Chapter 2

Quantitative Reverse Transcription-qPCR-Based Gene Expression Analysis in Plants

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Abstract

The investigation of gene expression is an initial and essential step to understand the function of a gene in a physiological context. Reverse transcription-quantitative real-time PCR (RT-qPCR) assays are reproducible, quantitative, and fast. They can be adapted to study model and non-model plant species without the need to have whole genome or transcriptome sequence data available. Here, we provide a protocol for a reliable RT-qPCR assay, which can be easily adapted to any plant species of interest. We describe the design of the qPCR strategy and primer design, considerations for plant material generation, RNA preparation and cDNA synthesis, qPCR setup and run, and qPCR data analysis, interpretation, and final presentation.

Key words Gene expression, Primer design, Reverse transcription, cDNA, qPCR, Reference gene, Cq value

1 Introduction

Gene expression analysis is used to identify the physiological context in which a gene is transcribed and the encoded protein produced in the cell. Precise quantification of expression levels can provide important clues to identify and verify the functions of key genes in cellular pathways and it helps to discriminate functional diversification within gene families. Molecular diagnostic tools based on gene expression are commonly developed to determine the physiological status of cells. Gene expression analysis is popular because of its versatility and applicability to model and non-model systems. Suitable transcript cDNA sequence information necessary for the gene expression assays is relatively easy and fast to obtain even for non-model systems.

Gene expression assays need to fulfill a number of prerequisites to ensure reliability. Importantly, the assay needs to be sensitive, quantitative, and specific for the gene of interest and reproducible across biological replicates and laboratories. When only a few genes are being investigated, fluorescence-based reverse transcription-quantitative

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PCR (RT-qPCR) has become the method of choice for most scientists as it meets all the above-mentioned requirements. Semiquantitative reverse transcription-PCR with analysis of PCR fragments by agarose gel electrophoresis or Northern blot hybridization is not considered appropriate today to reach firm conclusions.

Our protocol for a SYBR Green-based fluorescence RT-qPCR assay is easy to establish even in nonspecialist laboratories. SYBR Green fluorescence is measured after each cycle (real-time) and increasing levels of fluorescence during the qPCR are indicative of the amplification of double-stranded PCR fragments. The "quantification cycle" (Cq) value of a qPCR run is used for quantification and it reflects the time point and cycle during the exponential PCR phase at which a set threshold level of SYBR Green fluorescence is reached (Fig. 1a). The mass standard curve method can be used to correlate the Cq value with the initial amount of template present in the qPCR reaction (Fig. 1a, b). The expression data for the genes of interest are normalized to reference gene expression data to account for possible variations in input in different biological samples (termed normalized absolute gene expression). In this chapter we summarize important criteria to consider, present a strategy for establishing and performing a reliable and robust assay, and provide a framework for how to analyze and display the data. Detailed explanations and further guidelines for the usage of qPCR can be obtained from the MIQE (=Minimum Information for Publication of quantitative real-time PCR experiments) website at http://miqe.gene-quantification.info [1]. We use this RT-qPCR method as a diagnostic tool to determine the iron nutritional status of plant mutants and as a bioassay to study regulatory responses to iron deficiency, e.g. [2, 3].

2 Materials

Prepare all solutions using ultrapure water and analytical grade chemicals. Store solutions at room temperature or as indicated by the manufacturer. Handle kits and enzymes following the manufacturers' instructions. Use filter tips during RNA preparation, cDNA, and qPCR setup to avoid contamination of reagents in the reaction tubes. Use RNAse and DNAse-free reaction tubes.

2.1 DNA Sequence Analysis Tools

- DNA analysis: http://www.ebi.ac.uk
- Primer design: http://primer3.ut.ee

2.2 General Analysis of DNA and RNA

- Regular PCR: REDTaq ReadyMix PCR Reaction Mix (Sigma).
- TAE DNA gel electrophoresis: 40 mM Tris–HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.0. Prepare 50× TAE buffer stock and dilute to 1× TAE buffer ready to use. A 50× stock solution

