Chapter 20

Gene expression analysis by quantitative real-time PCR for floral tissues

Raquel Álvarez-Urdiola [1], Mariana Bustamante[1,*], Joana Ribes[1], and José Luis Riechmann[1,2,3]

[1] Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Edifici CRAG, Campus UAB, 08193 Cerdanyola del Vallès, Barcelona, Spain.

[2] Institució Catalana de Recerca i Estudis Avançats (ICREA), Pg. Lluís Companys 23, 08010 Barcelona, Spain.

[3] Corresponding author: joseluis.riechmann@cragenomica.es

[*] Present address: Roche Pharma Research and Early Development, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd., Basel, Switzerland

i. Running Head: qRT-PCR for floral tissues

ii. Summary

Real-time, or quantitative, reverse transcription polymerase chain reaction (qRT-PCR), is a powerful method for rapid and reliable quantification of mRNA abundance. Although it has not featured prominently in flower development research in the past, the availability of novel techniques for the synchronized induction of flower development, or for the isolation of cell-specific mRNA populations, suggests that detailed quantitative analyses of gene expression over time and in specific tissues and cell types by qRT-PCR will become more widely used. In this chapter, we discuss specific considerations for studying gene expression by using qRT-PCR, such as the identification of suitable reference genes for the experimental set-up used. In addition, we provide protocols for performing qRT-PCR experiments in a multi-well plate format (with the LightCycler® 480 system, Roche) and with nanofluidic arrays (BioMark™ system, Fluidigm), which allow the automatic combination of sets of samples with sets of assays, and significantly reduce reaction volume and the number of liquid-handling steps performed during the experiment.

iii. Key Words

Real-time PCR, qRT-PCR, quantitative PCR (qPCR), SYBR Green I dye

1. Introduction

Differential gene expression, over time or among different cell and tissue types, is central to the developmental processes of all organisms. In flower development studies, this aspect of gene function has usually been approached by using methods to characterize spatial patterns or domains of gene expression, such as *in situ* hybridization and promoter-reporter gene fusions. Several groups have also progressed in the characterization of flower development in different plant species using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analyses ***(1–6)***, although this technique has not traditionally featured prominently in flower development research. Nevertheless, as a result of the development of techniques for the synchronized induction of flower development and for the isolation of cell-specific mRNA populations, detailed quantitative analyses of gene expression over time and in specific tissues and cells are becoming more broadly used. QRT-PCR is a powerful method for rapid and reliable quantification of mRNA abundance, which involves three processes: i) the conversion of mRNA into cDNA via reverse-transcription; ii) the amplification of the resulting cDNA by PCR; and iii) the detection and quantification in real time of the synthesized PCR amplification products ***(7–9)***. The reliability of the data obtained in qRT-PCR experiments can be affected by several factors that impact on those processes, including template quality (RNA integrity ***(9,10)***), purity ***(9,11)*** and quantity, efficiency of the RT reaction, PCR primer design and efficiency of the PCR amplification ***(9)***. To compensate for between-sample variations in the amount of starting material and in the efficiency of the qRT-PCR process, expression levels of the genes of interest are reported relative to one or more reference genes that are presumed to be uniformly and stably expressed across the tissues or conditions tested in the experiment, and whose abundance reflects the total amount of mRNA present in each sample. Thus, the reliability of qRT-PCR analyses is largely affected by the suitability of the gene (or genes) that is selected as a

reference, *i.e.*, by whether or not such a gene really fulfils the requirements of a normalization control ***(12,13)***.

Housekeeping genes, which function in basic cellular processes and are expressed in all cells of an organism, have often been used as reference genes to normalize the data in qRT-PCR experiments (*e.g.*, genes such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor-1α (*EF-1α*), actin (*ACT*), or tubulin (*TUB*)). Although the initial evidence indicating that housekeeping genes are stably expressed was obtained using methods that are mostly qualitative (for instance, RNA gel-blots and end-point RT-PCR), subsequent studies demonstrated that in some circumstances their expression may be regulated or be unstable, showing changes in transcript levels throughout development or among different conditions or tissues. Besides, housekeeping genes are usually expressed at higher levels than the typical genes of interest. For these reasons, using them as reference genes may introduce biases in the results obtained by qRT-PCR ***(12,13)***. For example, in a series of experiments designed to assess traditional *Arabidopsis* reference genes (including *ACT2, ACT7, ACT8, ADENINE PHOSPHORIBOSYLTRANSFERASE 1* (*APT1*)*, EF1α, EUKARYOTIC TRANSLATION INITIATION FACTOR 4A1* (*eIF4A), TUB2, TUB6, TUB9, UBIQUITIN 4* (*UBQ4), UBQ5, UBQ10,* and *UBQ11*), it was found that *eIF4A* would appear to be stably expressed over the course of silique development when *APT1, UBQ5,* or *eF1α* were used to normalize the data, whereas its expression would appear quite variable when *TUB6* was used as reference gene ***(13)***.

In summary, the validity of "housekeeping" reference genes is not universal, and is highly dependent on the experimental conditions ***(12)***. Thus, the selection of appropriate reference genes for the normalization of qRT-PCR data has emerged as a crucial component for successful expression studies carried out with this technology, and statistical algorithms like *geNorm* **(14)** or *BestKeeper* ***(15)*** have been developed for that purpose (*see* **Note 1**).

Concomitantly, the use of genome-wide technologies (*i.e.*, initially DNA microarrays and subsequently RNA-Seq) to characterize gene expression changes across many different tissues and developmental stages, environmental conditions, or in response to biotic and abiotic stresses or perturbations, has resulted in very rich datasets (e.g.,, ***(16)***) that can be mined to identify novel, better suited reference genes for the desired experimental set-up. For instance, Czechowski *et al*. analysed a very large set of Arabidopsis data obtained with Affymetrix ATH1 GeneChip arrays to identify several hundred genes that outperform traditional reference genes in terms of expression stability throughout development and under a range of environmental conditions ***(17)***. Subsequent qRT-PCR experiments performed with a subset of those novel reference genes confirmed that they showed superior expression stability and lower absolute expression levels ***(17)*** (*see* **Note 2**). The results obtained in *Arabidopsis* have informed the selection of reference genes in other plant species, as the corresponding orthologous genes may also show stable expression (for an example in Leafy spurge, *see* ***(18)***). If candidate reference genes are selected based on orthology, however, their suitability needs to be confirmed experimentally, as such character is not always maintained across all experimental conditions in all organisms ***(9)*** (for instance, *see* (***19)***).

Candidate reference gene selections for various species, such as maize ***(20–23)***, rice ***(24–27)***, wheat ***(28–30)*** or strawberry ***(31,32)*** and for specific conditions, tissues, or developmental stages (e.g., rice anther development, wheat meiosis, or strawberry fruits) have also been published. In addition, a literature review by Joseph *et al.* compiled a collection of reference genes for Arabidopsis and other plant species ***(33)*** (*see* **Table 1**).

The approach of using genome-wide data to select reference genes has been further expanded and refined with *RefGenes*, an on-line tool that allows easy identification of condition-specific reference genes ***(34)***. *RefGenes* is based on the Genevestigator database of normalized and well-annotated microarray and RNA-Seq experiments and is accessible

through the Genevestigator web page (www.genevestigator.com). The appropriateness of using condition-specific reference genes is based on the observation that for each biological context a subset of stable genes exists that has a smaller variance than either commonly used reference genes or genes that were selected for their stability across all conditions ***(34)***. In other words, there is no gene that is universally stable, and the most appropriate set of reference genes for each biological context and specific experimental condition does vary. Through *RefGenes*, users can select the transcriptomic experiments that are most similar to their chosen experimental conditions (including tissue, developmental stage, treatment, etc.). Afterwards, the user indicates the set of target genes of interest (up to ten genes can be tested at once). A search is then triggered to identify those genes that have the lowest variance within the selected set of experiments and a range of expression that is similar to that of the target gene set. The result of the search is graphically displayed, showing the top 20-25 best candidate reference genes for the selected conditions. The behaviour of these candidate genes in the chosen (or in additional) tissues or experimental conditions can then be explored using the *Conditions* tool of Genevestigator ***(35)***.

It is worth noting that the novel candidate reference genes that are identified using *RefGenes* and the aforementioned algorithms (*geNorm* or *Bestkeeper*) should be validated for the specific biological conditions of the experiments to be performed, e.g., tissue type ***(36)***, growth conditions ***(24,37)***, stresses ***(22,38)***, treatments ***(39)***, etc. The evaluation of reference genes should be done by comparing the results with those obtained for other algorithms, experimentally, and preferably together with commonly used reference genes.

The use of *RefGenes* to select reference genes for flower development studies is illustrated in **Figures 1**,**2** and **3,** and in **Table 2**. Ten genes that participate in and/or are expressed at early stages of Arabidopsis flower development were used as target set to search for reference genes using the genome-wide expression profiling data available in Genevestigator

(*SUPERMAN -SUP*, At3g23130-, *LEAFY -LFY*, At5g61850-, *AGL24* -At4g24540-, *YABBY3 -YAB3*, At4g00180-, *APETALA2 -AP2*, AT4g36920-, *AGL42* -At5g62165-, *SHATTERPROOF2 -SHP2*, At2g42830-, *AGAMOUS -AG*, AT4g18960-, *SEPALLATA3 -SEP3*, At1g24260-, and *APETALA3 -AP3*, At3g54340-, *see* ***(40)***). *RefGenes* returns a list of candidate novel reference genes (**Figure 1**, **Table 2**), which in this chapter are then compared to traditional reference genes (list of genes from ***(17)***) and to reference genes for developmental processes (genes from ***(33)*** included in **Table 1**). The novel reference genes and the reference genes specifically selected for studying plant development are more stably expressed throughout all plant stages of development, and their mean expression level is generally lower than that of traditional reference genes, and thus closer to that of the typical genes of interest (*see* **Figure 2**). Besides, novel reference genes selected for flower development studies are more stably expressed in floral tissues than traditional reference genes and the reference genes selected for studying other developmental processes (**Figure 3**).

