plant biology

Plant Biology ISSN 1435-8603

RESEARCH ARTICLE

Evaluation of reference genes for RT-qPCR gene expression analysis in *Arabidopsis thaliana* exposed to elevated temperatures

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Keywords
RT-qPCR; reference genes; *Arabidopsis thaliana*; elevated temperature; microarray.

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Editor
E. Flemetakis

Received: 9 March 2021; Accepted: 8 December 2021

doi:10.1111/plb.13382

ABSTRACT

- Increases in environmental temperature are directly linked to the issue of climate change and are known to significantly disrupt plant growth and development. Studies of gene expression in plants commonly include RT-qPCR but the reliability of the method depends on the use of suitable reference genes for data normalization. Despite this, no reference genes have been validated specifically for experiments in *Arabidopsis thaliana* employing treatments with elevated temperature.
- Here, ten genes were selected for expression stability analysis based on the screening of available literature and microarray data from temperature-treated *A. thaliana*. Expression levels of candidate reference genes were measured in 12-day-old seedlings, rosette leaves and flower buds of 5-week-old *A. thaliana* plants exposed to five different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and their expression stabilities were assessed using four statistical algorithms (BestKeeper, geNorm, NormFinder and comparative ΔC_q method).
- This study provides reliable reference genes for use in *A. thaliana* RT-qPCR expression analyses employing elevated temperature treatments, namely *OGIO* and *PUX7* in seedlings, *UBC21* and *PUX7* in leaves, *TIP41* and *UBC21* in buds, and *TIP41* and *UBC21* in all three tissues combined.
- Orthologues of these genes can be of potential use in less studied plants, especially agricultural species heavily affected by climate change.

INTRODUCTION

Temperature is one of the major environmental factors affecting all stages of plant development and metabolism. Exposure to cold and heat can have short-term and long-term consequences on development of plants, including important crop species, which can in turn negatively affect global food production. The ongoing issue of climate change has instigated a flurry of articles studying the effect of temperature on plant adaptation to abiotic stress conditions. Gene expression analyses are commonly employed in these studies.

A state-of-the-art method for medium throughput gene expression analysis is reverse transcription quantitative PCR (RT-qPCR). Although a remarkably accurate and sensitive method, if not properly used, it can produce highly unreliable results. A number of articles has been published addressing common trends and issues with using RT-qPCR (Bustin 2002; Derveaux *et al.* 2010) and a set of guidelines entitled 'The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE)' has been issued to promote transparency of reported RT-qPCR results (Bustin *et al.* 2009). As stated in these papers, several crucial variables need to be controlled in order to ensure maximum reliability of RT-qPCR results, such as sample preparation and quality control, assay design, reference gene selection and validation, efficiency correction, normalization of gene expression data and biostatistical analysis (Derveaux *et al.* 2010).

A crucial tool in removing sample-related technical variation is the normalization step, in which expression of a target gene is normalized to, most commonly, expression of a reference gene. In a given set of experimental conditions, a reference gene should be stably expressed across all tested samples (Bustin 2002). Traditionally, reference genes such as *ACT2*, *EF1α*, *GAPDH*, *TUB6*, *UBI10*, *UBQ21* and *18S rRNA* have been selected based on their suspected housekeeping roles, which assume stable expression in all cells, treatments and between developmental stages. However, in the last two decades, substantial evidence has been collected showing expression variation of traditional reference genes in different conditions (Czechowski *et al.* 2005; Gutierrez *et al.* 2008). Due to increased need for adequate validation of reference genes in a given set of experimental conditions, several reference gene validation software packages have been designed, such as NormFinder (Andersen *et al.* 2004), geNorm (Vandesompele *et al.* 2009), BestKeeper (Pfaffl *et al.* 2004), comparative ΔC_q method (Silver *et al.* 2006) and others. Additionally, it has been shown that using more than one stably expressed reference gene renders the normalized data more accurate and the results statistically more significant (Vandesompele *et al.* 2002). Furthermore, according to the MIQE guidelines, three reference genes are suggested as optimal for generating reliable RT-qPCR results (Bustin *et al.* 2009).

Superior reference genes for use in RT-qPCR experiments investigating relative gene expression in conditions of non-

optimal temperature have been proposed for wheat (Paolacci *et al.* 2009), barley (Janská *et al.* 2013), rice (Xu *et al.* 2015), soybean (Gao *et al.* 2017), *Arabidopsis pumila* (Jin *et al.* 2019), strawberry (Liu *et al.* 2020) and many other plants. These papers offer a starting point for individual research laboratories to select reference genes and verify their suitability within their own experimental design. In the model organism *Arabidopsis thaliana*, reference genes related to temperature have only been marginally investigated, using microarray data obtained from seedlings grown at two different temperatures (Hong *et al.* 2010) and adult plants exposed to cold stress (Czechowski *et al.* 2005), or using RNA-seq data from leaves exposed to cold and heat treatment (Klepikova *et al.* 2016). Although these studies provide valuable information, they are, in themselves, far from a detailed analysis focused exclusively on non-optimal temperatures. Despite being an agriculturally unimportant species, *A. thaliana* will most likely remain a model plant for research of climate change impacts on plant growth and development, which makes a pool of reliable reference genes for use in RT-qPCR not only useful but also a necessary resource. To the best of our knowledge, no studies have been performed in *A. thaliana* investigating stability of reference genes over a range of temperatures and in different tissues.

Here, we report a systematic analysis of ten candidate reference genes of *A. thaliana*, in three different tissues (seedlings, rosette leaves, flower buds) exposed to five different temperatures (22°C, 27°C, 32°C, 37°C and 42°C). Candidate genes were selected after a literature review of commonly used *A. thaliana* reference genes and screening of publicly available microarray data obtained in experiments using *A. thaliana* tissues in conditions of non-optimal temperature. Expression stabilities of selected genes were estimated using four statistical algorithms (BestKeeper, geNorm, NormFinder and comparative ΔC_q method). Based on our results, we propose a set of reference genes for use in RT-qPCR gene expression analyses in different *A. thaliana* tissues after exposure to elevated temperatures.

MATERIAL AND METHODS

Plant material and experimental treatments

Seeds of wild-type *A. thaliana* (L.) Heynh ecotype Columbia-0 were washed in 70% ethanol, followed by surface-sterilization in 1% Izosan-G (Pliva, Zagreb, Croatia) and 0.1% Mucasol™ (Sigma-Aldrich, St. Louis, MO, USA). Seeds were rinsed in sterile water and plated on solid (0.7% w/v agar; Sigma-Aldrich) Murashige and Skoog (MS) growth medium (Murashige & Skoog 1962). Following stratification for 72 h at 4°C, plates were incubated at 24°C in long-day conditions (16-h light/8-h dark). After 12 days, seedlings were either immediately used for treatment or transferred to a mixture of white peat and perlite (Steckmedium KLASMANN; Klasmann-Deilmann, Geeste, Germany) and kept for 1 week at 24°C in short-day conditions (8-h light/16-h dark). Plants were then transferred to long-day conditions (16-h light/8-h dark) until they reached the age of 5 weeks. Temperature was maintained at 24°C and light intensity at 70 μmol $m^{-2}{\cdot}s^{-1}$. Previous analyses indicated that in our laboratory setting, light intensities higher than 100 μmol $m^{-2}{\cdot}s^{-1}$ cause accumulation of anthocyanins in *A. thaliana*, which commonly indicates that the plant is under light stress (Steyn *et al.* 2002). Therefore, we opted for a lower light intensity to ensure optimal growth. Temperature treatments were carried out in a plant growth chamber RK-500 CH (Kambič, Semič, Slovenia) 3 h after the start of the light period. Twelve-day-old seedlings on MS medium in sealed Petri dishes and 5-week-old plants in soil were incubated for 3 h at each of the five temperatures (22°C, 27°C, 32°C, 37°C and 42°C) at 70 μmol $m^{-2}{\cdot}s^{-1}$ light intensity. Ten seedlings, three rosette leaves (approximately 2 cm long from base to apex) from three individual plants and flower buds from six to 12 individual plants were pooled to comprise the 'seedling', 'leaf' and 'bud' samples, respectively. Two independent biological replicates were collected. Each sample of 'seedling', 'leaf' and 'bud' contained approximately 20, 80 and 20 mg of tissue, respectively. Plant samples were frozen in liquid nitrogen and stored at −80°C before RNA extraction.

