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Selection and validation of appropriate reference genes for real-time quantitative PCR analysis in *Momordica charantia*

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ABSTRACT

Real time quantitative reverse transcription PCR (RT-qPCR) has been attracting more attention for its high sensitivity in gene expression analysis. Given the widely use of RT-qPCR in normalization, it is playing a pivotal role for seeking suitable reference genes in different species. In current work, 12 candidate reference genes including Actin 2 (*ACT2*), Cyclophilin 2 (*CYP2*), Glyceraldehyde-3-phosphate dehydrogenase C2 (*GAPC2*), Elongation factor 1-α (*EF1-α*), Nuclear cap binding protein 20 (*NCBP20*), Serine/threonine-protein phosphatase PP2A (*PP2A*), Polypyrimidine tract-binding protein 1 (*PTBP1*), *SAND* family protein (*SNAD*), TIP41-like protein (*TIP41*), Tubulin beta-6 (*TUB6*), Ubiquitin-conjugating enzyme 9 (*UBC9*) and Glyceraldehyde-3-phosphatede-hydrogenase (*GAPDH*) were screened from the transcriptome datasets of *M. charantia*. Afterwards, GeNorm, NormFinder and BestKeeper algorithms were applied to assess the expression stability of these 12 genes under different abiotic stresses including drought, cold, high-salt, hormone, UV, oxidative and metal stress. The results indicated that 12 selected genes exhibited various stability across the samples under different external stress conditions, but *TIP41*, *PTBP1* and *PP2A* presented high stability among all the reference genes. To validate the suitability of the identified reference genes, the results of hormone subset were compared with RNA sequencing (RNA-seq) data, and the relative abundance of Ascorbate peroxidase 1 (*APX1*) was used to confirm the reliability of the results. This work assesses the stability of reference genes in *M. charantia* under different abiotic stress conditions, which will be beneficent for accurate normalization of target genes in *M. charantia*.

1. Introduction

M. charantia, also called “bitter melon” or “bitter gourd”, is a valuable plant which belongs to the Cucurbitaceae family (Jia et al., 2017). Its fruit can be used as material for food, and the extracts of various organs of this plant (stem, leaf and fruit) were found to have significant pharmacological effects on human body, including anti-diabetic, anthelmintic, antitumor and anti-inflammatory (Jiang et al., 2016; Raza et al., 2000; Sun et al., 2016). Recent researches have suggested that the polysaccharide extracts from *M. charantia* are involved in regulation of pancreatic cells secretion in type 2 diabetes (Ma et al., 2017; Zhang et al., 2018). Moreover, further investigations have confirmed the aqueous extracts from *M. charantia* have significant effects on low density lipoproteins and triglycerides in a suitable dose (Hossain et al., 2012; Ma et al., 2017; Saeed et al., 2017; Wehash et al., 2012). Nonetheless, few studies focused on the *M. charantia* genes and gene expression. The key genes involved in biosynthesis pathways of certain active components have not been completely understood. For example, genes involved in the biosynthesis of momorcharin are still obfuscating, despite various biological activities and pharmacological functions have been demonstrated (Manoharan et al., 2014; Pan et al., 2014). Furthermore, studies of gene transcription and expression patterns under multifarious exogenous regulators will be stagnated or severely hampered, since most of the genes remain unknown. For these reasons, there should be a detailed study or a suitable method to make a progress in the respect of gene finding and function verification of *M. charantia*.

Next Generation Sequencing (NGS) technology has a tremendous

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Table 1
Genes and primer pairs used for RT-qPCR in *M. charantia*.

Gene symbol	Description	Gene ID	*Arabidopsis* homolog locus	Primer sequence forward/reverse (5′-3')	Length (bp)	PCR efficiency	R^2
ACT2	Actin2	c8469_g2_i1	At3g18780	F: GTATGTTGCCATCCAAGCCG R:AGACGGAGGATAGCATGGGG	138	1.922	0.9995
CYP2	Cyclophilin 2	c29470_g1_i3	At4g33060	F: TCCAGCTCTGTCTCGAAGGC R: ATTCATCCGGGAAAACACCC	138	1.881	0.9998
EF1-α	Elongation factor 1-α	c30621_g1_i4	At1g07940	F:GACCAACGGAGGGAAAAAGC R: TGAGTTCGAGGTGCTTTGGC	139	1.903	0.9998
GAPC2	Glyceraldehyde-3-phosphate dehydrogenase C2	c29674_g2_i1	At1g13440	F: CATCGACAGTCGGAACACGG R: AGAGGTGGAAGAGCCGCTTC	130	1.899	0.9998
NCBP20	Nuclear cap binding protein 20	c30195_g1_i1	At5g44200	F: TTCTCGGGAAGATGCAGAGG R: ATTGCCTGCCATCCTGAAAC	107	1.895	0.9998
PP2A	Serine/threonine-protein phosphatase PP2A	c34515_g2_i1	At1g59830	F: TGTGAGGGACAAAGCTGTGG R: ATGAAACAGCCCACATGCAG	148	1.891	0.9998
PTBP1	Polypyrimidine tract-binding protein 1	c30609_g1_i6	At3g01150	F: TTGAGGGTTCTGATGCACGC R: TGCACCAATGCCTGAAATCC	121	1.891	0.9998
SNAD	SAND family protein	c25723_g1_i1	At2g28390	F: TAGCGGCAGGTCAGAGATGG R: ATCATCCTCATTGCCATGCC	124	1.891	0.9997
TIP41	TIP41-like protein	c61808_g1_i1	At4g34270	F: TGAGTTGGCTGACAATGGCA R: CGCAATGCATACGAGTGTCC	137	1.905	0.9998
TUB6	Tubulin beta-6	c33261_g2_i1	At5g12250	F: TCTGCTTCCGAACCCTCAAG R: GATTAACAGCGAGCTTCCGG	141	1.902	0.9998
UBC9	Ubiquitin-conjugating enzyme 9	c41261_g1_i1	At4g2796	F: TGTCACCATTCATTTCCCCC R: CAGGGCTCCACTGCTCTTTC	134	1.888	0.9998
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	c12972_g1_i2	At1g42970	F: CCTTGCACGCCTCAAGTCAC R: TTCGGCATTGTGAAGGGAAC	96	1.896	0.9998
APX1	Ascorbate peroxidase 1	c31196_g1_i1	At1g07890	F: ATCAGAACCTTTGGTAGCGTCG R: CGCTGTTGAGATTACAGGAGGG	109	1.912	0.9996

Unigenes were selected from the transcriptome of *M. charantia*. The mean PCR efficiency and the regression coefficient (R^2) for each primer pair were calculated by LinRegPCR software.

impact on genomic researches since it was created, and it has been proved to be a rapid and cost-effective method in analyzing the genome and transcriptome of model and non-model organisms, especially those with large and complex ones (Cao et al., 2015; Stone and Storchova, 2015). Furthermore, countless genes have been identified though the powerful performance of NGS. RT-qPCR is a technique used for exhaustive expression analysis of target genes on account of its high-throughput capabilities, quantitative accuracy, reproducibility and high sensitivity (Bustin et al., 2009; Zhao et al., 2015). For reliable and accurate analysis on expression of target gene, normalization of RT-qPCR requires an appropriate internal reference gene because the results are inevitably affected by several external or internal factors including RNA purity, DNA contamination, primer design, reverse transcription efficiency, cDNA quality examination and the PCR efficiency (Andersen et al., 2004; Bustin, 2002). To eliminate the discrepancies of the experimental results, one or more suitable reference genes are needed to improve the accuracy and credibility of RT-qPCR results (Aerts et al., 2004; Goidin et al., 2001; Vandesompele et al., 2002). Meanwhile, it is urgent for us to select and identify suitable reference genes for genetic and biological research.

