ORIGINAL PAPER

Systematic selection and validation of appropriate reference genes for gene expression studies by quantitative real-time PCR in pear

Yuanyuan Xu · Hui Li · Xiaogang Li ·
Jing Lin · Zhonghua Wang · Qingsong Yang ·
Youhong Chang

Abstract RT-qPCR is a widely used method in gene expression and transcriptome studies. Normalization based on reference genes is necessary to accurately analyze RT-qPCR data. Thus, an accurate and systematic evaluation of these reference genes before experiments are conducted is necessary. In this study, 18 candidate reference genes were evaluated under various experimental conditions covering a range of tissue types and cultivars, NaCl, CaCl$_2$ and temperature treatments, hormones (6-BA, ABA and NAA) and a set of osmotic stress (mannitol and PEG6000) treatments. Gene expression across 48 pear samples was evaluated using geNorm, NormFinder and BestKeeper statistical algorithms. Actin2/7 (*ACT2/7*), ubiquitin extension protein (*UBI*) and Yellow-leaf-specific gene 8 (*YLS8*) exhibited the most stable expression across all the pear samples tested. While in the other experimental groups, different sets of samples had their own best reference genes. In addition, the gene expression of *PbCBL7*, a member of the calcineurin B-like protein, was measured across all the 48 samples using the best three reference genes, it displayed variation in gene expression across different tissues and cultivars, and exhibited diverse up- or down-regulated expression patterns under various treatments, which indicate that *PbCBL7* may play a role in response to specific abiotic stress in pear. These results are valuable for future research on gene expression and abiotic stress tolerance in pear.

Abbreviations

ABA	Abscisic acid
bp	Base pair
NAA	1-Naphthaleneacetic acid
PEG6000	Polyethylene Glycol 6000
RT-qPCR	Quantitative real-time polymerase chain reaction
6-BA	6-Benzylaminopurine

Introduction

Gene expression analysis could improve our understanding of various biological processes and has become much more prevalent. RT-qPCR is a powerful tool widely used on gene expression analysis due to its high specificity, reproducibility, sensitivity, broad dynamic range and no post-PCR processing. These advantages allow a straightforward comparison and make it become the most appropriate choice for gene expression analysis (Garson et al. 2005; Li et al. 2010; Martin et al. 2008). Despite its numerous advantages, several serious flaws have been reported, including the lack of reliable reference genes for normalization (Obrero et al. 2011). To obtain reliable results, normalization is crucial for RT-qPCR studies to control for sample differences (Tong et al. 2009). Since the reference

Y. Xu · H. Li · X. Li · J. Lin (✉) · Z. Wang · Q. Yang ·
Y. Chang (✉)
Institute of Horticulture, Jiangsu Academy of Agricultural Sciences, Jiangsu Key Laboratory for Horticultural Crop Genetic Improvement, Nanjing 210014, People's Republic of China
e-mail: lj84390224@126.com

Y. Chang
e-mail: cyh@jaas.ac.cn

genes are exposed to the same preparation steps as the gene of interest, they are most frequently used among a set of methods proposed to normalize RT-qPCR data and to avoid the possible errors caused by the minor variations of the experimental conditions (Gutierrez et al. 2008; Han et al. 2013; Huggett et al. 2005; Schmidt and Delaney 2010; Zhu et al. 2013).

Ideal reference genes such as actin (*ACT*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), α- or β-tubulin (*TUA*, *TUB*) and ubiquitin (*UBQ*) are considered to be constitutively expressed and unaffected by experimental conditions. Thus, these genes are commonly used for normalization without any validation (Brunner et al. 2004; Chi et al. 2012; Marum et al. 2012). However, a few recent studies have shown that the widely used reference genes vary considerably in response to some specific experimental conditions (Marino et al. 2008; Morgante et al. 2011; Nicot et al. 2005), that if not chosen properly, the reference genes may be unsuitable for normalization purposes owing to large measurement errors (Dekkers et al. 2012; Obrero et al. 2011). Therefore, to ensure proper normalization, it must be demonstrated that the reference genes of choice have high expression stability independent of the varying experimental conditions.

In the recent decades, several studies on validating the importance of the reference genes for each particular experimental condition have been performed mainly on plants species including *Arabidopsis thaliana*, tobacco, tomato, chicory, peanut, potato, peach, soybean, cucumber, litchi, flax and zucchini (Chi et al. 2012; Czechowski et al. 2005; Expósito-Rodríguez et al. 2008; Huis et al. 2010; Jian et al. 2008; Maroufi et al. 2010; Nicot et al. 2005; Obrero et al. 2011; Remans et al. 2008; Schmidt and Delaney 2010; Tong et al. 2009; Wan et al. 2010; Zhong et al. 2011). To identify the suitable reference genes in a range of samples, statistical analysis procedures including geNorm, NormFinder and BestKeeper have been developed and applied (Andersen et al. 2004; Pfaffl et al. 2004; Vandesompele et al. 2002).