Selection of candidate reference genes

Identification of candidate reference genes that have stable expression independent of ambient temperature was based on the method proposed in Czechowski *et al.* (2005). Publicly available Arabidopsis ATH1 Genome Array data were downloaded from NCBI GEO or EMBL-EBI ArrayExpress databases (Table S1). From ten experimental series, encompassing 334 arrays in total, only the data obtained from *A. thaliana* Columbia-0 ecotype exposed to different temperatures were retained for further analysis. Treatment temperatures ranged from 4 to 40°C and light intensity varied between 35 and 400 μmol $m^{-2}{\cdot}s^{-1}$ (Table S1). For all experimental series, downloaded array data (CEL files), each containing information on 22 746 probes representing approximately 24 000 gene sequences (Affymetrix 2012), were processed using RMAExpress software (Bolstad *et al.* 2003). The probe intensities from CEL files of individual experimental series were quantile normalized and, to each of the probe sets, the median polish summarization was applied. The delogged normalized data were used for further analysis. For each probe in the experimental series, mean value (MV), standard deviation (SD) and coefficient of variation (CV) were calculated. When available, the Detection Call information from MAS-processed data was used to filter probes that had a 'Present' call in more than 70% of the arrays of the given experimental series (retaining around 12 500 probes). From these, the top 5% highest expressed probes (by mean value) were removed. When Detection Call information was not available, the 5% highest and the 30% lowest expressed probes were removed from further analysis, retaining 14 785 probes. The remaining probes were sorted by their CV values in ascending order, and a list of 100 top-ranked genes (Top100) was compiled for each experimental series, resulting in ten Top100 lists (Dataset S1). Also, Top500 and Top1000 lists were assembled (data not shown). Some genes appeared in several Top100 lists and based on the number of their appearances, most prevalent genes were selected for further analysis. Additionally, available literature was searched for RT-qPCR reference genes adhering to the following criteria: they belonged to traditional reference genes ('housekeeping genes') and/or they had already been identified as potential reference genes for experiments employing specific temperatures in *A. thaliana* (Hong *et al.* 2010). Literature-identified genes were cross-compared with Top100 lists and based on the

number of their appearances in the 10 lists, they were selected for further analysis together with microarray-identified genes. The final ten candidate reference genes selected from Top100 lists underwent stability assessment and reference gene validation.

In addition, the expression data for ten candidate reference genes were extracted from existing *A. thaliana* transcriptomic data available through the Arabidopsis RNA-seq database (ARS) platform (Zhang *et al.* 2020). The analysis consisted of samples of seedlings, leaves and flowers in control conditions (16–23°C) and at elevated temperatures ranging from 25 to 45°C (Table S2). The expression levels were calculated as fragments per kilobase of transcript per million mapped reads (FPKM).

Primer design

Candidate reference gene sequences with designated exons, introns, 5′ and 3′ untranslated regions of all known splicing variants were retrieved from the Phytozome database version 12.1 (Goodstein *et al.* 2012). Gene-specific RT-qPCR primers were designed using PerlPrimer software (Marshall 2004) with set criteria for amplicon length of 100–200 bp, T_m of 59 ± 1°C and GC content of 40–60%. Furthermore, primers were designed to span an exon–exon junction. For genes with multiple transcripts, primers were designed to bind all known transcripts, with resulting amplicons of identical sequence and size. The obtained primer sequences were 20- to 22-nucleotides long. The specificity of each primer pair was verified using NCBI Blast against the *A. thaliana* transcript database. Primers were ordered from Macrogen (Macrogen Europe, Amsterdam, the Netherlands). Primer specificity in amplifying a single product was confirmed using agarose electrophoresis after 40 cycles of PCR. All primer sequences are listed in Table S3.

Total RNA extraction and cDNA synthesis

Nitrogen-frozen tissues were homogenized twice for 8 s in Silver Mix Mixer (GC 900548) at 50 Hz using SiLibeads Typ S. Total RNA was extracted using a MagMAx Plant RNA Isolation Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. RNA purity and concentrations were determined using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and visualized after electrophoresis on a 1% agarose gel in TAE buffer (40 M Tris-acetate, 10 mM EDTA, pH 8.0) and GelStar (Lonza, Basel, Switzerland) staining by UV light. An aliquot of 1 µg isolated RNA was used for reverse transcription reaction in a total volume of 20 µl using 200 U RevertAid H Minus Reverse Transcriptase, 1× Reaction Buffer (Thermo Scientific), 20 U RiboLock RNase inhibitor (Thermo Scientific), 0.5 mM dNTPs (Sigma-Aldrich) and 5 µM Oligo(dT)18 primer (Thermo Scientific). The reverse transcription reaction mix was incubated for 5 min at 65°C, 45 min at 42°C and 15 min at 70°C.

Analysis with qPCR

The PCR reactions intended for stability analysis of candidate reference genes were performed in an optical 96-well plate using an Applied Biosystems 7500 Fast Real-Time PCR system. SYBR Green was used to monitor dsDNA synthesis. The reaction mixture contained 1× GoTaq qPCR Master Mix reagent, 130 nM forward and reverse primers and 20 ng cDNA in a total volume of 15 µl. The thermal profile was as follows: 50°C for 20 s, 95°C for 10 min, 40 cycles of 95°C for 10 s, and 58°C for 40 s, except for *OGIO*, *PP2AA3* and *DWA1* genes where the annealing/extension step was carried out at 60 °C. The presence of a single amplicon was confirmed as a single peak in the melting curve obtained after amplification using the following parameters: 40–95°C with ramp speed of $1°C \cdot min^{-1}$. For each primer set, C_q values and primer efficiencies were calculated from raw amplification data in the exponential phase of each individual amplification plot using LinRegPCR software (Ramakers *et al.* 2003).

Reference genes which were consistently top-ranked (*OGIO*, *PUX7*, *UBC21*, *TIP41*) or lowest-ranked (*MON1*, *GLR2*) across all tissues and tissue combinations analysed in this work were validated in a *DREB2A* relative gene expression analysis. PCR reactions were performed in strip tubes using a MIC qPCR Cycler (Bio Molecular Systems) with reaction mixtures prepared as described previously. The thermal profile was as follows: 95°C for 5 min, 40 cycles of 95°C for 10 s, and 60°C for 10 s, and the presence of a single amplicon was confirmed as described previously. The primers used for *DREB2A* were previously published in Morimoto *et al.* (2017). For each primer set, C_q values and primer efficiencies were calculated with the MIC qPCR Cycler software (Bio Molecular Systems Queensland, Australia). When multiple genes were used for normalization, geometric mean values were applied. Relative expression profiles were generated using the method described in Vandesompele *et al.* (2002). The complete code and packages used in this analysis are available in Code S1.

Expression stability analysis

The expression stabilities of candidate reference genes were determined using four validation algorithms (BestKeeper, geNorm, NormFinder and comparative ΔC_q method), based on data obtained from two biological replicates. General descriptions of validation algorithms can be found in Methods S1. BestKeeper and comparative ΔC_q method assess the stabilities of candidate reference genes directly from raw C_q values, while geNorm and NormFinder use relative quantities (RQ) as input. For each replicate separately, RQ values of individual samples were calculated from C_q values and amplicon efficiencies (E), using $RQ = E^{Cq\ min - Cq\ (sample)}$, where $C_{q\ min}$ corresponds to the sample with lowest C_q value within a replicate.

All analyses were performed and graphs were generated in R software 4.0.0. (R Core Team 2020) and RStudio 1.3.959 (RStudio Team 2015). For each candidate reference gene, correlation coefficient (BestKeeper), M value (geNorm), stability value (NormFinder) and meanSD (comparative ΔC_q method) were calculated for individual tissue samples (seedling, leaf, bud), two-tissue combinations and all three tissues combined. The BestKeeper function included in the ctrlGene package (Zhong 2019) was used for calculation of descriptive statistics and the correlation coefficients (Pfaffl *et al.* 2004). geNorm analysis and calculation of M value was performed using functions read.qPCR and selectHKs implemented in packages ReadqPCR and NormqPCR (Perkins *et al.* 2012). NormFinder analysis and calculation of stability value was performed using *r*. NormOldStab5.txt file downloaded from https://moma.dk/normfindersoftware (Andersen *et al.* 2004). For NormFinder analysis, groups were constituted by temperature treatment

(five groups) and intra-group (GroupSD) and inter-group (GroupDiff) C_q variations were estimated. The complete code and packages used in this analysis are available in Code S1.

Consensus reference gene ranking list

The RankAggreg algorithm was used to generate a consensus ranking list of ten candidate reference genes based on the four ranking lists obtained from BestKeeper, geNorm, NormFinder and comparative ΔC_q method. The RankAggreg package was used to perform aggregation of ordered rank lists and standardized weights using the Cross-Entropy Monte Carlo algorithm. The complete code in this analysis is available in Code S1.

