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EXPLORING VALID REFERENCE GENES FOR QUANTITATIVE REAL-TIME RT-PCR STUDIES OF HYDROGEN PEROXIDE SIGNALING IN *ARABIDOPSIS*

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Abstract

Hydrogen peroxide (H_2O_2) acts as a signaling molecule modulating the expression of various genes in plants. However, the reference gene(s) used for gene expression analysis of H_2O_2 signaling is still arbitrary. A reliable result obtained by quantitative real-time RT-PCR (RT-qPCR) highly depends on accurate transcript normalization using stably expressed reference genes, whereas the inaccurate normalization could easily lead to the false conclusions. In this report, by using geNorm and NormFinder algorithms, 12 candidate reference genes were evaluated and compared in root and shoot tissues of *Arabidopsis* upon different doses of H_2O_2. The results revealed that, in our experimental conditions, three novel reference genes (*TIP41-like*, *UKN*, and *UBC21*) were identified and validated as suitable reference genes for RT-qPCR normalization in both root and shoot tissues under oxidative stress. This conclusion was further confirmed by publicly available microarray data of methyl viologen and drought stress. In comparison with a single reference gene (*EF-1a*), the expression pattern of *ZAT12* modulated by H_2O_2, when using *TIP41-like*, *UKN*, and *UBC21* as multiple reference gene(s), was similar with the previous reports by using northern blotting. Thus, we proposed that these three reference genes might be good candidates for other researchers to include in their reference gene validation in gene expression studies under H_2O_2-related oxidative stress.

Key words: *Arabidopsis*, Quantitative real-time RT-PCR, geNorm, NormFinder algorithms.

Introduction

Oxidative stress, largely arising from an imbalance in the generation or removal of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and hydroxyl radical, is a great challenge faced by aerobic organisms (Desikan *et al.,* 2001; Mittler, 2002). Although ROS were originally thought to be toxic cellular metabolites (Matsumoto & Motoda, 2013; Tian *et al.,* 2013), it is now recognized that H_2O_2 acts as a signaling molecule in both plants and animals (Neill *et al.,* 2002; Veal *et al.,* 2007). In plants, H_2O_2 is generated in normal metabolism via the Mehler reaction in chloroplasts, electron transport in mitochondria, and photorespiration (glycolate oxidase) in peroxisomes (Neill *et al.,* 2002; Mittler *et al.,* 2004; Chang *et al.,* 2013). Besides, biotic and abiotic stresses also enhance H_2O_2 generation via enzymatic sources, such as plasma-membrane-localized NADPH oxidases or cell wall peroxidases (Vranová *et al.,* 2002). For example, more evidence indicates that abiotic stresses such as dehydration, extremes of temperature, salt stress, and excess irradiation can induce oxidative stress and initiate H_2O_2 signaling responses (Desikan *et al.,* 2000). To understand the complex regulatory gene networks involved in H_2O_2 signaling, therefore, there is considerable interest in monitoring the expression changes of key genes under oxidative stresses.

The zinc-finger protein *ZAT12* belongs to the zinc finger protein family, potentially involving in ROS signaling and responses to abiotic stress. Its expression is transcriptionally enhanced during osmotic, drought, salinity, temperature, oxidative, high-light stress, and wounding (Davletova *et al.,* 2005). Meanwhile, plants possess many unique, putative Ca^{2+} sensors, including a large family (50 in *Arabidopsis*) of calmodulin-like proteins termed CMLs. These proteins likely play important roles as sensors in Ca^{2+}-mediated developmental and stress response pathways. CML37 is one of the CMLs in *Arabidopsis*. The previous report illustrated that the expression of *CML37* was increased when *Arabidopsis* seedlings suffer from H_2O_2 treatment (Vanderbeld & Snedden, 2007). Together, both *ZAT12* and *CML37* play important roles in the H_2O_2 stress response, and were commonly analyzed in H_2O_2 stress condition (Desikan *et al.,* 2001; Rizhsky *et al.,* 2004; Vanderbeld & Snedden, 2007).

To date, quantitative real-time RT-PCR (RT-qPCR) is the best method available for assessing the expression trends of target genes, because of its higher sensitivity, specificity and broad quantification range (Gachon *et al.,* 2004; Dekkers *et al.,* 2012). It is important that the data of RT-qPCR should be normalized according to a constitutively and uniformly expressed gene, known as reference gene(s), whose abundance is strongly correlated to the total amounts of mRNA present in each sample (Huggett *et al.,* 2005; Skern *et al.,* 2005). Nevertheless, reference genes have often been adopted from the literatures without taking into account their specific tissue-dependent behaviors or the special design of the respective study. Unfortunately, the expression of traditional reference genes is not always stable (Tai *et al.,* 2009; Borges *et al.,* 2012; Manoli *et al.,* 2012; Rapacz *et al.,* 2012). Thus, a single endogenous gene can usually lead to erroneous normalization (Vandesompele *et al.,* 2002; Remans *et al.,* 2008).