The detection of product formation in real-time during the amplification reaction of qRT-PCR experiments is carried out by measuring the emission signal from either fluorescent double-stranded DNA-binding dyes (such as SYBR® Green I and EvaGreen®, see below), or template-specific fluorescent probes (such as the TaqMan® probe technology). A general protocol for using SYBR Green I dye in a qRT-PCR experiment performed in a LightCycler® 480 Real-Time PCR system (Roche) is provided in this chapter (equally suited real-time PCR machines are available from various manufacturers). In addition to standard real-time PCR systems, in which reactions are performed either in thin-wall PCR tubes or in multi-well plates, newer systems based on nanofluidc arrays (such as the BioMark™ system, Fluidigm) have been developed for high-throughput analyses. These arrays contain nanofluidic networks that allow the automatic combination of sets of samples with sets of

assays, significantly reducing reaction volume (and thus the amount of material needed to perform an assay) and the number of liquid-handling steps performed during the experiment. A protocol for a qRT-PCR experiment using EvaGreen® and the BioMark™ system is also provided.

2. Materials

2.1. Tissue Collection and RNA Extraction

1. RNase-free microcentrifuge tubes (1.5 mL)
2. Plastic pellet pestles for 1.5 mL microcentrifuge tubes (optional: a mixer motor or an electric drill)
3. Forceps (*e.g.,* Dupont size #5)
4. Liquid nitrogen
5. Vortex
6. Microcentrifuge
7. Spectrum Plant Total RNA Kit (Sigma-Aldrich) or an equivalent total RNA isolation kit or reagents (*see* **Note 3**)
8. Spectrophotometer (such as a Nanodrop)
9. Agilent Bioanalyzer and associated reagents (Agilent RNA 6000 Nano kit)

2.2. Reverse Transcription Reaction

1. High-Capacity cDNA Reverse Transcription Kit (*e.g.,* Applied Biosystems; other commercial kits are available, but the protocols provided below are based on this kit) containing dNTPs (100 mM), MultiScribe reverse transcriptase (50 U/mL), reverse transcription Random Primers, reverse transcription buffer (10×), RNase inhibitor (20 U/mL).
2. RNase-free PCR-tubes

3. Nuclease-free water.

2.3. Quantitative Real Time PCR – LightCycler® 480 System

1. LightCycler® 480 SYBR Green I Master (Roche Diagnostics; other commercial kits are available, but the protocols provided below are based on this kit): ready-to-use hot-start PCR mix containing FastStart Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP, instead of dTTP), SYBR Green I dye, and $MgCl_2$.
2. LC 480 Multiwell Plate 96 (Roche Diagnostics) (*see* **Note 4**)
3. Forward and reverse PCR primers at 100 μM each.
4. Nuclease-free water.

2.4. Quantitative Real Time PCR – BioMark™ System

1. TaqMan PreAmp Master Mix 2X (Applied Biosystems).
2. SsoFast EvaGreen SuperMix with Low ROX (Biorad): 2x real-time PCR mix, containing dNTPs, Sso7d fusion polymerase, $MgCl_2$, ROX passive reference dye and stabilizers.
3. 2X Assay Loading Reagent (Fluidigm)
4. 20X DNA Binding Dye Sample Loading Reagent (Fluidigm)
5. Exonuclease I *(E. coli)* (20,000 U/mL; New England Biolabs)
6. Exonuclease I Reaction Buffer 10X (New England Biolabs).
7. Forward and reverse PCR primers at 100 μM each.
8. Nuclease-free water.
9. TE Buffer: 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA (TEKnova)
10. DNA Suspension Buffer; 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA (TEKnova)
11. 48.48 Dynamic Array IFC (Fluidigm)

3. Methods

The performance of the primers for a qRT-PCR experiment is crucial for obtaining high-quality results, and several aspects must be considered for successful primer design (*see* **Note 5**). There are many on-line resources for primer design, some of which also provide access to a consultative design service, such as:

- Oligoarchitect: http://www.oligoarchitect.com/LoginServlet
- RealTimeDesign: https://www.biosearchtech.com/support/tools/design-software/realtimedesign-software
- QuantPrime: http://www.quantprime.de/
- IDT-qPCR: http://eu.idtdna.com/scitools/Applications/RealTimePCR/
- Primer3: http://primer3.sourceforge.net/
- Primer-BLAST: http://www.ncbi.nlm.nih.gov/tools/primer-blast/

3.1. Tissue Collection and RNA Extraction

RNA quality (integrity and purity) is a critical factor for qRT-PCR experiments.

1. Harvest at least 100 mg of the desired plant tissue (*e.g.,* inflorescences), into a 1.5 mL RNase-free microcentrifuge tube containing liquid nitrogen.

2. Grind the tissue to a fine powder with the pellet pestles (and a mixer motor), keeping the bottom of the tube immersed in liquid nitrogen throughout the grinding process to avoid RNA degradation (*see* **Note 6** and **Note 7**).

3. Follow the manufacturer's instructions for the RNA extraction kit.

4. Analyse the integrity of the isolated RNA using a Bioanalyzer (or by using the 3'/5' integrity assay, *see* ***(9)***) and determine the concentration by absorption at 260 nm (*e.g.,* with a Nanodrop spectrophotometer).

3.2. Reverse Transcription Reaction

The reverse transcription reaction to synthesize cDNA from the starting RNA material can be performed with various priming strategies, enzymes, and experimental conditions ***(8,9)***. However, to compare gene expression data across different experiments or laboratories, these variables should be kept constant, particularly ensuring that the same amount of RNA is added to each reaction (or that the enzyme/protocol used results in a proportional cDNA yield).

1. Prepare an RT master mix in a 1.5 mL tube:

Component	Volume (per reaction)
Water	4.2 µL
10x RT Buffer (1x)	2 µL
25x dNTP Mix (100 mM)	0.8 µL
10x RT Random Primers	2 µL
MultiScribe Reverse Transcriptase	1 µL

2. Add 10 µL of Master Mix to each individual PCR-tube. Then add 100-1000 ng of each RNA sample, in a volume of 10 µL. The final reaction volume is 20 µL. No-RT control reaction(s) should be included in the experiment.
3. Briefly centrifuge the tubes to collect the contents and to eliminate any air bubbles.

4. Place the tubes in a thermal cycler using the following conditions:

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25ºC	37ºC	85ºC	4ºC
Time	10 min	120 min	5 min	∞

5. Store cDNA samples at 4ºC (short term) or at -20ºC (for up to 6 months).

3.3. *Quantitative Real Time PCR - LightCycler® 480 System*

1. Set up the samples:

1.1. Every gene/primer-pair combination used in a qPCR should be tested to calculate primer efficiency (*see* **Note 8**).

1.2. The cDNA samples resulting from the RT reaction can be diluted in water, to obtain a final estimated concentration between 5 and 10 ng/µL (estimation based on the initial amount of RNA used in the RT reaction). This concentration range is ideal for the qRT-PCR. All amplification reactions should have a similar concentration of cDNA.

2. Before loading the PCR plate, and in order to minimize pipetting errors, it is important to prepare master mixes for each primer pair used. The accuracy of qPCR is highly dependent on accurate pipetting and thorough mixing of solutions. The protocol provided here uses SYBR® Green I chemistry, but other PCR-product detection chemistries could be used (*see* **Note 9**). To prepare the qPCR Master Mix, add components in the following order:

Component	**Volume (per reaction) for 96-well plate**

LC480 SYBR® Green I Master (2x) (Roche Diagnostics)	10 µL
Water	6.4 µL
Primer Forward (10 µM)	0.8 µL
Primer Reverse (10 µM)	0.8 µL

3. Loading the plate: Once all master mixes for each pair of primers are prepared, start loading the plate by adding first the Master Mix (18 µL) and then the cDNA samples (2 µL). Avoid producing bubbles. The final reaction volume in each well is 20 µL. Then add the No Template Control (NTC) and no-Reverse Transcription control (no-RT, or RT-) reactions (*see* **Note 10**). Seal the plate with LightCycler® 480 Sealing Foil by pressing it firmly to the plate surface, using your hand or a scraper. Sealing the plate properly is crucial to eliminate evaporation at high temperatures.

4. Place the multiwell plate in a standard swing-bucket centrifuge equipped with a rotor for multi-well plates with suitable adaptors. Balance it with a suitable counterweight (*e.g.*, another multi-well plate). Centrifuge the plate at 1,500 × *g* for 2 min.

5. Load the multi-well plate into the LightCycler® 480 Instrument and set-up the qPCR program (annealing temperature in the PCR is primer-dependent):

	Temperature	**Time**	**Acquisition**
Activation	95ºC	10 min	None
PCR (45 Cycles)	95ºC	10 s	None
	60ºC	30 s	None
	72ºC	30 s	Single
	95ºC	2 s	None

Melting	65ºC	15 s	None
	95ºC	-	Continuous
Cooling	40ºC	30 s	None

3.4. *Quantitative Real Time PCR - BioMark™ System*

BioMark System arrays allow for the automatic combination of sets of samples with sets of assays, significantly reducing reaction volume and the number of liquid-handling steps performed during the experiment. For instance, using the 48x48 array (as described in this protocol), 48 different samples (*e.g.*, time-points in a time-course experiment) can be tested with up to 48 different assays (*e.g.*, genes).

