121	qRT-PCR primer for *GUN4* (Southampton)
GUN4_R		TTGAAACGGCAGATACGG		
ADF2_F	*ADF2* (At3g46000)	CGATTTCGACTTTGTCACTGC	95	qRT-PCR primer for *ADF2* (Southampton) – reference gene
ADF2_R		TCATCTTGTCTCTCACTTTGGC		
YLS8_F	*YLS8* (At5g08290)	GCTGAAATATCCCGTGAACTG	93	qRT-PCR primer for *YLS8* (Southampton) – reference gene
YLS8_R		AATGGAGAACAACCGAAACAG		
YLS8_F	*YLS8* (At5g08290)	AAGGACAAGCAGGAGTTCATT	91	qRT-PCR primer for *YLS8* (Southampton) – reference gene
YLS8_R		AGTAATCTTTTGGAGCAATCACC		
TUB2_F	*TUB2* (At5g62690)	CCAGCTTTGGTGATTTGAAC	102	qRT-PCR primer for *TUB2* (Kyoto) – reference gene
TUB2_R		CAAGCTTTCGGAGGTCAGAG		
ACTIN_F	*ACT2* (At3g18780)	GGTAACATTGTGCTCAGTGGTG	201	qRT-PCR primer for *ACT2* (Sun *et. al* 2011) – reference gene
ACTIN_R		CTCGGCCTTGGAGATCCACATC		

Materials and Methods

Plant materials and growth conditions

The following *Arabidopsis* mutants were used in this study: *gun1-1* (Susek et al., 1993), *gun5-1* (Mochizuki et al., 2001), *gun6-1D* (Woodson et al., 2011), the T-DNA insertion lines *ptm-1* (SALK_013123, Sun et al., 2011) and *ptm-2* (SALK_073799), together with wild-type (WT) *Arabidopsis* Col-0. For experiments in Kyoto, WT (Col-0) and *ptm-1* (*ptm-1* OL) lines were also obtained from Lixin Zhang (Chinese Academy of Sciences). A second allele of *gun1* (SAIL_742_A11) was obtained from the Arabidopsis Biological Resource Center. The presence of the T-DNA insertion was confirmed by PCR (see Supplemental Table S1 for primers), and by sequencing. This line has been described in previous studies as *gun1* (Sun et al., 2011), or *gun1-2* (Dietzel et al., 2015), and we re-named it here as *gun1-103* (with permission of the corresponding author of Dietzel et al., 2015) as *gun1-2* had been used previously (Koussevitzky et al., 2007).

For Norflurazon (NF) experiments in Southampton, four different growth conditions were used: (1) seeds were sown onto half-strength Linsmaier and Skoog (LS) medium (Melford Laboratories, Ipswich, UK) supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar (pH5.7), with or without 5 µM NF, and grown in continuous low white light (25 $\mu mol.m^{-2}.s^{-1}$) for 7 d at 23 °C (Woodson et al., 2011; though note that Woodson et al used 0.6% (w/v) agar); (2) seeds were sown onto half-strength LS medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8), with or without 5 µM NF, and incubated in WL (120 $\mu mol.m^{-2}.s^{-1}$) for 2 h, then 4 d in the dark, followed by 3 d in WL, all at 23 °C (Sun et al., 2011); (3) seeds were sown onto half-strength Murashige and Skoog (MS) medium supplemented with 0.8% agar (pH 5.8), with or without 5 µM NF, and in the presence or absence of 1.5% (w/v) sucrose, and incubated in WL for 2 h, then 3 d in the dark, followed by 3 d in WL, all at 23 °C (McCormac and Terry, 2004); (4) seeds were sown onto half-strength Murashige and Skoog (MS) medium supplemented with 1% (w/v) agar (pH 5.8), with or without 1µM NF in the presence of 1 % (w/v) sucrose, and incubated

in WL for 2 h, then 2 d in the dark, followed by 3 d in WL (100 µmol.m^{-2}.s^{-1}), all at 22 °C.

