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Full Length Research Paper

Cloning and selection of reference genes for gene expression studies in *Ananas comosus*

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Full length mRNA sequences of *Ac-β-actin* and *Ac-gapdh*, and partial mRNA sequences of *Ac-18SrRNA* and *Ac-ubiquitin* were cloned from pineapple in this study. The four genes were tested as housekeeping genes in three experimental sets. GeNorm and NormFinder analysis revealed that *β-actin* was the most appropriate reference gene for qPCR analysis of callus under induction conditions and in different tissue types, meanwhile, *18SrRNA* was the most stable reference gene during organ development. *Gapdh* was the most unstable gene in all tested experimental sets. Transcript level analysis result of *AcSERK*1 in stressed callus normalized by *β-actin* and *18SrRNA* further confirmed that reference genes selected in this study were suitable for transcript level analysis of pineapple. The expression pattern of *AcSERK*1 during somatic embryogenesis normalized by *β-actin* coincided with the cytological features of calluses during somatic embryogenesis. These results will enable more accurate and reliable normalization of qPCR results for transcription analysis in pineapple.

Key words: Reference genes, qPCR, pineapple, geNorm, NormFinder.

INTRODUCTION

Gene transcript level analysis is significant for the understanding of gene expression pattern and the revealing of gene function. It is one of the foundations of modern molecular biology. Real time PCR, Northern blotting, ribonuclease protection assay (RPA), and gene chips are the main methods for the study of gene transcript level.

Quantitative real-time PCR (qPCR) is one of the most sensitive and accurate techniques for rapid quantification of gene transcript levels. However, several factors, such as the quantity of the initial material, the quality of the RNA, the efficiency of cDNA synthesis, primer performance, selection of reference genes, and the statistical analysis methods, can affect the accuracy of qPCR results in gene transcript level studies. The accuracy of qPCR results relies a lot on the adequate use of appropriate normalization techniques to compensate for the differences in sample amount, RNA isolation, reverse transcription efficiency and qPCR reaction.

Among the normalization approaches which have been proposed, the use of reference gene has become one of the most effective and convenient methods (Baudino, 2001; Malik, 2007; Qi, 2010). Ideal reference genes should be stably expressed under different experimental parameters and conditions (Rodriguez, 2008). Many studies used potential housekeeping genes (HKGs) as reference genes without validating their suitability for normalization (Bustin, 2002). However, many studies confirmed that the transcript levels varied depending on species, tissues, developmental stages and experimental conditions (Que et al., 2009; Spinsanti et al., 2006; Infante et al., 2008; Lin and Lai, 2010).

It is acknowledged that it is important and necessary to validate the expression stability of candidate reference genes in each specific experimental system before its use for normalization of the transcript level (Chari, 2010; Shen, 2010; Luo, 2010; Gresner, 2010; de Almeida, 2010). The use of inappropriate HKGs as reference genes can result in biased expression profiles (Xu, 2010).

Pineapple (*Ananas comosus*) is one of the three most

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important tropical fruit crops in the world. It is self-sterile and in practice, its propagation depends on the suckers. However, since there are only about five suckers on a pineapple plant per year, its propagation lags behind other seed propagated crops. Somatic embryogenesis is an effective regeneration protocol for plants.

We have established a somatic embryogenesis system under the induction of 2,4-dichlorophenoxyacetic acid (2,4-D) (He et al., 2007). Induction and synchronization of somatic embryos are the key traits for pineapple *in vitro* regeneration. Despite the morphological and anatomical studies (He et al., 2008, 2010) available, very little is known about the molecular mechanisms of somatic embryogenesis in pineapple. Studies showed that the expression variation of most somatic embryogenesis related-genes were inconspicuous (Nolan, 2003; Sharma et al., 2008). In this sense, it is very important to choose proper reference genes for gene transcript level analysis of somatic embryogenesis-related genes. So far, there is no report about the validation of the expression stability and the selection of reference genes for gene transcript level studies in pineapple. We have isolated *AcSERK*1 from pineapple (Ma et al., 2012). It belongs to the somatic embryogenesis receptor-like kinase (SERK) gene family. Studies confirmed that the SERKs played an important role during somatic embryogenesis and were receptors of many internal and environmental signals (Albrecht et al., 2008; Baudino et al., 2001; Huang et al., 2010, Sharma et al., 2008). It was reported that some SERKs partially mediated defense signal transduction leading to stress resistance (Hu et al., 2005; Song et al., 2008; Huang et al., 2010).

We isolated and examined the expression of four commonly used control genes including *β-actin*, *gapdh*, *18SrRNA* and *ubquitin* by the qPCR technique; actin is one of the most abundant proteins in eukaryotic cells. It is highly conserved and participates in more protein-protein interactions than any known protein (Dominguez and Holmes, 2011). Gapdh is a key enzyme in the glycolytic pathway, and it catalyzes the oxidation and subsequent phosphorylation of substrate aldehydes to acyl-phosphates, resulting in the production of adenosine triphosphate through the electron transport chain (Mozdziak et al., 2004). Ribosomal ribonucleic acid (rRNA) is one of the structural materials for ribosomes, which help build proteins. Ubiquitin has been suggested to play a key role in a wide variety of essential cellular functions ranging from differential regulation of gene expression to protein degradation (Monia et al., 1990).