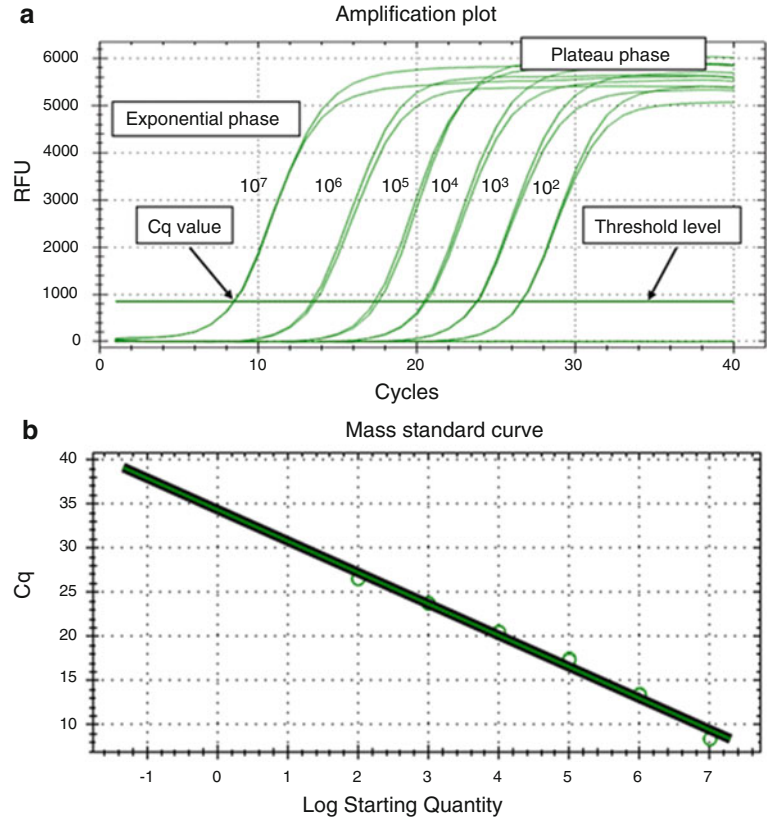

Fig. 1 Principles of qPCR using the mass standard curve method. (**a**) Amplification plot of a mass standard dilution series. Relative fluorescence units (=RFU) were measured during the qPCR run at the end of each of 40 cycles. In the beginning of the exponential phase quantification cycle (=Cq), values are recorded as the time point when a set threshold level of RFU is reached. Later, the RFU reach a plateau phase. The numbers 10^7–10^2 indicate the starting quantity in molecules per reaction. The reactions were performed in duplicate technical replicates. (**b**) Linear mass standard curve derived from the data in (**a**). Starting quantities of unknown samples are calculated based on the measured Cq values. The images were produced with the CFX Manager software (Bio-Rad)

is prepared by dissolving 242 g Tris base in water, adding 57.1 mL glacial acetic acid, and 100 mL of 500 mM EDTA (pH 8.0) solution, and bringing the final volume up to 1 L. For a 1 % gel dissolve 1 g agarose in 100 mL 1× TAE buffer by boiling, add 3 μL DNA staining dye (GelRed, Biotium, or equivalent) and pour agarose gel. For all steps of the agarose gel electrophoresis setup and run, consult the information provided by the agarose gel electrophoresis equipment supplier or molecular biology standard protocols [4]. For size determination use a DNA molecular weight marker with an appropriate

size range. DNA staining is visible under UV light (*see* **Note 1**) and documented with a gel documentation system.

- DNA gel extraction: GeneJET Gel Extraction Kit (Thermo Scientific).
- DNA/RNA quantification: Measure the nucleic acid concentration by UV spectrophotometry. The $OD_{260} = 1$ corresponds to 50 ng double-stranded DNA or 40 ng single-stranded RNA per microliter in a 1 cm cuvette. The ratios of the values for nucleic acid versus protein absorption peaks (OD_{260}/OD_{280}) are indicative of purity and should be above 1.5. To reduce the amount of nucleic acid material needed for measuring, a microvolume spectrophotometric device such as the NanoDrop (Thermo Scientific), Nano Quant plate of a microplate reader (Tecan) or equivalent can be used.
- PCR oligonucleotide primers.

2.3 Reverse Transcription- qPCR

- RNA preparation: peqGOLD Plant RNA kit (Peqlab) or equivalent.
- DNase I, RNase-free.
- RNase- and DNase-free PCR grade water.
- Oligo dT_{18}.
- cDNA synthesis: RevertAid First Strand cDNA synthesis kit (Thermo Scientific) or equivalent.
- Ribolock RNase inhibitor (Thermo Scientific) or equivalent.
- qPCR: Dynamo Flash SYBR Green qPCR kit (Thermo Scientific) or equivalent.
- Oligonucleotide primers for PCR reactions can be ordered from multiple companies.
- Colored 200 μL 8-tube PCR strips.
- Optical sealing tape for 96-well PCR plates.
- 96-well plates for qPCR, adapted for the available real-time PCR device.
- Real-time PCR cycler (CFX96, Bio-Rad or equivalent).

3 Methods

A flowchart of the RT-qPCR procedure is depicted in Fig. 2a. External calibration standards allow absolute quantification of template present at the start of the PCR (standard curve method) and serve as positive controls to monitor PCR assay variation over time.

Several points require special attention by the researchers when performing RT-qPCR and we address them in the following sections. These include the design of appropriate oligonucleotide