In recent years, several studies revealed that different reference genes should be used for RT-qPCR normalization in different types of plants under different oxidative conditions. For example, Le *et al.* (2012) showed that *F-box protein family* (*F-box*) and *60s ribosomal protein* (*60s*) genes are the most suitable reference genes in both dehydrated and salt-stressed

soybean root or shoot tissues. As for cold stress, *insulin-degrading enzyme* (*IDE*) and *60s* genes are the most suitable reference genes in soybean roots, while *F-box* and *actin 27* (*ACT27*) is the best pair in shoots. By contrast, Zhu *et al.* (2013) reported that *JX272646* and *SAND family protein* (*SAND*) are the most stably expressed genes in *Caragana intermedia* under salt stress. The combination of *JX272645*, *JX272646*, and *Protein phosphatase 2A* (*PP2A*) genes is suitable for PEG-treated roots, whereas *TIP41-like family protein* (*TIP41-like*) and *PP2A* are appropriate for PEG-treated shoots. Furthermore, *SAND* and *Elongation factor -1α* (*EF-1α*) are the most stably expressed genes in cold-treated leaves. Considering these results are mainly generated from different sets of candidate genes at the beginning of each validation, it is hard to compare all of the present results in an individually examined oxidative stress. An important point for identifying suitable reference genes is thus the selection of candidate genes used in the validation procedure, which should be already assessed as good ones regarding their expression stability. The database published by Czechowski *et al.* (2005) gives a great of help to choose suitable reference genes for RT-qPCR analyses. In *Arabidopsis*, some novel reference genes but not the traditional ones, has become the superior choice for validation and quantitation of gene expression under a broad range of developmental and environmental conditions. These reference genes are suitable candidates for RT-qPCR normalization under oxidative conditions. Moreover, as H_2O_2 signaling is involved in all of the oxidative stresses, identifying the suitable reference gene(s) in H_2O_2 signaling may open a new window for gene expression studies of all oxidative stresses.

The goal of this study was to explore suitable reference genes upon H_2O_2 treatment with different concentrations in *Arabidopsis* root and shoot tissues. According to the database published by Czechowski *et al.* (2005), the stability of 12 candidate reference genes was examined and compared. Using the available algorithms geNorm (Vandesompele *et al.,* 2002) and NormFinder (Andersen *et al.,* 2004), 3 novel reference genes were identified more stably expressed than traditional one(s) in H_2O_2 signaling and its related oxidative stress. The expression stability of these reference genes was further confirmed. Finally, this work supported the idea that the systematic validation of reference genes when using RT-qPCR approach in plant cells, should never be underestimated.

Materials and Methods

Plant material and growth conditions: The wild-type (Columbia, Col-0) Arabidopsis (*Arabidopsis thaliana*) were applied during the experiments. Seeds were surface-sterilized for 20 min and washed three times with sterilized water, then cultured in Petri dishes with half-strength Murashige and Skoog (MS, pH 5.8) solid medium containing 1% (w/v) sucrose. Plates containing seeds were kept at 4°C for 2 d, and then transferred into a growth chamber with a 16/8 h (day/night) regimes at 22°C and 120 μmol m^{-2} s^{-1} irradiation. 10-d-old *Arabidopsis* seedlings were transferred to half-strength MS liquid medium containing 1 mM and 20 mM H_2O_2. Seedlings without H_2O_2 treatment were regarded as a control. Root and shoot tissues samples were collected after 0 h, 1 h, 3 h of H_2O_2 treatment, and directly frozen in liquid nitrogen and stored at -80°C until further analysis.

RNA isolation and quality control: Total RNA was extracted from root and shoot tissues with Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions. The RNA was further dissolved in DNase-treated distilled water. Concentration of each RNA sample was analyzed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Only the RNA samples with A260/280 ratio between 1.9 and 2.1, and A260/230 ratio greater than 2.0 were applied for further analysis (Nolan *et al.,* 2006; Kumar *et al.,* 2013). The integrity of RNA samples was checked through gel electrophoresis by resolving the samples on 1.2 % agarose gel in 1×TBE buffer at 100 V.

cDNA synthesis and real-time RT-PCR (RT-qPCR) analysis: By using an oligo(dT) primer and M-MLV reverse transcriptase (BioTeke, Beijing, China). cDNA was synthesized from 2 μg of total RNA. RT-qPCR experiments were performed using a Mastercycler® ep *realplex* real-time PCR system (Eppendorf, Hamburg, Germany) with SYBR pre-mixture kit (BioTeke, Beijing, China). Combined with the background information related to the reference genes for RT-qPCR normalization from recent reports in H_2O_2-treated *Arabidopsis* (Table 1) and the previous corresponding descriptions (Czechowski *et al.,* 2005), 12 genes (*ACT2*, *Clathrin*, *EF-1α*, *F-box*, *GAPDH*, *PPR*, *SAND*, *TIP41-like*, *UBC21*, *UBQ10*, *UKN*, and *YLS8*) as the candidate reference genes were chosen in this study (Table 2). Additionally, the primer pairs 5'-TGACGGTGGCGATCAAAAAC-3' and 5'-AGCGTCG TTGTTAGGCTTCT-3' were used for *ZAT12* (At5g59820); 5'-CCGCCTAAGAGACTAACGCA-3' and 5'-TAGCGGAAGCAGCTCGTTAAA-3' were used for *CML37* (At5g42380). Similar to previous report (Kumar *et al.,* 2013), the efficiency and specificity of all the primers were checked by both melting curve analysis and agarose gel electrophoresis.

The amplification of all genes were performed by the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s and 68°C for 15 s, The melting curves were analyzed at 60°C to 95°C in increments of 0.5°C every 5 s (Rivera-Vega *et al.,* 2012; Hanafy *et al.,* 2013; Lai *et al.,* 2014). Negative controls were included to confirm the suitability of the assay conditions. Samples of all reference genes were evaluated for RT-qPCR analysis using three independent biological replicates and each replicate was performed in at least triplicate.

Table 1. Background information related to the reference genes for RT-qPCR normalization from recent reports in H_2O_2-treated *Arabidopsis*.