The expression stability of the four genes in three experimental sets was validated by geNorm and Norm-Finder programs. The results show that *β-actin* was the most appropriate reference gene for qPCR of pineapple callus under induction conditions and in different tissue types, meanwhile, *18SrRNA* was the most stable reference gene during developmental stages. *gapdh* was the most unstable gene in all tested experimental sets.

MATERIALS AND METHODS

Sample collection

Three sets of samples as follows were used to isolate RNA for the expression stability study of the four candidate genes; 1) callus were harvested at five, 10, 15, 20, 25, 30, 35, 40 and 45 days after cultured on 2,4-D containing medium to induce somatic embryogenesis; 2) calyxes were taken at the time points of 0, seven, 15, 30 and 60 days of flower bloom; and 3) stem, calyx, petal, stylus and ovary were harvested at the first day of flower bloom.

To test the suitability of the putative reference genes, four different induction conditions were used: high temperature (40°C) treatment for 24 h, low temperature (4°C) treatment for 24 h, culturing in NaCl (3% w/v) containing medium for 24 h, culturing in ethylene (1000 $mg \cdot L^{-1}$) containing medium for 24 h, and after injury treatment for 24 h. RNA was isolated and the expression levels of *AcSERK1* normalized by the selected reference genes were analyzed.

All the tissue samples obtained were quick-frozen immediately in liquid nitrogen and then stored at -80°C for later use. Three biological replicates of each sample were used for RNA isolation.

Total RNA extraction, quality controls

Total RNA was isolated using Plant RNAiso kit (Takara, Japan) according to the manufacturer's instructions. To remove the contaminating genomic DNA, we treated total RNA with RNase-free DNase I (Takara, Japan). Nucleic acid concentrations were measured at 260 nm with a BioPhotometer (Eppendorf, Germany). Purity of the total RNA was determined by the A260/280 and A260/230 ratio and its integrity was tested by electrophoresis using 1% formaldehyde denaturing agarose gel.

cDNA synthesis and quality confirmation

The first-strand complementary DNA (cDNA) was synthesized by reverse transcribing 2 µg of total RNA with Oligo dT Primer in a final reaction volume of 20 µl using M-MLV RTase Synthesis Kit (Takara, Japan) according to the manufacture's instructions. The cDNA was diluted according to the ratio of 1 µl cDNA to 9 µl nuclease-free water. The primers P23/P24 of *AcSERK1* (5'-GAAGTT/CCATCTTGGCCAGC-3' and 5'-ACCCAGTCC/AAGAAGCATGAC-3'), which can amplify a target with an intron from genomic DNA, were used to confirm that the cDNA samples were free of genomic DNA.

Cloning of four candidate reference genes

Primers used for the isolation of the four candidate reference genes are listed in Table 1. Degenerate primers were designed to amplify the conserve fragments of these genes. Then, 5' and 3' sequences of these genes were amplified by rapid amplification of cDNA ends (RACE) using the 3'-Full RACE Core Set Ver.2.0 kit and 5'-Full RACE Core Set Ver.2.0 kit (Takara, Japan) according to the manufacturer's instructions. The PCR production was purified and ligated into pMD19-T vector (Takara, Japan). The positive clones were sequenced by Sangon Co (China). Sequences were edited, aligned and analyzed using DNAMAN and CLUSTAL software tools. Specific primers were designed according to the results of the RACE to amplify the full-length cDNA sequences.

Table 1. Primer sequences for the cloning of four candidate genes in pineapple.

Symbol	Target	Primer name	Primer sequence (5'-3')
gapdh	Conserved fragment	gapdhup	CATGGSSAAGATYAAGATCGG
		gapdhdv	AGATGCTKGACCTGYTGTCACC
	5' fragment	gapdh5raceout	AAGGGTCTTAGAATCCTTCACCT
		gapdh5racein	AAGGGATCATTGACGGCGACGAG
	3' fragment	gapdh3raceout	TGCATTGAATGGGAAACTGACCG
		gapdh3racein	TACGACTATGTTCCCTGGTATTGC
	Full length	gapdhqcup	CTCTGTATTCTCCATCTCCATTTTCGTCTC
		gapdhqcdv	GCCAAATCCCAATTAAACTCTGATCACTT
β-actin	Conserved fragment	actinup	CAGTGGTCGTACAACTGGTAT
		actindv	ATCCTCCAATCCAGACACTGT
	5' fragment	actin5raceout	ATCCCAGTTGCTGACAATGCCGTGC
		actin5racein	CTCTTGGACTGGGCCTCATCCACCC
	3' fragment	actin3raceout	TGCGGGTATTCACGAGACCACTT
		actin3racein	TACGACTATGTTCCCTGGTATTGC
	Full length	actinqcup	GCTCTCTTAGGATCCATAAAAAATG
		actinqcdv	CAATCCAGGAAATTTAGAAGCACTT
18SrRNA	Conserve fragment	18Sup	CATCATTCAAATATCTGCCCTATCAA
		18Sdv	CAGCCTTGCGACCATACTCCC
ubquitin	Conserved fragment	ubup	TTGTGTTCTCTTTTGAAGTTC
		ubdv	CAAACCACGAGCATTCAAGTT
	3' fragment	ub3raceout	GCCTGCGTCCTGTTCTATGAA
		ub3racein	CGTCTCGTGTTCTTTGCTGTGGGTC