For Lincomycin (Lin) experiments in Southampton, two different growth conditions were used: (1) seeds were sown onto half-strength Linsmaier and Skoog (LS) medium (Melford Laboratories, Ipswich, UK) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8), with or without 0.5 mM Lin, incubated in WL for 2 h, and grown in continuous darkness for 5 d at 22 °C (Sun et al., 2011); (2) seeds were sown onto half-strength Murashige and Skoog (MS) medium supplemented with 1% (w/v) sucrose and 1% agar (pH 5.8), with or without 0.5 mM Lin and incubated 2 d in dark, followed by 3 d in WL, all at 22 °C. For experiments in Kyoto, seeds were sown onto MS 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 under continuous white light (100 µmol.m^{-2}·sec^{-1}) at 23°C.

DNA extraction, genotyping and sequencing

Genomic DNA was prepared from rosette leaves as described previously (Edwards et al., 1991). The *ptm-1* and *ptm-2* mutants were genotyped by PCR using primers listed in Supplemental Table S1. DNA fragments were analysed using QIAxcel system (Qiagen, Hilden, Germany). Amplicons were sequenced to confirm the precise T-DNA insertion sites, as shown in Supplemental Figure S1. *ptm-2* has at least two inverted T-DNA fragments, with left border (LB) sequences located at each end.

RNA extraction and qRT-PCR

Total RNA was extracted from whole seedlings using the Agencourt Chloropure System (Beckman Coulter, Miami, USA) following the manufacturer's instructions (Kyoto), or according to McCormac et al. (2001) (Southampton). cDNA was synthesised with oligo(dT)$_{12-18}$ using Transcriptor first-strand synthesis kit (Roche, Basel, Switzerland) according to the manufacturer's instructions (Kyoto), or with oligo(dT) and random nonamer primers using the nanoScript2 reverse transcription kit (Primerdesign, Southampton, UK) according to the

manufacturer's instructions (Southampton). Quantitative real-time PCR (qRT-PCR) in Kyoto was performed using LightCycler 480 SYBR Green I Master (Roche) and a LightCycler 96 (Roche) with the following standard thermal profile: 95°C for 5 min, followed by 40 cycles of 95°C for 5 s, 55°C for 10 s, and 72°C for 20 s. qRT-PCR in Southampton was performed using PrecisionPLUS and PrecisionFAST Sybr Green mastermix (Primerdesign) and a StepOnePlus™ Real Time PCR System (Applied Biosystems, Foster City, USA), with the following thermal profile: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min or 95 °C for 2 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s when using the Fast Sybr Green mastermix. Primers used for qRT-PCR are listed in Supplemental Table S1 and further experimental details are provided in the accompanying MIQE checklist.

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MIQE checklist

Seedlings lacking the PTM protein do not show a *genomes uncoupled (gun)* mutant phenotype

Mike T. Page, Sylwia M. Kacprzak, Nobuyoshi Mochizuki, Haruko Okamoto, Alison G. Smith and Matthew J. Terry

A Experimental design

1. Definition of experimental and control groups (E)

Southampton: For NF experiments, three different growth conditions were used: (1) seeds were sown onto half-strength Linsmaier and Skoog (LS) medium (Melford Laboratories, Ipswich, UK) supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar (pH5.7), with (experimental) or without (control) 5 µM NF, and grown in continuous low white light (LWLc, 25 µmol m-2 s-1) for 7 d at 23 °C (Woodson et al., 2011); (2) seeds were sown onto half-strength LS medium supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8), with (experimental) or without (control) 5 µM NF, and incubated in WL (120 µmol m^{-2} s^{-1}) for 2 h, then 4 d in the dark, followed by 3 d in WL, all at 23 °C (Sun et al., 2011); (3) seeds were sown onto half-strength Murashige and Skoog (MS) medium supplemented with 0.8% agar (pH 5.8), with (experimental) or without (control) 5 µM NF, and in the presence or absence of 1.5% (w/v) sucrose, and incubated in WL for 2 h, then 3 d in the dark, followed by 3 d in WL, all at 23 °C (McCormac and Terry, 2004). For Lin experiments in Southampton, two different growth conditions were used: (1) seeds were sown onto half-strength Linsmaier and Skoog (LS) medium (Melford Laboratories, Ipswich, UK) supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar (pH 5.8), with or without 0.5 mM Lin, incubated in WL for 2 h, and grown in continuous darkness for 5 d at 22 °C (Sun *et al*., 2011); (2) seeds were sown onto half-strength Murashige and Skoog (MS) medium supplemented with 1% (w/v) sucrose and 1% (w/v) agar (pH 5.8), with or without 0.5 mM Lin and incubated 2 d in dark, followed by 3 d in WL, all at 22 °C.