Reference gene	H_2O_2 treatments (mM)	Samples	Key references
ACT2	0.05–50	Suspension cells	Volkov *et al.,* 2006
	10	Seeds	Liu *et al.,* 2010
	10	Whole seedlings and leaves	Wu *et al.,* 2012
ACT11	20	Leaves	Xu *et al.,* 2011
EF-1α	0.5	Roots	Sundaravelpandian *et al.,* 2013
UBA	0.5	Roots	Wang *et al.,* 2010
UBC	20	Leaves	Ng *et al.,* 2013
UBQ10	2	Whole seedlings	Wang *et al.,* 2013
	0.05–50	Shoots	Pucciariello *et al.,* 2012

Data processing: Two statistical algorithms geNorm (Vandesompele *et al.,* 2002) and NormFinder (Andersen *et al.,* 2004) were used to evaluate the stability of the candidate reference genes. Assessment of expression levels were based on the number of amplification cycles needed to reach a specific threshold (quantification cycle; Cq) in the exponential phase of PCR (Bustin *et al.,* 2010). For both programs, all Cq values were converted into relative quantities before inputting into software. The relative expression levels of corresponding genes were calculated relative to the maximum abundance in different samples. The highest relative quantify for each gene was set to 1.0. The geNorm algorithm (Vandesompele *et al.,* 2002) calculates a stability measure (*M*) for each gene and then the pairwise variation (*V*) of this gene with the others. It creates a stability ranking according to their stability. NormFinder algorithm (Andersen *et al.,* 2004) estimates intra- and inter-group variation by using a statistical and mathematical model. The variation in the expression for each gene can be measured directly (Chi *et al.,* 2012).

The heatmap of gene expression is obtained from Bio-Array Resource (BAR, http://bar.utoronto.ca/) using the default parameters and the microarray data supplied by Toufighi *et al.* (2005).

Results

A variety of reference genes in H_2O_2-treated *Arabidopsis*: H_2O_2 is the simplest peroxide compound and has been ubiquitously used for testing oxidative responses (Cheng *et al.,* 2013; González-Sánchez *et al.,* 2013). After searching related studies in primary research journals in plant biology (The Plant Cell, Plant Physiology, etc.), we try to obtain the reference genes used for RT-qPCR normalization upon H_2O_2 treatment in *Arabidopsis*. Fortunately, there were at least six different reference genes were found to be used for RT-qPCR analyses (Table 1). We also noticed that there is no fixed internal control gene used for oxidative conditions, neither in different concentrations of H_2O_2 treatment nor in different tissues. Among the reference genes listed in Table 1, *ACT2* seems to be the most common internal control gene for RT-qPCR normalization upon H_2O_2 treatments, followed by *UBQ10*. Since the studies that use inappropriate reference genes may result in misapprehensive gene expression model and incorrect results, a systematic evaluation of the stability of reference genes should be performed for studying oxidative responses in *Arabidopsis*.

Expression profiling of candidate reference genes for oxidative stress in *Arabidopsis*: Twelve candidate genes that represent different gene families and functional classes, were selected for RT-qPCR validation under oxidative conditions. These include 5 traditional housekeeping genes (*ACT2*, *EF-1α*, *GAPDH*, *UBC21*, and *UBQ10*) and 7 novel reference genes (*Clathrin*, *F-box*, *PPR*, *SAND*, *TIP41-like*, *UKN*, and *YLS8*) according to the database published by Czechowski *et al.* (2005) (Table 2). Meanwhile, oxidative conditions were achieved by adding 1 mM and 20 mM H_2O_2 into half-strength MS liquid medium (Desikan *et al.,* 2001). Total RNA from root and shoot tissues were isolated after treating with H_2O_2 for 0 h, 1 h, and 3 h, respectively.

To obtain an overview of the relative abundance of candidate reference genes, the average of Cq values were analyzed for each gene across all the tested root and shoot tissues (Fig. 1). SYBR green-based RT-qPCR analysis illustrated that various candidate reference genes displayed different levels of abundance. The average Cq values ranged from 18 to 32, while the most of values lying between 21 and 28. *UBQ10* and *ACT2* showed higher transcript than other genes, while *Clathrin* was the least expressed gene in the all tested samples. Analysis in transcript abundance of all tested samples revealed that each tested gene exhibited higher transcript levels in shoot tissues than roots. The more significant differences could be observed in *GADPH* and *EF-1α* transcripts. Therefore, we separated the results from root and shoot tissues into two groups in the subsequent analysis.

Analysis of candidate reference gene expression stability: There is an important software program named geNorm algorithm which has became a popular and useful method to evaluate the expression stability of candidate reference genes due to its function of standardize analysis (Vandesompele *et al.,* 2002; Gutierrez *et al.,* 2008). The geNorm algorithm relies on the principle that the expression ratios calculated by logarithmically transformed between two ideal reference genes, should be consistent, and each gene's average expression stability (*M*) value which reflects the expression stability of the gene compared to the others, was calculated by the average of pairwise standard deviation between the tested genes. The candidate reference genes were ranked by geNorm based on their *M* value. Genes with the lower *M* value means the higher stability, while the higher *M* value indicates the lower stability (Vandesompele *et al.,* 2002).

Table 2. Candidate reference genes used in this study. The primers sequences were described by Czechowski *et al.* (2005).