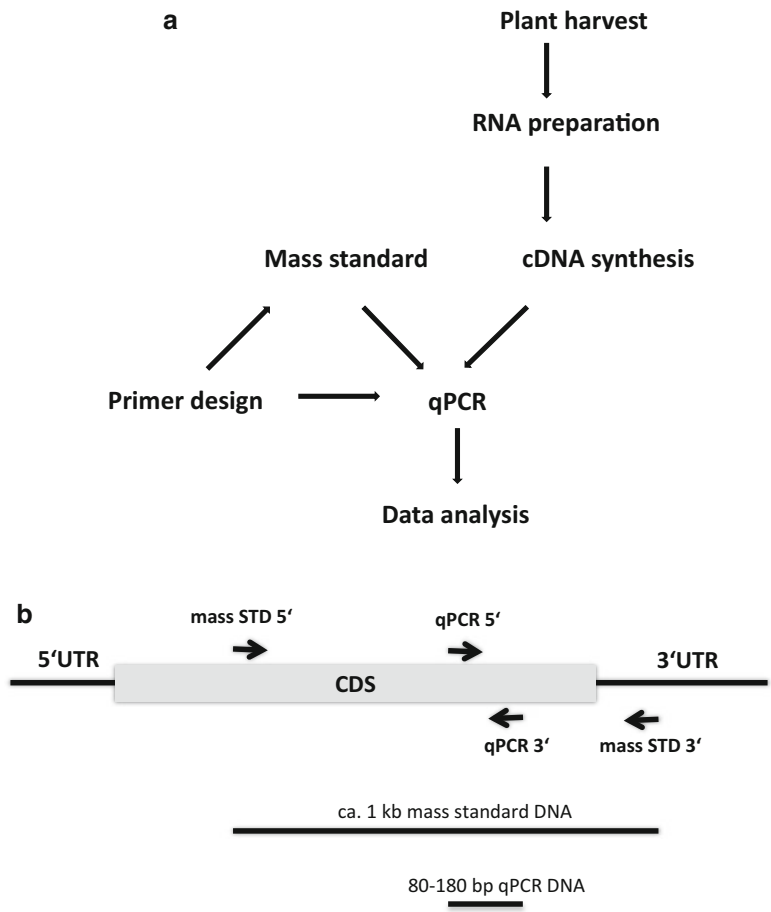

Fig. 2 Experimental flowchart and primer design. (**a**) Experimental flowchart to determine gene expression levels using RT-qPCR and the mass standard curve method. (**b**) Primer design. A schematic representation of a cDNA is shown with coding sequence (CDS) and 5′ and 3′ untranslated regions (UTR). qPCR and mass standard (mass STD) oligonucleotide primer binding sites are indicated by *arrows*. The amplification products are depicted below. The qPCR fragment must be contained within the mass standard DNA fragment

primers for qPCR to achieve high PCR efficiency, the choice of reference genes for normalization of biological samples, a work plan for generation of suitable plant material in biological replicates prior to RT-qPCR, handling and pipetting, the quality of materials and consumables, and finally, thorough analysis of qPCR data.

3.1 Design of qPCR Strategy and Oligonucleotide Primers

1. Select genes of interest and reference genes and obtain DNA sequence information (*see* **Note 2**). Use DNA analysis software tools and analyze cDNA sequence for translation start and stop codons and for conserved regions among related cDNA sequences obtained from the species to spot unique regions

optimal for qPCR primer design, at best in the vicinity of the 3′ end (Fig. 2b). Consider splicing and alternative splicing events.

2. Use the primer3 software tool to design qPCR 5′ and 3′oligonucleotide primers in non-conserved regions of the cDNA with the following characteristics: amplification of ca. 80–180 bp cDNA fragments, 60 °C annealing, low risk of hairpin, and primer dimer formation (Fig. 2b). Conduct a BLAST search with the oligonucleotide primer sequences against all expressed sequences available from the species or a close relative to check that oligonucleotide primer binding sites are not present in transcribed sequences.
3. Use the primer3 software tool to design mass standard 5′ and 3′oligonucleotide primers to produce external calibration standard DNA fragments. The mass standard oligonucleotide primer binding sites need to encompass the qPCR target regions and should allow production of linear ca. 1 kb mass standard DNA fragments (Fig. 2b).

3.2 Mass Standard Preparation for qPCR

1. Amplify mass standard DNA fragments from 1 μL template (*see* **Note 3**) with 1 μL of 10 μM 5′ and 3′ mass standard oligonucleotide primers in a regular 20 μL PCR reaction.
2. Use 15 μL of the PCR reaction and separate the DNA fragments by TAE agarose gel electrophoresis (1 % gel). With a razor blade and under UV light, dissect a small agarose cube containing the mass standard PCR fragment and purify the DNA using any of the commercially available gel extraction kits. The final volume of the purified mass standard DNA is typically 20–50 μL. Measure the DNA concentration of the purified mass standard DNA fragment solutions and use 3–5 μL to run a gel electrophoresis to confirm the correct size of the purified DNA fragments.
3. Determine the molar concentrations of the purified mass standard PCR fragment solutions and calculate the concentrations in number of DNA molecules/μL using the molar conversion tool at http://molbiol.edu.ru/eng/scripts/01_07.html.
4. Dilute the purified mass standard DNA fragment solutions in an initial 1:100 and subsequent 1:10 steps to obtain a dilution series ranging from 10^7, 10^6, 10^5, 10^4, 10^3 to 10^2 DNA molecules per 10 μL. Prepare 800–1000 μL of each dilution in 1.5 mL reaction tubes.
5. Dispense 50 μL of each of the six dilutions of the series into 8mer PCR strips and freeze until use (*see* **Note 4**).

3.3 Test of qPCR Oligonucleotide Primers by PCR

1. Perform a regular 20 µL PCR reaction (40 cycles) with qPCR 5′ and 3′ oligonucleotide primers and use 1 µL of purified mass standard DNA (10^7 molecules/10 µL dilution) as template.
2. Check 15 µL of the PCR reaction by TAE DNA agarose gel electrophoresis (using a 2.5 % gel) for the presence of a PCR fragment of the expected size. In case of absent PCR product or presence of unexpected PCR fragments, try to optimize PCR parameters (Mg and primer concentration, annealing temperature). If this fails, you will need to design new primers.
3. Test PCR efficiency of the qPCR primers for amplification of the mass standard series using qPCR. This step can be combined with and is described in Subheadings 3.6 and 3.7.

3.4 Generation of Plant Material

1. Grow plants to obtain three independent biological replicates of all sample conditions (*see* **Note 5**).
2. Harvest plant material, shock-freeze with liquid nitrogen, and if needed store at −80 °C (*see* **Note 6**).

3.5 RNA Preparation and Reverse Transcription

A critical point in RNA preparation and reverse transcription is to avoid damage by active RNases (*see* **Note 7**). For all steps of reverse transcription, prepare master mixes to achieve similar reaction conditions in all samples (*see* **Note 8**).