Primer design

According to the conserved sequences of *gapdh, β-actin, 18SrRNA* and *ubiquitin* in other plants, such as *Oryza sativa*, *Setaria italica*, *Zea mays*, *Hordeum vulgare*, *Glycyrrhiza uralensis*, etc., deposited at NCBI database, we designed specific primers (Table 1) for the isolation of these genes. The expression stability of the four genes was evaluated by qPCR. Primers were designed based on the cloned sequences using DNAMAN software 6.0 (Lynnon Biosoft). All oligonucleotides were synthesized by Sangon Co (China). The gene name, gene description, accession number, primer sequence, product size, PCR efficiencies, and annealing temperatures are provided in Table 2. The primers for qPCR analysis of *AcSERK1* were designed based on the cDNA sequence of *AcSERK1* we cloned before. Specificity of the amplifications was verified by 1.5% agarose gel electrophoresis and at the end of the PCR run, by melt curve analysis with a temperature gradient from 65 to 94°C.

qPCR analyses

qPCR was performed on the Bio-rad iQ5 real time PCR System (Biorad, USA) using THUNDERBIRD SYBR qPCR Mix (Toyobo, Japan). Each amplifications was performed in a 20 µl final volume containing 10 µl of 2×SYBR Mix; 1.0 µl of 10× diluted cDNA sample (about 0.01 µg RNA); 2 µl of specific primer pair mix at 2.5 µM and 7 µl of ddH_2O. Reactions were performed in triplicates for technical replicates. Amplification was carried out with the following cycling parameters: heating for 2 min at 95°C, 40 cycles of denaturation at 95°C for 10 s, annealing for 20 s at 52°C, and extension at 72°C for 35 s. Analysis of the relative gene expression data was conducted using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Negative control with no sample was included for each primer pair.

Statistical analysis

The PCR efficiency and quantification cycle (Cq) value were calculated by iQ5 System Software (Biorad). The calculated results are shown in Table 2 and Figure 3. The expression stability was evaluated by both geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) programs.

***AcSERK*1 mRNA expression analysis**

The qPCR analysis of the expression of *AcSERK*1 in pineapple callus under different stressful conditions was used to validate the normalization based on the reference genes we selected in this study. The qPCR primers of *AcSERK*1 are listed in Table 2. We also used the selected reference gene to analyze the expression pattern of *AcSERK*1 during somatic embryogenesis.

RESULTS

Cloning of the four candidate reference genes

In order to design proper primers for qPCR, we isolated

Table 2. Primer sequences, product size, PCR efficiencies, and annealing temperatures of the candidate reference genes.

Symbol	Accession number	Primer sequence (5'-3')	Product size (bp)	PCR efficiency (%)	Annealing temperature (°C)
18S rRNA	*JN129389*	F: ATGGTGGTGACGGGTGAC R: CAGACACTAAAGCGCCCGGTA	162	99.2	58
β-actin	*HQ148720*	F: CTGGCCTACGTGGCACTTGACTT R: CACTTCTGGGCAGCGGAACCTTT	135	99.3	63
gapdh	*HM768296*	F: CTTTAACATCATTCCCAGCAGCACT R: GGTAATCTCAAAGGCATCTTGGG	216	100.1	61
ubquitin	*JN129388*	F: TTGGCTGACTACAATATCCAGAAGG R: GACCCACAGCAAAGAACACGA	182	98.8	59
AcSERK1	*HM236375*	F: TCTTGGTGTTCCACCCCCTTGCCAG R:GGAACCAACGTACCAGACAGTGCTGC	217	99.7	63