1. Specific Target Amplification (STA): This step is recommended to increase the number of copies of target DNA.

1.1. STA Primer Mix (500nM):

1.1.1. Pool together 1 µL aliquots of all 100 µM primer sets to be included in the STA reaction (up to 100 different assays).

1.1.2. Add DNA Suspension Buffer to make the final volume 200 µL.

1.1.3. Vortex to mix and briefly spin reaction tube.

1.2. STA Pre-Mix:

1.2.1. In a DNA-free hood, prepare a Pre-Mix for the STA reaction:

Component	**Volume (per reaction)**
TaqMan PreAmp Master Mix	2.5 µL

500 nM pooled STA Primer Mix	0.5 µL
Water	0.75 µL

1.2.2. Add 3.75 µL of STA Pre-Mix for each sample in a 96-well plate.

1.2.3. Add 1.25 µL of cDNA (at 10-20 ng/µl to each reaction well, making a final volume of 5 µL. Include a no-PreAmplification control: add water instead of cDNA.

1.2.4. Seal the plate properly. Then, vortex and briefly spin the plate.

1.3. STA thermal cycle reaction:

1.3.1. Place the plate into the thermal cycler and run the following program (annealing temperature in the PCR is primer-dependent):

	Activation	**16 cycles**		**Hold**
Temperature	95ºC	95ºC	60ºC	4ºC
Time	10 min	15 s	4 min	∞

1.3.2. Eliminate the unincorporated primers from the STA amplification reaction. Prepare Exonuclease Mix as follows:

Component	**Per 5 µL Sample**
Water	1.4 µL
Exonuclease I Reaction Buffer	0.2 µL
Exonuclease I (20 units/ µL)	0.4 µL

1.3.3. Add 2 µL of Exonuclease Mix to each 5 µL STA reaction. Vortex, centrifuge, and place in a thermal cycler.

	Digest	Inactivate	Hold
Temperature	37ºC	80ºC	4ºC
Time	30 min	15 min	∞

1.3.4. Dilute the STA reaction to an appropriate final product concentration, as shown below. A minimum dilution of 5-fold should be used.

	Volume of water or TE Buffer		
Volume of STA Rx	5-fold dilution	10-fold dilution	20-fold dilution
7 µL	18 µL	43 µL	93 µL

Store diluted STA products at -20°C or use immediately for on-chip PCR.

2. Sample and Assay Mix preparation:

2.1. Prepare Sample mix as shown below:

Component	Volume per inlet with overage (µL)
2x SsoFast EvaGreen Supermix with Low ROX	3.0
20x DNA Binding Dye Sample Loading Reagent	0.3

2.2. In a new 96-well plate aliquot 3.3 µL of Sample mix and add 2.7 µL of each STA and Exo I-treated and diluted sample.

2.3. Seal the plate properly. Then, vortex and spin plate. Keep on ice.

2.4. Prior to preparing the Assay mix, combine the two primers of each primer pair making a final concentration of 20 µM.

2.5. Prepare Assay mix as shown below:

Component	Volume per inlet with overage (µL)
2x Assay Loading Reagent	3.0
1x DNA Suspension Buffer	2.4

2.6. In a new 96-well plate, aliquot 5.4 µL of Assay mix and add in 1 µL of the 100 µM combined forward and reverse primers primer pair mix. The final concentration of each primer pair is 5 µM in the inlet and 500 nM in the final reaction.

2.7. Seal the plate properly. Then, vortex and spin the plate. Keep on ice.

3. Priming the 48x48 Dynamic Array™ IFC

3.1. Inject control line fluid into each accumulator on the chip. Load the chip within 60 min of priming (refer to instrument manufacturer's instructions for details).

3.2. Remove and discard the blue protective film from the bottom of the chip.

3.3. Place the chip into the IFC controller for the 48x48 Dynamic Array IFC.

3.4. Run the Prime script for the 48x48 Dynamic Array IFC.

3.5. Pipette 5 µL of each assay and 5 µL of each sample into their respective inlets on the chip. Avoid creating bubbles while vortexing and when transferring reagents to the IFC, failure to do so may result in a decrease in data quality

3.6. Place the chip to the IFC controller and run the Load Mix program.

3.7. After the program has run, take out the chip from the IFC controller and remove any dust particle from the chip surface.

3.8. Place the chip in the Biomark System and run the following program (annealing temperature in the PCR is primer-dependent):

	Activation	30 Cycles		Melting	
Temperature	95ºC	96ºC	60ºC	60ºC	95ºC
Time	60 s	5 s	20 s	3 s	1ºC / 3 s

3.5. Data Analysis

Different methodologies can be used for determination of the Quantification Cycle, Cq ***(41)*** (previously referred to as Ct/Cp/take off point):

- The threshold cycle method measures the Cq at a constant fluorescence level. These constant threshold methods assume that all samples have the same amplicon DNA concentration at the threshold fluorescence. The strength of this method is that it is extremely robust, but the threshold value needs to be adjusted for each experiment.
- The second derivative method calculates Cq as the second derivative maximum of the amplification curve. It is not user-dependent and is widely used.

Before performing the actual analysis, it is important to validate the data according to a variety of criteria (preferably following the Minimum Information for Publication of Real Time PCR Experiments: MIQE guidelines, (*see* **Note 11**, ***(41)***). In particular:

- Check amplification curves. A normal amplification plot has 3 distinct phases: linear baseline, exponential and plateau.
- Check controls (RT-, NTC).
- Check that the slope of the standard curve is between -3.2 and -3.5.
- Check technical replicates. They should be within 0.5 Cq of each other.

- Check melting peaks (when using a binding dye, or probes such as Molecular Beacons or Scorpions that are not hydrolysed during the reaction) to verify that single, specific amplification products have been synthesized in the reaction.

3.5.1 Absolute quantification.

Absolute quantification relies on measurement to a standards curve constructed using the real-time PCR data obtained from amplification of these standards of known concentrations of template. Commonly, standards are derived from purified dsDNA plasmid, in vitro-transcribed RNA or in vitro-synthesized ssDNA. A standard curve (plot of Cq value against log of amount of standard) is generated using different dilutions of the standard. The Cq value of the target is compared with the standard curve, allowing calculation of the initial amount of the target. It is important to select an appropriate standard for the type of nucleic acid to be quantified. This method requires having the same efficiency of amplification in all reactions (reactions with experimental samples and reactions with the external standards). When using absolute quantification for determination of mRNA concentration, it is usual to correct absolute copy number of the specific target relative to absolute copy number of one or more reference genes.

3.5.2 Relative quantification.

Relative quantification relies on comparing the expression level of a target gene relative to a reference gene between a control sample and the test samples. Normalization to reference genes is the most common method for controlling for variation in qRT-PCR experiments. It is used to measure the relative change in

mRNA expression levels. Many mathematical models are available. Most common relative quantification methods are:

a) Pfaffl model ***(42)***: combines gene quantification and normalization into a single calculation (equation 1). This model adjusts the amplification efficiencies (E) from target and reference genes in order to correct differences between the two assays.

$$(1)\ \text{Ratio} = \frac{(E_{target})^{\Delta Cq\ target\ (control-sample)}}{(E_{reference})^{\Delta Cq\ reference\ (control-sample)}}$$

b) $2^{-\Delta\Delta Cq}$ method ***(43)***: This is a simpler version of the first model. Target and control amplification efficiency (E_{target} and $E_{reference}$) are assumed to be maximum (100%, *i.e.*, a value of 2, indicating amplicon doubling during each cycle) (equation 2). In addition, the relative expression of the target in all test samples is compared to that in a control or calibrator sample

$$(2)\ \text{Ratio} = 2^{-[\Delta Cq\ Sample - \Delta Cq\ control]}$$

4. Notes

1. *geNorm* is a widely used algorithm to determine the most stable reference from a given set of candidate genes on the basis of the *M* value (the *M* value is the internal control gene-stability measure, defined as the average pair-wise variation of a particular gene with all other control genes; genes with the lowest M values have the most stable expression) ***(18)***. *geNorm* calculates and compares the *M* value of each pair of genes, and eliminates the gene with the highest *M* value, and then repeats this process with the remaining genes until the pair of

genes with the lowest *M* value is identified. Thus, the genes forming this pair are considered as optimal reference genes among the initial candidate set.

2. The genome-wide analyses performed by Czechowski *et al.* led to the identification of many novel reference gene candidates, with purportedly better expression characteristics than traditional reference genes ***(17)***. In these analyses the SD/MV ratio (SD/mean expression value, *i.e.*, the coefficient of variation, or CV) for each gene in all the given experimental conditions (developmental series, abiotic stress series, hormone series, nutrient starvation and re-addition series, diurnal series, light series and biotic stress series) is calculated. The gene that has the lowest CV value is considered as the gene with the most stable expression, and therefore a potential reference gene. Through these analyses, 25 reference genes, including 20 novel and 5 traditional ones, were recommended ***(17)***. These genes were then validated by qRT-PCR and their expression stability ranked using the *geNorm* algorithm.