RESULTS

Selection of candidate reference genes

Expression profiling data (ATH1 Genome Array datasets) obtained on several tissues of *A. thaliana* exposed to different temperature treatments (4–40°C) (Table S1) were used to select candidate reference genes with minimum expression variation. Probes retrieved from each of the ten experimental series were sorted by coefficient of expression variation (CV), resulting in ten Top100 lists (Dataset S1). The criteria for selection of candidate reference genes from Top100 lists was the number of their appearances within all ten lists, with highest priority given to genes with the greatest number of appearances. Moreover, literature was reviewed for commonly used reference genes and 12 genes were selected. Of these 12, five were traditionally used reference genes (*ACT2*, *GAPDH*, *TUB6*, *UBC21* and *UBI10*) and seven were novel reference genes (*MON1*, *PP2AA3*, *PTB1*, *RHIP1*, *RPF3*, *TIP41* and *YLS8*), which have been introduced into literature within the last decade. Here, all novel reference genes were selected based on recommendations published in Hong *et al.* (2010). Expression stabilities of these 12 literature-identified genes were individually assessed within the Top100 lists obtained from microarray expression data. Again, the highest priority was given to genes with greatest number of appearances.

Seven genes in total appeared three times in Top100 lists: *AKR2B*, *AT3G45560*, *DWA1*, *OGIO*, *PUX7*, *TRAPPC6* and literature-identified *PP2AA3*. Of these genes, *DWA1*, *OGIO*, *PUX7*, *TRAPPC6* and *PP2AA3* were selected for further analysis. *AT3G45560* was not selected due to low expression, and *AKR2B* was discarded because its sequence similarity with *AT4G35450* (CDS identity 81.92%) did not allow primer design according to previously set criteria (specifically, encompassing an exon/exon junction). Next, 70 genes in total appeared two times in Top100 lists, and *GLR2* was selected as a candidate for further analysis. To perform this selection, we pooled and analysed microarray data from all experimental series and obtained an all-comprehensive list of most stable genes. Here, *GLR2* was positioned highest relative to the other 69 genes (data not shown). In addition to *PP2AA3,* four literature-identified genes, *MON1*, *RHIP1*, *TIP41* and *UBC21*, were selected for further analysis. Other literature identified genes were discarded for the following reasons. *RPF3* appeared on Top100 lists, but was discarded because it lacks introns, which prevented RT-qPCR primer design according to the aforementioned criteria. *ACT2*, *GAPDH*, *UBI10* and *YLS8* were among the top 5% highest expressed genes in all experimental series, while *PTB1* and *TUB6* never ranked within the top 1000 genes (sorted by CV). In all ten experimental series, *PTB1* and *TUB6* had an average CV of 23.5% and 19.3%, respectively. All ten selected candidate reference genes had an average CV below 15% in all experimental series. Although the number of appearances in Top100 lists was the main criterion for selection of candidate reference genes, we additionally assessed their number of appearances in Top500 lists. All selected genes and the numbers of their appearances in Top100/500 lists are provided in Table 1.

In addition, expression levels of candidate reference genes were evaluated *in silico* using RNA-seq data deposited in the ARS database (Zhang *et al.* 2020). The samples used were different *A. thaliana* tissues exposed to a wide range of temperatures (16–23°C in control samples and 25–45°C in treated samples). For all ten genes, expression was relatively stable between control and heat stress conditions (Fig. S1).

Expression variation of candidate reference genes

Ten candidate reference genes (*DWA1*, *OGIO*, *PUX7*, *TRAPPC6, GLR2, PP2AA3, MON1, RHIP1*, *TIP41* and *UBC21)* were evaluated for gene expression stability using RT-qPCR. Gene expression was assessed in three types of tissue: 12-day-old seedlings, as well as rosette leaves and flower buds of 5-week-old plants. Seedlings and plants were exposed to a range of elevated temperatures (22°C, 27°C, 32°C, 37°C and 42°C) for 3 h. The quality of total extracted RNA was verified before reverse transcription, with $A_{260/280}$ ratio of all RNA samples approximately 2 and the $A_{260/230}$ ratio 2.0–2.2. Also, electrophoresis showed distinct bands of 18S and 28S ribosomal RNA (Fig. S2). Successful primer design for the ten candidate reference genes was tested with RT-qPCR. The presence of a single peak in a melting curve was taken to indicate amplification of a single amplicon and absence of primer dimer formation (Fig. S3A). Furthermore, amplification of a qPCR product of expected size was confirmed with agarose gel electrophoresis (Fig. S3B). Two biological replicates (independent experiments) of each temperature-treated tissue were prepared (15 independent tissue samples per replicate) and two technical replicates were used in RT-qPCR. After averaging technical replicates, a dataset of 300 C_q values was obtained (Table S4).

Based on the analysis of average C_q values, *UBC21* was the gene with highest expression in all three individual tissues (seedling, leaf and bud) exposed to five different temperatures. Two genes with lowest expression in individual tissues were *GLR2* (seedling and bud) and *MON1* (leaf) (Fig. 1A). When examining all three tissues combined, average C_q values of candidate reference genes ranged from 21.95 to 25.82. Again, the gene with highest expression was *UBC21* (C_q mean = 21.95) and the least expressed gene was *GLR2* (C_q mean = 25.82) (Fig. 1B). Beside lowest expression, *GLR2* and *MON1* had the highest overall C_q value dispersion (Fig. 1B), indicating lower stability in different experimental conditions. The assessment of relevant RNA-seq data gave similar results (Fig. S1). Again, the least expressed gene was *GLR2*, while the top five highest expressed genes were the same as in RT-qPCR analysis but in a slightly altered order (*RHIP1*, *PP2AA3*, *PUX7*, *UBC21* and *OGIO*). Moreover, expression stability did not highly differ between RT-qPCR and RNA-Seq analysis, with exception of *GLR2*, which was surprisingly stable *in silico*.

Table 1. RT-qPCR candidate reference genes selected for evaluation of gene expression stability in *A. thaliana* exposed to elevated temperatures. The table lists each gene's symbol, accession number, name and function. Top100/500 denotes the number of times a gene appeared in lists of top 100 or top 500 most stable genes, as calculated from ten microarray experimental series (for details, see Table S1 and Dataset S1).

Gene symbol (Accession no.)[a]	Gene name	Function[a,b]	Top100/500
TRAPPC6 (At3g05000)	Transport protein particle (TRAPP) component 6	Endoplasmic reticulum to Golgi vesicle-mediated transport, pollen tube development, regulation of GTPase activity, response to abscisic acid	3/6
PUX7 (At1g14570)	Plant UBX domain-containing protein 7	Encodes a nuclear UBX-containing protein that can bridge ubiquitin to AtCDC48A	3/5
OGIO (At5g51880)	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Oxidoreductase activity	3/5
DWA1 (At2g19430)	DWD (DDB1-binding WD40 protein) hypersensitive to ABA 1	Gene silencing by RNA, negative regulation of ABA-activated signalling pathway, production of ta-siRNAs involved in RNA interference, protein ubiquitination	3/3
GLR2 (At2g17260)	Glutamate receptor 2	Cellular calcium ion homeostasis, response to light stimulus, stomatal movement	2/3
PP2AA3 (At1g13320)	Protein phosphatase 2A subunit A3	Subunit of protein phosphatase 2A (PP2A), regulation of phosphorylation	3/5
RHIP1 (At4g26410)	RGS1-HXK1 interacting protein 1	Cellular response to glucose stimulus, regulation of glucose-mediated signalling pathway	2/4
TIP41 (At4g34270)	TIP41-like family protein	TOR signalling, regulation of phosphoprotein phosphatase activity, signal transduction	1/4
MON1 (At2g28390)	Monensin sensitivity 1 - SAND family protein	Intracellular protein transport, late endosome to vacuole transport, plant organ development, vacuole organization	0/1
UBC21/PEX4 (At5g25760)	Ubiquitin-conjugating enzyme 21/ Peroxin 4	Fatty acid beta-oxidation, peroxisome organization, protein import into peroxisome matrix, protein ubiquitination	1/3

[a]The Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org/).
[b]The Universal Protein Resource (UniProt) database (https://www.uniprot.org/).