Reference genes, also called housekeeping genes, are expressed stably in different tissues, physiological status of the organisms and under different environmental stimuli. Besides, an ideal reference gene should be expressed at a constant level in all tissues no matter how the experimental conditions change (Vandesompele et al., 2002). Traditional reference genes like *GAPDH*, *18S rRNA* and *ACT* are commonly used in many relevant researches (Goidin et al., 2001; Zhu and Altmann, 2005). Nevertheless, this conformity has recently been repudiated by a growing number of studies demonstrating that the traditional reference genes are expressed diversely in different tissues under various external experimental conditions (Expósito-Rodríguez et al., 2008; Jian et al., 2008; Nicot et al., 2005). With the aim of counteracting for the weaknesses of traditional reference genes under some experimental conditions, several new ideal reference genes expressing at a constant level have been determined as candidates for normalization. Owing to the importance of gene normalization, a great number of studies attempted to identify the most suitable reference genes among species and tissues under various kinds of experimental conditions, such as pig tissues (Nygard et al., 2007), peach (Tong et al., 2009), cucumber (Wan et al., 2010), *Arabidopsis thaliana* (Czechowski et al., 2005), tomato (Løvdal and Lillo, 2009), soybean (Hu et al., 2009), *C. elegans* (Hoogewijs et al., 2008), banana (Chen et al., 2011), *C. intybus* (Maroufi et al., 2010), potato (Nicot et al., 2005), neutrophils (Zhang et al., 2005), sugarcane (Iskandar et al., 2004), peanut (Jiang et al., 2011) and rice (Kim et al., 2003). However, systematic evaluation of the selection of reference genes in *M. charantia* under multifarious external conditions has not been reported, which means it is essential to select and validate some stable reference genes in *M. charantia* with the purpose of promoting target gene normalization.

In the current study, 12 reference genes, which were selected based on the transcriptome dataset of *M. charantia* (data not published), were identified to investigate the most appropriate candidates for gene normalization. In order to make a comprehensive analysis of the reference genes stability, the plants were exposed to several external experimental treatments including H_2O_2, NaCl, PEG6000, $CuSO_4$, MeJA, cold and UV. The raw RT-qPCR data were analyzed using three Excel-based statistical algorithms including geNorm (Vandesompele et al., 2002), NormFinder (Vandesompele et al., 2002), BestKeeper (Pfaffl et al., 2004), and then the RefFinder was used to make a comprehensive stability ranking of these reference genes. For futher confirmation of the suitability of selected candidates, part of the ranking results were compared with RNA-seq based gene expression profiling. Meanwhile, the relative expression level of a target gene named *APX1* was analyzed by RT-qPCR using the most and least stable reference genes recognized in this work to confirm the reliability of data. In a word, the results of this study would accelerate the process of the researches on *M. charantia*, especially on the genes involving compounds synthesis.

2. Results

2.1. Assessment of amplification specificity in M. charantia and PCR efficiency analysis

In this study, 12 candidate reference genes (*ACT2*, *CYP2*, *NCBP20*, *EF1-α*, *GAPC2*, *PTBP1*, *PP2A*, *SNAD*, *GAPDH*, *TUB6*, *UBC9* and *TIP41*) were screened from *M. charantia* transcriptome datasets through a local tblastn, and the corresponding genes from The Arabidopsis Information Resource (TAIR) database (http://www.arabidopsis.org) was used as templates. The gene symbol, *Arabidopsis* homolog locus, amplicon length, PCR efficiency and correlation coefficients (R^2) are presented in Table 1. The RT-qPCR products ranged from 96 to 148 bp. The PCR products which were validated by 1.5% agarose gel electrophoresis exhibited high specificity as only one single band was observed (Fig. S1). In addition, the primer pairs specificity was further confirmed by the melt curve analysis with only one single peak formed (Fig. S2). To avoid error amplification, the PCR products were inserted into pMD19-T Vector (Takara, Dalian, China) for sequencing and the results are listed in Table S1. According to the manual of LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009), the mean PCR efficiencies of each primer pair were distributed from 1.881 to 1.922, and the optimal range of amplification efficiency (E) should be distributed from 1.900 to 2.050 (Ruijter et al., 2009). Likewise, the linear R^2 (correlation coefficients) were ranged from 0.9995 to 0.9998 (Table 1).

2.2. Expression profile of reference genes

To analyze the transcript abundance of each reference gene, the cycle threshold (Ct) values of candidate reference genes under different treatments were examined using RT-qPCR assay as the relative abundance of these genes can be reflected in the form of the cycle threshold values, which means gene with lower Ct value represents higher transcript abundance. The mean Ct values of the reference genes were distributed from 15.95 to 21.26, most were distributed between 19 and 20. *UBC9* was the most abundant candidate among all the reference genes because the lowest average Ct value 15.95 ± 1.41 (mean ± SD). Conversely, *CYP2* (21.02 ± 1.06) and *EF1-α* (21.26 ± 1.23) were the least expressed genes (Fig. 1 and Table S2). Additionally, *PTBP1* showed a low variability with the lowest SD value of 0.76, suggesting that it may be the most stable gene under different treatments, while SNAD, with SD value of 2.17, showed the most variable level of expression (Fig. 1 and Table S2). Generally, the Ct values can intuitively show both the stability and expression levels of candidate reference genes. However, for further evaluation of the stability of the 12 selected genes across all the experimental samples, it requires a more systematical analysis of the data.

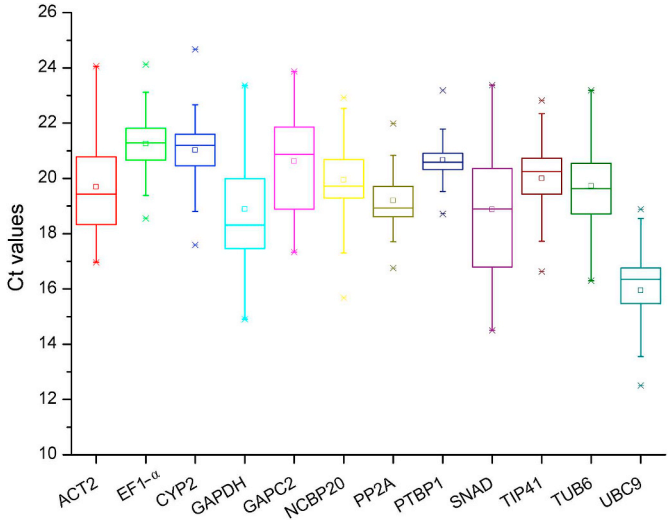

Fig. 1. Distribution of raw Ct values of candidate reference genes across all of the experimental samples. The lines across the box-plot represent the median. Boxes indicate the interquartile range, whiskers represent the lowest and highest Ct values respectively.