3. There are specific plates and films for the LC480 system that have been designed to ensure the best heat transfer from the thermal block and minimal autofluorescence, which is important to achieve a good signal-to-noise ratio in the detection of amplification products. In this protocol, we suggest using the LC 480 Multiwell Plate 96 from Roche.

4. The RNA preparation should be free of contaminating genomic DNA, so we recommend using a previously tested commercial kit for RNA isolation (*see* **Note 10**).

5. For primer design, it is important to consider the following points: (1) PCR products should be short (ideal length is from 70 to 250 bp); (2) The gene-specific forward and reverse primers should have similar melting temperatures (Tm) and length; (3) Primers should be between 15 and 25 nucleotides long and with a G/C content of around 50%. (4) Primers should have low or no self-complementarity to avoid the formation of primer dimers; (5) For

the same reason, avoid pairs of primers that show sequence complementarity at their 3' ends; (6) Primers that span introns or cross intron/exon boundaries are advantageous because they allow to distinguish amplification from cDNA or from contaminant genomic DNA. Primers should be ordered with desalt purification. Primer stock solutions should be prepared with DNase/RNase-free water. Make aliquots to avoid contamination and repeated freezing/thawing. Original stock of PCR primers should be stored at -20ºC and working dilutions at 4ºC for up to 2 weeks.

6. The presence of liquid nitrogen inside the microcentrifuge tubes during tissue grinding should be avoided, to prevent potential loss of tissue by nitrogen spill, or by the popping of the tube if closed with liquid nitrogen inside. Tubes can be pre-chilled in liquid nitrogen. As an alternative for grinding the tissue, mortar and pestle could be used instead of pellet pestles and an electric drill.

7. Both fresh and frozen (-80ºC) tissue can be used as starting material, and ground plant material can be stored at -80°C before RNA purification. However, do not allow the frozen material to thaw before grinding or before the first solution of the RNA purification procedure is added.

8. Make a 4-step dilution series (1:4 dilutions) from cDNA samples. To evaluate the efficiency of the PCR reaction, it is important to generate at least one standard curve for each primer pair. A standard curve graph is made by plotting the Ct/Cp values on the y-axis and the logarithm of the input amounts on the x-axis. The slope of the line of this plot will give the efficiency of the reaction according to the equation $E = [10^{\wedge(-1/slope)}] - 1$; slope should be between -3.2 and -3.5 and $R^2>0.98$.

9. SYBR® Green I and EvaGreen® are the most used dye chemistries, due to cost and simple optimization process. However, these dyes bind to any double-stranded DNA formed in the reaction, including primer-dimers and other non-specific reaction products, which may result in an overestimation of the target concentration. Other methods, such as hydrolysis probes, may also be used. Probe-based qRT-PCR relies on the sequence–specific detection of a desired PCR product. It utilizes a fluorescently-labelled target-specific probe, which results in increased specificity and sensitivity.

10. No template controls (NTC) should be included for each pair of primers tested to ensure that there is no reagent contamination. In these control reactions, water is added instead of sample, so no amplification is expected. In case the NTC reaction shows the synthesis of amplification products (*i.e.*, the presence of a contaminant), measures such as pipette decontamination, using new primers aliquots, or thorough bench cleaning might be necessary. No reverse transcription controls (no-RT, or RT-) are used to detect the presence of contaminant genomic DNA in the RNA samples. If the RT- reaction shows the synthesis of amplification products, the corresponding RNA samples should be treated with DNase prior to their use in the reverse transcription reaction. If the primers were designed to span an intron or an intron/exon boundary, it is not necessary to perform a no-RT control.

11. MIQE Guidelines ***(41)***. The MIQE guidelines were published in response to the recognition that several publications contain little information that describes the qPCR or that gives the reader the opportunity to determine the quality of the experiment. The result of these omissions is that several publications contain misleading conclusions based on inadequate quality control of the technical process. The MIQE guidelines contain a step-by-step guide and checklist, which leads the experimenter through the process of experiment validation. This has the additional function of providing a framework for publication analysis

by peer reviewers and journal editors. Several publishing houses are now requiring that MIQE guidelines are followed for papers containing qPCR data.

Acknowledgements

Work in the authors' laboratory was supported by grant BFU2014-58289-P (funded by MICIN/AEI/ 10.13039/501100011033 and by "ERDF A way of making Europe") and by grant 2017SGR718 (from the Agencia de Gestió d'Ajuts Universitaris I de Recerca) to JLR, and by institutional grant SEV-2015-0533 (funded by MCIN/AEI/10.13039/501100011033) and by the CERCA Programme / Generalitat de Catalunya. R.A. is supported by fellowship PRE2018-084278 funded by MCIN/AEI/ 10.13039/501100011033 and by "ESF Investing in your future". We acknowledge the contributions of Jian Jin, Oriol Casagran and Tania Nolan to the previous, first edition of this chapter.

5. References

1. Aamir M, Karmakar P, Singh VK et al (2021) A novel insight into transcriptional and epigenetic regulation underlying sex expression and flower development in melon (Cucumis melo L.). Physiol Plant 173(4):1729–1764. doi:10.1111/ppl.13357

2. Haider S, Bashir MA, Habib U et al (2021) Phenotypic Characterization and RT-qPCR Analysis of Flower Development in F1 Transgenics of Chrysanthemum × grandiflorum. Plants 10(8):1681. doi:10.3390/plants10081681

3. de Moura SM, Rossi ML, Artico S et al (2020) Characterization of floral

morphoanatomy and identification of marker genes preferentially expressed during specific stages of cotton flower development. Planta 252(4):71. doi:10.1007/s00425-020-03477-0

4. Deng MH, Zhao K, Lv JH et al (2020) Flower transcriptome dynamics during nectary development in pepper (Capsicum annuum L.). Genet Mol Biol 43(2): e20180267. doi:10.1590/1678-4685-GMB-2018-0267

5. Moschin S, Nigris S, Ezquer I et al (2021) Expression and Functional Analyses of Nymphaea caerulea MADS-Box Genes Contribute to Clarify the Complex Flower Patterning of Water Lilies. Front Plant Sci 12:730270. doi:10.3389/fpls.2021.730270

6. Serra-Picó M, Hecht V, Weller JL et al (2022) Identification and characterization of putative targets of VEGETATIVE1/FULc, a key regulator of development of the compound inflorescence in pea and related legumes. Front Plant Sci 13:765095. doi:10.3389/fpls.2022.765095

7. Bustin SA, Benes V, Nolan T, Pfaffl MW (2005) Quantitative real-time RT-PCR – a perspective. J Mol Endocrinol 34(3):597–601. doi:10.1677/jme.1.01755

8. Kubista M, Andrade JM, Bengtsson M et al (2006) The real-time polymerase chain reaction. Mol Aspects Med 27(2–3):95–125. doi:10.1016/j.mam.2005.12.007

9. Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. Nat Protoc 1(3):1559–1582. doi:10.1038/nprot.2006.236.

10. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 27(2–3):126–39. doi:10.1016/j.mam.2005.12.003.

11. Nolan T, Hands RE, Ogunkolade W, Bustin SA. SPUD: A quantitative PCR assay for

the detection of inhibitors in nucleic acid preparations. Anal Biochem 351(2):308–310. doi: 10.1016/j.ab.2006.01.051

12. Gutierrez L, Mauriat M, Pelloux J, Bellini C, Van Wuytswinkel O (2008) Towards a Systematic Validation of References in Real-Time RT-PCR. Plant Cell 20(7):1734–1735. doi:10.1105/tpc.108.059774

13. Gutierrez L, Mauriat M, Guénin S et al (2008) The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol J 6(6):609–618. doi:10.1111/j.1467-7652.2008.00346.x

14. Vandesompele J, De Preter K, Pattyn F et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):RESEARCH0034. doi:10.1186/gb-2002-3-7-research0034 4

15. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper-Excel-based tool using pair-wise correlations. Biotechnol Lett 26(6):509–515. doi: 10.1023/b:bile.0000019559.84305.47

16. Schmid M, Davison TS, Henz SR et al (2005) A gene expression map of Arabidopsis thaliana development. Nat Genet 37(5):501–506. Available from: https://www.nature.com/articles/ng1543

17. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible W-R. Genome-Wide Identification and Testing of Superior Reference Genes for Transcript Normalization in Arabidopsis. Plant Physiol [Internet]. 2005;139(September):5–17. doi:10.1038/ng1543

18. Chao WS, Doğramaci M, Foley ME et al (2012) Selection and Validation of Endogenous Reference Genes for qRT-PCR Analysis in Leafy Spurge (Euphorbia esula). PLoS One 7(8):e42839. doi:10.1371/journal.pone.0042839

19. Caldana C, Scheible WR, Mueller-Roeber B, Ruzicic S. A quantitative RT-PCR platform for high-throughput expression profiling of 2500 rice transcription factors. Plant Methods 3:7. doi:10.1186/1746-4811-3-7

20. Alves Oliveira D, Tang JD, Warburton ML (2021) Reference Gene Selection for RT-qPCR Analysis in Maize Kernels Inoculated with Aspergillus flavus. Toxins 13(6):386. doi:10.3390/toxins13060386

21. Galli V, da Silva Messias R, dos Anjos e Silva SD, Rombaldi CV (2013) Selection of reliable reference genes for quantitative real-time polymerase chain reaction studies in maize grains. Plant Cell Rep 32(12):1869–1877. doi:10.1007/s00299-013-1499-x