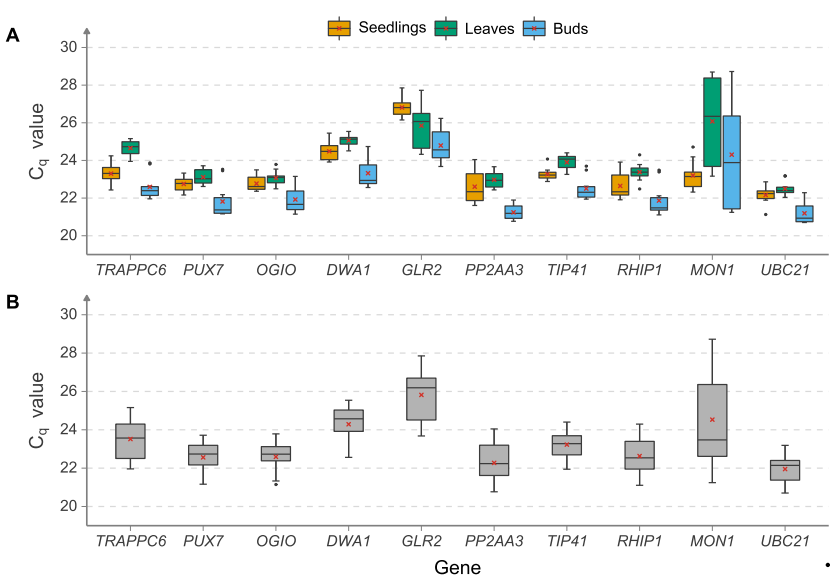

Fig. 1. Expression profiles of ten candidate reference genes in *A. thaliana* tissues exposed to elevated temperatures. Seedlings, leaves and flower buds were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and RT-qPCR analysis performed. C_q value variation was calculated for (A) samples of individual tissues (N = 10 per gene/tissue) and (B) samples of all tissues combined (N = 30 per gene). Boxes indicate 25th/75th percentiles, the horizontal line in the box represents median value and the vertical lines mark 5th and 95th percentiles. Outliers are marked with a dot and average values are marked with a red cross.

Evaluation of expression stability in temperature-treated tissues

In this experiment, three types of tissue (12-day-old seedlings, and rosette leaves and flower buds of 5-week-old plants) had been treated with five different temperatures (22°C, 27°C, 32°C, 37°C and 42°C). Stability of candidate reference genes relative to temperature treatment was assessed in all three tissues and their combinations.

To assess the expression stability of candidate reference genes in seedlings, leaves and buds of *A. thaliana* after

temperature treatments, the obtained C_q data were analysed with four validation algorithms: BestKeeper, geNorm, NormFinder and comparative ΔC_q method. The stability ranking of candidate reference genes differed between the four algorithms. However, even though the final ranking order was different, genes that were shown to be most stable by one algorithm also retained that characteristic in other algorithms. For example, in seedlings, *OGIO* was ranked third, first, second and third by BestKeeper, geNorm, NormFinder and comparative ΔC_q, respectively. Similarly, *TRAPPC6* ranked eighth, seventh, eighth and seventh, respectively (Table 2).

Analysis of C_q variation was performed in BestKeeper for individual tissues and all tissues combined. In seedlings, SD values of all candidate reference genes remained below 1 and the average CV value was lowest (1.9%) compared to that in leaves and buds (Table S5). Genes with an SD value larger than 1 are generally not considered suitable reference genes (Pfaffl *et al.* 2004). Here, two genes had SD values higher than 1,

Table 2. Stability ranking of ten candidate reference genes in *A. thaliana* tissues exposed to elevated temperatures. Seedlings, leaves and flower buds were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and RT-qPCR analysis performed. Genes were ranked according to expression stability values '*r*', 'meanM', 'Stability' and 'MeanSD', as implemented in BestKeeper, geNorm, NormFinder and comparative ΔC_q method, respectively. Comprehensive ranking derived from RankAggreg is based on individual rankings of the four algorithms. Data are shown for individual tissues and all tissues combined. For descriptions of expression stability values calculated by each algorithm, see Methods S1.

		BestKeeper		geNorm[a]		NormFinder		Comparative ΔC_q		Rank aggreg
Tissue	Rank	Gene	*r*	Gene	meanM	Gene	Stability	Gene	MeanSD	Gene
Seedlings	1	*DWA1*	0.980*	*OGIO/PUX7*	0.159	*DWA1*	0.10	*TIP41*	0.645	*OGIO*
	2	*MON1*	0.964*			*OGIO*	0.14	*PUX7*	0.672	*PUX7*
	3	*OGIO*	0.956*	*TIP41*	0.184	*PUX7*	0.16	*OGIO*	0.674	*DWA1*
	4	*RHIP1*	0.884*	*DWA1*	0.197	*UBC21*	0.18	*UBC21*	0.705	*TIP41*
	5	*GLR2*	0.875*	*UBC21*	0.228	*TIP41*	0.19	*GLR2*	0.723	*GLR2*
	6	*PUX7*	0.873*	*GLR2*	0.252	*GLR2*	0.23	*DWA1*	0.725	*UBC21*
	7	*TIP41*	0.828*	*TRAPPC6*	0.264	*RHIP1*	0.27	*TRAPPC6*	0.768	*RHIP1*
	8	*TRAPPC6*	0.792*	*RHIP1*	0.287	*TRAPPC6*	0.29	*RHIP1*	0.845	*MON1*
	9	*PP2AA3*	0.656*	*MON1*	0.306	*MON1*	0.30	*MON1*	0.901	*TRAPPC6*
	10	*UBC21*	0.516	*PP2AA3*	0.340	*PP2AA3*	0.40	*PP2AA3*	0.993	*PP2AA3*
Leaves	1	*PP2AA3*	0.882*	*UBC21/MON1*	0.167	*UBC21*	0.14	*DWA1*	0.789	*UBC21*
	2	*OGIO*	0.679*			*TIP41*	0.18	*OGIO*	0.815	*PUX7*
	3	*TRAPPC6*	0.570	*PUX7*	0.209	*PUX7*	0.19	*UBC21*	0.821	*OGIO*
	4	*RHIP1*	0.553	*OGIO*	0.283	*MON1*	0.22	*TRAPPC6*	0.824	*PP2AA3*
	5	*PUX7*	0.551	*TIP41*	0.316	*PP2AA3*	0.22	*PUX7*	0.826	*TIP41*
	6	*UBC21*	0.525	*PP2AA3*	0.337	*OGIO*	0.23	*TIP41*	0.829	*TRAPPC6*
	7	*TIP41*	0.428	*TRAPPC6*	0.353	*TRAPPC6*	0.25	*PP2AA3*	0.843	*MON1*
	8	*DWA1*	0.355	*DWA1*	0.367	*DWA1*	0.29	*RHIP1*	0.868	*DWA1*
	9	*MON1*	0.341	*RHIP1*	0.398	*RHIP1*	0.35	*GLR2*	1.383	*RHIP1*
	10	*GLR2*	0.084	*GLR2*	0.437	*GLR2*	0.41	*MON1*	2.414	*GLR2*
Buds	1	*RHIP1*	0.975*	*TIP41/UBC21*	0.182	*TIP41*	0.13	*PP2AA3*	1.055	*TIP41*
	2	*DWA1*	0.972*			*UBC21*	0.16	*UBC21*	1.123	*UBC21*
	3	*PUX7*	0.970*	*RHIP1*	0.235	*RHIP1*	0.20	*TIP41*	1.150	*RHIP1*
	4	*UBC21*	0.940*	*DWA1*	0.258	*TRAPPC*	0.21	*TRAPPC6*	1.186	*DWA1*
	5	*TIP41*	0.922*	*TRAPPC6*	0.281	*DWA1*	0.23	*OGIO*	1.195	*TRAPPC6*
	6	*MON1*	0.881*	*PUX7*	0.299	*OGIO*	0.32	*DWA1*	1.242	*PUX7*
	7	*PP2AA3*	0.870*	*MON1*	0.318	*MON1*	0.33	*GLR2*	1.282	*PP2AA3*
	8	*TRAPPC6*	0.868*	*PP2AA3*	0.352	*PUX7*	0.33	*RHIP1*	1.294	*OGIO*
	9	*OGIO*	0.851*	*OGIO*	0.382	*PP2AA3*	0.34	*PUX7*	1.328	*MON1*
	10	*GLR2*	0.571	*GLR2*	0.430	*GLR2*	0.37	*MON1*	3.073	*GLR2*
All tissues	1	*DWA1*	0.959*	*DWA1/PUX7*	0.271	*TIP41*	0.13	*OGIO*	1.306	*TIP41*
	2	*RHIP1*	0.938*			*UBC21*	0.14	*UBC21*	1.319	*UBC21*
	3	*PUX7*	0.938*	*TIP41*	0.295	*PUX7*	0.15	*TIP41*	1.324	*RHIP1*
	4	*TIP41*	0.919*	*UBC21*	0.310	*DWA1*	0.16	*PUX7*	1.368	*DWA1*
	5	*OGIO*	0.914*	*TRAPPC6*	0.327	*TRAPPC6*	0.18	*DWA1*	1.423	*TRAPPC6*
	6	*TRAPPC6*	0.893*	*OGIO*	0.352	*OGIO*	0.18	*RHIP1*	1.426	*PUX7*
	7	*UBC21*	0.879*	*RHIP1*	0.365	*MON1*	0.19	*PP2AA3*	1.452	*PP2AA3*
	8	*PP2AA3*	0.827*	*MON1*	0.379	*PP2AA3*	0.20	*TRAPCC6*	1.490	*OGIO*
	9	*MON1*	0.609*	*PP2AA3*	0.394	*RHIP1*	0.22	*GLR2*	1.612	*MON1*
	10	*GLR2*	0.499*	*GLR2*	0.424	*GLR2*	0.22	*MON1*	2.687	*GLR2*

[a]This version of geNorm does not differentiate between the top two positions within a ranking.
*p-value < 0.05.