2.3. Candidate reference genes expression stability analysis

For the purpose of further evaluating the RT-qPCR results of the candidates exposed to different stresses, all of the Ct values were corrected and pre-treated into a suitable form which can be recognized and analyzed by geNorm, NormFinder and BestKeeper.

2.3.1. geNorm analysis

GeNorm analysis is an algorithm using the expression stability value (M) to compare the stability of reference genes. According to the manual, the Ct values of each sample were changed into relative expression levels by using formula $2^{-\Delta Ct}$ (ΔCt = each corresponding Ct value - the minimum Ct value). Knowing that pairwise variation (V) can be interpreted from calculating the standard deviation (SD) of the elements composed of the logarithm of each gene comparing with other genes, we can figure out the expression stability value by taking the arithmetic mean of V (Vandesompele et al., 2002). As shown in Fig. 2, all candidate reference genes exhibited various stability under different treatments. For instance, *SNAD*, *GAPDH, CYP2* displayed unsatisfactory performances in stability among the selected genes. More specifically, *PTBP1* and *EF1-α* were more stable than other reference genes in control group. In the low temperature group, *TIP41* and *EF1-α* were certified to be the most stable genes, but *ACT2*, *CYP2*, *GAPDH* seemed to have similar stability. *PTBP1*, *PP2A* and *TIP41* performed well in stability than others in $CuSO_4$ treatment group. Similarly, both *TIP41* and *PTBP1* were more stable under H_2O_2 treatment. In MeJA group, most candidates seemed to be good reference genes except *SNAD*, *CYP2* and *ACT2*. Interestingly, the top 5 candidates in NaCl group showed lower M value than the rest. Additionally, most candidates, except *SNAD* in PEG6000 group and *CYP2*, *GAPDH* in UV group, were considered as the best stabilized reference genes.

Apart from evaluating the stability of gene expression through average expression stability values, another function of geNorm algorithm is that it determines the best number of reference genes used for an accurate normalization by calculating the Pairwise variation (Vn/n+1) (Vandesompele et al., 2002). The threshold of Vn/n+1 was 0.15, a value < 0.15 indicates that no significant influence was generated to the normalization even adding one more gene. In this study, we calculated all the Vn/n+1 values using geNorm (Fig. 3 and Table S3), the V2/3 values of all the samples exposed to various stresses showed coincident values lower than 0.15, implying two reference genes were enough to perform a reliable normalization in these groups. Although 0.15 is not a strict threshold parameter to determine the optimal number of reference genes prepared for the normalization (Paolacci et al., 2009; Vandesompele et al., 2002), one more reference gene should be more advisable for accurate normalization in RT-qPCR analysis. In this work, most of Vn/n+1 values were obviously lower than 0.15 except V10/11 and V11/12 in UV subset (Fig. 3), suggesting that three or more reference genes could be considered for accurate normalization.

2.3.2. NormFinder analysis

Similar to geNorm algorithm, NormFinder uses formula $2^{-\Delta Ct}$ to transform the format of Ct values of each gene from RT-qPCR into relative expression levels, then the stability of each gene will be analyzed. Lower expression stability value represents a higher stability. The rankings of all candidate genes calculated by NormFinder algorithm were presented in Table 2. For all samples subsets, it could be easily seen that *PTBP1* and *TIP41* were the most stable genes or at least ranked

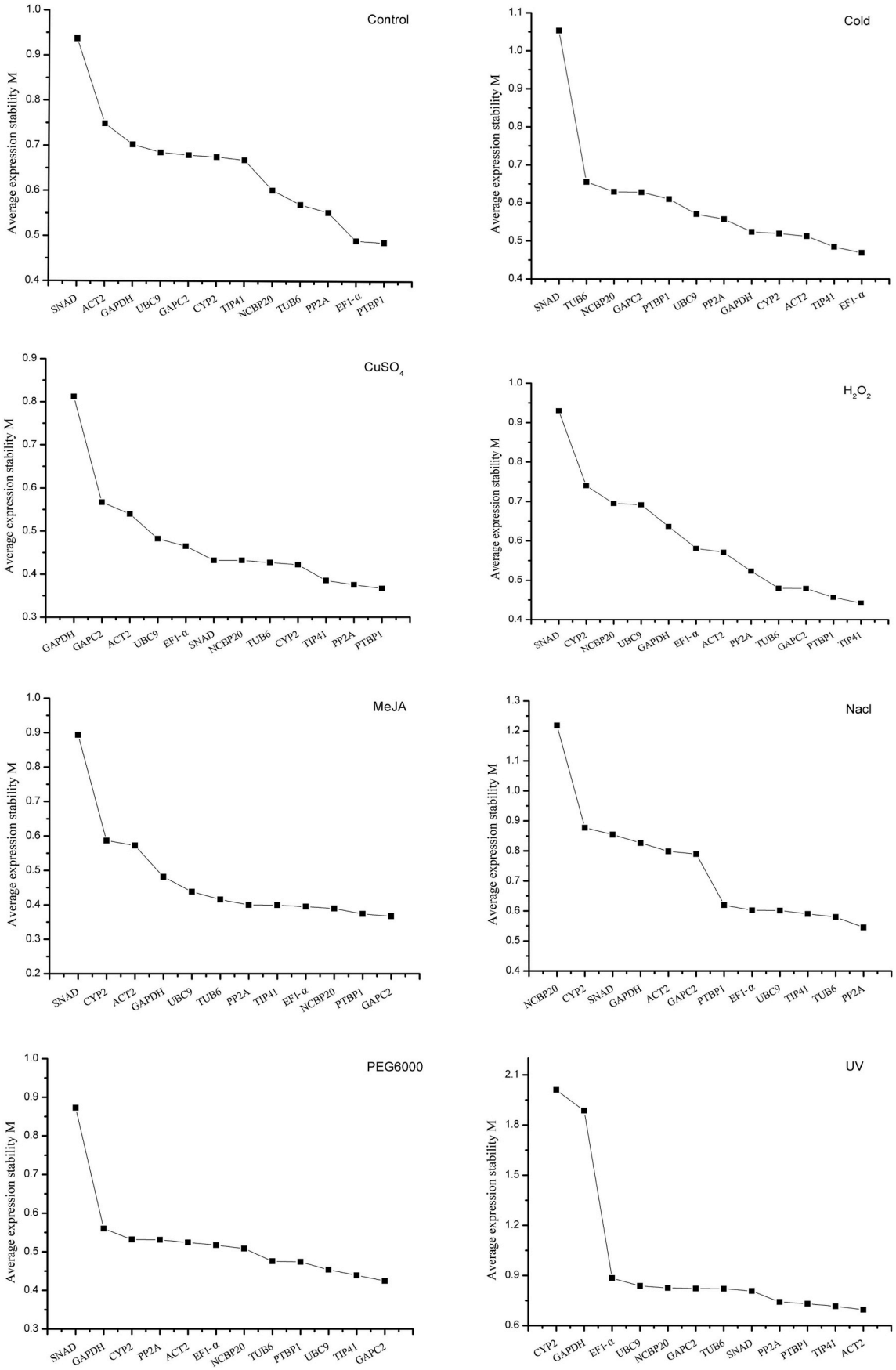

Fig. 2. Expression stability of 12 candidate genes in *M. charantia* calculated by geNorm analysis. Average expression stability values (M) of the candidate reference genes were calculated by the geNorm. The lowest M-value indicates the most stable gene. The least stable genes are on the left, and the most stable on the right.