22. Lin Y, Zhang C, Lan H, Gao S et al (2014) Validation of Potential Reference Genes for qPCR in Maize across Abiotic Stresses, Hormone Treatments, and Tissue Types. PLoS One 9(5):e95445. doi:10.1371/journal.pone.0095445

23. Manoli A, Sturaro A, Trevisan S et al (2012) Evaluation of candidate reference genes for qPCR in maize. J Plant Physiol 169(8):807–815. doi:10.1016/j.jplph.2012.01.019

24. Auler PA, Benitez LC, do Amaral MN et al (2017) Selection of candidate reference genes and validation for real-time PCR studies in rice plants exposed to low temperatures. Genet Mol Res 16(2):16029695. doi:10.4238/gmr16029695

25. Auler PA, Benitez LC, do Amaral MN et al (2017) Evaluation of stability and validation of reference genes for RT-qPCR expression studies in rice plants under water deficit. J Appl Genet 58(2):163–177. doi:10.1007/s13353-016-0374-1

26. Ji Y, Tu P, Wang K et al (2014) Defining reference genes for quantitative real-time PCR analysis of anther development in rice. Acta Biochim Biophys Sin (Shanghai) 46(4):305–312. doi:10.1093/abbs/gmu002

27. Bevitori R, Oliveira MB, Grossi-de-Sá MF et al (2014) Selection of optimized candidate reference genes for qRT-PCR normalization in rice (Oryza sativa L.) during Magnaporthe oryzae infection and drought. Genet Mol Res 13(4):9795–9805. doi:10.4238/2014

28. Garrido J, Aguilar M, Prieto P (2020) Identification and validation of reference genes for RT-qPCR normalization in wheat meiosis. Sci Rep 2020;10(1):2726. doi:10.1038/s41598-020-59580-5

29. Tenea GN, Peres Bota A, Cordeiro Raposo F, Maquet A (2011) Reference genes for gene expression studies in wheat flag leaves grown under different farming conditions. BMC Res Notes 4:373. doi:10.1186/1756-0500-4-373

30. Wu D, Dong J, Yao YJ et al (2015) Identification and evaluation of endogenous control genes for use in quantitative RT-PCR during wheat (Triticum aestivum L.) grain filling. Genet Mol Res 14(3):10530–10542. doi:10.4238/2015.September.8.15

31. Zhang Y, Peng X, Liu Y et al (2018) Evaluation of suitable reference genes for qRT-PCR normalization in strawberry (Fragaria × ananassa) under different experimental conditions. BMC Mol Biol 19(1):8. doi:10.1186/s12867-018-0109-4

32. Galli V, Borowski JM, Perin EC et al (2015) Validation of reference genes for accurate normalization of gene expression for real time-quantitative PCR in strawberry fruits using different cultivars and osmotic stresses. Gene 554(2):205–214. doi:10.1016/j.gene.2014.10.049.

33. Joseph JT, Poolakkalody NJ, Shah JM (2018) Plant reference genes for development and stress response studies. J Biosci 43(1):173–187. doi:10.1007/s12038-017-9728-z

34. Hruz T, Wyss M, Docquier M et al (2011). RefGenes: identification of reliable and condition specific reference genes for RT-qPCR data normalization. BMC Genomics 12:156. doi:10.1186/1471-2164-12-156

35. Wang M, Bhullar NK (2021) Selection of Suitable Reference Genes for qRT-PCR Gene Expression Studies in Rice. In: Bandyopadhyay A, Thilmony R (eds) Rice Genome Engineering and Gene Editing. Methods in Molecular Biology, vol 2238. Humana, New York, NY, pp 293–312. doi:10.1007/978-1-0716-1068-8_20

36. Zheng T, Chen Z, Ju Y et al (2018) Reference gene selection for qRT-PCR analysis of flower development in Lagerstroemia indica and L. speciosa. PLoS One 13(3):e0195004. doi:10.1371/journal.pone.0195004

37. Škiljaica A, Jagić M, Vuk T et al (2022) Evaluation of reference genes for RT-qPCR gene expression analysis in Arabidopsis thaliana exposed to elevated temperatures. Plant Biol 24(2):367–379. doi:10.1111/plb.13382

38. Xu W, Dong Y, Yu Y et al (2020) Identification and evaluation of reliable reference genes for quantitative real-time PCR analysis in tea plants under differential biotic stresses. Sci Reports 10(1):2429. doi:10.1038/s41598-020-59168-z

39. Joseph JT, Poolakkalody NJ, Shah JM (2019) Screening internal controls for expression analyses involving numerous treatments by combining statistical methods with reference gene selection tools. Physiol Mol Biol Plants 25(1):289-301. doi:10.1007/s12298-018-0608-2

40. Wellmer F, Alves-Ferreira M, Dubois A et al (2006). Genome-Wide Analysis of Gene

Expression during Early Arabidopsis Flower Development. PLoS Genet 2(7):e117. doi:10.1371/journal.pgen.0020117.eor

41. Bustin SA, Benes V, Garson JA et al (2009) The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clin Chem 55(4):611–622. doi:10.1373/clinchem.2008.112797

42. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 29(9):e45. doi:10.1093/nar/29.9.e45

43. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. Methods 25(4):402–408. doi:10.1006/meth.2001.1262

Figure Captions

Figure 1. Example of output results obtained when using the *RefGenes* tool (Anatomy-Inflorescence category in Genevestigator) with a set of floral regulatory genes (*SUP* (AT3G23130), *LFY* (AT5G61850), *AGL24* (AT4G24540), *YAB3* (AT4G00180), *AP2* (AT4G36920), *AGL42* (AT5G62165), *SHP2* (AT2G42830), *AG* (AT4G18960), *SEP3* (AT1G24260), and *AP3* (AT3G54340)).

Figure 2. Expression characteristics during plant development of some commonly used and novel reference genes in Arabidopsis inflorescences. **A)** Traditional reference genes: *GAPDH* (AT3G26650, GAPA1), *ACT2* (AT3G18780), *UBQ10* (AT4G05320), *TUBB6* (AT5G12250), *TUBA5* (AT5G19780) ***(17)***. **B)** Reference genes for developmental processes: *AP2M* (*AT5G46630*), *AT1G58050*, *AT4G26410*, *AT4G34270* (33). **C)** Novel reference genes based on the expression of floral regulatory genes: *AT2G28390*, *AT5G15710*, *VPS45 (AT1G77140)*, *AT5G10700*, and *CLT2* (*AT4G24460*).

Figure 3. Expression characteristics in different floral tissues of some commonly used and novel reference genes in Arabidopsis inflorescences. **A)** Traditional reference genes. **B)** Reference genes for developmental processes. **C)** Novel reference genes based on the expression of floral regulatory genes (as in **Figure 2**).

Tables

Table 1. Arabidopsis general reference genes according with their expression stability under different conditions ***(33)***. Primer sequences indicated in the table correspond to those used in the original experiment, as referenced in ***(33)***.

Table 2. Candidate novel reference genes for Arabidopsis proteins and peptides expressed in floral tissues identified using *RefGenes*. These genes were selected using as search set a list of floral regulatory genes.

Table 1

Accession number	Gene	Primers (5'-3')	Conditions	
At1g50010	α-Tubulin	GATGTACCGTGGTGATGTC GAGCCTCTGAAAATTCTCC	Abiotic stress	Sulfate starvation, salt, drought, ABA
AT3G18780	Actin 2	CTTGCACCAAGCAGCATGAA CCGATCCAGACACTGTACTTCCTT	Abiotic stress	Dehydration, cold, salt, oxidative, exposure to high light intensity
		TATGTGGCTATTCAGGCTGT TGGCGGTGCTTCTTCTCTG	Abiotic stress	Salt, mannitol, drought and cold
		ATGCCATCCTCCGTCTTGAC CGCTCTGCTGTTGTGGTGAA	Biotic stress	*A. tumefaciens, H. schachtii, B. cinerea, P. syringae* pv.*maculicola, P. syringae* pv. *tomato*
At3g53750	Actin 3	GAGGCTCCTCTTAACCCAA TACAATTTCCCGCTCTGC	Abiotic stress	Salt stress, drought stress, ABA
At1g49240	Actin 8	TATGTGGCTATTCAGGCTGT TGGCGGTGCTTCTTCTCTG	Abiotic stress	Salt, mannitol, drought and cold
		GGTGATGGTGTGTCT ACTGAGCACAATGTTAC	Biotic stress	*A. tumefaciens*
At1g13440	GAPDH	TTGGTGACAACAGGTCAAGCA AAACTTGTCGCTCAATGCAATC	Abiotic stress	Salt, mannitol, drought and cold
At2g41540	GAPDH	GAAGCAAGGCAAAGAAAT GAAGCAAGGCAAAGAAAT	Biotic stress	*A. tumefaciens*
At5g25760	UBC21	TTCAAATGGACCGCTCTTATCA AAACACCGCCTTCGTAAGGA	Biotic stress	*A. tumefaciens*
At1g64230	UBC28	TCCAGAAGGATCCTCCAACTTCCTG CAGT ATGGTTACGAGAAAGACACCGCCTG AATA	Abiotic stress	Salt, osmotic, temperature, radiomimetic, oxidative, UV, Zebularine, Trichostatin A, Sodium butyrate
At3g62250	UBQ5	GTAAACGTAGGTGAGTCC GACGCTTCATCTCGTCC	Abiotic stress	Drought, mannitol and salt
		GACGCTTCATCTCGTCC GTAAACGTAGGTGAGTCC	Biotic stress	*B. cinerea; P. syringae pv. maculicola, P. syringae pv. tomato*
At5g62690	Tubulin 2	CTCTGACCTCCGAAAGCTTGC TCACCTTCTTCATCCGCAGTT	Abiotic stress	Sucrose, NaCl, mannitol, paclobutrazol, hormonal
		AGCAATACCAAGATGCAACTGCG TAACTAAATTATTCTCAGTACTCTTC C	Biotic stress	*B. cinerea; P. syringae pv. maculicola, P. syringae pv. tomato*
At5g15710	F-BOX	TTTCGGCTGAGAGGTTCGAGT GATTCCAAGACGTAAAGCAGATCAA	Abiotic stress	Metal stress
At5g08290	YLS8	TTACTGTTTCGGTTGTTCTCCATTT CACTGAATCATGTTCGAAGCAAGT	Abiotic stress	Metal stress
At2g28390	SAND	AACTCTATGCAGCATTTGATCCACT TGATTGCATATCTTTATCGCCATC	Abiotic stress	Metal stress
		AACTCTATGCAGCATTTGATCCACT TGATTGCATATCTTTATCGCCATC	Biotic stress	*P. infestans, A. laibachii*
At5g60390	EF1-α	TGAGCACGCTCTTCTTGCTTTCA GGTGGTGGCATCCATCTTGTTACA	Abiotic stress	Metal stress
AT5G46630	AP2M (CACS)	TCGATTGCTTGGTTTGGAAGAT GCACTTAGCGTGGACTCTGTTTGAT C	Development	Different tissues, organs, developmental stages, and genotypes