Gene symbol	Gene name	*Arabidopsis* homolog locus	Primer sequences (5'→3')
ACT2	Actin2	AT3G18780	F- CTTGCACCAAGCAGCATGAA R- CCGATCCAGACACTGTACTTCCTT
Clathrin	Clathrin adaptor complex subunit	AT5G46630	F- TCGATTGCTTGGTTTGGAAGAT R- GCACTTAGCGTGGACTCTGTTTGATC
EF-1α	Translation elongation factor 1 alpha	AT5G60390	F- TGAGCACGCTCTTCTTGCTTTCA R- GGTGGTGGCATCCATCTTGTTACA
F-box	F-box family protein	AT5G15710	F- TTTCGGCTGAGAGGTTCGAGT R- GATTCCAAGACGTAAAGCAGATCAA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase C2	AT1G13440	F- TTGGTGACAACAGGTCAAGCA R- AAACTTGTCGCTCAATGCAATC
PPR	Pentatricopeptide repeat superfamily protein	AT5G55840	F- AAGACAGTGAAGGTGCAACCTTACT R- AGTTTTTGAGTTGTATTTGTCAGAGAAAG
SAND	SAND family protein	AT2G28390	F- AACTCTATGCAGCATTTGATCCACT R- TGATTGCATATCTTTATCGCCATC
TIP41-like	TIP41-like family protein	AT4G34270	F- GTGAAAACTGTTGGAGAGAAGCAA R- TCAACTGGATACCCTTTCGCA
UBC21	Ubiquitin-conjugating enzyme 21	AT5G25760	F- CTGCGACTCAGGGAATCTTCTAA R- TTGTGCCATTGAATTGAACCC
UBQ10	Ubiquitin10	AT4G05320	F- GGCCTTGTATAATCCCTGATGAATAAG R- AAAGAGATAACAGGAACGGAAACATAGT
UKN	Unknown	AT4G26410	F- GAGCTGAAGTGGCTTCCATGAC R- GGTCCGACATACCCATGATCC
YLS8	Mitosis protein YLS8	AT5G08290	F- TTACTGTTTCGGTTGTTCTCCATTT R- CACTGAATCATGTTCGAAGCAAGT

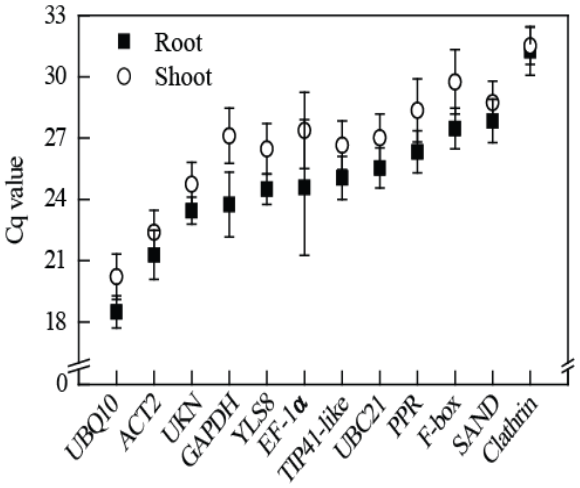

Fig. 1. Average of quantification cycle (Cq) values for the 12 candidate reference genes used in this study. 10-d-old *Arabidopsis* seedlings were transferred to half-strength MS liquid medium containing 0 mM (control), 1 mM, and 20 mM H_2O_2. Total RNA was isolated from root and shoot tissues after loading H_2O_2 for 0 h, 1 h, and 3 h, respectively. Values were given in the form of RT-qPCR quantification cycle numbers across all tested samples.

Among the H_2O_2-treated samples, *TIP41-like* and *SAND* were ranked as the most stable reference genes by using geNorm analysis, followed by *UBC21* in root tissues (Fig. 2A). Meanwhile, *TIP41-like* and *UBC21* were observed to be the most stable genes, followed by *SAND* in shoot tissues of our experimental conditions (Fig. 2B). To give an insight into the suitable reference genes in whole tissue samples, we combined the results obtained from root and shoot tissues. Finally, *TIP41-like* and *UBC21* were regarded as the two most stable reference genes for further RT-qPCR normalization under H_2O_2 stressed conditions, followed by *UKN* and *SAND* (Fig. 2C). In contrast, *EF-1α* and *Clathrin* ranked lower than any other reference genes, and both of them were suggested to be excluded from stringent normalization (Fig. 2).

Although the most stable reference genes had been ranked as the appropriate choice for normalization, the use of two or more reference genes could be more accurate and credible. The geNorm software also calculate the pairwise variations (Vn/n+1) between two sequential normalization factors to evaluate the necessity of adding further reference genes. Pairwise variation cut-off V value of 0.15 is a threshold (Vandesompele *et al.,* 2002), below which the inclusion of an additional

reference gene is not required. The pairwise variation analyses show that *V* values were less than 0.15 in this set of samples until *V*7/8 (Fig. 2D). According to these criteria, it indicated that the use of two reference genes was sufficient to normalize gene expression in our experimental conditions. However, when considering total samples, the *V*3/4 and *V*6/7 values were much lower than that of *V*2/3, while no such obvious difference was observed between *V*3/4 and *V*4/5, and between *V*6/7 and *V*5/6. Therefore, considering operation capacity and convenience, the combination of three reference genes was recommended to be as a optimal choice.

As geNorm dependents on an elimination procedure to select a stable pair of reference genes, there is a small risk when using this algorithm (Infante *et al.,* 2008). If the genes are co-regulated genes, geNorm would easily give erroneous suggestions. To avoid introducing unnecessary bias, all of the data were re-assessed by NormFinder. This is a mathematical model which takes into account intra- and inter-group variations for normalization factor calculations. It ranks the entire tested gene according to their stability values, and the most stable gene have the lowest values (Andersen *et al.,* 2004). Interestingly, we found that the ranking generated by this algorithm (Fig. 3) was similar with those determined by geNorm (Fig. 2). *TIP41-like* was still the highest ranked reference gene in both roots (Fig. 3A) and shoots (Fig. 3B). The stability value of *TIP41-like* was 0.018, 0.059, and 0.054 in roots, shoots, and total samples, respectively. When evaluated across all the tested samples, the best combination obtained from NormFinder analysis in H_2O_2-treated plants was *TIP41-like* and *UKN*, and the stability value of which was 0.041. Therefore, *TIP41-like* and *UKN* were considered as the best pair for RT-qPCR normalization upon H_2O_2 treatment (Fig. 3C). Moreover, *EF-1α* was clearly not a reliable reference gene in our experimental conditions.