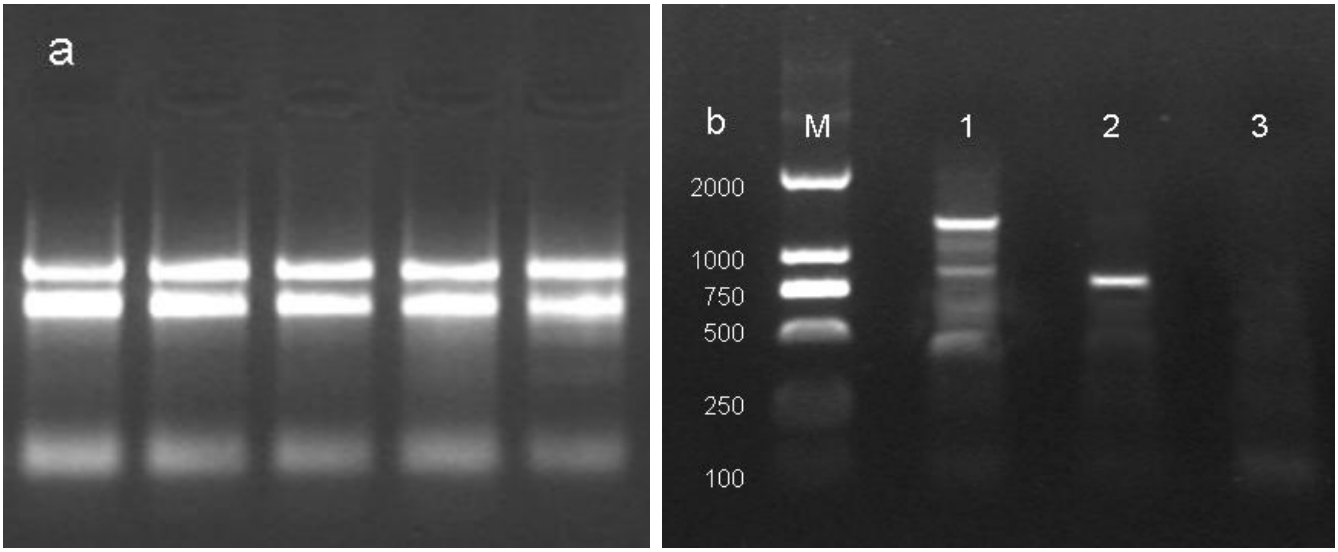

Figure 1. a) Confirmation of the integrity of RNA isolated; b) PCR result of primer pair P23/P24. M, Marker; 1, amplification result of genomic DNA; 2, amplification result of cDNA; 3, negative control without template.

the mRNA sequence of *β-actin*, *gapdh*, *ubiquitin* and *18SrRNA* using the primers listed in Table 1. The NCBI accession numbers of these sequences are listed in Table 2. *Ac-β-actin* was composed of 1169 bp, containing an 1134 bp open reading frame. The identities of *Ac-β-actin* to other actins were 85 to 88%. *Ac-gapdh* was composed of 1259 bp, containing 1014 bp open reading frame. The identities of *Ac-gapdh* to other *gapdh*s were 83 to 85%. A 835 bp fragment of *Ac-18SrRNA* was isolated, the identities of it to other *18SrRNAs* were 97 to 99%. A 319 bp fragment of *Ac-ubiquitin* was isolated; the identities of *Ac-ubiquitin* to other *ubiquitins* were 90 to 95%.

PCR efficiency and amplification specificity

The quality and integrity of RNA is essential in gene expression analysis. The integrity of RNA was confirmed by agarose gel electrophoresis. Only the RNA samples with A260/A280 ratio between 1.9 and 2.1, A260/A230 ratio higher than 2.0 and with integral three bands (Figure 1a) were used for further analysis. The amplification results of P23/P24 showed a belt of 1200 bp from genomic sample and an 810 bp product from cDNA sample (Figure 1b). It confirmed that the cDNA samples were free of genomic DNA. The melt curve analysis (Figure 2) showed that all the primers used in the study

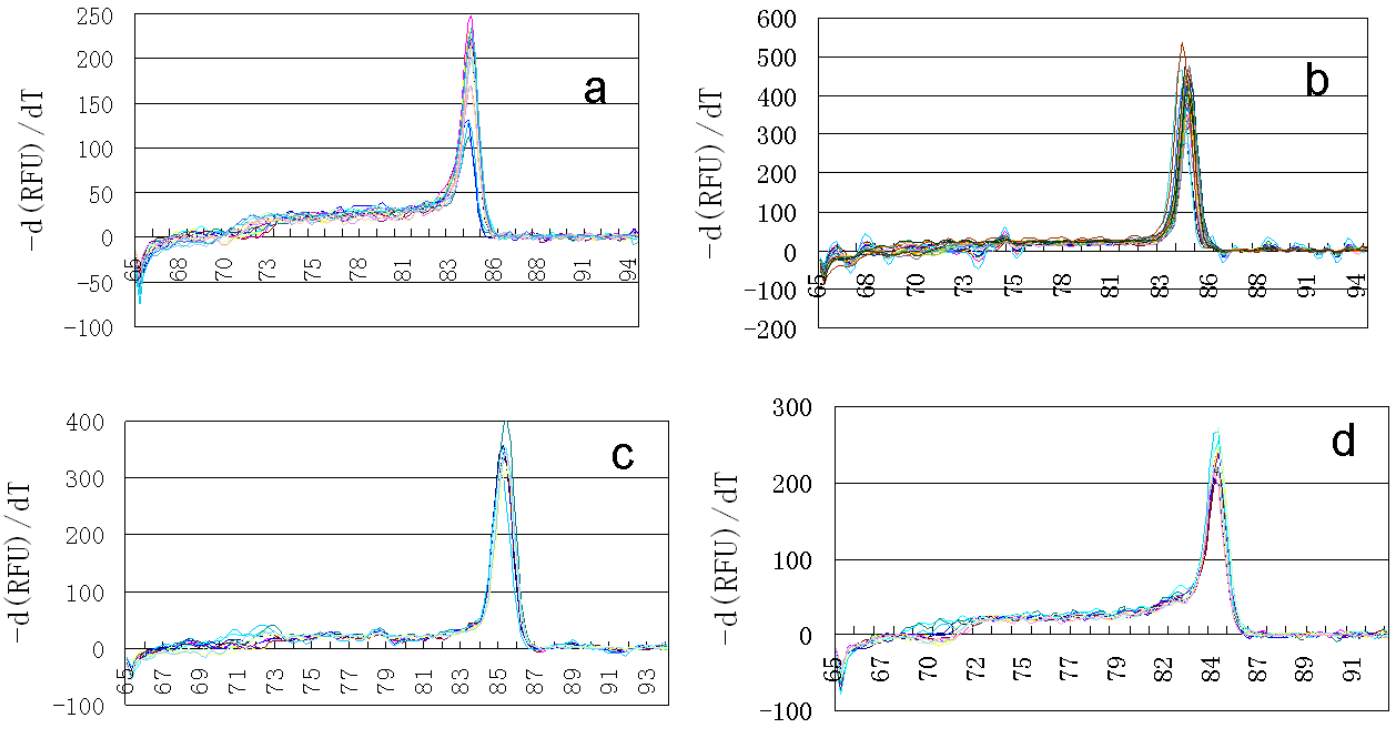

Figure 2. Melt curve of the four candidate reference genes; a) 18S rRNA, b) *gapdh*, c) β-actin, d) ubiquitin.

generated a single PCR product of the desired size from the various cDNAs. It indicated the good specificity of all of the primer pairs used in this study. A standard curve was created for each analyzed gene using a set of dilutions with five orders of magnitude. The significant linear relationships (r^2>0.98) and PCR efficiency values (ranged from 98 to 101%) are listed in Table 2.