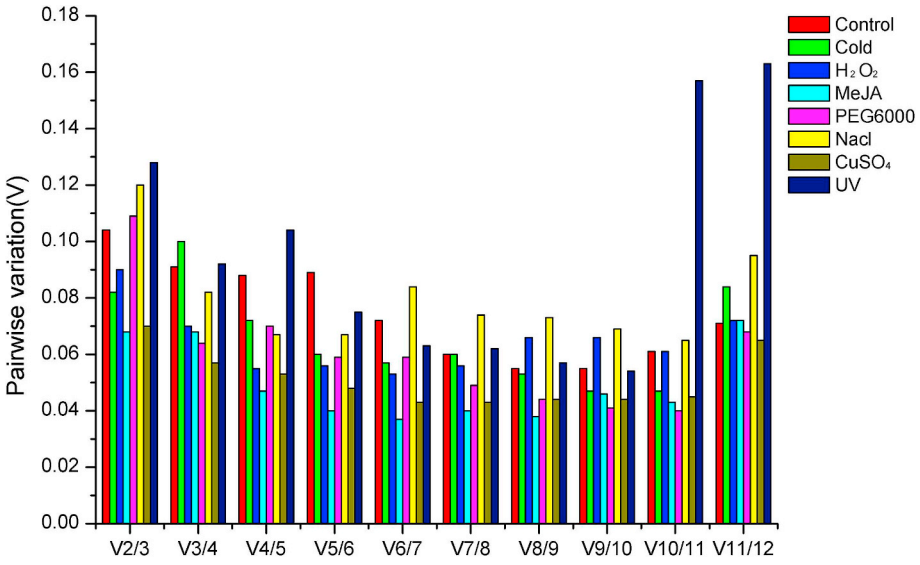

Fig. 3. Pairwise variation V (Vn/n+1) values of candidate reference genes calculated by using geNorm in the *M. charantia*. Different treatments are marked as square frame with different colors. The cut-off value is 0.15 and used to determine the optimal number of candidate reference genes for RT-qPCR normalization. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

secondly of all the genes in different treatment groups. Intriguingly, in control, low temperature, oxidative stress and hormone group, the stability of the first two genes were totally the same with that of geNorm analysis, which validated the reliability of geNorm. In the other four groups (drought treatment, NaCl stress, $CuSO_4$ and UV), *GAPC2* (0.009), *PP2A* (0.005), *PP2A* (0.005) and *TIP41* (0.005) were the most stable genes, respectively, as lowest values were presented (Table 2). Among the least stable reference genes, *SNAD* showed a highest value in most groups (5 out of 8 groups), which suggested that this gene was not a credible choice.

2.3.3. BestKeeper analysis

BestKeeper analysis can directly calculate the raw data of Ct values rather than transfer into relative expression levels (Pfaffl et al., 2004). As is shown in Table 3, the stability of each gene could easily be identified through sequencing the coefficient of variation (CV) and the standard deviation (SD). As far as the lowest CV ± SD is acquired, the relating reference genes cloud be identified as the most stable one. Besides, genes will be considered as unacceptable if the SD values are greater than 1. In $CuSO_4$ stress, MeJA treatment and H_2O_2 treatment subsets, *SNAD* was demonstrated to be the most stable gene exhibiting the lowest CV ± SD values at 0.5 ± 0.08, 1.04 ± 0.21 and 2.26 ± 0.47, respectively. In the control, drought stress conditions, *EF1-α* and *NCBP20* were the best reference genes with CV ± SD values at 0.69 ± 0.15 and 0.55 ± 0.11, respectively. What makes us attentive is that in UV-treated subset, *TIP41* (0.6 ± 0.13), *PTBP1* (1.17 ± 0.25) were considered as the most stable genes, showing consistency with the results of NormFinder analysis. Additionally, *ACT2* and *CYP2* displayed the highest stability among all candidates in the treatment of NaCl and Cold, respectively (Table 3).

2.3.4. RefFinder analysis

To implement a comprehensive analysis on the results obtained from the former three Excel-based algorithms, an auxiliary tool named RefFinder was utilized (http://150.216.56.64/referencegene.php#) to calculate the Ct values of each candidate gene and re-rank the

Table 2
Expression stability of the candidate reference genes calculated by NormFinder software.

Rank	Control	Cold	H_2O_2	MeJA	PEG6000	NaCl	$CuSO_4$	UV
1	*PTBP1*	*EF1-α*	*TIP41*	*GAPC2*	*GAPC2*	*PP2A*	*PP2A*	*TIP41*
	0.004	0.008	0.003	0.006	0.009	0.005	0.005	0.005
2	*EF1-α*	*TIP41*	*PTBP1*	*PTBP1*	*TIP41*	*TIP41*	*PTBP1*	*PTBP1*
	0.005	0.015	0.004	0.006	0.012	0.011	0.006	0.006
3	*PP2A*	*CYP2*	*TUB6*	*NCBP20*	*PTBP1*	*TUB6*	*TIP41*	*ACT2*
	0.015	0.015	0.009	0.010	0.014	0.012	0.009	0.008
4	*TUB6*	*ACT2*	*GAPC2*	*TIP41*	*TUB6*	*EF1-α*	*CYP2*	*PP2A*
	0.016	0.016	0.009	0.010	0.015	0.014	0.011	0.009
5	*NCBP20*	*GAPDH*	*PP2A*	*EF1-α*	*EF1-α*	*PTBP1*	*SNAD*	*GAPC2*
	0.020	0.019	0.012	0.012	0.016	0.017	0.013	0.016
6	*CYP2*	*PP2A*	*EF1-α*	*PP2A*	*NCBP20*	*UBC9*	*NCBP20*	*NCBP20*
	0.023	0.02	0.019	0.012	0.017	0.021	0.014	0.017
7	*GAPC2*	*PTBP1*	*ACT2*	*TUB6*	*CYP2*	*GAPC2*	*TUB6*	*TUB6*
	0.024	0.023	0.024	0.013	0.018	0.032	0.015	0.019
8	*TIP41*	*GAPC2*	*NCBP20*	*UBC9*	*UBC9*	*ACT2*	*EF1-α*	*EF1-α*
	0.024	0.025	0.026	0.018	0.018	0.034	0.017	0.024
9	*GAPDH*	*UBC9*	*GAPDH*	*CYP2*	*ACT2*	*CYP2*	*ACT2*	*SNAD*
	0.03	0.026	0.03	0.023	0.019	0.036	0.021	0.031
10	*ACT2*	*NCBP20*	*CYP2*	*GAPDH*	*GAPDH*	*GAPDH*	*UBC9*	*UBC9*
	0.032	0.027	0.03	0.026	0.022	0.037	0.021	0.031
11	*UBC9*	*TUB6*	*UBC9*	*ACT2*	*PP2A*	*SNAD*	*GAPC2*	*GAPDH*
	0.035	0.03	0.037	0.027	0.023	0.04	0.028	0.088
12	*SNAD*	*SNAD*	*SNAD*	*SNAD*	*SNAD*	*NCBP20*	*GAPDH*	*CYP2*
	0.044	0.046	0.042	0.045	0.041	0.064	0.037	0.093

Table 3
Expression stability of the candidate reference genes calculated by BestKeeper software.