Kyoto: Seeds were sown onto MS 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 under continuous white light (WLc, 100 µmol m^{-2} sec^{-1}) for 4 d at 23°C.

2. Number within each group (E)

For each biological replicate, each sample was a pool of approximately 100 seedlings. Each experiment was performed on at least three independent occasions (three biological replicates).

3. Assay carried out by the core or investigator's laboratory? (D)

The majority of the experiments were performed in Southampton (UK), with the data shown in Figure 3 and Figure S1C,D generated in Kyoto (Japan), as outlined in the manuscript.

4. Acknowledgment of authors' contributions (D)

The experiments were designed by Mike Page, Sylwia Kacprzak, Nobuyoshi Mochizuki and Matthew Terry, and executed by Mike Page, Sylwia Kacprzak and Nobuyoshi Mochizuki.

B Sample

1. Description (E)

Cotyledon tissue from *Arabidopsis thaliana* seedlings grown as in section A1. Every effort was made to exclude other tissues (such as seed coats and excessive hypocotyl tissue).

2. Volume/mass of sample processed (D)

Each sample consisted of approximately 100 seedlings.

3. Microdissection or macrodissection (E)

Cotyledon tissue was macrodissected from seedlings using dissecting scissors (cat. no. S274, TAAB, Aldermaston, UK).

4. Processing procedure (E)

Cotyledon tissue was dissected and immediately transferred to a sterile 1.5 ml tube on liquid nitrogen.

5. If frozen, how and how quickly? (E)

Tissue was frozen by transferring freshly dissected tissue to an open 1.5 ml tube suspended in a beaker of liquid nitrogen. Tissue was frozen within 10 s of dissection.

6. If fixed, with what and how quickly? (E)

Tissue not fixed.

7. Sample storage conditions and duration (E)

Samples were stored at -80 °C. Generally samples were stored for less than one week, but never for more than one month.

C Nucleic acid extraction

1. Procedure and/or instrumentation (E)

Southampton: Samples were homogenised in 500 µl extraction buffer (100 mM NaCl, 10 mM Tris pH7.0, 1 mM EDTA, 1% (w/v) SDS). After the addition of 150 µL phenol (pH 4.8), samples were vortexed vigorously. 250 µL chloroform was then added and the samples again vortexed vigorously. After centrifugation (16,100 x *g*, 5 min, 4°C), the upper aqueous phase was transferred to a new tube containing 450 µL ice-cold 4 M LiCl. RNA was precipitated overnight at 4°C. After centrifugation (16,100 x *g*, 20 min, 4 °C), pellets were resuspended in 300 µL DNase buffer (10 mM Tris pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) and 1 µL DNase was then added and samples incubated at 37 °C for 25 min. Samples were mixed with 500 µL phenol:chloroform:isoamyl alcohol (25:24:1), pH 6.7 and vortexed vigorously. After centrifugation (16,100 x *g*, 5 min, 4 °C), the aqueous upper phase was

mixed with 750 µL 95% ethanol:5% 3 M sodium acetate, pH 5.2 and RNA precipitated at -20 °C for 1 h. After centrifugation (16,100 x *g*, 20 min, 4 °C), RNA pellets were air dried for 5 min and resuspended in 50 µL TE.