Variations in the expression of candidate reference genes

Transcripts of the four candidate reference genes were detected in all the 16 samples by qPCR. The mean Cq values of three biological replicates of all the samples at same cDNA input are shown in Figure 3. Analysis of the raw expression levels in all the samples showed that the transcript levels of the four genes were significantly impacted by experimental conditions, developmental stages and tissue types. *β-actin* showed the lowest Cq values in all tested samples indicating the highest transcript levels. Contrarily, *ubiquitin* showed the highest Cq values. Some Cq values of *ubiquitin* were a little more than 30, but it can still be included by the standard curve. This indicated that the Cq values still obey the linearity of the PCR reaction, and it would not compromise the accuracy of the analysis. The other candidate reference genes were expressed at moderate levels. Each candidate gene displayed similar transcript levels in the respective experimental condition tested in this study. It can be assumed that all of the candidate genes were relatively stable in the tested experimental conditions.

Analysis of gene expression stability and reference genes selection

Different statistical methods based on respective principles can potentially yield discrepant results for different experimental data sets (Lin and Lai, 2010). Therefore, we compared two different statistical approaches in this study.

GeNorm program calculates an average expression stability value (*M*) based on the average pairwise variation existing between all pairs of the candidate genes. All the *M* value of the four candidate genes were below the default limit of *M*=1.5. According to the geNorm program, which calculates an average expression stability value (*M*) based on the average pairwise variation existing between all pairs of the candidate genes (Vandesompele et al., 2002), the expression stability of the four candidate genes in the three experimental sets was as follows (Table 3): 1) *β-actin* and 1*8S rRNA* were the most stable genes in callus under the induction of 2,4-D, from most stable (lowest *M* value) to least stable (highest M value): *β-actin* /1*8S rRNA* > *ubiquitin* > *gapdh*; 2) in different developmental stages of calyx, from most stable to least stable: *18S rRNA* / *β-actin* > *ubiquitin* > *gapdh*; therefore, for these two situations, *18S rRNA* and *β-actin* were the most stable genes; 3) in different tissue types, *β-actin* and *ubiquitin* were the most stable genes.

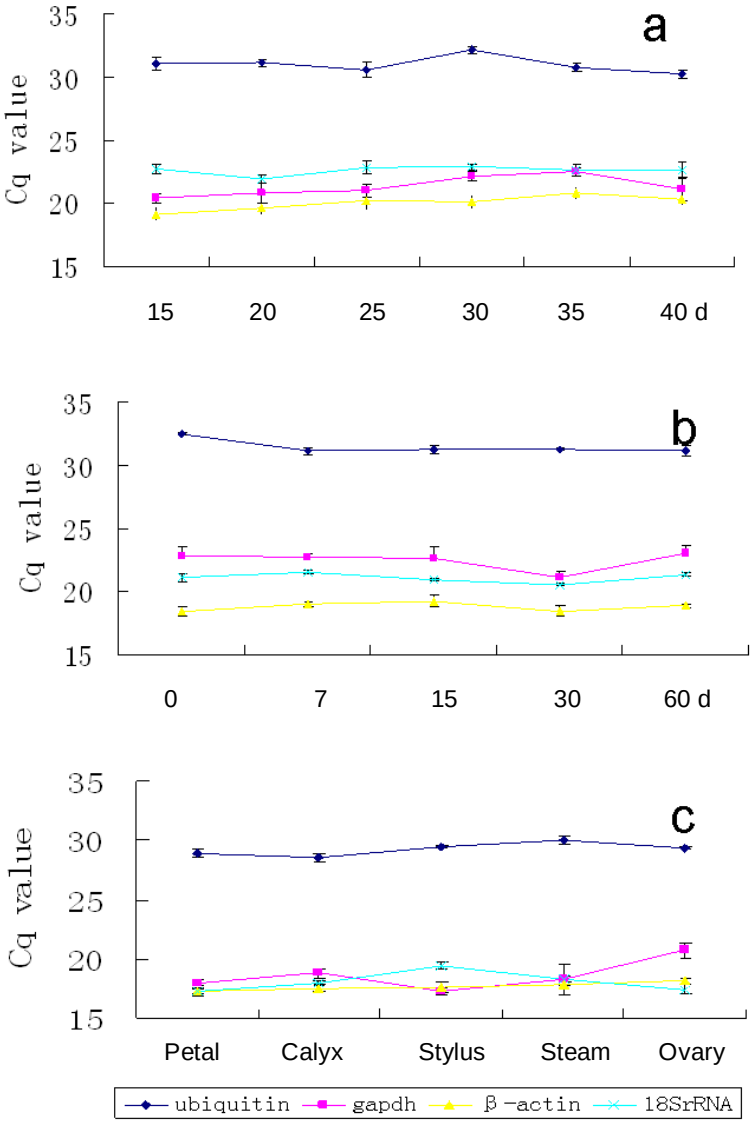

Figure 3. RNA transcription levels of candidate reference genes tested, compared at same cDNA inputs, and presented as mean value of Cq in different samples. Cq values are mean of three biological replicates; a) time duration of 2,4-D induction (in days), b) developmental stages of calyx in (days), c) different organs. 2,4-D, 2,4-Dichlorophenoxyacetic acid.