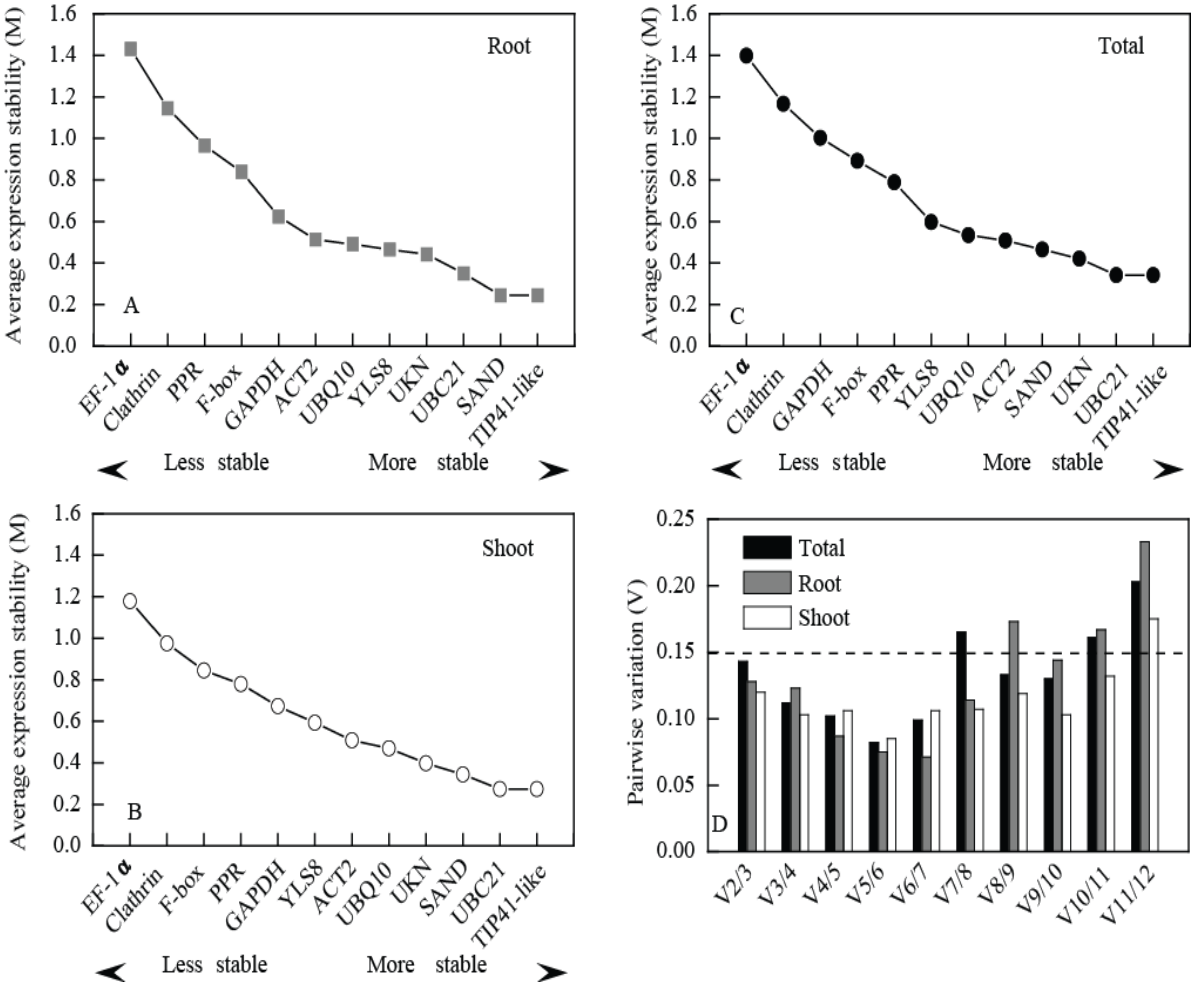

Fig. 2. Average expression stability values (*M*) and pairwise variation (*V*) analysis of candidate reference genes under oxidative conditions by geNorm. 12 candidate reference genes were amplified in cDNA samples from root and shoot tissues under 0 mM (control), 1 mM, and 20 mM H_2O_2 treatments. A lower *M* indicated more stable expression. Mean expression stability following stepwise exclusion of the least stable gene from root tissues (A), shoot tissues (B), or total samples (C), respectively. The *V*n/n+1 measured the effect of adding additional reference genes on the normalization factor for these treatments (D). The dash line denotes 0.15 cut-off *V* value. Calculations were performed as described in "Materials and methods" section.