Kyoto: Total RNA was extracted from whole seedlings using the Agencourt Chloropure System (Beckman Coulter, Miami, USA) following the manufacturer's instructions.

2. Name of kit and details of any modifications (E)

Kyoto: The Agencourt Chloropure System (Beckman Coulter, A47949) was used to extract RNA from plant tissue (Kyoto).

3. Source of additional reagents used (D)

Sodium chloride, Fisher, cat. no. S/3120/60
Tris buffer, Fisher, cat. no. T/P630/60
EDTA, Sigma, cat. no. E5134
SDS, Calbiochem, cat. no. 428015
Phenol (pH 4.8), Sigma, cat. no. P4682
Chloroform, Sigma, cat. no. 288306
Lithium chloride, Sigma, cat. no. L9650
Magnesium chloride, Sigma, cat. no. M8266
Calcium chloride, VWR, cat. no. 100703H
RQ1 RNase-free DNase, Promega, cat. no. M6101
DNase I recombinant, RNase-free, Roche, cat. no. 04 716 728 001
Phenol:chloroform:isoamyl alcohol (25:24:1, pH 6.7), Fisher, cat. no. BP1752I
Ethanol, Fisher, cat. no. E/0650DF/P17
Sodium acetate pH 5.2, Alfar Aesar, cat. no. J63560

4. Details of DNase or RNase treatment (E)

Southampton: Precipitated pellets were resuspended in 300 µL DNase buffer (10 mM Tris pH 7.5, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$) and 1 µL RNase-free DNase was then added and samples incubated at 37 °C for 25 min.

Kyoto: Nucleic acids bound on SPRI-magnet beads were suspended in 10 µL of DNase solution (40 mM Tris-HCl, 10 mM NaCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, pH7.9, 2 U RNase-free DNase).

5. Contamination assessment (DNA or RNA) (E)

Primer pairs were designed to span introns where possible. Contamination with gDNA would therefore generate a second larger product, which would be detected during melt-curve analysis. No gDNA contamination was detected.

6. Nucleic acid quantification (E)

This was performed using a NanoDrop (i.e. spectrophotometrically).

7. Instrument and method (E)

Quantification was performed using a NanoDrop ND-1000 (Thermo Scientific). A 2 µL drop of each sample was loaded onto the instrument, after blanking with TE buffer.

8. Purity (A_{260}/A_{280}) (D)

Purity was determined. For all samples, purity was between 1.98 and 2.10.

9. Yield (D)

Total yield was between 15-75 µg, depending on sample type/treatment given.

Kyoto: Total yield was between 4-5µg, depending on sample type/treatment given.

10. RNA integrity: method/instrument (E)

Not determined.

11. RIN/RQI or C_q of 3' and 5' transcripts (E)

Not determined.

12. Electrophoresis traces (D)

Not determined.

13. Inhibition testing (C_q dilutions, spike, or other) (E)

Not determined.

D Reverse transcription

1. Complete reaction conditions (E)

Southampton: Annealing step – 2 µg total RNA was used per sample, and mixed with 1 µL 40 µM random nonamer and 1 µL 20 µM oligo dT primer, with the volume made up to 10 µL with RNase/DNase free water. Samples were incubated at 65 °C for 5 min, then transferred immediately to ice.

Extension step – 5 µL 4x nanoScript2 buffer, 1 µL dNTP mix (10 mM each), 3 µL RNase/DNase free water and 1 µL nanoScript2 reverse transcriptase were then added to the samples. These reagents were mixed together in a mastermix in the order given here, prior to adding to the samples. Samples were incubated at 25 °C for 5 min, 42 °C for 20 min, and 75 °C for 10 min.

Kyoto: Extension step - 2.25 µL RNase/DNase free water, 4 µL 5x Transcriptor reverse transcriptase reaction buffer, 2 µL dNTP mix (10 mM each), 1 µL 0.5 µg/µL oligo dT primer, 0.25 µL Protector RNase Inhibitor, and 0.5 µL Transcriptor reverse transcriptase were added to 10 µL of the RNA samples. These reagents were mixed together in a mastermix in the order given here, prior to adding to the samples (total volume 20 µL). Samples were incubated at 55 °C for 30 min, and 85 °C for 5 min. Then, the reactions were mixed with 20-40 µL of NFW for qPCR analysis.