At1g58050	Helicase	CCATTCTACTTTTTGGCGGCT TCAATGGTAACTGATCCACTCTGAT G	Development	Different tissues, organs, developmental stages, and genotypes
AT4G26410	Expressed	GAGCTGAAGTGGCTTCCATGAC GGTCCGACATACCCATGATCC	Development	Different tissues, organs, developmental stages, and genotypes
AT4G34270	TIP41-like	GTGAAAACTGTTGGAGAGAAGCAA TCAACTGGATACCCTTTCGCA	Development	Different tissues, organs, developmental stages, and genotypes

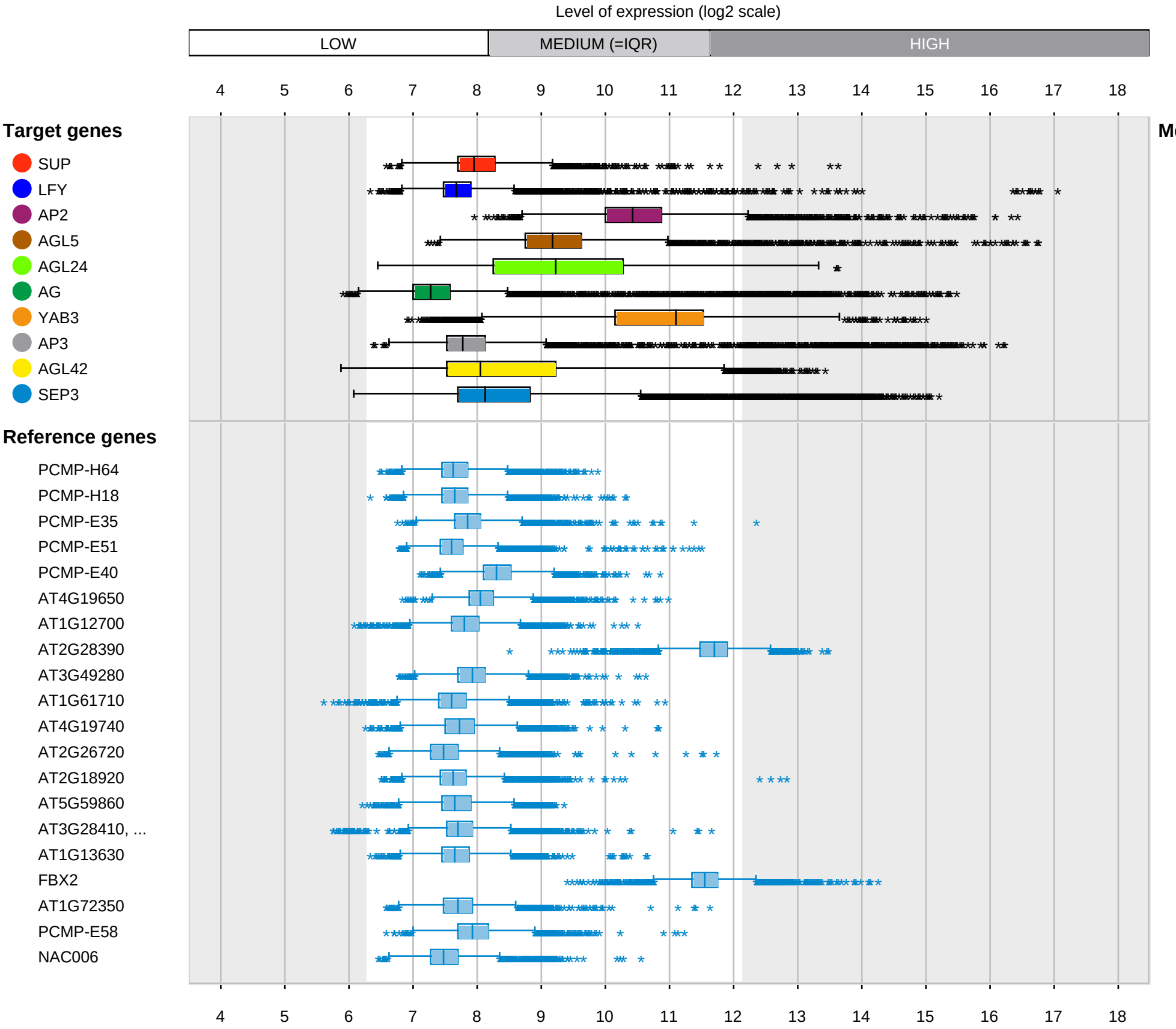
Table 2

Gene	Annotation	Search set
AT2G28390	SAND family protein (MON1)	Floral regulatory genes
AT5G15710	Galactose oxidase/kelch repeat superfamily protein	Floral regulatory genes
AT1G77140	Vacuolar protein sorting 45 (VPS45)	Floral regulatory genes
AT5G10700	Peptidyl-tRNA hydrolase II (PTH2) family protein	Floral regulatory genes
AT4G24460	CRT (chloroquine-resistance transporter)-like transporter 2 (CLT2)	Floral regulatory genes
AT5G22760	PHD finger family protein (DDP2)	Floral regulatory genes
AT5G11380	1-deoxy-D-xylulose 5-phosphate synthase 3 (DXPS3)	Floral regulatory genes
AT5G04270	DHHC-type zinc finger family protein (PAT15)	Floral regulatory genes
AT1G50170	Sirohydrochlorin ferrochelatase B (SIRB)	Floral regulatory genes
AT3G59000	F-box/RNI-like superfamily protein	Floral regulatory genes
AT2G36480	ENTH/VHS family protein	Floral regulatory genes
AT5G52880	F-box family protein	Floral regulatory genes
AT5G65620	Zincin-like metalloproteases family protein (TOP1)	Floral regulatory genes
AT5G60750	CAAX amino terminal protease family protein. Encodes a chloroplast endoproteinase required for photosynthetic acclimation to higher light intensities (SCO4)	Floral regulatory genes
AT5G64970	Mitochondrial substrate carrier family protein	Floral regulatory genes
AT3G61180	RING/U-box superfamily protein	Floral regulatory genes
AT2G41790	Insulinase (Peptidase family M16) family protein	Floral regulatory genes
AT5G13050	5-formyltetrahydrofolate cycloligase (5FCL)	Floral regulatory genes
AT5G04920	EAP30/Vps36 family protein (VPS36)	Floral regulatory genes
AT3G59770	SacI homology domain-containing protein / WW domain-containing protein (SAC9)	Floral regulatory genes