1. Grind the frozen material to fine powder (*see* **Note 9**).
2. Use a maximum of 100 mg ground plant material and isolate total RNA according to the supplier's protocol. This procedure usually results in 50 µL purified RNA.
3. Determine RNA concentration (*see* **Note 10**).
4. Label individual 200 µL PCR tubes (*see* **Note 11**).
5. In each 200 µL PCR tube, combine 500–1000 ng RNA and RNase- and DNase-free PCR grade water in a maximum volume of 7 µL to obtain similar RNA amounts in all samples. Include a negative cDNA control sample devoid of RNA.
6. For DNase I treatment, prepare a DNase I reaction master mix containing per reaction a total of 3 µL (0.5 µL DNase-free water, 1.0 µL DNase I buffer, 1 µL of 1 u/µL DNase I, 0.5 µL of 40 u/µL Ribolock). Add 3 µL of this DNase I reaction mix to the 7 µL RNA and incubate at 37 °C for 30 min (total reaction volume 10 µL).
7. For denaturing and oligo dT primer annealing, prepare an oligo dT primer annealing master mix containing per reaction 1 µL of 100 µM oligo dT_{18} primer and 1 µL of 25 mM EDTA. Add 2 µL of this oligo dT primer annealing mix to the reactions from **step 6** and incubate for 10 min at 65 °C, then at 4 °C until further use (total reaction volume 12 µL).

8. For cDNA synthesis prepare a cDNA synthesis master mix by combining per reaction 1.0 µL DNase-free water, 4.0 µL reverse transcription buffer, 2 µL of 10 mM each dNTP, 0.5 µL of 40 u/µL Ribolock, and 0.5 µL of 200 u/µL M-MuLV RTase, as according to the suppliers. Add 8 µL of this cDNA synthesis mix to the reactions from **step 7** and incubate for 2 h at 42 °C, followed by 10 min at 70 °C (total reaction volume 20 µL).
9. Add 180 µL of RNase- and DNase-free PCR grade water to each cDNA sample and mix well. Store these cDNA stocks long term at −20 °C.
10. For qPCR, dilute each cDNA stock 1:10, e.g., by combining in a 1.5 mL reaction tube 350 µL RNase- and DNase-free PCR grade water, 10 µL yellow color indicator (from qPCR kit), and 40 µL cDNA, mix well. Dispense 50 µL of the cDNA dilutions into 8mer PCR strips and store at −20 °C (*see* **Note 12**).

3.6 qPCR Setup and Run

Prepare a master mix for qPCR and simultaneously perform the qPCR reactions for the cDNA samples, the cDNA negative control, a qPCR negative control, and the mass standards in technical replicates (*see* **Note 13**).

1. Plan and program the plate setup and qPCR conditions using the qPCR software of the available real-time PCR cycler (see example in Fig. 3a, b).
2. Prepare the qPCR master mix by combining per reaction 9.6 µL qPCR commercial 2× mix (containing SYBR Green and blue color indicator; mix thoroughly before use), 0.2 µL of each 15 µM 5′ and 3′oligonucleotide primer, mix well.
3. Dispense 10 µL of the qPCR master mix into the bottom of each well of the PCR plate (*see* **Note 14**). Check for even loading of the wells.
4. Transfer 10 µL of the samples (diluted cDNA, cDNA control, and mass standard dilution series) from the 8mer PCR strips into the plate using a 10 µL multichannel pipet (*see* **Note 15**). Check even loading and color indicator change to green.
5. Tap the plates gently at the bench, seal with optical tape by slowly placing the tape from the middle to the edges of the plate, fix with sealing tool, centrifuge the plate in a plate centrifuge. Ready PCR plates can be stored at 4 °C in the dark for several hours.
6. Place the plate into the qPCR machine and start the run as programmed. Save the optical data in a file.

Fig. 3 (continued) using a 10 µL multichannel pipet. (**b**) PCR program; step 1 is the initial denaturation (3:00 min, 95 °C); steps 2–4 comprise denaturing (0:10 min; 95 °C), annealing (0:15 min; 60 °C), and elongation (0:20 min; 72 °C) and are repeated 39 times with fluorescence data acquisition after each cycle indicated by a *camera sign*; step 6 serves the melt curve data collection (0:05 min; 65–95 °C with gradual increase of 0.5 °C) with regular data acquisition at each increment. The image was produced with the CFX Manager software (Bio-Rad)

a

qPCR master mix / 32 reactions / gene

9.6 µL*32 =	308	µL 2X qPCR commercial mix
0.2 µL*32 =	6.4	µL qPCR 5’ primer, 15 µM
0.2 µL*32 =	6.4	µL qPCR 3’ primer, 15 µM

1

V= 80 µL

2

	1	2	3	4	5	6	7	8	3
A	C1	C1	C1	C1	10^7	10^7	10^7	10^7	10^7
B	C2	C2	C2	C2	10^6	10^6	10^6	10^6	10^6
C	C3	C3	C3	C3	10^5	10^5	10^5	10^5	10^5
D	T1	T1	T1	T1	10^4	10^4	10^4	10^4	10^4
E	T2	T2	T2	T2	10^3	10^3	10^3	10^3	10^3
F	T3	T3	T3	T3	10^2	10^2	10^2	10^2	10^2
G	H_2O	H_2O	H_2O	H_2O	NC	NC	NC	NC	NC
	GOI		*REF*		*GOI*		*REF*		