2. Amount of RNA and reaction volume (E)

Southampton: 1 - 2 µg total RNA was added to the reverse transcription reaction per sample. The total reaction volume was 20 µL.

Kyoto: 0.5-1.5 µg total RNA was added to the reverse transcription reaction per sample. The total reaction volume was 20 µL.

3. Priming oligonucleotide (if using GSP) and concentration (E)

Not using GSP.

4. Reverse transcriptase and concentration (E)

Southampton: nanoScript2 reverse transcriptase at 160 U/µL.

Kyoto: Transcriptor reverse transcriptase at 20 U/µL.

5. Temperature and time (E)

See section D1.

6. Manufacturer of reagents and catalogue numbers (D)

Southampton: Primerdesign (Southampton, UK). Catalogue number = RT-nanoScript2.

Kyoto: Roche Applied Science (Mannheim, Germany). Catalogue number = 04897030001.

7. C_qs with and without reverse transcription (D)

See table below for the mean C_q values (two technical replicates per reaction) for a selection of genes included in this study. The samples contained template from cDNA synthesis reactions with reverse transcriptase (+RTase) or without RTase (-RTase), or with NFW instead of template (NTC). The quantification threshold was kept constant for all samples. NAD = no amplification detected (i.e. fluorescence remained below the threshold). The RNA sample used in this experiment was purified from Col-0 seedlings grown in LWLc for 7 d.

Gene	**C_q +RTase**	**C_q -RTase**	**C_q NTC**
CHLH	16.59	NAD	35.33
GUN4	17.83	36.71	NAD
CA1	15.27	35.70	34.28

8. Storage conditions of cDNA (D)

cDNA stored at -20 °C.

E qPCR target information

1. Gene symbol (E)

See Supplemental Table S1.

2. Sequence accession number (E)

See Supplemental Table S1.

3. Location of amplicon (D)

Not included.

4. Amplicon length (E)

See Supplemental Table S1.

5. In silico specificity screen (BLAST, and so on) (E)

All primers underwent a BLASTn search against the entire *A. thaliana* cDNA database to check for specificity.

6. Pseudogenes, retropseudogenes, or other homologs? (D)

No.

7. Sequence alignment (D)

Not determined.

8. Secondary structure analysis of amplicon (D)

Not determined.

9. Location of each primer by exon or intron (if applicable) (E)

Not applicable.

10. What splice variants are targeted? (E)

No splice variants are targeted.

F qPCR oligonucleotides

1. Primer sequences (E)

See Supplemental Table S1.

2. RTPrimerDB identification number (D)

Sequences not submitted to RTPrimerDB.

3. Probe sequences (D)

Probes not used – SYBR green assays used here.

4. Location and identity of any modifications (E)

No modifications.

5. Manufacturer of oligonucloetides (D)

Sigma-Aldrich

6. Purification method (D)

Desalted.

G qPCR protocol

1. Complete reaction conditions (E)

Southampton: Each reaction contained 0.5 µL cDNA, 5 µL PrecisionPLUS SYBR green mastermix or PrecisionFAST SYBR and 2.5 µL of primer mix (containing forward and reverse primers each at 2 µM), with the volume made up to 10 µL with NFW.

Kyoto: Each reaction contained 2 µL of diluted cDNA, 7.5 µL LightCycler 480 SYBR Green I Master and 0.3 µL of primer mix (containing forward and reverse primers each at 10 µM), with the volume made up to 15 µL with NFW.

2. Reaction volume and amount of cDNA/DNA (E)

The total reaction volume was 10 µL, and 0.5 µL cDNA was used per reaction.

Kyoto: The total reaction volume was 15 µL, and 2 µL of diluted cDNA was used per reaction.