Rank	Control	Cold	H_2O_2	MeJA	PEG6000	NaCl	$CuSO_4$	UV
1	*EF1-α*	*CYP2*	*SNAD*	*SNAD*	*NCBP20*	*ACT2*	*SNAD*	*TIP41*
CV ± SD	0.69 ± 0.15	1.23 ± 0.25	2.26 ± 0.47	1.04 ± 0.21	0.55 ± 0.11	0.73 ± 0.14	0.5 ± 0.08	0.6 ± 0.13
2	*PTBP1*	*PP2A*	*NCBP20*	*EF1-α*	*PTBP1*	*PP2A*	*ACT2*	*PTBP1*
CV ± SD	1.04 ± 0.21	1.65 ± 0.3	2.71 ± 0.56	2.43 ± 0.53	1.49 ± 0.31	1.58 ± 0.3	0.97 ± 0.21	1.17 ± 0.25
3	*PP2A*	*TUB6*	*PP2A*	*NCBP20*	*GAPC2*	*TUB6*	*PP2A*	*PP2A*
CV ± SD	1.34 ± 0.25	1.94 ± 0.34	2.94 ± 0.58	2.69 ± 0.54	1.57 ± 0.34	1.73 ± 0.32	1.05 ± 0.21	1.23 ± 0.26
4	*NCBP20*	*PTBP1*	*CYP2*	*PTBP1*	*TIP41*	*PTBP1*	*PTBP1*	*TUB6*
CV ± SD	1.47 ± 0.29	2.12 ± 0.43	2.99 ± 0.66	2.7 ± 0.55	2.12 ± 0.42	2.07 ± 0.42	1.16 ± 0.24	1.23 ± 0.28
5	*TUB6*	*EF1-α*	*PTBP1*	*TIP41*	*EF1-α*	*EF1-α*	*CYP2*	*NCBP20*
CV ± SD	1.71 ± 0.34	2.4 ± 0.46	3.12 ± 0.67	2.74 ± 0.55	2.14 ± 0.46	2.14 ± 0.44	1.18 ± 0.25	1.37 ± 0.3
6	*ACT2*	*ACT2*	*GAPC2*	*UBC9*	*GAPDH*	*CYP2*	*TIP41*	*ACT2*
CV ± SD	1.73 ± 0.32	2.49 ± 0.44	3.74 ± 0.82	2.81 ± 0.46	2.15 ± 0.39	2.31 ± 0.48	1.55 ± 0.31	1.4 ± 0.33
7	*GAPC2*	*TIP41*	*TIP41*	*GAPC2*	*CYP2*	*SNAD*	*UBC9*	*GAPC2*
CV ± SD	2.47 ± 0.52	2.71 ± 0.47	3.86 ± 0.8	2.83 ± 0.6	2.24 ± 0.47	2.49 ± 0.45	1.59 ± 0.25	1.66 ± 0.39
8	*CYP2*	*UBC9*	*TUB6*	*TUB6*	*TUB6*	*TIP41*	*TUB6*	*SNAD*
CV ± SD	2.67 ± 0.57	2.8 ± 0.36	4.08 ± 0.85	3.45 ± 0.66	2.57 ± 0.52	2.78 ± 0.54	1.63 ± 0.32	2.27 ± 0.34
9	*SNAD*	*GAPDH*	*EF1-α*	*PP2A*	*UBC9*	*GAPC2*	*NCBP20*	*EF1-α*
CV ± SD	2.68 ± 0.53	3.04 ± 0.53	4.68 ± 1.02	3.45 ± 0.64	2.63 ± 0.43	2.87 ± 0.54	1.68 ± 0.35	2.38 ± 0.55
10	*TIP41*	*NCBP20*	*ACT2*	*CYP2*	*SNAD*	*UBC9*	*EF1-α*	*UBC9*
CV ± SD	2.73 ± 0.56	3.36 ± 0.61	5.05 ± 0.99	3.62 ± 0.76	2.66 ± 0.5	3.8 ± 0.6	1.92 ± 0.4	2.81 ± 0.49
11	*GAPDH*	*SNAD*	*UBC9*	*GAPDH*	*ACT2*	*GAPDH*	*GAPDH*	*GAPDH*
CV ± SD	2.88 ± 0.51	3.39 ± 0.74	5.44 ± 0.91	4.1 ± 0.71	2.76 ± 0.52	4.67 ± 0.82	2.73 ± 0.58	3.92 ± 0.88
12	*UBC9*	*GAPC2*	*GAPDH*	*ACT2*	*PP2A*	*NCBP20*	*GAPC2*	*CYP2*
CV ± SD	3.23 ± 0.53	3.44 ± 0.63	5.63 ± 1.08	4.52 ± 0.84	2.98 ± 0.56	4.99 ± 0.94	2.86 ± 0.54	6.93 ± 1.44

candidate reference genes through analyzing the geometric mean of each single gene rankings calculated by three algorithms (Tang et al., 2017) (Tables S4 and S5). As was revealed, the most stable genes calculated by RefFinder were approximate to that of geNorm and NormFinder barring BestKeeper analysis in control, low temperature, oxidative stress, hormone, drought stress and salt stress subsets. In metal stress and UV subsets, *PP2A* and *PTBP1* respectively exhibited high stability among candidates, which was slightly different from the outcomes of geNorm, NormFinder and BestKeeper analysis.

2.4. Reference gene validation

For the aim of validating the ranking results of the 12 candidate genes, the stability of the reference genes under hormone stress were compared with expression profiling of *M. charantia* samples based on RNA-seq results. The Fragments Per Kilobase Million (FPKM) of each reference gene were exhibited in Table S6. The expression levels and quantities of unigenes could be estimated according to the FPKM values and the CV of FPKM represents the variability in gene expression (Ma et al., 2016). As depicted in Fig. 4A, the CV values of *PTBP1*, *GAPC2* and *NCBP20* were lower than those of other genes, which indicated a more stable expression. According to the results, the rankings of these three genes were similar with the ranking results of geNorm and NormFinder (Fig. 4B), which validated the reliability of the results once again. Besides, *SNAD* seemed not compliant as a suitable reference gene due to the CV value under the stimulation of MeJA was much higher than other genes.