V= 50 µL
diluted cDNA

V= 50 µL
mass standards

b

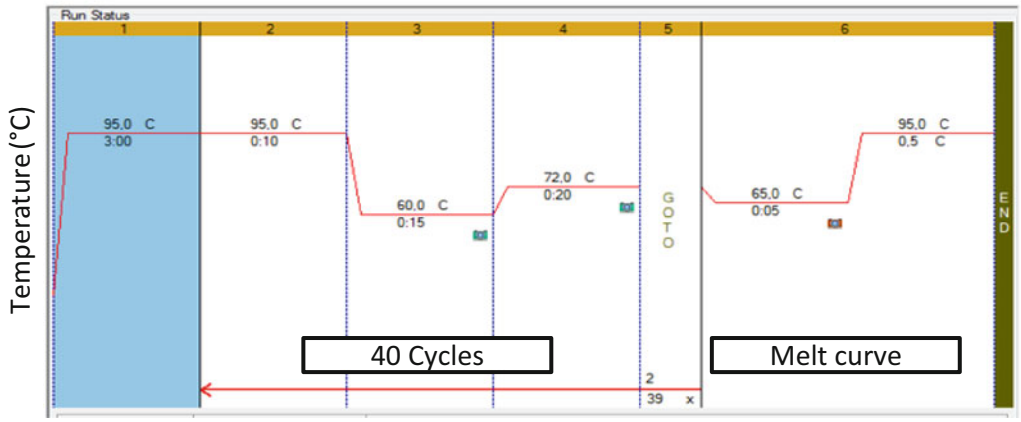

Fig. 3 Setup of qPCR. (**a**) Plate setup and pipetting scheme. The plate setup shows the arrangement of samples in a qPCR microplate, with three biological replicates of the control (C1–C3) and treated samples (T1–T3), the negative cDNA control (H_2O), the mass standards 10^7–10^2, and a qPCR negative control (=NC). Two different qPCR reactions are to be performed (*GOI*, gene of interest, *REF*, reference gene). All reactions are to be conducted in technical duplicates. The preparation of a qPCR master mix is provided as an example. 80 µL of the qPCR master mixes is transferred to the wells of an 8mer strip, with *GOI* qPCR master mix in positions 1, 2, 5, 6, and *REF* qPCR master mix in the positions 3, 4, 7, 8. The *circled numbers* indicate the order of pipetting steps to transfer 10 µL of the qPCR master mixes, the diluted cDNA samples, and the mass standards from 8mer strips into the plate

3.7 qPCR Data Analysis and Presentation of Gene Expression Data

1. Check qPCR data thoroughly by melt curve analysis (*see* **Note 16**; Fig. 4c, d), check for reliability of Cq values and technical replicate amplification (*see* **Note 17**; Fig. 4e), check PCR efficiency and mass standard curve (*see* **Note 18**; Fig. 1a, b).
2. Export raw data into Excel. Name samples appropriately and remove unnecessary columns and rows to keep only the means of the technical replicate initial template numbers for the biological samples as the raw data (Fig. 5).
3. Perform the analysis steps by following the outline in Fig. 5. Briefly, proceed with subtraction of negative cDNA water control values (*see* **Note 19**). Calculate normalization factors based on the reference gene amplification data (*see* **Note 20**). Normalize gene of interest data using the normalization factors. Calculate average and standard deviation based on the biological replicate data. Subject the data to statistical analysis (*see* **Note 21**). Represent the final results in a bar diagram (*see* **Note 22**).

4 Notes

1. UV light is dangerous. Wear eye and skin protection.
2. The choice of reference genes is critical for data interpretation. Generalization about the number of reference genes needed for analysis is difficult. It is important to search for a reference gene whose expression does not vary across the experimental conditions that are studied. The probability of finding such a reference gene is highest when only one well-defined plant part or tissue is under investigation, e.g., roots. However, if there is high variation across the biological samples, e.g., when comparing expression in different parts of the plants or when comparing physiological situations that severely disturb development, the use of only one reference gene may not be reliable and there is a need for additional reference genes. In this case, the expression data of multiple reference genes are usually averaged for calculation of normalization factors. The selection of reference genes requires prior experiments or the mining of transcriptomic data. Typical reference genes are those encoding general cellular functions, like tubulin, ubiquitin, and elongation factor. In Fig. 4a, b we present qPCR amplification plots for two reference genes across biological samples (treatment and control). It can be seen that reference gene #2 shows a lower degree of variability than reference gene #1 and should therefore be preferred (Fig. 4a, b; *see* later **Note 20**).
3. Mass standard templates can be cDNA, recombinant plasmid, or genomic DNA.