GLR2 and *MON1* in leaves and in all three tissues combined, and *MON1* in buds. However, because elimination of these genes did not significantly alter the BestKeeper ranking order (data not shown), *GLR2* and *MON1* were not removed from the analysis.

The M values of all evaluated candidate reference genes calculated by geNorm were below the threshold value of 0.5 (Hellemans *et al.* 2007), both in individual tissues and in tissue combinations (Table 2, Table S6). This indicates that, according to geNorm, all selected genes are of adequate stability and are therefore suitable for use as reference genes. The version of geNorm used in this work outputs a stability ranking list in which two genes share the position of the most stable gene. Therefore, according to geNorm, most stable genes were *OGIO*/*PUX7* (0.159) in seedlings, *UBC21*/*MON1* (0.167) in leaves and *TIP41*/*UBC21* (0.182) in buds. In all three tissues combined, *DWA1*/*PUX7* (0.271) was considered most stable. Furthermore, geNorm pairwise variation analysis was used to assess the number of reference genes necessary for accurate RT-qPCR analysis. One of the analysis outputs was the pairwise variation value ($V_{2/3}$) between the normalization factors calculated for the first two (NF_2) and three (NF_3) genes in a geNorm ranking (in individual tissues and all three tissues combined). In all cases, the $V_{2/3}$ values were below the threshold value of 0.15, indicating that the use of geometric means of two top-ranked reference genes would be as sufficient for data normalization as the geometric mean of three or more genes (Fig. 2).

NormFinder uses an inter-group variation to circumvent the effect of co-regulated genes on stability evaluation. In our case, groups were constituted by temperature (five groups). The most stable gene was *DWA1* (0.10) in seedlings, *UBC21* (0.14) in leaves and *TIP41* (0.13) in buds (Table 2). In all tissues combined, *TIP41* (0.13) was considered most stable by NormFinder (Table S6). Moreover, all top-ranked genes also showed the lowest inter-group variation (Table S7).

When all genes were compared against one another using the comparative ΔC_q method and then ranked by mean SD values, the most stable gene was *TIP41* (0.645) in seedlings, *DWA1* (0.789) in leaves and *PP2AA3* (1.055) in buds (Table 2). In all three tissues combined, the most stable gene was *OGIO* (1.306). Based on this ranking, significant ΔC_q deviation was present for *MON1* and *PP2AA3* in seedlings, *MON1* and *GLR2* in leaves, *MON1* and *PUX7* in buds and *GLR2* and *MON1* in all tissues combined (Fig. S4), implying lower expression stability of these genes in the indicated tissues.

Comprehensive ranking for each individual tissue (seedling, leaf and bud) and tissue combinations was obtained with RankAggreg. In this ranking, the most stable candidate reference genes in seedlings were *OGIO* and *PUX7*. This is in accordance with the rankings obtained using individual algorithms, where *OGIO* appeared four times and *PUX7* three times among the top three most stable genes. Although *PUX7* was ranked in sixth place in the BestKeeper analysis, its SD value (0.34) was still one of the lowest of all SD values in the BestKeeper analysis. In the RankAggreg ranking, the most stable candidate reference genes in leaves were *UBC21* and *PUX7*. In individual algorithms, *UBC21* and *PUX7* appeared three and two times in the top three positions, respectively. In buds, the most stable genes according to RankAggreg were *TIP41* and *UBC21*. Both genes appeared three times in the top three positions of the four algorithms. In the ranking obtained using data from all three tissues combined, *TIP41* and *UBC21* were genes with the highest stability (Table 2).

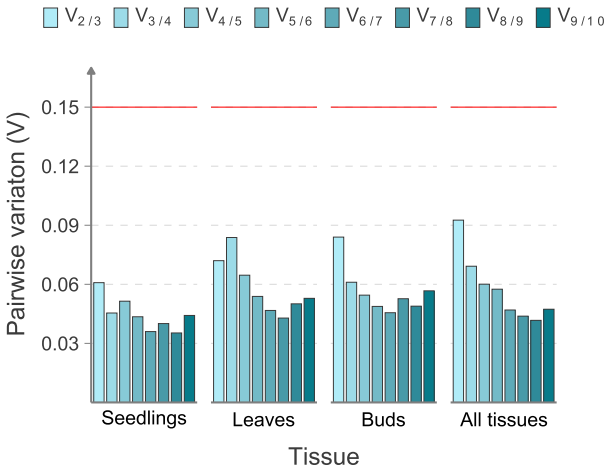

Fig. 2. Pairwise variation ($V_{n/n+1}$) analysis between normalization factors NF_n and NF_{n+1} (n = number of genes used to calculate NF). Seedlings, leaves and flower buds of *A. thaliana* were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and RT-qPCR analysis performed. Ten candidate reference genes were ranked by expression stability in geNorm for each individual tissue and all three tissues combined (see Table 2). Pairwise variation analysis illustrates the optimal number of consecutively ranked reference genes needed for accurate normalization of RT-qPCR data. Gene groups with pairwise variation values below 0.15 (horizontal red line) are sufficient for normalization. Data are shown for individual tissues and all tissues combined.

In addition, the stabilities of candidate reference genes were analysed in two-tissue combinations (seedlings and leaves, seedlings and buds, and leaves and buds). In all two-tissue combinations, *UBC21* and *GLR2* were the highest and the lowest expressed gene, respectively (Table S5). C_q value variations of candidate reference genes remained low even in different two-tissue combinations, with the majority of the genes having an SD value below 1. The two exceptions were *MON1* in all two-tissue combinations and *TRAPPC6* in the combination of leaves and buds (Table S5). In the RankAggreg analysis, *PUX7* and *UBC21* were the most stable genes in seedlings and leaves, *DWA1* and *TIP41* in seedlings and buds, and *UBC21* and *TIP41* in leaves and buds (Table S6). Pairwise variation values of V2/3 calculated by geNorm were less than 0.15. This indicates that for accurate normalization, the use of two top-ranked genes would be sufficient for all two-tissue combinations (Fig. S5).

Validation of selected reference genes

To evaluate the reliability of selected reference genes, we used single or multiple reference genes to normalize the expression of a heat-inducible gene, *DREB2A*. Relative expression levels of *DREB2A* upon temperature treatment were evaluated in individual tissues (seedling, leaf and bud) and all three tissues combined. Both for individual tissue analysis and group tissue analysis, data normalization was performed using the two respective top-ranked and one lowest-ranked reference gene according to Table 2. The heat-induced rise in expression levels of *DREB2A* was most prominent during the most intense

temperature treatment (42°C) in all three tissues and was higher in seedlings than in leaves and buds of *A. thaliana* (Figs 3 and 4). When either of the two top-ranked reference genes was used to normalize the data (*OGIO* or *PUX7* for seedlings, *UBC21* or *PUX7* for leaves and *TIP41* or *UBC21* for buds), the resulting expression levels of *DREB2A* were similar. However, using lowest-ranked reference genes for data normalization (*MON1* for seedlings and *GLR2* for leaves and buds) resulted in a deviation of *DREB2A* gene expression levels, compared to levels reported using top-ranked genes (Fig. 3A–C).