3. Primer, (probe), Mg^{2+}, and dNTP concentrations (E)

Southampton: Primers were at a final concentration of 0.25 µM. Probes were not used. The final Mg^{2+} concentration was 5 mM, and the final concentration of each dNTP was 0.25 mM.

Kyoto: Primers were at a final concentration of 0.2 µM. Probes were not used. The final Mg^{2+} concentration and the final concentration of each dNTP are not disclosed by the manufacturer.

4. Polymerase identity and concentration (E)

Southampton: PrecisionPlus thermostable Taq polymerase at 0.05 U/µL and PrecisionFAST polymerase that contains point mutation(s) that increase the reaction rate.

Kyoto: FastStart Taq DNA Polymerase – concentration not disclosed by the manufacturer.

5. Buffer/kit identity and manufacturer (E)

Southampton: PrecisionPLUS qPCR Mastermix and PrecisionFAST qPCR Mastermix, manufactured by Primerdesign (Southampton, UK), catalogue numbers = PrecisionPLUS-SY and PrecisionFAST-SY.

Kyoto: LightCycler 480 SYBR Green I Master (Roche Applied Science, Mannheim, Germany. Catalogue number = 04887352001.

6. Exact chemical composition of the buffer (D)

Southampton: The PrecisionPLUS SYBR mastermix contains a Tris buffer (the exact composition of the buffer was classified as proprietary information by the manufacturer).

Kyoto: The chemical composition of the buffer is not disclosed by the manufacturer.

7. Additives (SYBR Green I, DMSO, and so forth) (E)

A final concentration of 1x SYBR Green was included in the mastermix described in section G5 and G6. No other additives were included.

8. Manufacturer of plates/tubes and catalog number (D)

Southampton: 96-well semi-skirted, low-profile, raised rim, white qPCR plates were supplied by Starlab (cat. no. E1403-7709). Plates were sealed with polyolefin Star-Seal, X-clear seals manufactured by Starlab (cat. no. E2796-9795).

Kyoto: 96-well semi-skirted, low-profile, raised rim, clear qPCR plates were supplied by Roche (LightCycler® 480 Multiwell Plate 96, clear, cat. no. 05102413001). Plates were sealed with LightCycler® 480 Sealing Foil (Roche, cat. no. 04729757001).

9. Complete thermocycling parameters (E)

Southampton: Ramp speeds were set to 100%. Plates were incubated at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s (or 3 s for FAST) and 60 °C for 1 min (or 30 s for FAST). Fluorescence was determined at the end of each cycle. Melt curve analysis was performed at the end of each run – 60 °C to 92 °C, in 0.5-0.6 °C increments. Two technical replicates of each reaction were performed in each run.

Kyoto: Ramp speeds were set to 100%. Plates were incubated at 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 20 s. Fluorescence was determined at the end of each cycle. Melt curve analysis was performed at the end of each run – 60 °C to 92 °C, in 0.5 °C increments.

10. Reaction setup (manual/robotic) (D)

Manual.

11. Manufacturer of qPCR instrument (E)

Southampton: StepOnePlus, manufactured by Applied Biosystems.

Kyoto: LightCycler 96, manufactured by Roche Applied Science.

H qPCR validation

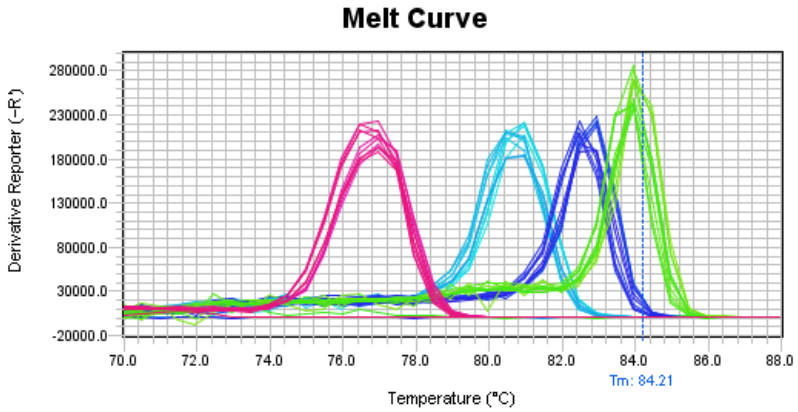
1. Evidence of optimization (from gradients) (D)

Primers were designed to have the same T_m. As such, all qPCRs were performed using the same annealing temperature.