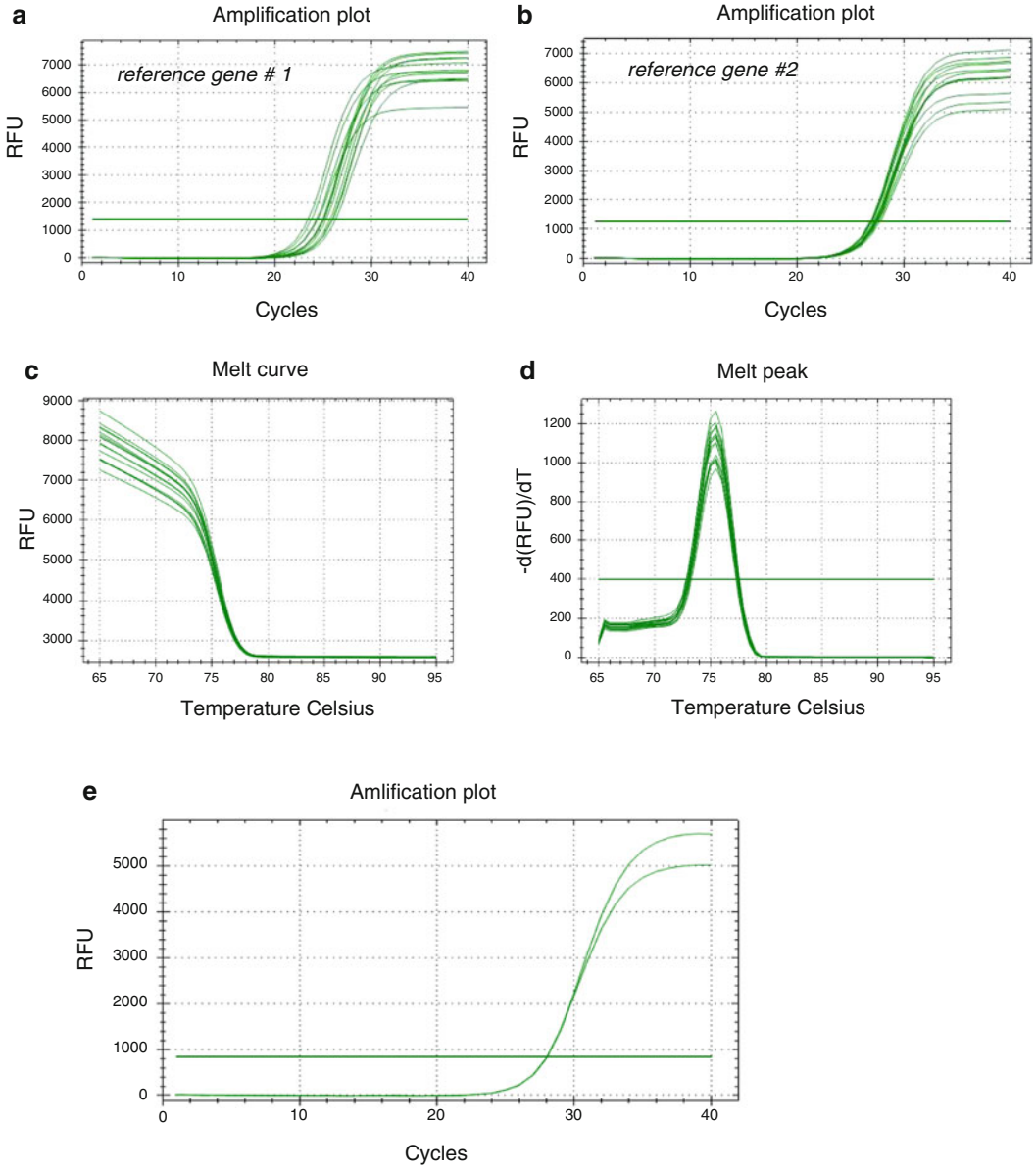

Fig. 4 qPCR data control. (**a**, **b**) Amplification plots for reference genes #1 and #2 across multiple biological samples. Reference gene #2 would be selected due to low variation in the Cq values. (**c**) Melt curve analysis. The decrease of RFU with increasing temperature is caused by melting of double-stranded DNA and loss of SYBR Green fluorescence. (**d**) Melt peak analysis. A single peak indicates melting in a narrow temperature range indicating that a single DNA fragment was present in the samples. (**e**) Amplification plot of two technical replicates; due to $\Delta Cq < 0.3$ an average Cq value is used for data analysis. A description of the amplification plots is provided in Fig. 1. The images were produced with the CFX Manager software (Bio-Rad)