Dataset: 10562 samples from data selection: ATH all

Search Space: Gene

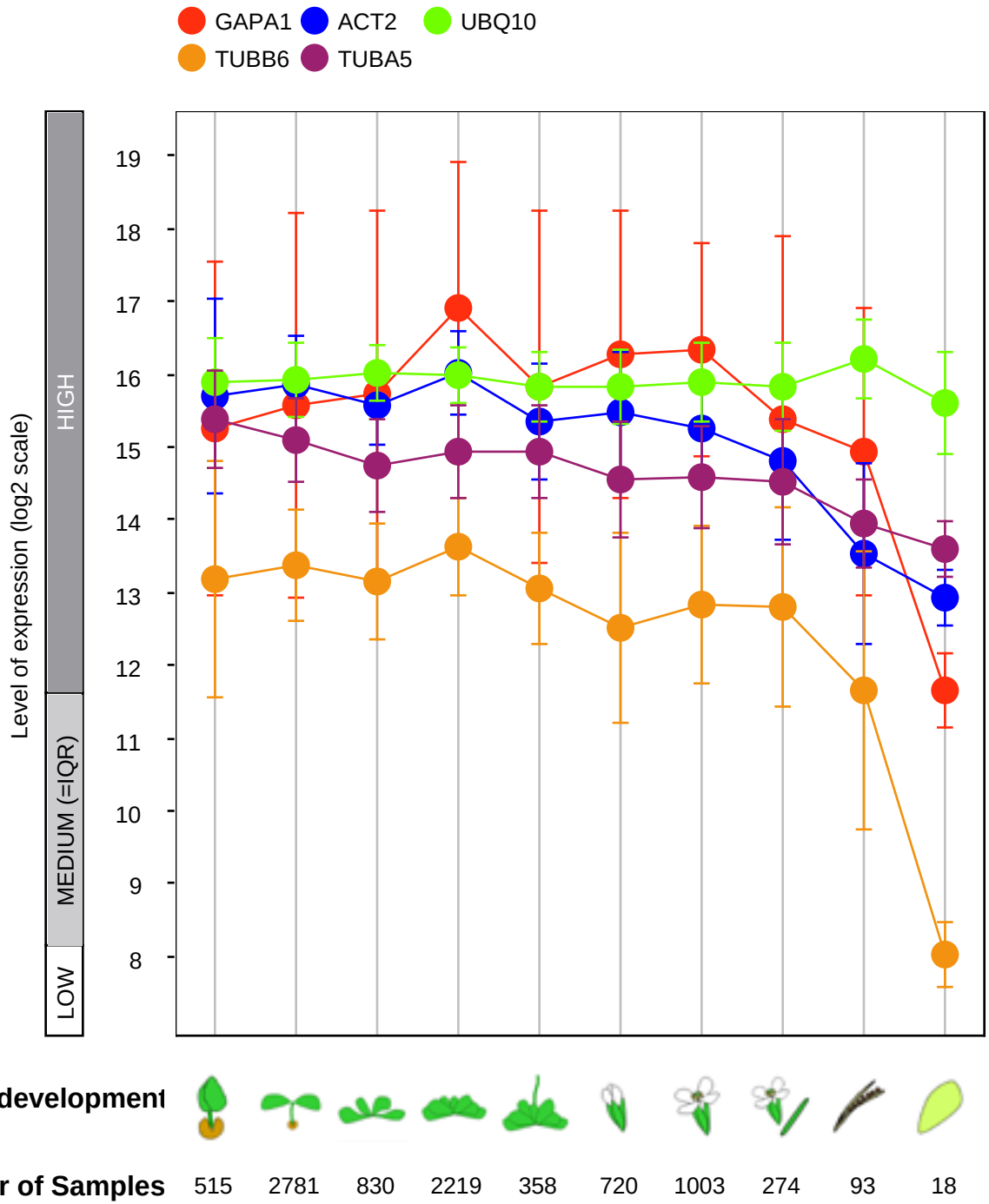
Found 20 measure(s) of 21 gene(s)



created with GENEVESTIGATOR

Dataset: 10 developmental stages from data selection: ATH all

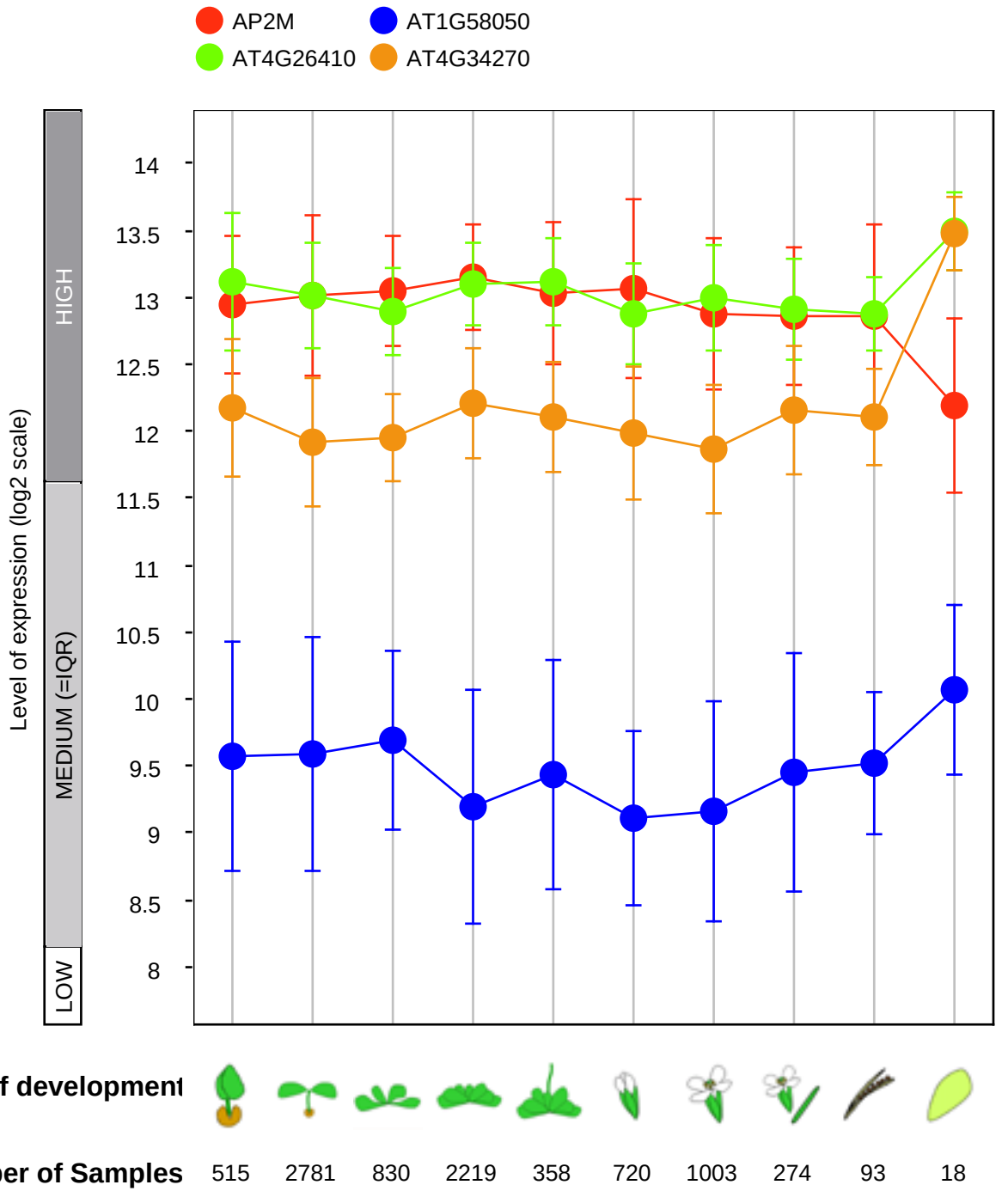
Showing 5 measure(s) of 5 gene(s) on selection: Traditional HK Czechowski et al. (17)



created with GENEVESTIGATOR

Dataset: 10 developmental stages from data selection: ATH all

Showing 4 measure(s) of 4 gene(s) on selection: HK Joseph et al. (20)