To go deeply into the reliability of the results, the normalized fold expression of *APX1* under Cold, NaCl and MeJA treatments were analyzed. Ascorbate peroxidase is a hydrogen peroxide-scavenging enzyme that is specific to plants and algae and is indispensable to protect chloroplasts and other cell constituents from damage by hydrogen peroxide and hydroxyl radicals produced from it (Asada, 1992). Previous studies demonstrated that *APX* could be regulated by various factors including low temperature, H_2O_2, NaCl and MeJA (Lu et al., 2007; Sharma et al., 2012). As is shown in Fig. 5, the expression level of *APX1* was only slightly up-regulated using *EF1-α*, *TIP41* and *CYP2* as reference genes during low temperature treatment, but 12.73-fold change was observed using *SNAD* for reference. Similar outcomes could be found in the NaCl subsets, where a slight difference appeared between *PP2A* + *TUB6* and *PP2A* + *TUB6* + *TIP41*, while a significant difference was found when using the most unstable gene (*NCBP20*) as the internal control. Under MeJA treatment, inconsistent relative abundances of *APX1* were revealed among *GAPC2*, *NCBP20*, *EF1-α* and *SNAD* reference genes. The results indicated that the external stress conditions where the species were treated should be considered when selecting a suitable reference gene.

3. Discussion

As an advanced and commonly used technique, RT-qPCR acts as a pivotal part in quantifying the relative abundance of target genes as well as improving the quantification accuracy of target genes in various kinds of species (Dekkers et al., 2012; Evans et al., 2008; Maltseva et al., 2013; Pinto et al., 2012). Nevertheless, selecting unsuitable reference genes may cause unfaithful or wrong conclusions for normalization of genes (Gutierrez et al., 2008; Li et al., 2016). Therefore, selecting suitable reference genes meticulously would be essential for target gene normalization in different species under biotic or abiotic stresses.

Despite the importance of some compounds extracted from *M. charantia,* no related reference genes have been investigated now. In current work, 12 reference genes were screened from the transcriptome dataset of *M. charantia*. The Ct values of RT-qPCR in *M. charantia* exposed to different stresses were systematically analyzed by geNorm, NormFinder, and BestKeeper, and the findings of this study suggested that genes should be selected flexibly for specific milieu. Besides, the rankings of genes were not definitely the same even under the same conditions due to the discrepant calculation principles of three statistical algorithms. In most cases, the genes stability results calculated by geNorm and NormFinder were similar, such as *PTBP1* in control group. In cold, osmotic, salt, oxidative and hormone stress subsets, *EF1-α*, *GAPC2*, *PP2A*, *TIP41* and *GAPC2* were ranked at the most stable position in a decreasing order. However, the most stable gene calculated by geNorm in metal stress subset was slightly different from that of NormFinder, which determined *PP2A* as the most stable gene (Fig. 2 and Table 2). Interestingly, despite similar rankings results could be found when comparing the data obtained from geNorm and NormFinder, the conclusion of BestKeeper reveal a great difference. For instance, in oxidative stress subset and hormones subsets, *SNAD* was validated to be the best reference gene in BestKeeper (Table 3), whereas

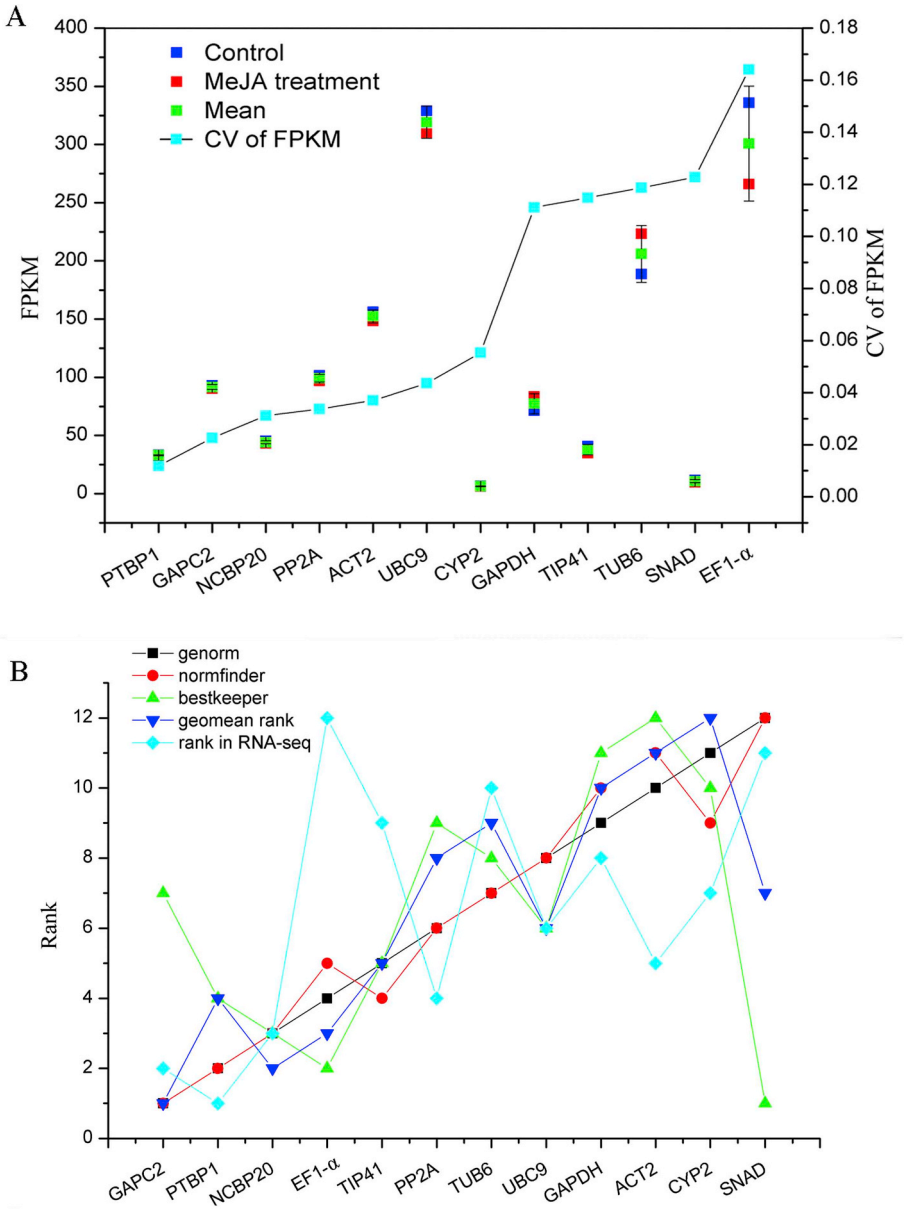

Fig. 4. Validation of RT-qPCR results through comparison with RNA-seq expression profiles. (A) Stability rankings of candidate genes by CV of FPKM in RNA-seq. A lower CV value indicates gene with more stable expression. (B) Correlation analysis between ranking of MeJA treatment subset by RT-qPCR and the ranking of RNA-seq. CV, coefficient of variation; FPKM, fragments per kilobase of exon model per million mapped reads.

ranked at the final position by geNorm (Fig. 2). Additionally, the final rankings of 12 candidate genes were generated by conducting a comprehensive tool RefFinder, which analyzed the geometric mean of rankings of each single gene calculated by three algorithms (Tang et al., 2017; Xie et al., 2012). The lower the geometric mean is, the more stable the gene is. (Table S4 and S5).