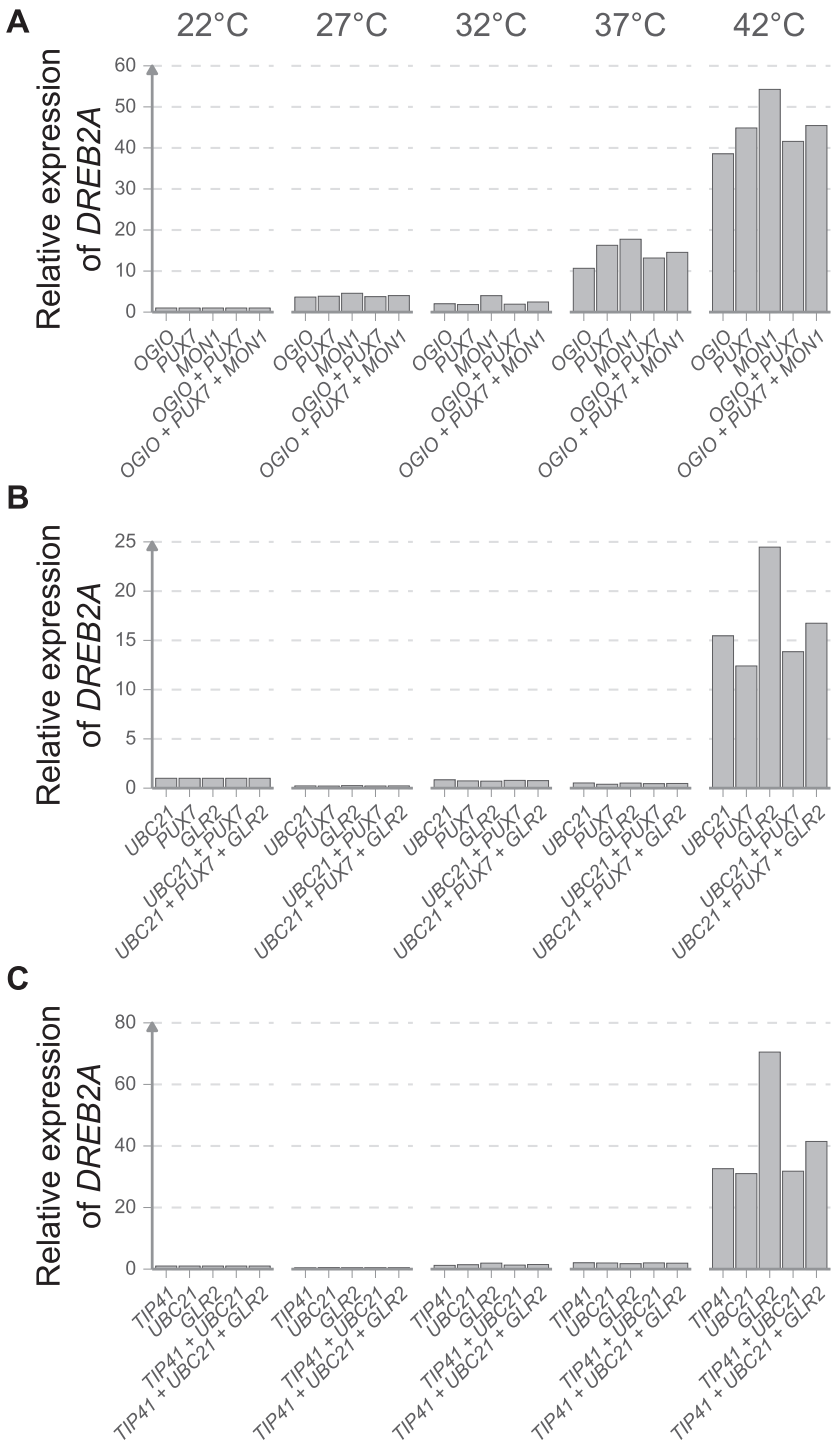

Fig. 3. Validation of selected reference genes in individual *A. thaliana* tissues exposed to elevated temperatures. Seedlings, leaves and flower buds were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and expression levels of *DREB2A* were calculated using single or multiple reference genes for normalization. *DREB2A* expression was normalized for (A) seedlings, using top-ranked genes *OGIO* and *PUX7* and low-ranked *MON1*, (B) leaves, using top-ranked genes *UBC21* and *PUX7* and low-ranked *GLR2* and (C) buds, using top-ranked genes *TIP41* and *UBC21* and low-ranked *GLR2*. For each tissue, *DREB2A* expression was calibrated to its expression in sample '22°C'. Results of one independent experiment are shown.

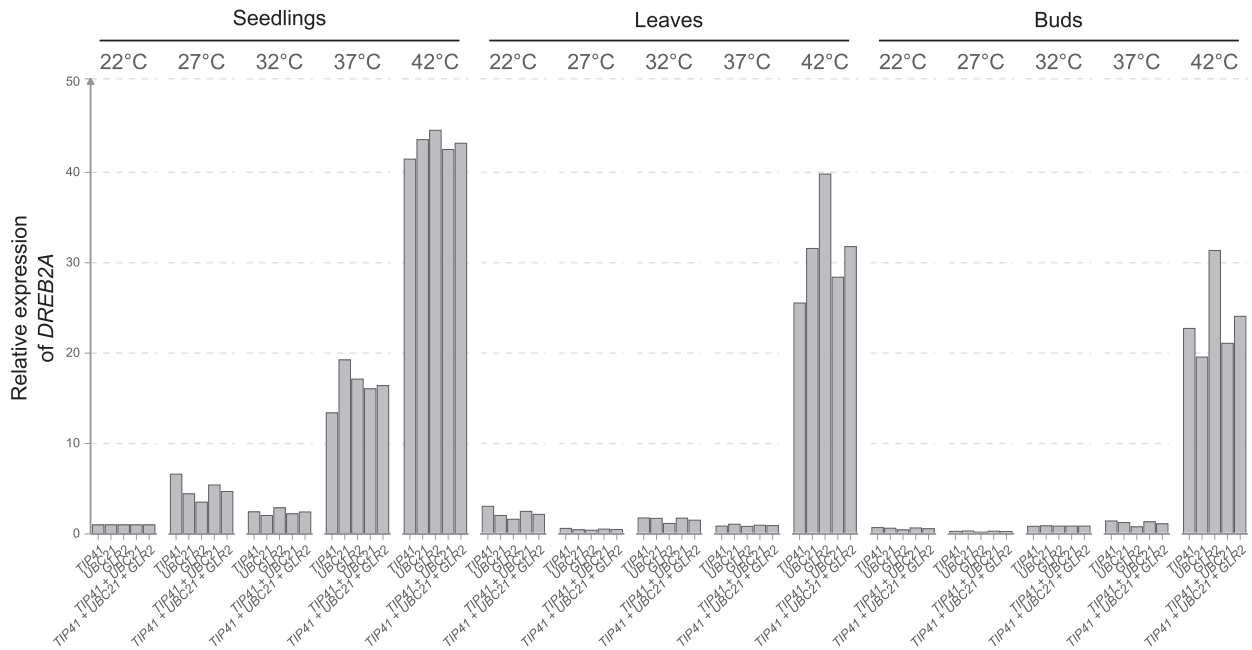

Fig. 4. Validation of selected reference genes in a group analysis of combined *A. thaliana* tissues exposed to elevated temperatures. Seedlings, leaves and flower buds were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and expression levels of *DREB2A* were calculated using single or multiple reference genes for normalization. *DREB2A* expression was normalized in a group analysis using two top-ranked genes, *TIP41* and *UBC21,* and one low-ranked gene, *GLR2*. Expression of *DREB2A* was calibrated to its expression in sample 'seedlings at 22°C'. Results of one independent experiment are shown.

This deviation was most striking in leaves and buds, where tenth-ranked *GLR2* was used. For example, in buds at 42°C, relative gene expression level of *DREB2A* was 31.02 and 32.6 when normalized with *TIP41* and *UBC21*, respectively, and 70.5 when normalized with *GLR2* (Fig. 3C). The deviation in *DREB2A* expression levels was somewhat less apparent in seedlings, where *MON1* was used as representative of a low-ranked gene. This might be accounted for by the relatively high stability values of *MON1*, which ranked eight but still had similar values to those of the first-ranked *OGIO*. This was especially evident in the BestKeeper analysis, where both *MON1* and *OGIO* had a high Pearson correlation coefficient ($r > 0.9$). For comparison, in buds, the *r*-value of the first-ranked reference gene *TIP41* was 0.922 and the last-ranked gene *GLR2* was 0.571. A similar pattern of *DREB2A* expression was observed when group analysis was performed for all three tissues combined (Fig. 4). Here, the two top-ranked genes were *TIP41* and *UBC21* and the lowest-ranked gene was *GLR2*. Again, relative expression levels of *DREB2A* remained comparable when *TIP41* and *UBC21* were used for data normalization and deviated when *GLR2* was used.

As stated previously, geNorm analysis indicated that two reference genes were sufficient for data normalization in seedlings, leaves and buds of *A. thaliana* treated with elevated temperatures. When geometric mean data of the two most stable genes were used for normalization of *DREB2A* expression data, relative gene expression levels were more accurately represented than when only a single gene was used. This was evident for all tissues and tissue combinations analysed. However, the provided correction was subtle, probably due to both genes being of similarly high stability. The importance of using multiple reference genes and the corrective effect of highly stable genes was more apparent in combination with the least stable genes. When the low-ranked gene was individually used for normalization, *DREB2A* expression appeared artificially enhanced, but when it was used in combination with top-ranked genes, this deviation was largely corrected, with expression levels pulled back toward the range provided by top-ranked genes (Figs 3 and 4).

