

MIQE checklist for Phil. Trans. R. Soc. B. article

Overexpression of chloroplast-targeted ferrochelatase 1
results in a *genomes uncoupled* chloroplast-to-nucleus
retrograde signalling phenotype

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MIQE checklist

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A Experimental design

1. Definition of experimental and control groups (E)

Seedlings were grown for 7 d in continuous white light (LWLC; $25 \mu\text{mol m}^{-2} \text{s}^{-1}$, 23 °C) on half-strength Linsmaier and Skoog medium, 1% (w/v) sucrose, 1% (w/v) agar, supplemented with either 5 μM Norflurazon (NF) (**experimental group**) or 0.1% DMSO (**control group**).

2. Number within each group (E)

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

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

Yes

4. Acknowledgment of authors' contributions (D)

The experiment was designed by Mike Page and Matthew Terry, and executed by Mike Page.

B Sample

1. Description (E)

Cotyledon tissue from 7 d LWLC-grown (see section A1) *Arabidopsis thaliana* seedlings. 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)

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 MgCl₂, 0.5 mM CaCl₂) 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 buffer (10 mM Tris pH 8.0, 1mM EDTA).

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

No kit used.

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

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)

Precipitated pellets were resuspended in 300 μ L DNase buffer (10 mM Tris pH 7.5, 2.5 mM MgCl₂, 0.5 mM CaCl₂) and 1 μ L RNase-free DNase was then added and samples incubated at 37 °C for 25 min.

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 μ L 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 μ g, 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)

Annealing step – 2 μ g total RNA was used per sample, and mixed with 1 μ L 40 μ M random nonamer and 1 μ L 20 μ M oligo dT primer, with the volume made up to 10 μ L 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.

2. Amount of RNA and reaction volume (E)

2 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)

nanoScript2 reverse transcriptase at 160 U/µL.

5. Temperature and time (E)

See section D1.

6. Manufacturer of reagents and catalogue numbers (D)

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

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 nuclease-free water (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
<i>FC1</i>	15.50	30.62	31.10
<i>CHLH</i>	16.59	NAD	35.33
<i>GUN4</i>	17.83	36.71	NAD
<i>CA1</i>	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 Supplementary Table S3.

2. Sequence accession number (E)

See Supplementary Table S3.

3. Location of amplicon (D)

Not included.

4. Amplicon length (E)

See Supplementary Table S3.

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 Supplementary Table S3.

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 oligonucleotides (D)

Sigma-Aldrich.

6. Purification method (D)

Desalted.

G qPCR protocol

1. Complete reaction conditions (E)

Each reaction contained 0.5 μL cDNA, 5 μL PrecisionPLUS SYBR green mastermix 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.

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.

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

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.

4. Polymerase identity and concentration (E)

PrecisionPlus thermostable Taq polymerase at 0.05 U/ μL .

5. Buffer/kit identity and manufacturer (E)

PrecisionPLUS qPCR Mastermix, manufactured by Primerdesign (Southampton, UK), catalogue number = PrecisionPLUS-SY.

6. Exact chemical composition of the buffer (D)

The PrecisionPLUS SYBR mastermix contains a Tris buffer (the exact composition of the buffer was classified as proprietary information 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 sections G5 and G6. No other additives were included.

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

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).

9. Complete thermocycling parameters (E)

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 and 60 °C for 1 min. Fluorescence was determined at the end of each cycle. Melt curve analysis was performed at the end of each run from 60 °C to 92 °C in 0.5 °C increments. Two technical replicates of each reaction were performed in each run.

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

Manual.

11. Manufacturer of qPCR instrument (E)

StepOnePlus, manufactured by Applied Biosystems.

H qPCR validation

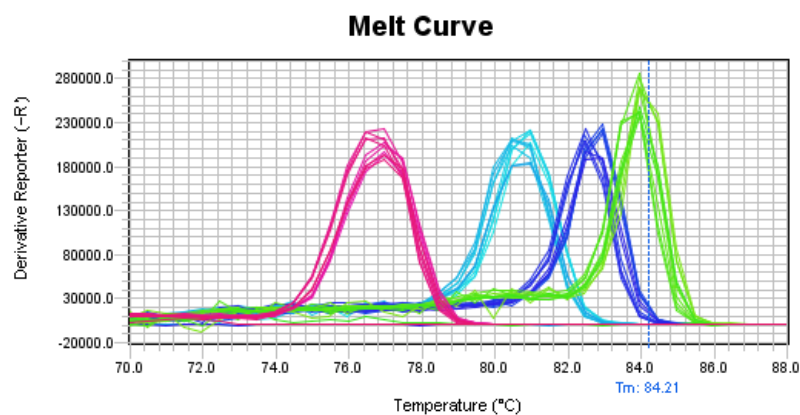
1. Evidence of optimization (from gradients) (D)

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:

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

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)

StepOne software, v2.3 (Applied Biosystems).

2. Method of C_q determination (E)

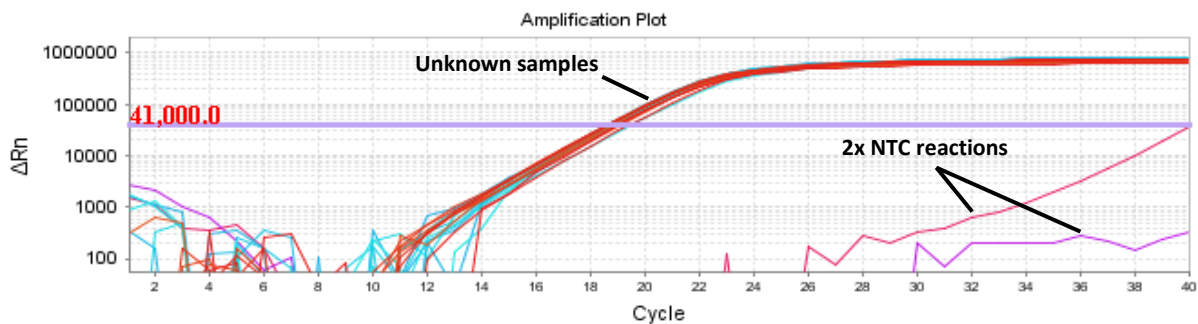
A threshold was applied to the amplification plots. This was the same for all primer pairs on each run, and was applied in the logarithmic region of signal increase. The resulting C_q s were used to generate relative expression levels using the $\Delta\Delta C_q$ method.

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 amplification at threshold level). When 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)

Two reference genes were used for all experiments. These reference genes gave very similar expression profiles for the samples in each experiment, and so it was decided that a third reference gene was unnecessary. The reference genes used were *ACTIN DEPOLYMERISING FACTOR 2* (*ADF2*, At3g46000) and *YELLOW-LEAF-SPECIFIC GENE 8* (*YLS8*, At5g08290). *ADF2* and *YLS8* were identified as excellent reference genes for NF screens through analysis of independent microarray data from Col-0 seedlings grown with/without NF (Page et al, 2017, *New Phytol.* **213**, 1168–80).

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)

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).

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 as evidenced 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.