The *M* value from the lowest to the highest is: *β-actin*/*ubiquitin* < *18S rRNA* < *gapdh*.

Pairwise variations (V_n/V_{n+1}) were calculated by geNorm. The V2/3 values of the three experiment sets were all smaller than the cutoff threshold of 0.2. The results indicate that using two genes as internal controls could strongly reduce the variability, and it is sufficient for accurate normalization in pineapple (Figure 4).

NormFinder (Andersen et al., 2004) ranked the candidate reference genes according to their expression variation between intergroups and intragroups. According to it, *β-actin* and *18S rRNA* were the best normalization factors with the lowest stability values of 0.005, 0.008 and 0.013 respectively in the three experimental sets (Table 3). The expression stability order of the four candidate genes analyzed by NormFinder was coincident with the results of geNorm program. Stability of *gapdh* was the lowest among four candidate genes in the three experimental sets.

AcSERK1 expression

Use of *β-actin* and *18S rRNA* was evaluated as normalization genes to analyze the transcript level of *AcSERK*1 in pineapple callus under the following stressful

Table 3. Candidate genes ranked according to their expression stability, estimated using Normfinder and geNorm.

2,4-D induction			Different tissue types			Developmental stages		
Gene name	**Stability value (NormFinder)**	**Stability value (geNorm)**	**Gene name**	**Stability value (NormFinder**	**Stability value (geNorm)**	**Gene name**	**Stability value (NormFinder)**	**Stability value (geNorm)**
β-actin	0.005	0.519	*β-actin*	0.008	0.512	*18SrRNA*	0.013	0.516
18SrRNA	0.019	0.562	*ubiquitin*	0.008	0.572	*β-actin*	0.028	0.596
ubiquitin	0.040	0.648	*18SrRNA*	0.062	0.659	*ubiquitin*	0.032	0.824
gapdh	0.055	0.603	*gapdh*	0.077	0.747	*gapdh*	0.041	0.662
Best gene	*β-actin*	*β-actin*	Best gene	*β-actin*	*β-actin*	Best gene	*18SrRNA*	*18SrRNA*

conditions: high or low temperature, ethane, NaCl, and injury. The results show that *AcSERK*1 expression pattern normalized by *β-actin* was strongly agreed with the result of *18S rRNA* (Figure 5). Under NaCl-stressed conditions, the *AcSERK*1 transcript level was six to seven folds to that of low temperature treatment. The transcript levels under the induction of high temperature and ethane were close to those of low temperature. The similarity of *AcSERK*1 expression pattern normalized by *β-actin* and *18S rRNA* confirmed that the reference genes we selected in this study are suitable for the transcript level analysis of pineapple.

β-actin was used as normalization factor to analyze expression pattern of *AcSERK*1 during somatic embryogenesis in pineapple. The results show that there were two peaks of expression at different times (Figure 6). The first peak of expression occurred at 10 to 15 days of culture in 2,4-D containing medium, and at that time, many cells have acquired embryogenic competence. The second peak of expression is observed at 40 days of culture in 2,4-D containing medium. At this stage, there were many competent cells, meristematic cell clusters and globular embryos in the callus. These results coincided with the appearance of the cytological features of calluses under 2,4-D induction to form somatic embryo (He et al., 2010). The results suggest that accumulation of 2,4-D was sensed by cells and could induce the expression of *AcSERK*1, indicating that this gene may play a role in induction of embryogenesis of pineapple callus.

DISCUSSION

The expression level of a stable reference gene should vary minimally even between different experiments (Dean et al., 2002). Our results show that each experimental condition may demand a specific set of reference genes, thereby emphasizing the importance of validating reference genes for each experimental condition. To our knowledge, this study was the first attempt to validate a set of candidate reference genes under *in vitro* induction conditions, in different tissue types and during developmental stages for qPCR normalization in pineapple.

The expression stability analysis of the candidate reference genes showed that there were apparent differences in the stability between the four genes in three experimental conditions. This is coincident with the view of Luo (2010), de Almeida (2010), Gresner (2010) and Shen (2010). Similar to the result of Gresner (2010), the stability order ranked by geNorm closely matched the result of NormFinder, but in *Populus* (Xu, 2010) and *Brassica rapa L.ssp.pekinensis* (Qi, 2010), the ranking result of geNorm and NormFinder was different. This indicated that the candidate reference genes expressed more stably in pineapple. It was known that *β-actin* is a good reference gene for its stable expression in many species (Hu et al., 2009; Luo, 2010), but not stable in others, such as rice (Jain, 2006). In this study, *β-actin* was the most stable gene in the tested samples. Some studies (Chari, 2010; Shen, 2010; Gresner, 2011) showed that *gapdh* was a good reference gene, but in pineapple, its expression level was so slow and unstable, that it was not suitable as reference gene in the tested samples, as described for water lily (Luo, 2010). It was reported that *gapdh* expression changed apparently with age and nutrition status (Mozdziak et al., 2003) and varied in the oocytes matured in low oxygen (Bermejo-Alvarez et al., 2010), This study with pineapple further confirmed that gene expression stability often varies with species, tissue types and developmental stages.