DISCUSSION

Temperature is one of the most important environmental factors affecting the rate of plant development, growth and productivity (Hatfield & Prueger 2015). It significantly impacts the rates of photosynthesis and respiration, which in turn influence biomass yield and seed ripening, consequently affecting seed weight and germination. On a subcellular level, changes in environmental temperature greatly influence gene expression, metabolite synthesis or degradation and can shift the plant's entire developmental programme (Lawlor 2005; Pyl *et al.* 2012; Posch *et al.* 2019). The currently accepted global climate change model predicts warmer temperatures overall and more intense temperature extremes in the upcoming years (Meehl *et al.* 2007). In the light of these environmental changes and their effects on plant growth and development, the scientific community has increased their attention on molecular mechanisms which enable plants to recognize stressful events and generate an appropriate stress response. Large numbers of these studies include expression analyses of genes encoding potential regulators and other major players in the molecular cascades of the stress response. The method of choice for medium-scale

gene expression analyses is RT-qPCR, which offers both speed and sensitivity. However, the reliability of RT-qPCR results largely depends on correct implementation of the method which, among other procedural requirements, demands the use of suitable reference genes. Inaccurate normalization of RT-qPCR data can cause misinterpretation of gene expression results, which can then lead to false conclusions and mislead the work of biologists, breeders, geneticists, agronomists and climate scientists developing strategies to cope with a rapidly changing environment.

Detailed validation studies proposing reference genes for use in RT-qPCR experiments which employ different temperature treatments and tissue types are available for numerous plant species (Paolacci *et al.* 2009; Janská *et al.* 2013; Xu *et al.* 2015; Gao *et al.* 2017; Jin *et al.* 2019; Liu *et al.* 2020). However, they are essentially non-existent for *A. thaliana*, despite its cult status in molecular biology. Czechowski *et al.* (2005) completed the first detailed study based on microarray data, in which several reference genes suitable for use in RT-qPCR experiments on *A. thaliana* are identified and validated. The study includes data from different development stages, abiotic and biotic stresses, hormone stress and nutrient stress (Czechowski *et al.* 2005). This was followed by another extensive validation study which, among other experimental series, includes data on seedlings exposed to 16°C and 23°C (Hong *et al.* 2010). In both papers, the results are based on a single tissue and no more than two temperatures (Hong *et al.* 2010), or cold (4°C), but not heat stress (Czechowski *et al.* 2005). Moreover, in Czechowski *et al.* (2005), to validate reference gene stability, cold stress samples were pooled together with 19 other samples obtained from different plant tissues, stresses or growth conditions, and no reference genes were proposed specifically for cold treatment. A relatively recent RNA-seq analysis of *A. thaliana* gene expression profiles across different organs and different abiotic stresses also provides a list of stably expressed genes; however, this analysis is based on more than 80 tissue and treatment samples, of which temperature treatment includes only leaves incubated at 4°C or 42°C (Klepikova *et al.* 2016).

Here, we propose for the first time a set of reference genes for use in RT-qPCR gene expression analysis in different *A. thaliana* tissues (seedlings, rosette leaves and flower buds) exposed to a range of elevated temperatures (22°C, 27°C, 32°C, 37°C and 42°C). Candidate reference genes were selected after an exhaustive literature analysis and from microarray data obtained in different *A. thaliana* tissues exposed to a range of temperatures, from 4°C to 40°C. Because gene rankings obtained after microarray data analysis depend on experimental conditions (databases) used (Guenin *et al.* 2009), candidate reference genes selected in this way can introduce inconsistencies if applied in different experimental contexts. However, microarray data certainly contribute to narrowing down a pool of potential reference genes specific for a given set of conditions, which can then be validated employing standard RT-qPCR reference gene validation methods (Czechowski *et al.* 2005). Such practice was applied in this work, with particular attention given to the selection process. Using available microarray data, we first ranked genes based on their expression stability under varying temperature conditions, expressed as coefficients of gene expression variation (CV values). Next, we removed genes with low transcript abundance (30% lowest expressed genes) because they generally show higher variation (Fan *et al.* 2009) and are difficult to accurately detect and quantify using microarray data (Shendure 2008). Further, we removed genes with high expression (5% highest expressed genes) because large discrepancies in transcript abundances between reference and target gene can lead to false data interpretation (Hruz *et al.* 2011). Finally, ten lists of the 100 most stable genes were obtained. Six genes were selected from these lists, namely *DWA1*, *OGIO*, *PP2AA3, PUX7*, *TRAPPC6* and *GLR2,* all having the highest numbers of appearances in the Top100 lists. In addition 12 commonly used reference genes were identified from screened literature and, with consideration of their ranking within the Top100 lists, four genes were selected: *MON1, RHIP1*, *TIP41* and a traditional reference gene, *UBC21*. Traditional reference genes should not be *a priori* rejected, as some have been confirmed as stable in certain conditions, such as *GAPDH* in seedlings of *Arabidopsis pumila* exposed to 40°C and 4°C, and *TUBα* and *EF1α* in seedlings of strawberry exposed to 38°C and 4°C, respectively (Jin *et al.* 2019; Liu *et al.* 2020). Because of the scrutiny of the selection process, all ten selected genes had high probability of being validated as stable reference genes in conditions of non-optimal temperature. Moreover, *MON1, PP2AA3*, *RHIP1* and *TIP41* are proposed as stable in the analysis comprising data from more than one tissue, namely rosette leaves and flowers (Hong *et al.* 2010), which implied stability of these genes not only across different temperatures but also across different temperature-treated tissues. Out of the ten analysed candidate reference genes, five appear on stability rankings provided by Klepikova *et al.* (2016) in an RNA-seq analysis, namely *TIP41*, *MON1*, *PP2AA3*, *PUX7* and *UBC21*. This analysis, however, did not include temperature-treated tissues other than leaves at 42°C, but did include various other development stages exposed to different abiotic stresses (Klepikova *et al.* 2016).

Here, the recommended gene combinations for use in gene expression normalization of RT-qPCR data in experiments employing elevated temperature treatments were as follows: *OGIO* and *PUX7* in seedlings, *UBC21* and *PUX7* in leaves, *TIP41* and *UBC21* in flower buds, and *TIP41* and *UBC21* in all three tissues combined. Interestingly, *UBC21*, a traditional reference gene, was shown as the optimal reference gene in three out of four scenarios. This indicates that traditional reference genes still hold high potential for use in normalization of RT-qPCR data. In two out of four scenarios, namely in buds and all three tissues combined, *TIP41* was recommended as an optimal reference gene. *TIP41* has been more frequently used in recent years (Gentric *et al.* 2020; Škiljaica *et al.* 2020) and was shown to be stable in different tissues and under various abiotic stresses (Czechowski *et al.* 2005; Hong *et al.* 2010). Furthermore, two microarray selected genes, *OGIO* and *PUX7,* were recommended as optimal reference genes in seedlings. *PUX7* was additionally recommended in leaves, and both genes ranked highly for all three tissues combined.

We also assessed expression levels and stability of candidate reference genes *in silico* using available RNA-seq data. The order of genes by expression level and expression stability was comparable for both *in vitro* and *in silico* analysis. An interesting exception was *GLR2*, which showed higher stability *in silico* but was found to be the least stable gene in experimental conditions described here and was also predicted to be among the less stable genes by the microarray data analysis. The majority of the RNA-seq samples were from seedlings (57%), where

GLR2 was experimentally shown to be most stable, and the minority were from inflorescences (5%), where *GLR2* was experimentally shown to be least stable, which might have caused the discrepancy in the expected and confirmed expression stability. Nevertheless, this additionally emphasizes the need for experimental validation of each reference gene under a given set of conditions and laboratory settings, despite high performance in a theoretical analysis.

To test the validity of selected reference genes, we conducted a relative gene expression analysis of *DREB2A,* a heat-inducible gene with a well-known pattern of gene expression induction upon temperature treatment (Morimoto *et al.* 2013). Expression of *DREB2A* was analysed in different *A. thaliana* tissues (seedling, rosette leaf and flower bud) exposed to a range of elevated temperatures (22°C, 27°C, 32°C, 37°C and 42°C). To normalize the data, we used top-ranked and lowest-ranked reference genes and then compared the outcomes. While normalization with top-ranked genes produced similar results across all tissues and tissue combinations analysed, the use of low-ranking genes reported higher *DREB2A* relative gene expression. The overestimation of *DREB2A* expression was largely corrected when top-ranked genes were combined with the low-ranking genes for normalization. Therefore, although researchers should in all cases aim to select the most stable reference genes, using multiple reference genes can indeed mitigate errors introduced by a poorly selected reference gene. This could prove to be especially useful in experiments where more than one treatment and/or environmental condition is investigated, or in field research where multiple conditions naturally fluctuate throughout the course of a single experiment. Selection of several reference genes would at least partially extenuate possible destabilizing effects of a multivariate experiment. Although the MIQE guidelines suggest the use of three reference genes when performing RT-qPCR gene expression analysis (Bustin *et al.* 2009), our results, obtained with the geNorm algorithm indicate that using two stable reference genes would be sufficient for accurate data normalization in an experiment employing elevated temperature treatments.

