MIQE checklist

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

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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

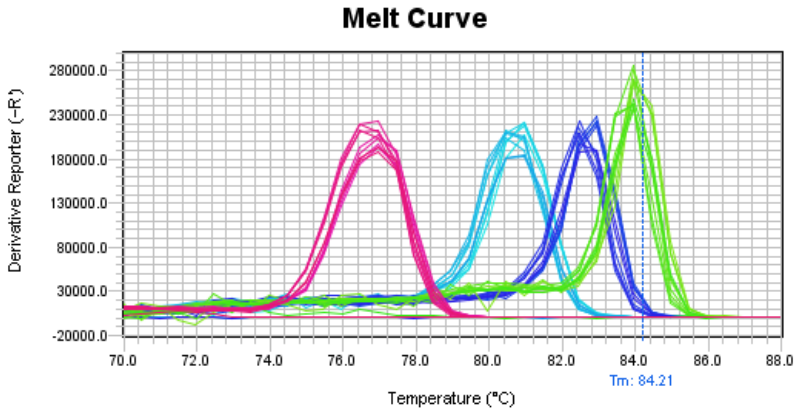
1. Evidence of optimization (from gradients) (D)

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.

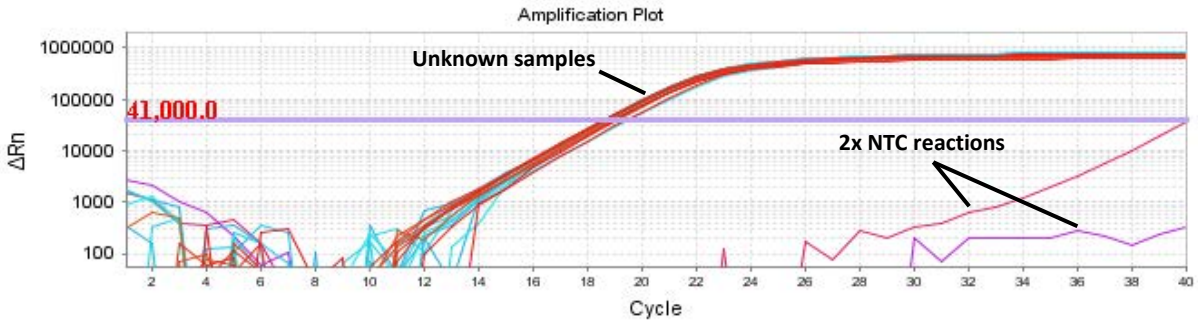
3. Outlier identification and disposition (E)

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.