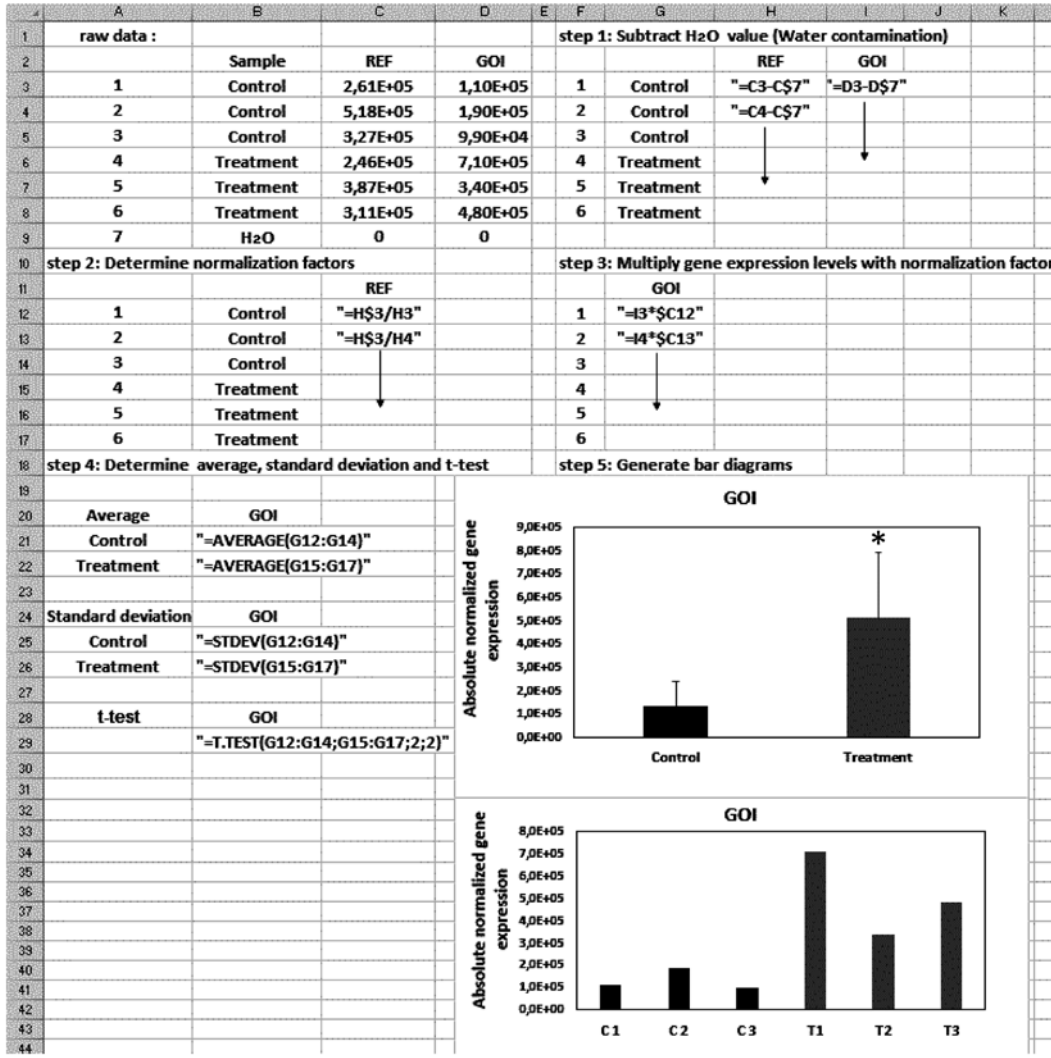

Fig. 5 Data interpretation using Excel. An Excel data sheet with calculation tools is shown. The raw data correspond to the initial starting quantities in the 10 µL cDNA samples (control and treatment, three biological replicates) and the negative cDNA control (H_2O), and are the average of two technical replicates for the gene of interest (GOI) and the reference gene (REF). The data are processed and normalized as indicated inside the figure. Mean and standard deviation values are calculated, and a *t*-test (unpaired, two-tailed) is performed. The final data are displayed in the form of bar diagrams reflecting the absolute normalized gene expression of GOI in the two experimental conditions control and treatment, representing either the average values, standard deviation and * indicating significant difference with $p < 0.05$ or the individual values

4. A volume of 50 µL is sufficient to set up four 10 µL qPCR reactions (Fig. 3a). For convenience prepare up to 15 mass standard strips per gene and store the unused ones at −20 °C. Discard unused solutions after thawing. Specifically colored PCR strips are useful to easily distinguish the mass standards corresponding to different genes.

5. The plant material itself accounts to a large extent for the variability in gene expression analyses. At least three independent biological replicates are required for publication and for statistical treatment of gene expression data. Try to minimize variation in the source of genetic material, growth conditions, harvesting time, and age of plants and if possible pool material from 10 or more plants in a single biological replicate to achieve low levels of standard deviations among the three replicates. Repeat the entire experiment including biological replicates at least two or three times.
6. Note that storage at −80 °C may affect the stability of some RNA species.
7. Some types of RNases are very stable, omnipresent on hands and working materials, and released upon cell disruption. RNase contamination can occur easily in laboratories where bacterial plasmid and genomic DNA preparations are conducted since some of the preparation solutions may contain added RNase. RNA extraction buffers may contain toxic substances like phenol, guanidinium thiocyanate (GTC), β-mercaptoethanol, and/or other effective denaturing and reducing agents to block RNase activity (wear gloves). In some commercial RNA extraction kits, the buffer composition is not described (e.g., peqGold kit). Generally, RNA is safeguarded in the deep-frozen state (−80 °C or lower) and inside RNA extraction buffer. For subsequent steps of RNA purification, RNA elution and reverse transcription, change gloves, use filter tips, clean the work surface area and materials (better reserve special equipment for RNA handling). Open and close the tubes at the very tip of the cap with two fingers without contaminating with RNase from the thumb.
8. When preparing master mixes, take into account volume loss after multiple pipetting steps, e.g., prepare a master mix of 11 reactions for ten reactions.
9. A simple way to grind plant material in liquid nitrogen is to use either a mortar and pestle or an automated homogenizer stick fitting into a plastic reaction tube. Working with liquid nitrogen is dangerous and protection of hands, body, face, and eyes is needed. Closing of tubes should be avoided while working with liquid nitrogen since evaporation of liquid nitrogen may cause explosion of tubes at room temperature. Plant material should never be allowed to thaw in the absence of RNA extraction buffer. If available, use the Precellys homogenizer machine (or equivalent) as it allows safe and more reproducible homogenization results. In this case, add 450 µL RNA extraction buffer and ceramic Precellys beads (for Arabidopsis roots and leaves use 50 beads of 1.4 mm; testing of appropriate number and mix of, e.g., 1.4 and 2.8 mm beads may be required for

other plant species) to the 100 mg frozen intact tissue inside 2 mL Precellys tubes. Proceed immediately for cell disruption using the Precellys homogenizer (3 × 60 s, interrupted by 30 s breaks).