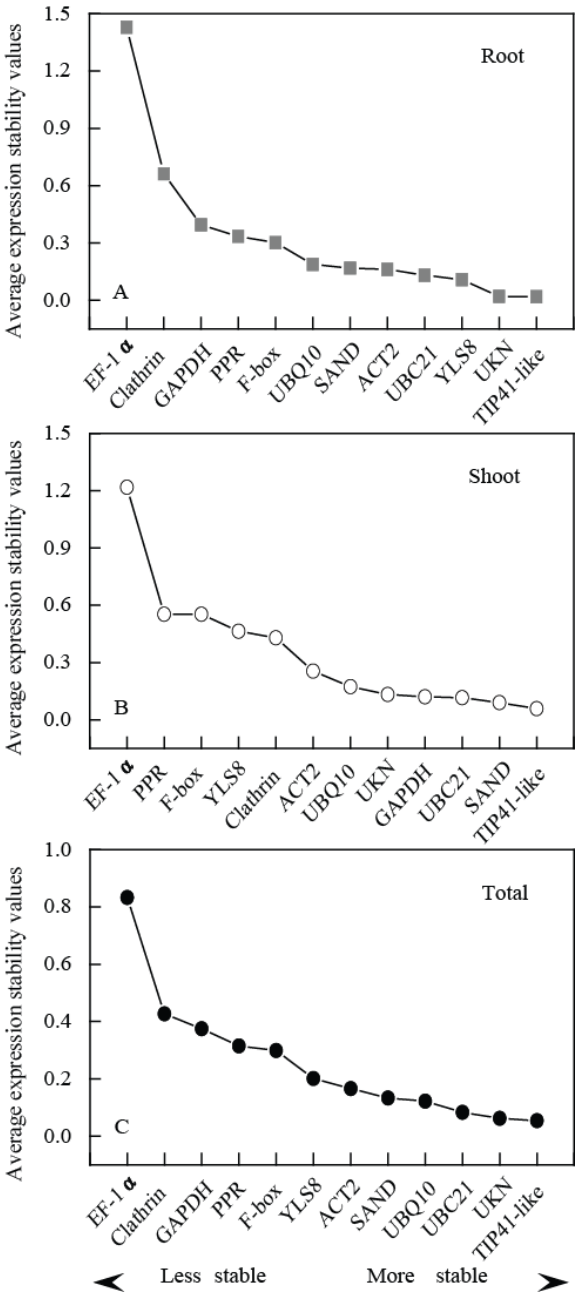

Fig. 3. Ranking of candidate reference genes based on stability values calculated by NormFinder. 12 candidate reference genes were amplified in cDNA samples from root and shoot tissues under 0 mM (control), 1 mM, and 20 mM H_2O_2 treatments. Relative quantifications of root tissues (A), shoot tissues (B), or total samples (C) were performed respectively, as described in "Materials and methods" section.

Verification of reference genes by referring to microarray data: To further confirm the results from H_2O_2 treatments, the stability of all candidate reference genes were compared according to published microarray experiments. By using the e-Northern tool provided by the Bio-Array Resource for *Arabidopsis* Functional Genomics (http://bar.utoronto.ca/), the relative gene expressions of 11 candidate reference genes (*PPR* gene is not included in this database) during methyl viologen treatment in *Arabidopsis* root and shoot tissues were investigated (Fig. 4A). The heatmap of gene expression at *Arabidopsis* growth stage 1.02 showed that the relative gene expression of *TIP41-like* and *UBC21* (ranged from -0.1 to 0.2), and *UKN* (ranged from -0.2 to 0.1), were more stable than the majority of tested candidate reference genes when exposed to 10 µM methyl viologen. Meanwhile, the maximal changes were observed in the expression levels of *ACT2* (ranged from -0.4 to 0.4), while *UBQ10* gene seemed to be constantly decreased by oxidative stress. Although the expression levels of *EF-1α* were not greatly changed in this data set, it consistently decreased after 6 h of oxidative treatment in both shoot and root tissues.

Since H_2O_2 signaling is also related to drought-induced oxidative stress (Desikan *et al.,* 2001), the stability of above mentioned candidate reference genes were investigated again under drought stress conditions (Fig. 4B). Similar to the results produced from methyl viologen treatments (Fig. 4A), microarray data also showed that the relative gene expression of *TIP41-like* (ranged from -0.1 to 0.2) and *UKN* (ranged from -0.2 to 0.1), were still more stable than the majority of tested candidate reference genes under drought conditions. By contrast, the relative expression of *ACT2* increased after 0.25 h of drought treatment in shoot tissues and then rapidly decreased, with the changes from 50% gain to 40% loss within 3 h of drought stress. Furthermore, a biphasic change along 24 h of drought treatment was observed in relative expression of *EF-1α.* Together, combined with the results from geNorm and NormFinder analyses (Figs. 2 and 3), these results indicated that *TIP41-like*, *UKN*, and *UBC21*, rather than *EF-1α* or *ACT2*, were the suitable reference genes for RT-qPCR normalization in H_2O_2 signaling.

Validation of reference gene(s): To demonstrate the usefulness of the above validated candidate reference genes in RT-qPCR, the relative expression levels of two *Arabidopsis* H_2O_2-induced genes, *ZAT12* and *CML37* were analyzed using single (*EF-1a*) and multiple reference gene(s) (*TIP41-like*, *UKN,* and *UBC21*) in our experimental conditions. In this study, the expression levels were assessed under H_2O_2 stress for different treat time points (Fig. 5). When *TIP41-like*, *UKN*, and *UBC21* were used for normalization, the expression of *ZAT12* under H_2O_2 treatment increased strongly at 0.5 h and 1 h, and then decreased rapidly at 3 h. These were similar with the previous results by using RNA blotting, showing the expression of *ZAT12* was abruptly increased and declined within 2 h, following a further decline after 4 h treatment with H_2O_2 (Rizhsky *et al.,* 2004). Meanwhile, *CML37* expression levels were progressively increased during 3 h treatment when using multiple reference gene(s) for normalization. Comparatively, the increased *ZAT12* and *CML37* transcripts were slowed down during the beginning period of treatment (1 h) by using the single reference gene (*EF-1a*).