Based on the analysis results, we used *β-actin* and *18S rRNA* as normalization genes to analyze the *AcSERK1*expression under stressful conditions.

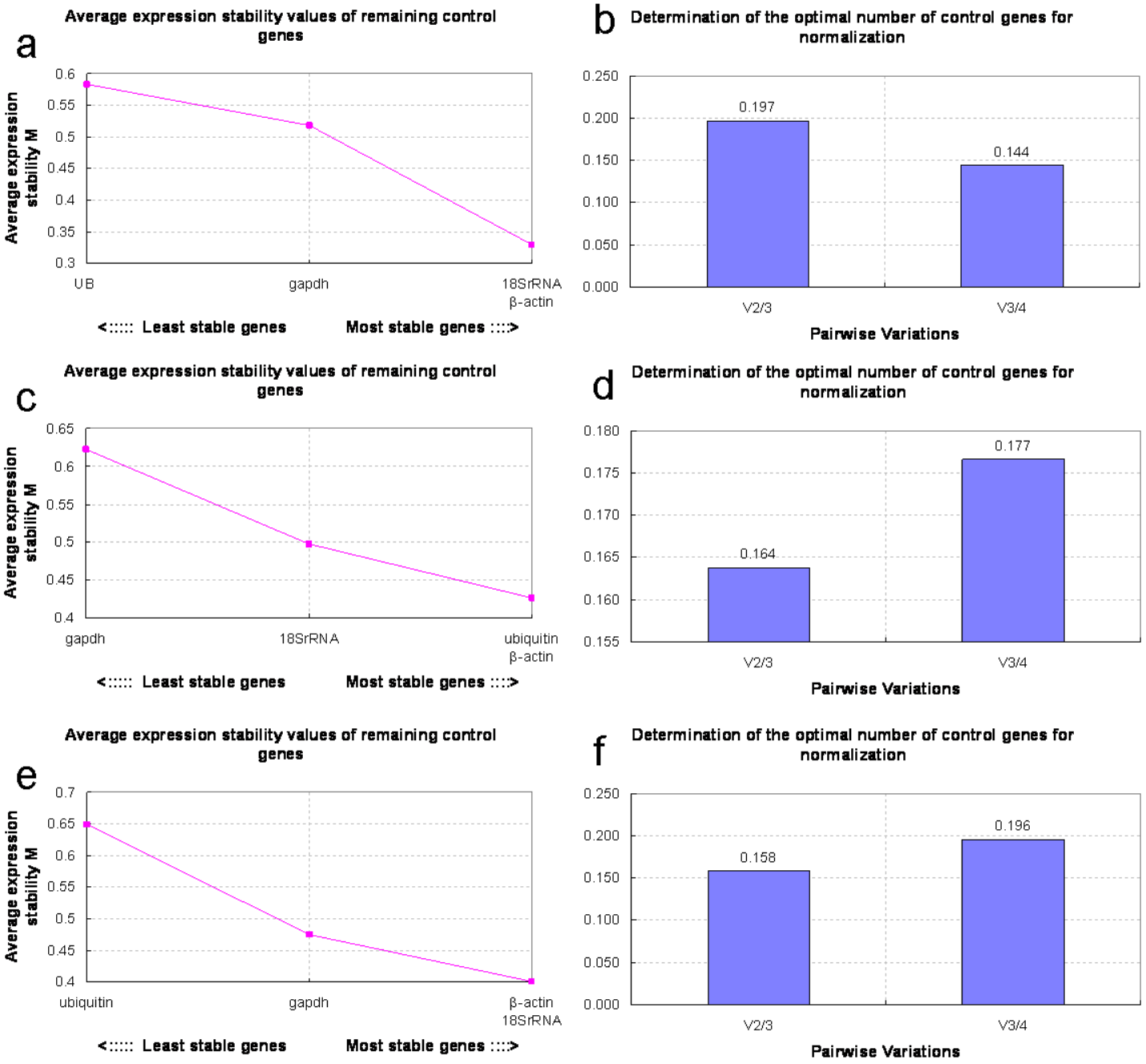

Figure 4. Ranking of gene stability and combination of genes for normalization factor calculation evaluated by geNorm; a, b) time duration of 2,4-D induction; c, d) developmental stages of calyx; e, f) different organs; a, c and e) the M value was calculated for each gene, and the least gene was automatically excluded for the next round of calculations. b, d and f) Determination of the optimal number of reference genes for normalization was conducted. The V value defines the pairwise variation between two sequential normalization factors. 0.2 is set as the cut-off,

The high similarity of the results derived by the two normalization genes suggest that the reference genes we selected in this study was optimal for the transcription analysis of pineapple, then, *β-actin* was used to normalize the expression pattern of *AcSERK1* during embryogenesis. The expression data obtained was reasonable. It demonstrated that *AcSERK1* expression was accumulated during somatic embryogenesis, and *AcSERK1* expression could be effectively induced by 2,4-D. It was reported that 2,4-D was necessary and effective for the induction of somatic embryogenesis of pineapple (He et al., 2007), and *SERK* could be used as a mark gene for somatic embryogenesis (Baudino et al., 2001; Hecht et al., 2001). These results indicate that the analysis results