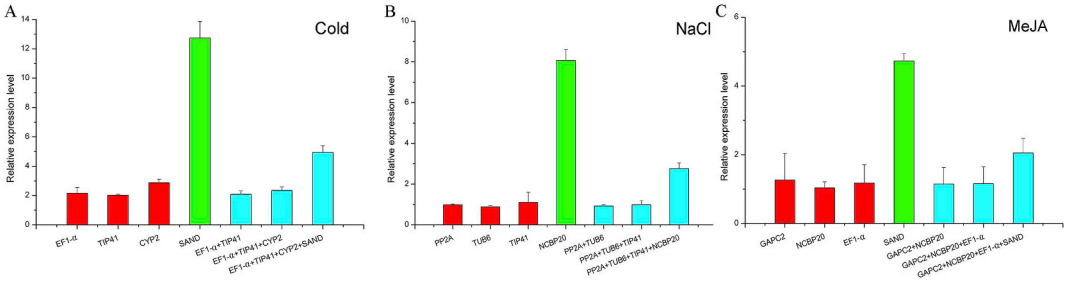

Fig. 5. Relative expression level of *APX1* was normalized using identified candidate reference genes under different treatments. (A) Expression level was normalized using most and least stable reference genes under cold treatment. (B) Four reference genes with three stable and one least stable were used to normalize the expression level of *APX1* under NaCl treatment. (C) The relative expression level of *APX1* was normalized by using the four reference genes under hormone treatment. Values shown are relative expression levels ± SD (n = 3).

The most natural and general functions of reference genes include participating in basic and ubiquitous cellular processes and expressing cellular structure components. Genes like *ACT*, *EF1-α* and *18S rRNA* are considered to be expressed constantly among different species and under environmental stimuli (Chandna et al., 2012). However, it should be pointed out that previous researches have demonstrated that most stable internal reference genes are not always stably expressed in many species or under some specific experimental conditions (Iskandar et al., 2004; Ohl et al., 2005; Rubie et al., 2005; Silver et al., 2006; Yperman et al., 2004). In this study, for instance, the results have proved that *GAPC2* was the optimal reference gene in osmotic stress and hormones subsets, whereas the result of metal stress indicated that *GAPC2* was not reliable enough to be a candidate reference gene as it almost ranked at the final position. Besides, despite *EF1-α* did well in control and low temperature subsets, the same conclusions could not be drawn from both UV and metal stress subsets. When compared with the previous studies, *EF1-α* was stably expressed under cold stress in tomato (Løvdal and Lillo, 2009), the same conclusion could be found in *M. charantia* under the same condition. *PTBP1* was the most stable gene in copper sulfate subset, and a similar consequence could also be found in *Peucedanum praeruptorum* (Zhao et al., 2016). *TUB6* was demonstrated to be a reliable reference gene in *M. charantia* by the stimulation of high concentration of salt stress, but it was reported to be the penultimate stable gene calculated by NormFinder in *Lycoris aurea* under salt stress (Ma et al., 2016), which reminds us that enough consideration about species and experimental conditions should be taken into account when selecting a suitable reference gene.

The most stable reference genes figured out by three algorithms could be appropriate choices under specific experimental conditions, but the second or the third gene had a similar stable characteristic, which means more than one reference gene are suitable for accurate normalization of RT-qPCR data in different conditions. Previous researches have demonstrated that one single reference gene may result in inaccurate relative quantitation of target genes (Gu et al., 2011; Veazey and Golding, 2011). Therefore, to solve this problem, 0.15 was recommended as an ideal threshold value by geNorm algorithm to screen the minimum number of reference genes needed for accurate normalization across different external stress conditions (Vandesompele et al., 2002). The result was exhibited in Fig. 3, where the V2/3 values among samples under specific conditions showed coincident values below 0.15, implying that two references should be needed for reliable normalization in these groups. However, evidence of 0.15 working as a reference value comes from several studies, showing that proposed 0.15 is an ideal and theoretical threshold rather than an absolute one and a higher V value has been reported (Marum et al., 2012; Tian et al., 2015; Wan et al., 2010).

In this study, similar results corresponding with previous studies could be obtained that *APX1* could be regulated by low temperature, hormone and salt stress (Lu et al., 2007; Sharma et al., 2012). Our results indicated the relative abundances of target gene *APX1* were slightly different when using the most stable genes figured out by former algorithms. However, a significantly different outcome could be found if the reference genes were the least stable ones. Hence, it should be necessary to choose suitable reference genes for accurate RT-qPCR results. Meanwhile, for further verification of the best reference genes, we compared the CV values of the FPKM of 12 candidate reference genes between control and MeJA treatment groups in our RNA-seq data (Fig. 4A and Table S6). At the same time, CV values of 12 candidates were also compared with our results obtained by geNorm, NormFinder and BestKeeper (Fig. 4B) and the overall result revealed a general consistency. Given that there were only two biological repeats of RNA-seq data, some potential inaccuracy might emerge in CV values of 12 candidates and the verification may become unconvincing. However, it did not undermine the final results of the experiments because the sequencing data were only used for the verification in the following validating experiments, instead of being directly regarded as conclusion. For such reason, the data could only be considered as preliminary, which indicated the trends of CV values of the FPKM values of 12 candidate reference genes.

4. Conclusions

In summary, 12 reference genes were screened from the *M. charantia* transcriptome and were investigated to select the most stable candidates under a wide range of stress conditions. The results revealed that 12 selected candidate genes exhibited different stability in *M. charantia* under specific experimental conditions. *TIP41* was the most stable candidate across all the reference genes, followed by *PTBP1* and *PP2A*. Besides, *SNAD* was proved not a good choice in most circumstances because of its low stability. Additionally, the results were further validated by comparing with the expression profiles based on the RNA-seq, and the relative abundance of *APX1* was applied once again to confirm the reliability of the results. Generally speaking, the fundamental research on gene expression in *M. charantia* deserved further effort in multiple abiotic stress conditions. More importantly, it could provide guidelines to obtain best reference genes in other plant species.

5. Experimental

5.1. Plant material, growth conditions and stress treatments

The seeds of *M. charantia* used in this study were purchased from GanXin Seed Co. Ltd, Jiangxi Province, China. Seeds with similar size were germinated in plastic pots containing vermiculite sand and nutrient soil. All seeds were grown in greenhouses with relative constant condition at 25 °C, 3000 lux of light intensity (16/8 h light/darkness) and a relative humidity of 50.0% ± 5% until collected. After 7 days' maintenance, seeds were germinated and seedlings were irrigated with Hoagland's solution weekly. Two weeks after germination, seedlings with four leaves were prepared for various experimental treatments. For drought treatment, plants were subjected to 30% PEG6000 solution (w/v, polyethylene glycol, Sangon, China) for 24 h. For low temperature treatment, plants were placed in greenhouse at temperature of 4 °C for 24 h 100 mL of 300 mM NaCl was applied to simulate a high-salt circumstance and plants were also incubated for 24 h. For hormone treatment, a same volume of MeJA (100 μM) solution was applied for 24 h according to the method described before (Ma et al., 2016). For the ultraviolet rays (UV), plants irrigated by 100 mL of distilled water were exposed to UV for 24 h. Oxidative stress was performed by exposing seedlings to 100 mL of 500 mM H_2O_2 for 24 h. Metal stress was performed by using 300 mM $CuSO_4$ for 24 h (Zhao et al., 2016). And the seedlings of control groups were irrigated with 100 mL of distilled water. Each external experimental treatment contains three biological replicates. The leaves of samples were harvested and frozen in liquid nitrogen before mRNA degrading, and then stored at −80 °C.