2. Specificity (gel, sequence, melt, or digest) (E)

Melt curve analysis was performed on every reaction at the end of every run as described in section G9.

See below for an example of a typical melt curve analysis result. This result was obtained after a qPCR run to amplify *ADF2* (light blue), *GUN4* (dark blue), *CA1* (green) and *YLS8* (pink). NTC reactions are flat lines around zero on the y-axis. The melt curve was assessed between 60 °C – 92 °C as described in section G9. The x-axis on the graph below was trimmed to 70 °C – 88 °C to aid visualisation of the curves here – no other peaks were observed outside of this range.



3. For SYBR Green I, C_q of the NTC (E)

No fluorescence signal was observed in the majority of NTCs. Where signal was seen, the C_q of the NTC was at least 13 cycles later than the C_q of the experimental samples.

4. Calibration curves with slope and *y* intercept (E)

This was performed for all primer pairs, using a 2-fold serial dilution of WT (Col-0) untreated cDNA. Two technical replicates of each reaction were performed, with each calibration curve performed twice. The *x*-axis was plotted on a log10 scale, and the *y*-axis plotted on a linear scale.

5. PCR efficiency calculated from slope (E)

PCR efficiency (PE) was calculated from the slope using the following formula:

$$PE = 2.718^{(-1/slope)}$$

PCR efficiency was determined twice independently for each primer pair. The PE used downstream for each primer pair was the mean of these two calculations.

6. CIs for PCR efficiency or SE (D)

Not determined.

7. r^2 of calibration curve (E)

Determined for all primer pair calibration curves, and was always higher than 0.990.

8. Linear dynamic range (E)

A linear range of at least three orders of magnitude was observed for all primer pairs. For all primer pairs, the calibration curve's linear interval included the interval for the target nucleic acids being quantified.

9. C_q variation at LOD (E)

LOD not reached in calibration curves, but well outside the interval for the target nucleic acids being quantified.

10. CIs throughout range (D)

Not determined.

11. Evidence for LOD (E)

See section H9.

12. If multiplex, efficiency and LOD of each assay (E)

Not multiplex.

I Data analysis

1. qPCR analysis program (source, version) (E)

Southampton: StepOne software, v2.3 (Applied Biosystems).

Kyoto: Light Cycler 96 Software v1.1 (Roche Applied Science).

2. Method of C_q determination (E)

A threshold was applied to the amplification plots. This was the same for all primer pairs on each run, and was applied in the logarithmic region of signal increase. The resulting C_qs were used to generate relative expression levels using the $\Delta\Delta C_q$ method.

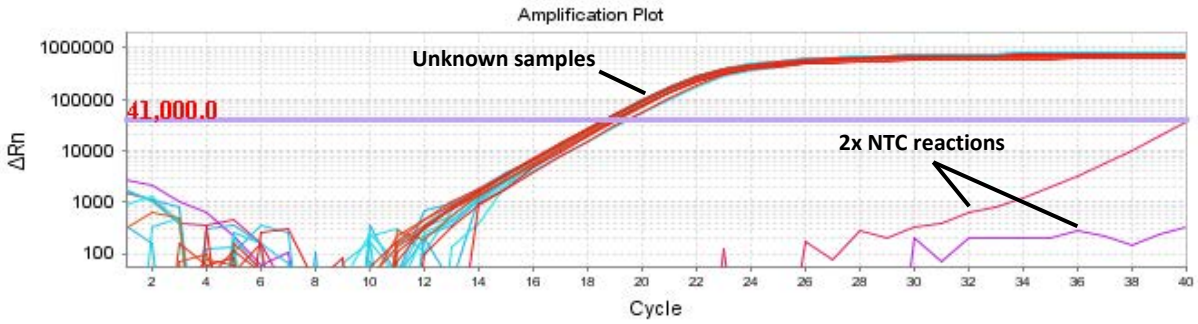
3. Outlier identification and disposition (E)

Reactions with abnormal attributes (melt curve with multiple peaks, large variation between technical replicates) were discarded.