10. For unexperienced operators it is advisable to check the quality of the RNA by running 5 μL in a TAE gel electrophoresis (1 % gel). Use only thoroughly cleaned electrophoresis materials and TAE buffer reserved for RNA electrophoresis purposes. Discrete bands for ribosomal and Rubisco RNA species indicate a high quality of RNA. If available use the Agilent Bioanalyzer or equivalent device to determine RNA quality and quantity. Note that different methods for determining nucleic acid concentrations have inherent sources of mistakes. It is therefore important for the subsequent steps of RT-qPCR that the concentrations of all biological RNA samples are determined in parallel with the same technique to ensure a low level of variation in the RNA input.
11. 8mer PCR strips are problematic for this step since vortexing is not possible and risk of cross-contamination is high. The cDNA samples can be kept long term. Therefore, label reaction tubes with a tape stating "date," "cDNA sample name," and "number" and prepare for long-term storage a sealed plastic bag with a print-out of the experimental description, date and detailed description of the samples.
12. Dilution of the cDNA stocks is necessary for qPCR since it is more precise to dispense 10 μL rather than 1 μL. A volume of 50 μL of diluted cDNA sample is sufficient for four qPCR reactions, namely two technical replicates × two genes (Fig. 3a). For convenience prepare up to eight cDNA strips from the 400 μL diluted cDNA samples and store the unused ones at −20 °C until needed. Stored strips should be allowed to thaw only once and then be discarded. Long-term storage of diluted cDNA samples beyond 6 months is not recommended. After the 6 months, prepare new diluted cDNA from the cDNA stocks. Specifically colored strips are convenient for handling in order to distinguish different cDNA samples (e.g., samples 1–8 in yellow, samples 9–16 in blue).
13. In case that the total number of samples exceeds the well number in a 96-well plate, include interplate calibration standards (e.g., commercially available through TATAA Biocenter) to account for plate to plate qPCR variations and allow to integrate the results of multiple plates. At least two reliable technical replicates are required. Technical replicate variation is most frequently a consequence of inappropriate handling and imprecise pipetting. To ensure best repeatability of qPCR, use only high-quality 10 or 20 μL pipets and 10 μL multichannel pipets and perform an initial training of pipet handling. Use thin pipetting tips for small volumes and carefully check upon every pipetting

step the expected volume in the pipet tip. Check the expected volume also inside the PCR plate after transferring the solutions. Use only 2× qPCR reaction mixes since transferring 10 µL introduces fewer errors than transferring smaller volumes.

14. Dispense the qPCR master mix either with a 10 or 20 µL pipet sequentially into each well or transfer a higher volume into an 8mer PCR strip for dispensing with a 10 µL multichannel pipet (shown in Fig. 3a).
15. Hold the pipet in an angle to dispense the template solution onto the top side of the wells, without touching the qPCR mix. This procedure allows using the same tips to load multiple wells or plates with the same templates.
16. The melt curve peak provides hints to the integrity and length of the PCR product. A single peak indicates melting of the PCR products at one specific temperature, indicating that there is a single PCR product present. In case of multiple peaks the PCR conditions and primer design have to be improved prior to qPCR and any meaningful analysis.
17. Assess technical replicate amplification for all samples one by one by judging the ΔCq values. For low Cq values (<20) a ΔCq deviation up to 0.3 is acceptable. Higher Cq values especially those above 25 may show per se a higher deviation between the technical replicates and a ΔCq of 0.5 is acceptable and for Cq values above 30, ΔCq 0.5–1.0 is acceptable. Samples with inappropriate amplification and high technical replicate deviations need to be removed from the analysis and the entire qPCR experiment repeated. Consistently large variations in ΔCq values between technical replicates will not allow to reach valid conclusions, qPCR has to be optimized, and precision of pipetting improved (*see* **Note 13** for recommendations). In case that all gene expression data can only be based on high Cq values >30, a meaningful conclusion is drastically compromised. In this case, reconsider and improve RNA purification and test alternative kits for cDNA synthesis and qPCR.
18. PCR efficiency is usually calculated with the mass standard curve. The mass standard curve shows an inverse linear correlation between Cq value and template input (Fig. 1b). This is usually the case during the linear dynamic range and when PCR efficiency is close to 1 (100 %). A 100 % PCR efficiency indicates full doubling of PCR fragments at each cycle. In case of a poor PCR efficiency below 90 %, check different conditions for amplification with oligonucleotide primers. Most primer pairs function optimally in equimolar ratios of 150 nM in a qPCR. In case of low PCR efficiency, perform a primer matrix to find more optimal primer concentrations and ratios [5] and test a temperature gradient for optimal annealing temperatures. Lack of success denotes the need to design new primers.

19. In case of a contamination that prevents proper interpretation of qPCR results, repeat qPCR and/or cDNA synthesis. In our experience, thorough working does not lead to contamination of the negative cDNA controls.
20. In the example (Fig. 4a, b), a low level of variation of expression was found for reference gene #2 resulting in low variation of the Cq values, in contrast to reference gene #1. Reference gene #2 is thus the preferred reference gene for qPCR data analysis.
21. In the presented outline (Fig. 5), we performed *t*-test (unpaired, two-tailed) for statistical analysis. Due to the low number of biological replicates, it is also transparent and acceptable to present the data for individual biological replicates instead. In case of many different biological samples and multiple genes to be studied, it is convenient to use an ANOVA-based statistical tool, e.g. [6]. Further considerations about statistical analysis are discussed at http://www.nature.com/collections/qghhqm.
22. We suggest representing the data as absolute normalized gene expression rather than normalized initial transcript amounts. Application of the standard curve method for deducing initial template amounts in the cDNA samples is based on the assumption of accurate reverse transcription and qPCR. This accuracy can be determined during the RT-qPCR procedure by adding internal RNA and DNA spikes to the samples, commercially available through TATAA Biocenter or equivalent.

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