5.2. Total RNA isolation and cDNA synthesis

Approximately 100 mg of frozen samples were used to RNA extraction using Spectrum Plant Total RNA Kit (Sigma, USA). The quality and purity of the total RNA were analyzed by NanoDrop spectrophotometer 2000 (Thermo Scientific, USA). The integrity of RNA samples was confirmed by 1.5% agarose gel. RNAs with an $A_{260}/_{280}$ ratio between 1.8 and 2.2 as well as $A_{260/230}$ higher than 2.0 were used for further synthesis. RNA samples were pretreated with RNase-free DNase I (Takara Biotechnology, Dalian, China) before being used in reverse transcription for the purpose of eliminating the influence of DNA contamination. According to the instructions (HiScript Q RT SuperMix for qPCR, Vazyme, China), 1 μg of total RNA was used for cDNA synthesis in a total volume of 20 μL. Then, 10 times diluted cDNA was used for RT-qPCR studies.

5.3. Potential candidate reference gene selection and primer design

We have performed transcriptome sequencing of *M. charantia* seedlings using 454 sequencing technology, the samples were exposed to 0 μM MeJA (control), 10 μM MeJA and 100 μM MeJA for 24 h, each condition contains two replicates. The results could be found in NCBI with the accession number SRP165810. According to the previous studies of reference genes in multiple species, 12 candidate genes were selected with the purpose of identifying the most stable reference genes of *M. charantia* exposed to multiple environmental conditions. The potential unigenes were screened and selected through a local blast (TBLASTN) using the built-in program in Bioedit Sequence Alignment Editor. The corresponding homologs of these 12 reference genes were selected from TAIR database, only the unigene with lower E-value and higher bit score among the results was selected. To avoid genomic DNA contamination, primers were designed across exon boundaries and exon analysis was processed using AlignX program in vector NTI advance 11.5. All primer pairs were designed abide by the following criteria: the amplicon lengths of each gene from 100 to 150 bp, GC contents of 40–60%, primer lengths between 20 and 22 bp, temperature difference of each primer pair was lower than 1 °C, and melting temperature (Tm) in a range of 59–62 °C. All information about the primer pairs designed in this study are exhibited in Table 1 and Table S6.

5.4. Real-time quantitative PCR analysis

In order to improve the accuracy and proficiency of RT-qPCR, PCR was carried out using the cDNA as template, and primer pairs for the reference genes are shown in Table 1. The size of PCR products were checked by 1.5% (w/v) agarose gel electrophoresis, the PCR products were inserted into pMD19-T Vector (Takara, Dalian, China) for sequencing. qPCR analysis was carried out on the LightCycler 480 (Roche) using Hieff™ qPCR SYBR® Green Master Mix (No Rox) (YEASEN, Shanghai, China). The reaction mixture contained 2 μL 10 times diluted cDNA, 10 μL Hieff™ qPCR SYBR® Green Master Mix (No Rox), 0.4 μL each primer (10 μM) in a total volume of 20 μL. The reactions were incubated under following cycling conditions: 5 min at 95 °C, 40 cycles of 10 s at 95 °C, 20 s at 55 °C and 20 s at 72 °C, followed by 1 cycle of 95 °C for 1s, 60 °C for 60s, and 95 °C for 15s. Three technical replicates and three biological replicates were set for each cDNA and the raw Ct values of all samples were listed in Table S2. To obtain the qPCR amplification efficiency of primer pairs, the relationship between fluorescence and cycle values was quantified and calculated by LC480 Conversion and LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009). Melting curve analysis were also achieved to check the specificity of each primer pair.

5.5. Statistical analysis of gene expression stability

To make an intuitive analysis of the stability of each gene performed across all of the experimental conditions, geNorm, NormFinder as well as BestKeeper were performed to analyze the Ct values. For both geNorm and NormFinder analysis, all the raw Ct values achieved by RT-qPCR were changed into relative quantities using a formula: $2^{-\Delta Ct}$ (ΔCt = each corresponding Ct value - the minimum Ct value), then the stability of each gene was available after the values of $2^{-\Delta Ct}$ were imported into programs. According to manual of geNorm, the expression stability value (M) of each reference gene was generated by comparing pairwise variation (V) of that gene with all the other reference genes. The threshold parameter (M = 1.5) was set to determine the stability of each gene, and low M values are attributed to high stability. Pair-wise variation analysis (Vn/n + 1) was calculated using geNorm to determine the optimal number of reference genes required for reliable normalization. The threshold value of Vn/n + 1 was set as 0.15, a (Vn/n + 1) value below this threshold value indicated that there is no necessary to add more reference genes for reliable normalization (Vandesompele et al., 2002). NormFinder, similarly but not the same, used ANOVA-based model to estimate intra- and inter-group variation (Andersen et al., 2004). Identical with geNorms, the candidate gene with lower average expression stability value is the most stable reference gene. However, the BestKeeper analysis, unlike geNorm and NormFinder, uses the CV ± SD as the parameters to screen the most stable reference genes, and gene with lower CV ± SD value possesses higher stability. After a comprehensive analysis of the rankings obtained from these three algorithms, it would be intuitive to assess the optimal reference genes in different external experimental conditions.

5.6. Validation of reference genes

For validating the ranking results of the 12 selected reference genes calculated by three algorithms, the gene rankings under MeJA treatment were compared with the expression profiles of 12 candidates in RNA-seq, and the samples of RNA-seq were exposed to 100 μM MeJA for 72 h. The CV values of FPKM represents the variation of unigene expression, in light of which lower CV of FPKM reveals higher gene stability. Furthermore, according to the candidate genes identified in the study, the relative abundances of *APX1* of the NaCl and MeJA groups were quantified by RT-qPCR for further confirmation of the reliability of RT-qPCR results, while the samples without treatments were used as controls. Three biological and technical replicates were performed to obtain the qPCR data. The relative expression data were calculated according to the $2^{-\triangle Ct}$ method (Livak and Schmittgen, 2001) and presented as relative expression level. The sequence of *APX1* was obtained by aligning with *AtAPX1* from TAIR database.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

Z.L.W and Y.C.Z designed the project, Z.L.W, Y.H.L performed the experiments and wrote the manuscript. Z.L.W, J.Y.C, Y.L.H, H.F.L interpreted and analyzed the data; All authors read and approved the final manuscript. This Project funded by National Natural Science Foundation of China (Grant No. 81703637 and 81573564), China Postdoctoral Science Foundation (2016M601922, 2018T110577) and Natural Science Fund in Jiangsu Province (BK20170736).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2019.04.010.

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