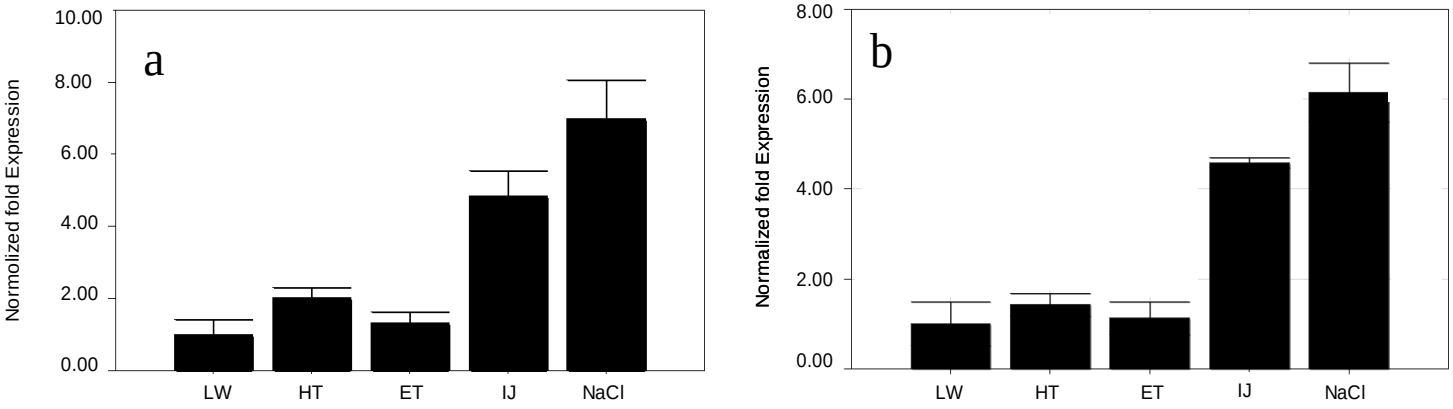

Figure 5. The expression pattern of *AcSERK1* in callus under induction conditions; a) expression pattern of *AcSERK1* using *β-actin* as reference gene; b) expression pattern of *AcSERK1* using *18S rRNA* as reference gene (LW, low temperature; HT, high temperature; ET, ethane; NaCl; IJ, injury).

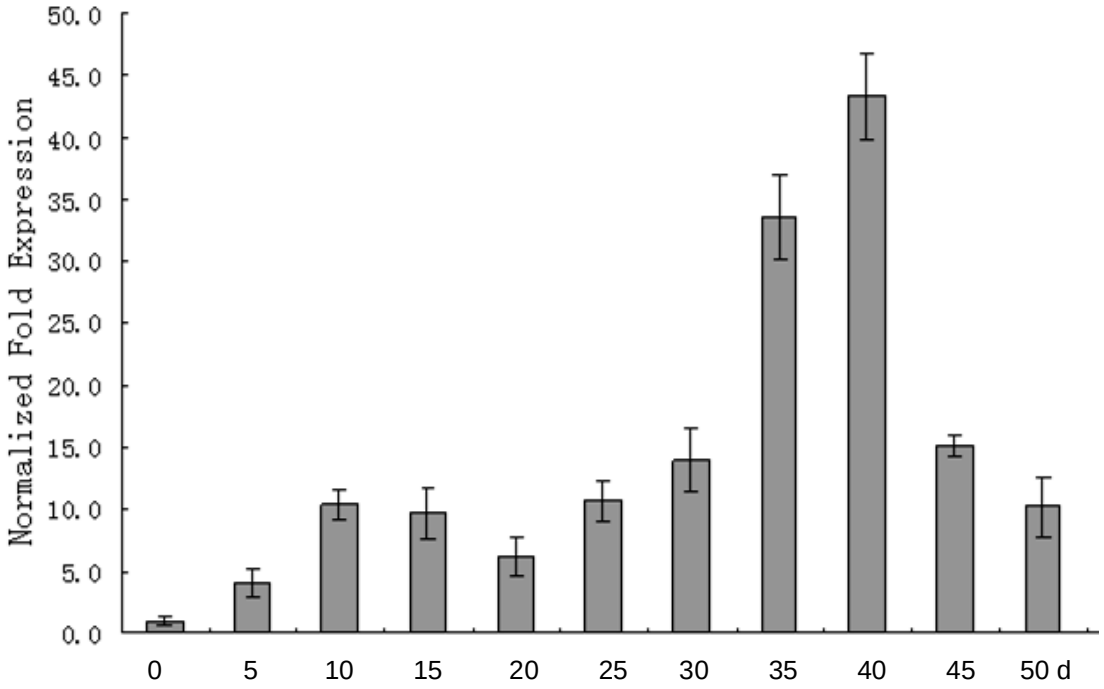

Figure 6. Relative expression of *AcSERK1* measured using *β-actin* as reference gene in callus cultured on 2,4-D containing medium to induce somatic embryo.

normalized by the selected reference gene can reflect the real situation of gene expression in pineapple. The validation results will enable more accurate and reliable normalization of qPCR results for transcription analysis in pineapple.

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