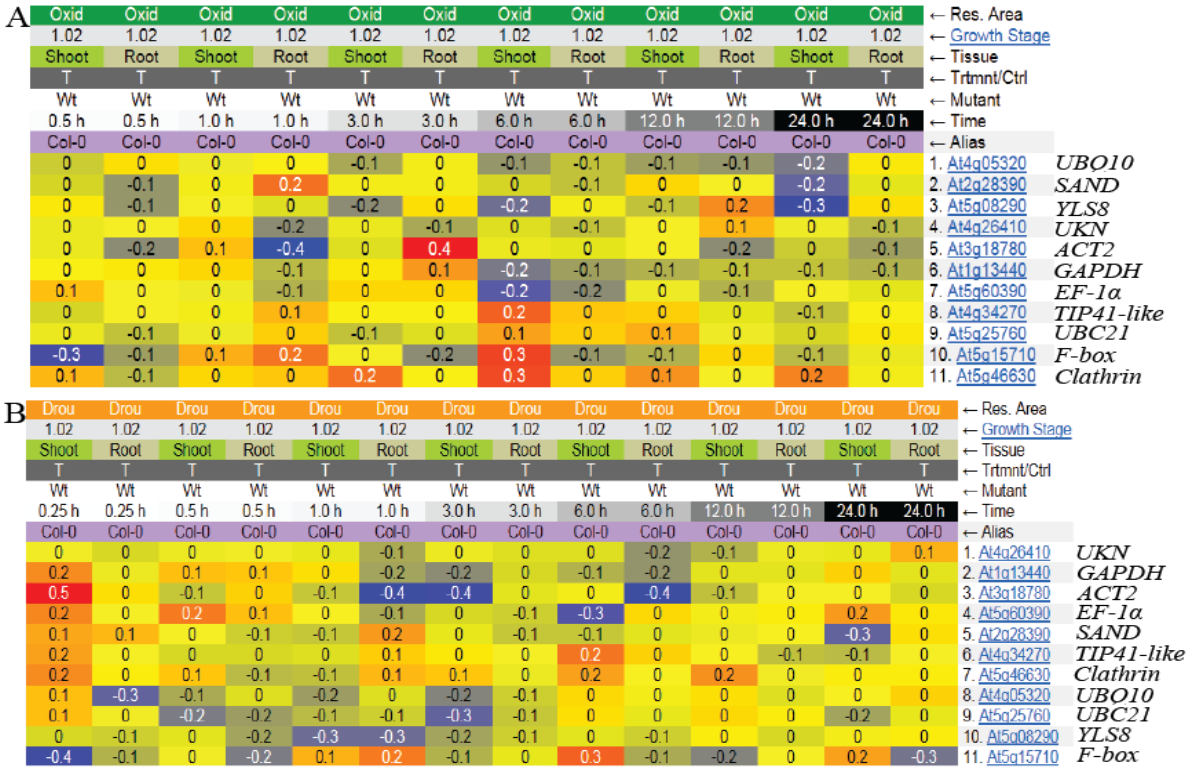

Fig. 4. Relative expression of candidate reference genes for research on oxidative and drought stresses obtained from microarray data sets. (A) Time-course analysis of 10 µM methyl viologen stress responses in root and shoot tissues of *Arabidopsis* seedlings. (B) Time-course analysis of the drought stress responses in *Arabidopsis* root and shoot tissues. Relative expression values of 11 candidate reference genes were obtained from Bio-Array Resource (Toufighi *et al.,* 2005). Relative expression of *PPR* gene was not found in this database.

Discussion

Previously, a database of reference genes has been identified in *Arabidopsis* (Czechowski *et al.,* 2005). This set contains putative reference genes for transcript normalization under different developmental stages, biotic and abiotic stresses, hormonal treatments and nutrient stress conditions. Since H_2O_2 is the intermediary signaling molecule related to all of these conditions, the choice of suitable reference gene(s) for RT-qPCR normalization in H_2O_2 signaling was assessed in this investigation.

In fact, the related reference genes in H_2O_2-treated *Arabidopsis* were variable (Table 1). According to the database published by Czechowski *et al.* (2005), 12 candidate reference genes were evaluated in both *Arabidopsis* root and shoot tissues when loading different doses of H_2O_2 (Table 2; Fig. 1). In our experimental systems, by using geNorm and NormFinder algorithms, we identified and validated 3 novel reference genes (*TIP41-like*, *UKN*, and *UBC21*) for transcriptional analysis under H_2O_2 treatment in *Arabidopsis* (Figs. 1-3). Publicly available microarray data of methyl viologen treatment confirmed this conclusion (Fig. 4A). Methyl viologen is a widely used herbicide which can induce superoxide anion production in the chloroplasts, thereafter leading to H_2O_2 accumulation (Xu *et al.,* 2012). The relationship between methyl viologen and H_2O_2 indicated that these 3 novel reference genes can be used for RT-qPCR normalization under oxidative stress. Interestingly, when we checked the microarray data of drought stress, the relative expression of *TIP41-like* and *UKN* were still more stable than the majority of tested candidate reference genes (Fig. 4B). It is known that drought stress enhances H_2O_2 production in different cellular compartments. Photorespiration is likely to account for over 70% of total H_2O_2 production under drought stress conditions (Noctor *et al.,* 2002). Therefore, these results strengthened the possibility that *TIP41-like*, *UKN*, and *UBC21* could be regarded as suitable reference genes for RT-qPCR analysis in H_2O_2 signaling and/or its related oxidative stressed conditions.

Although slight differences were found in the results from geNorm and NormFinder analyses, we concluded that *TIP41-like* was the most stable reference gene shown in H_2O_2-related oxidative stress (Figs. 2 and 3). Similarly, *TIP41-like* was also identified as the most stable reference gene across different tissues and developmental stages in other plants such as tomato (Expósito-Rodríguez *et al.,* 2008) and bamboo (Fan *et al.,* 2013). Moreover, it was suggested that *TIP41-like* exhibited the highest expression stability, not only under iron- and nitrogen-related stress (Han *et al.,* 2013; Warzybok & Migocka, 2013), but also under other different abiotic stress conditions (Czechowski *et al.,* 2005). Therefore, *TIP41-like* appeared to be a novel "super-stable" reference gene for RT-qPCR normalization in a wide range of experimental settings.