Although this work was focused on validation of reference genes in conditions of elevated temperature (22–42°C), for the selection of candidate reference genes, we have used microarray data obtained from experiments using both low and elevated temperature treatments. Therefore, the selected candidate reference genes could serve as a starting pool for validation of stable reference genes in conditions of decreased temperature. For instance, *MON1, RHIP1* and *TIP41* were the top three stable reference genes in a dataset retrieved from experiments employing, among others, cold stress (Czechowski *et al.* 2005), which indicates their likelihood of being validated as suitable reference genes for normalization of RT-qPCR data following low temperature treatments.

Moreover, orthologues of the ten reference genes validated in this work could serve as candidate reference genes in other plant species. This has already been demonstrated in seeds of *A. thaliana* and *Solanum lycopersicum*, where six out of seven reference genes previously confirmed as stable in *A. thaliana* were also stable in *S. lycopersicum* (Dekkers *et al.* 2012). Similarly, in different development stages and in the cambial region of *A. thaliana* and hybrid aspen, two out of four reference genes considered stable in *A. thaliana* were also found to be stable in hybrid aspen (Gutierrez *et al.* 2008).

In conclusion, reference genes validated for use in *A. thaliana* RT-qPCR experiments employing non-optimal temperatures could greatly contribute to the fast-growing field studying the adaptive capabilities of plants facing temperature extremes and the effects of climate change on their growth and development. Although no reference gene is universal (Hruz *et al.* 2011) and the presence of reference gene orthologues in another species does not automatically imply their stability in that species, available lists of temperature-stable reference genes in the model plant *A. thaliana* can offer a good starting point for researchers investigating plants for which there are only limited available microarray data, many of which belong to economically important crop species.

AUTHOR CONTRIBUTIONS

NB conceived the study and together with DLL, supervised the research. LM designed the experiments, performed RNA-seq analysis, reference gene selection and data analysis. TV performed plant growth, treatments and sampling. MJ performed RNA isolation and RT. AŠ designed primers, performed qPCR and LinReg analysis. LM and AŠ wrote the article with input from all authors. All authors have read and approved the manuscript.

ACKNOWLEDGEMENTS

This work was funded by the Croatian Science Foundation (grant IP-2016-06-6229) and European Regional Development Fund (grant KK.05.1.1.02.0005).

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Publicly available microarray data (Affymetrix Arabidopsis ATH1 Genome Array) screened for selection of candidate reference genes for use in RT-qPCR-based gene expression analyses of tissues exposed to non-optimal temperatures. Experiment accession numbers and sample IDs retrieved from the NCBI GEO or EMBL-EBI ArrayExpress databases and the number of arrays. For each experimental series, developmental stage and treatment condition (temperature treatment and treatment duration) are provided. Plants used in all experimental series were Columbia-0 wild type unless otherwise stated. The databases were accessed and data downloaded on 12–15 April 2019.

Table S2. The sample information of temperature-treated tissues with publicly available RNA-seq data used for analysis of expression stability of candidate reference genes. Listed are project and sample accession numbers, as well as treatment conditions (temperature and treatment duration) and tissue type. Plants used in all experimental series were Columbia-0 wild type. The data were accessed and downloaded from the Arabidopsis RNA-seq database (Zhang *et al.* 2020).

Table S3. Primer sequences for candidate reference genes and *DREB2A* with average primer efficiency, amplicon size in base pairs (bp) and number of known transcript variants.

Table S4. C_q values of ten candidate reference genes in *A. thaliana* tissues exposed to elevated temperatures. Seedlings,

leaves and flower buds of *A. thaliana* were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and RT-qPCR analysis performed. Raw amplification data were analysed in LinReg software and C_q values of two technical replicates per sample were averaged. Results of two independent experiments (biological replicates R1 and R2) are shown.

Table S5. Descriptive statistics of C_q values of ten candidate reference genes in *A. thaliana* tissues exposed to elevated temperatures. Seedlings, leaves and flower buds of *A. thaliana* were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and RT-qPCR analysis performed. Standard deviation (SD) and coefficient of variation (CV) of C_q values were calculated in BestKeeper. Data are shown for individual tissues, all tissues combined and two-tissue combinations.

Table S6. Stability ranking of ten candidate reference genes in two-tissue combinations of *A. thaliana* tissues exposed to elevated temperatures. Seedlings, leaves and flower buds of *A. thaliana* were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and RT-qPCR analysis performed. Genes were ranked according to expression stability values '*r*', 'meanM', 'Stability' and 'MeanSD', as implemented in BestKeeper, geNorm, NormFinder and comparative ΔC_q method, respectively. Asterisks denote probability value calculated by BestKeeper, $P < 0.05$. Comprehensive ranking derived from RankAggreg is based on rankings of the four individual algorithms. Data are shown for two-tissue combinations. For descriptions of expression stability values calculated by each algorithm see Methods S1.

Table S7. Intra-group (GroupSD) and inter-group (GroupDiff) C_q variation estimates of ten candidate reference genes in *A. thaliana* tissues exposed to elevated temperatures. Seedlings, leaves and buds of *A. thaliana* were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and RT-qPCR analysis performed. C_q variation estimate analysis was performed in NormFinder, with groups formed by temperature treatment. Data are shown for individual tissues, all tissues combined and two-tissue combinations.

Method S1. Descriptive assessment of validation algorithms.

Fig. S1. Expression levels of ten candidate reference genes obtained using *A. thaliana* transcriptomic data available through the Arabidopsis RNA-seq database (Zhang *et al.* 2020). Expression data include samples of seedlings, leaves and flowers in control conditions (16–23°C) and at elevated temperatures ranging from 25 to 45°C (Table S2). Expression level values are indicated as fragments per kilobase of transcripts per million mapped reads (FPKM). Boxes indicate the 25th/75th percentiles; the horizontal line in the box represents median value and the vertical lines mark 5th and 95th percentiles. Average values are marked with a red cross.

Fig. S2. Quality assessment of RNA extracted from *A. thaliana* tissues exposed to elevated temperatures. Twelve-day-old seedlings, and rosette leaves and flower buds from 5-week-old plants of *A. thaliana* were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) followed by RNA extraction. Integrity of RNA was confirmed by agarose gel electrophoresis and RNA purity was assessed by determination of $A_{260/280}$ and $A_{260/230}$ ratios. Results of two independent experiments (R1 and R2) are shown.

Fig. S3. Specificity of RT–qPCR amplification. (A) Dissociation curves of ten candidate reference genes showing individual peaks, based on two technical replicates of 15 cDNAs from three different tissues and five temperature treatments of *A. thaliana*. (B) Agarose gel showing single qPCR products of ten candidate reference genes, with expected size (bp) denoted above bands.

Fig. S4. ΔC_q dispersion in pairwise comparisons of ten candidate reference genes from *A. thaliana* tissues exposed to elevated temperatures. ΔC_q dispersion was calculated by comparative ΔC_q method in (A) seedlings, (B) leaves, (C) buds, (D) all tissues (E) seedlings and leaves, (F) seedlings and buds and (G) leaves and buds. Boxes indicate $25^{th}/75^{th}$ percentiles, the horizontal line in the box represents median value and the vertical lines mark 5^{th} and 95^{th} percentiles. Average values are marked with a black cross

Fig. S5. Pairwise variation ($V_{n/n+1}$) analysis between normalization factors NF_n and NF_{n+1} (n = number of genes used to calculate NF). Seedlings, leaves and flower buds of *A. thaliana* were incubated for 3 h at different temperatures (22°C, 27°C, 32°C, 37°C and 42°C) and RT-qPCR analysis was performed. Ten candidate reference genes were ranked by expression stability in geNorm for two-tissue combinations (see Table S6). Pairwise variation analysis illustrates the optimal number of consecutively ranked reference genes needed for accurate normalization of RT-qPCR data. Gene groups with pairwise variation values below 0.15 (horizontal red line) are sufficient for normalization. Data are shown for two-tissue combinations.

Code S1. The complete code used for selection and validation of reference genes used for RT-qPCR gene expression analysis in *A. thaliana* exposed to elevated temperatures. Data used for the analysis are available at https://github.com/Edlenil/Reference-genes.

Dataset S1. Top 100 genes retrieved from microarray data ranked by expression stability. Supports Table 1. Listed are probe numbers corresponding to Affymetrix ATH1 Genome Array, accession numbers and gene descriptions. For each gene, mean value (MV), standard deviation (SD) and coefficient of variation (CV) are listed. When available, present call percentage (Present %) from detection call data is listed. The list is sorted and ranked by CV. Microarray experimental series' names correspond to Table S1. Literature-selected genes are in blue. Genes that appear three or two times in the Top100 lists are in yellow or green, respectively.

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