4. Results for NTCs (E)

The majority of NTCs gave no C_q value (no amplication at threshold level). Where a NTC did give a C_q, it was always at least 13 cycles later than the C_q of the experimental samples.

See graph below for an example (amplifying an amplicon of *ADF2* from 9 unknown samples (2 technical replicates) and 2 NTC reactions).



5. Justification of number and choice of reference genes (E)

Southampton: Three reference genes were used for all experiments. These reference genes gave very similar expression profiles for the samples in each experiment. The reference genes used were *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000), *YELLOW-LEAF-SPECIFIC GENE 8* (*YLS8*, At5g08290) and *ACTIN 2 (ACT2*, At3g18780). *ADF2* was identified through analysis of independent microarray data from Col-0 seedlings grown with/without NF (Page et al., 2016) and *YLS8* has been proposed before as a candidate reference gene due to its stable expression profile across a variety of experimental conditions in *Arabidopsis* (Czechowski et al., 2005) and also verified in seedlings grown on NF (Page et al., 2016). *ACT2* was used previously by Sun et al. (2011) and was included in this study as a third reference gene for a more robust comparison with that work.

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol 139: 5-17

Page MT, McCormac AC, Smith AG, Terry MJ (2016) Singlet oxygen initiates a plastid signal controlling photosynthetic gene expression. New Phytol DOI: 10.1111/nph.14223

Sun X, Feng P, Xu X, Guo H, Ma J, Chi W, Lin R, Lu C, Zhang L (2011) A chloroplast envelope-bound PHD transcription factor mediates chloroplast signals to the nucleus. Nature Commun 2: 477

Kyoto: *TUB2* (At5g62690) was used for all experiments. *TUB2* gives a very similar expression profile with other reference genes *YLS8* (At5g08290) and *UBQ10* (At4g05320) in the RT-qPCR analysis of the retrograde signalling assay.

6. Description of normalisation method (E)

The $\Delta\Delta C_q$ method was used. The ΔC_q between the control sample (Col-0, untreated) and the experimental samples was calculated. The primer efficiency was then raised to the power of the ΔC_q for each sample for each gene of interest. These values were then divided by the same values for the reference genes to generate $\Delta\Delta C_q$ values.

7. Number and concordance of biological replicates (D)

Southampton: Three biological replicates were performed independently (experiment performed at different times) for each experiment. Each biological replicate was run separately on the qPCR instrument. Concordance of biological replicates can be seen by examining the standard error bars on graphs in the manuscript (generally excellent).

Kyoto: Five biological replicates were performed independently (5 different plant groups were harvested from 5 different plates, followed by RNA extraction and RT-qPCR.)

8. Number and stage (reverse transcription or qPCR) of technical replicates (E)

Two technical replicates of each sample/primer pair combination were run at the qPCR stage.

9. Repeatability (intraassay variation) (E)

There was strong agreement between technical replicates, with the average ΔC_q between technical replicates approximately 0.1.

10. Reproducibility (interassay variation, CV) (D)

CV not determined. The reproducibility between biological replicates was excellent, evident by the small standard error bars given on graphs in the manuscript. Each sample was a pool of approximately 100 seedlings and so a small number of outliers in a sample would be averaged out by the large number of non-outliers.

11. Power analysis (D)

Not determined.

12. Statistical methods for results significance (E)

Student's *t*-tests were performed between the mean relative expression values of Col-0 and each sample to determine if differences were significant (two-tailed test, $p<0.05$).

13. Software (source, version) (E)

SigmaPlot (v12.5, Sigmaplot software Inc.).

14. Cq or raw data submission with RDML (D)

Raw data not submitted to RDML.