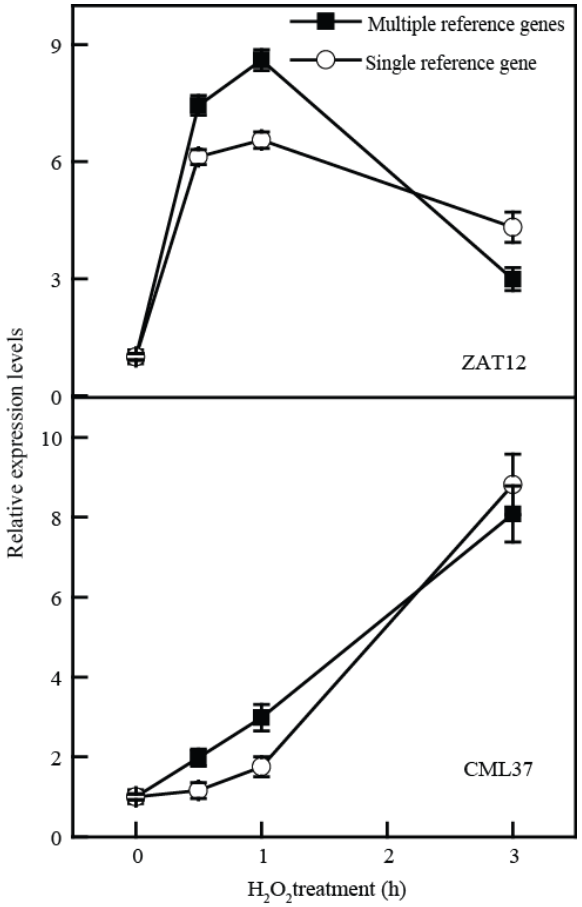

Fig. 5. Relative expression levels of *ZAT12* and *CML37* during H_2O_2 treatment conditions using single or multiple reference gene(s) for normalization. 10-day-old *Arabidopsis* seedlings were transferred to half-strength MS liquid medium containing 20 mM H_2O_2 for 3 h. Total RNA was isolated from root tissues at the indicated time points. Single reference gene (*EF-1a*) and multiple reference genes (*TIP41-like, UKN, and UBC21*) were respectively used for normalization. Values are mean.

Unfortunately, normalization to a single endogenous gene can not meet the needs of academic rigor, since it leads to relatively large errors (Vandesompele *et al.,* 2002). Subsequently, the pairwise variation analyses (Fig. 2D) showed that two reference genes were enough for reliable RT-qPCR normalization in gene expression studies of H_2O_2 signaling, because *V*2/3 was less than 0.15 in this set of samples. But this cut-off *V* value can not be taken as an absolute rule, while the *V*3/4 values were much lower than that of *V*2/3. Although lower values were also obtained by using four or five reference genes, too many reference genes was not a form of rational economic behavior. Thus, we proposed that the combination of three reference genes (*TIP41-like*, *UKN*, and *UBC21*), was the optimal choice for RT-qPCR normalization in H_2O_2-related oxidative stress.

To illustrate the suitability of the reference genes revealed in the present study, the expression pattern of *ZAT12* and *CML37*, two H_2O_2-induced marker genes, by using single and multiple reference gene(s) for normalization, were analyzed and compared. These results (Fig. 5) illustrated that the expression levels of two genes using *EF-1α* for normalization were different from the results using *TIP41-like*, *UKN*, and *UBC21* as reference gene(s). More importantly, the expression of H_2O_2-induced *ZAT12*, reported by using RNA northern blotting (Rizhsky *et al.,* 2004), was similar with the change pattern of RT-qPCR analysis using *TIP41-like*, *UKN*, and *UBC21* for normalization. These results clearly showed that suitable reference gene presents a crucial role in accurate validate the data of RT-qPCR, and the combination of *TIP41-like*, *UKN*, and *UBC21* might provide a strong foundation for determining transcript quantification under H_2O_2 treatment.

Comparatively, we also noticed that *EF-1α* might be not a reliable reference gene, at least in our experimental conditions (Figs. 1-5). These results can be supported by the fact that EF-1α plays a vital role in expediting the execution of the apoptotic program when subjected to oxidative stress (Chen *et al.,* 2000). Moreover, EF-1α is responsible for actin interactions and bundling, which is required for regulation of the actin cytoskeleton and cell morphology (Gross & Kinzy, 2005). Although *ACT2* is the most common internal control gene for RT-qPCR normalization in H_2O_2-treated *Arabidopsis* (Table 1), it was still suggested to be excluded from stringent normalization according to microarray data analysis in this study. It was also reported that a lethal UV dose of 50 kJ/m^2 can induce the expression of *metacaspase-8* (*AtMC8*) by 80-fold when using *18S* as a reference gene, while using *ACT2* instead, would result in a calculated induction of 120-fold because UVC down-regulates *ACT2* (He *et al.,* 2008). Actually, ROS signaling to actin aggregation has been documented in yeast (Franklin-Tong & Gourlay, 2008). The reorganization of actin microfilaments stimulated by increased ROS suggests that it is an early target for oxidative stress in animal cells (Dalle-Donne *et al.,* 2001). In plants, Wilkins *et al.* (2011) also showed that ROS and nitric oxide (NO) mediate actin reorganization and programmed cell death in the self-incompatibility response of *papaver*. Thus, actin may be a target for H_2O_2 and NO signals, which makes it not suitable for RT-qPCR normalization in H_2O_2 signaling studies.

Conclusions

In summary, our work evaluates the appropriate choice of reference genes for gene expression analyses of H_2O_2 signaling. We showed that some traditional reference genes (*EF-1α*, *ACT2*) currently used, might be rather unstable expressed during H_2O_2 stressed conditions. Most importantly, the combination of *TIP41-like*, *UKN*, and *UBC21* is suggested to be the best choice of reference genes for exploring H_2O_2 signaling in *Arabidopsis*. Therefore, we proposed that these reference genes can be suitable candidates for other researchers to include in their reference gene validation in gene expression studies under H_2O_2-related oxidative stress. Together, above results provide a valuable evidence for the importance of adequate reference genes in RT-qPCR normalization, insisting on the use of suitable reference gene validation in all transcriptional researches.

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