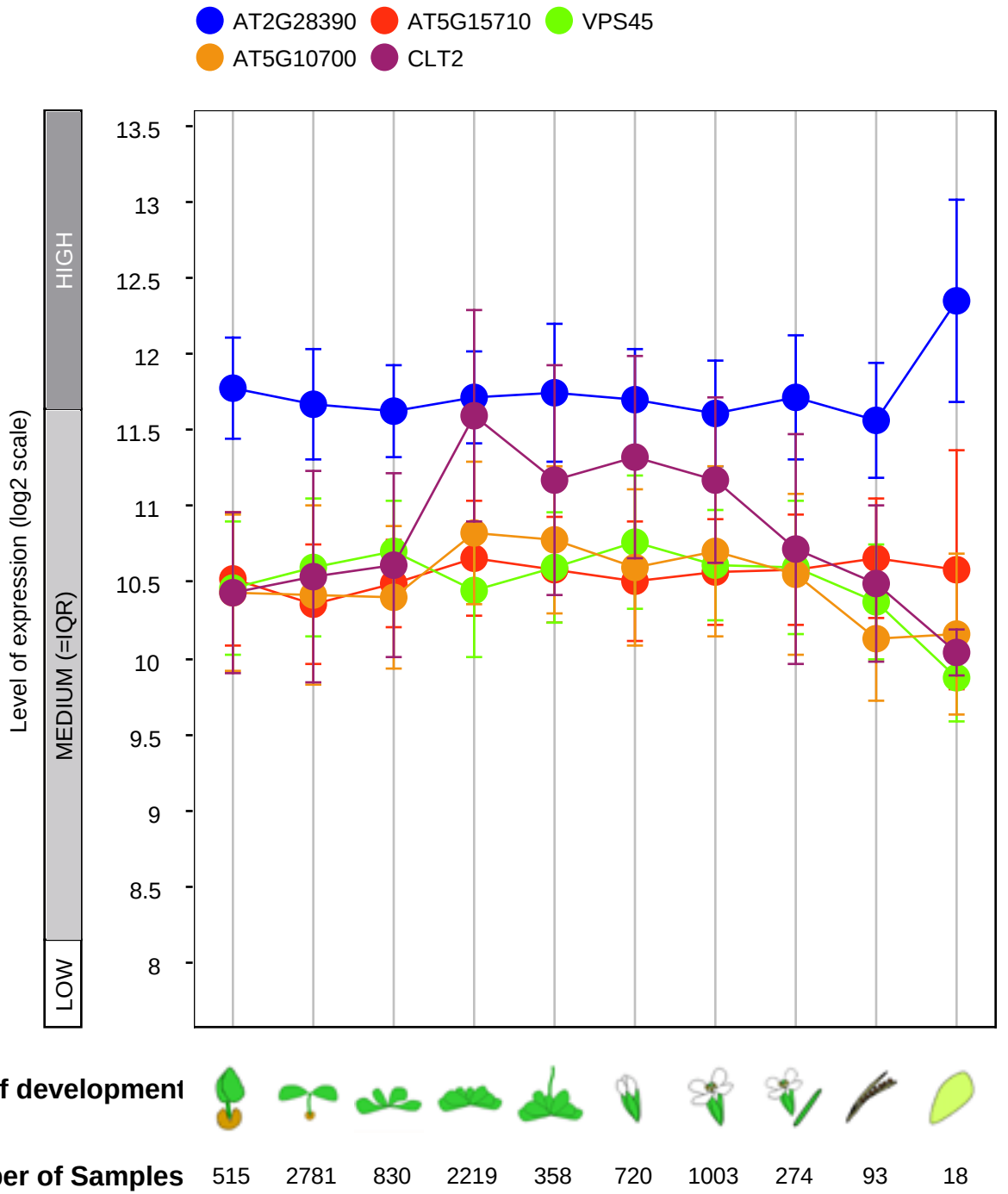
Stage of development

Number of Samples 515 2781 830 2219 358 720 1003 274 93 18

created with GENEVESTIGATOR

Dataset: 10 developmental stages from data selection: ATH all

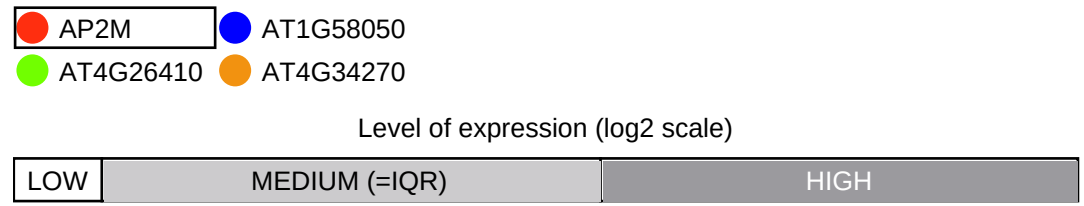
Showing 5 measure(s) of 5 gene(s) on selection: HK for flower development



created with GENEVESTIGATOR

Dataset: 127 anatomical parts from data selection: ATH all

Showing 4 measure(s) of 4 gene(s) on selection: HK Joseph et al. (20)



Level of expression (log2 scale)

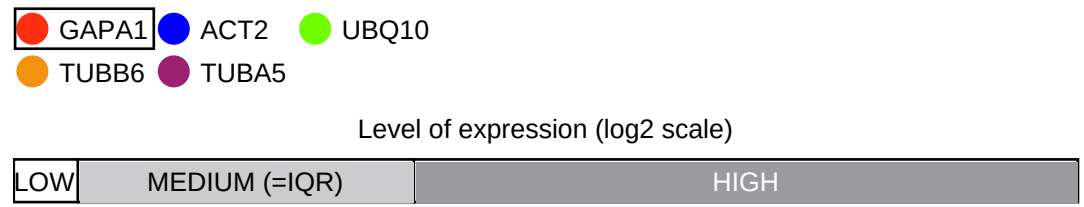
Arabidopsis thaliana (127)

LOW | MEDIUM (=IQR) | HIGH

	samples	avg. expr.
callus	31	13.06
▶ cell culture / primary cell	714	13.67
▶ seedling	2331	12.86
▼ inflorescence	801	12.74
▼ raceme	327	12.86
▼ flower	324	12.86
▼ stamen	118	13.18
▼ anther	52	13.76
pollen	52	13.76
abscission zone	15	13.64
▼ pistil	40	13.25
▼ carpel	11	12.42
stigma	3	12.23
▼ ovary	6	12.69
ovule	2	13.35
petal	6	12.75
sepal	6	12.63
pedicel	3	12.76
▶ silique	372	12.67
▶ shoot	4606	13.06
▶ roots	1081	13.40

Dataset: 127 anatomical parts from data selection: ATH all

Showing 5 measure(s) of 5 gene(s) on selection: Traditional HK Czechowski et al. (17)



Level of expression (log2 scale)

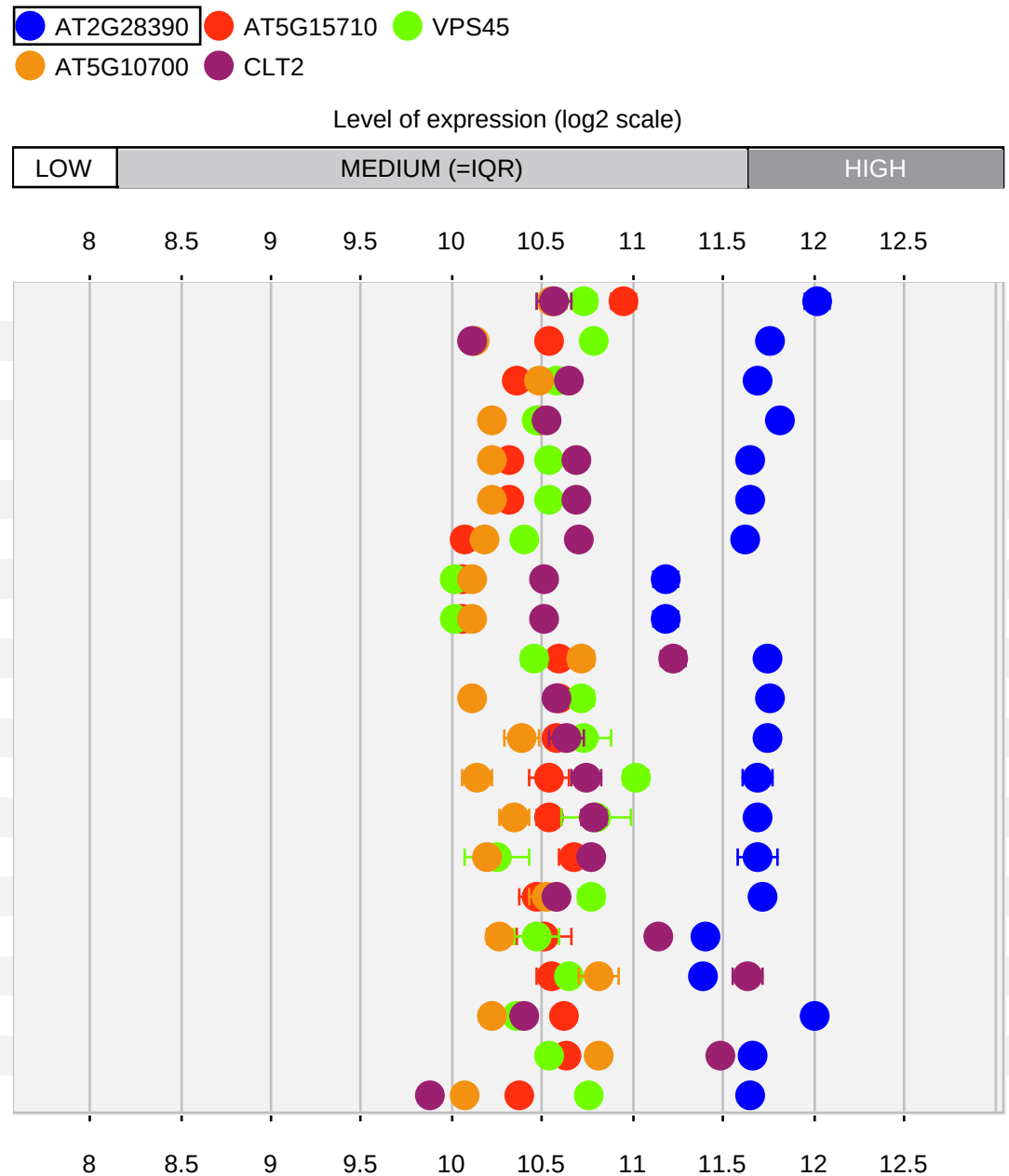
Arabidopsis thaliana (127)

LOW | MEDIUM (=IQR) | HIGH

	samples	avg. expr.
callus	31	13.41
▶ cell culture / primary cell	714	12.69
▶ seedling	2331	16.63
▼ inflorescence	801	14.52
▼ raceme	327	14.75
▼ flower	324	14.74
▼ stamen	118	12.41
▼ anther	52	9.43
pollen	52	9.43
abscission zone	15	16.36
▼ pistil	40	15.74
▼ carpel	11	15.12
stigma	3	15.49
▼ ovary	6	15.21
ovule	2	14.92
petal	6	15.42
sepal	6	16.11
pedicel	3	16.47
▶ silique	372	13.92
▶ shoot	4606	17.05
▶ roots	1081	10.60

Dataset: 127 anatomical parts from data selection: ATH all

Showing 5 measure(s) of 5 gene(s) on selection: HK for flower development



Arabidopsis thaliana (127)

	samples	avg. expr.
callus	31	12.02
▶ cell culture / primary cell	714	11.76
▶ seedling	2331	11.68
▼ inflorescence	801	11.82
▼ raceme	327	11.64
▼ flower	324	11.64
▼ stamen	118	11.62
▼ anther	52	11.18
pollen	52	11.18
abscission zone	15	11.74
▼ pistil	40	11.75
▼ carpel	11	11.74
stigma	3	11.69
▼ ovary	6	11.69
ovule	2	11.69
petal	6	11.72
sepal	6	11.40
pedicel	3	11.38
▶ silique	372	12.00
▶ shoot	4606	11.66
▶ roots	1081	11.65