The pear (*Pyrus* L.), which belongs to the subfamily Pomoideae in the Rosaceae family, is a popular and commercial crop. It is also the third most important fresh fruit species which has been widely cultivated in the world (Wu et al. 2013). The birch-leaf pear (*Pyrus betulifolia* Bunge) originated in China and is one of the most widely used rootstock in pear production. It has a strong adaptability and is tolerant to cold, drought, waterlogging and infertility. Increasing the stress resistance of pear will improve its production and quality (Chang et al. 2012b). However, studies on plant stress resistance have focused on crops including *Arabidopsis*, tomato and barley (Jakab et al. 2005; Jiang et al. 2012; Pandey et al. 2011; Visioni et al. 2013). Understanding the expression patterns of some key genes in

the stress tolerance pathway of the pear would give insight into the mechanisms that underlie the processes from germination to harvest, and may help to reveal the changes in gene expression under various conditions. In addition, with the release of the pear genome sequence, functional genomics research on the pear based heavily on gene expression analysis is becoming more popular and will facilitate the understanding of pear development (Wu et al. 2013). Recently, RT-qPCR has always been reported in gene expression analyses in pear (Zhang et al. 2011, 2013), but up to now, no comprehensive or systematic evaluation of candidate housekeeping genes has been undertaken in pear.

In the present study, the expression profiles of 18 candidate reference genes, including Actin2/7(*ACT2/7*), α-tubulin (*TUA*), translation elongation factor 2 (*TEF2*), 18S ribosomal RNA (*18SrRNA*), ubiquitin extension protein (*UBI*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), elongation factor 1 alpha (*EF-1α*), Glyceraldehyde-3-phosphate dehydrogenase C subunit 1 (*GAPC1*), Actin 11 (*ACT11*), ubiquitin-conjugating enzyme 8 (*UBC8*), Yellow-leaf-specific gene 8 (*YLS8*), TIP41-like family (*TIP41*), Protein phosphatase 2A subunit A2 (*PP2A*), F-box family protein (*F-box*), Pentatricopeptide repeat superfamily protein (*PPR2*), Sand family protein (*SAND*), ubiquitin-conjugating enzyme 21 (*UBC21*) and ubiquitin 10 (*UBQ10*) were evaluated using RT-qPCR analysis to determine the optimal reference genes across 48 pear cDNA samples. The expression levels of these internal control genes were tested in different pear tissues and cultivars, and within a range of abiotic stress and hormone treatments. To evaluate the expression stability of reference genes, three common statistical methods, geNorm, NormFinder and BestKeeper, were used.

In addition, our previous work on *PbCBL7* gene, a member of calcineurin B-like protein, has revealed that this gene can enhance *E. coli* tolerance towards a variety of stresses (data not shown). Given that the newly identified *PbCBL7* gene may play an important role in anti-adversity stress in pear, the newly identified reference gene combination in this study was further used to examine and normalize its expression profiles. The results obtained will provide guidelines for reference gene selection for gene expression analysis under different experimental conditions in pear and also provide reference for future analysis on suitable RT-qPCR normalization conditions in other crops.

Materials and methods

Plant materials

We obtained the birch-leaf pear (*Pyrus betulifolia* Bunge), an important rootstock that is highly resistant to salt, from

the national germplasm orchard of Institute of Horticulture of Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu, China. Pear samples of varying genotypes and tissues and from various conditions (hormonal treatments (ABA, 6-BA and NAA), stress treatments (low and high temperature, NaCl, PEG6000, CaCl$_2$ and mannitol)) were obtained.

For the experimental group comprising different cultivars, the flesh of 'Yuanhuang (YH)', 'Sucui1 (SC1)'and 'Cuiguan (CG)' pears was collected at a stage equivalent to commercial ripeness, which is at about from 11.9 to 14.4 % soluble solids concentration (SSC) for the selected fruits from 8-year-old trees and free from visual symptoms of any disease or blemishes. These samples were directly stored at −80 °C for further use. The peel and leaves were collected from the fruits and 8-year-old trees, respectively, of 'YH', 'SC1', 'CG' 'Xizilu (XZL)' and 'Cuilu (CL)'. For the experimental group comprising different tissue types, young leaf, old leaf, petiole, peel and flesh samples were taken from 8-year-old 'Sucui2 (SC2)' trees growing under natural conditions. For the experimental group comprising samples subjected to a range of abiotic stress including NaCl, Mannitol, CaCl$_2$, PEG6000 and temperature treatments, seedlings of *P. betulifolia* Bunge were grown in 1/2 MS nutrient solution containing NaCl solution at varying concentrations (0, 100, 200, 300 and 400 mM at room temperature for 6 h), mannitol solution at varying concentrations (0, 100 and 200 mM at room temperature for 6 h), 100 mM CaCl$_2$, or 10 % (w/v) PEG6000 solution at varying time points (2 h, 6 h, 12 h, 24 h). Seedlings were also exposed to high (42 °C) and low temperatures (4 °C) for 6 h, and seedlings which were exposed to room temperature (24 °C) are used as the control. The leaves were collected following these experimental treatments. For the experimental group comprising samples subjected to different hormone treatments, seedlings of *P. betulifolia* Bunge were grown in 1/2 MS nutrient solution containing different concentrations of hormones (6-BA (20 μM), ABA (50 μM), NAA (10 μM)) at various duration (2 h, 6 h, 12 h and 24 h). Leaves harvested at 0 h for each treatment were treated as controls. Plant materials were collected in triplicate, frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from 100 mg of samples using an EASYspin Plus Plant RNA mini kit (Aidlab Biotech, China). DNase I (TaKaRa, Japan) was added to eliminate any trace of genomic DNA contamination, according to the manufacturer's procedures. The integrity of the RNA samples was evaluated by 1.2 % (w/v) agarose gel electrophoresis, and the A$_{260/280}$ and A$_{260/230}$ nm absorption ratios were determined using a BioPhotometer Plus (Eppendorf, Germany) to estimate RNA concentration and quality. Clear bands corresponding to 28S and 18S rRNA and the absence of a smear indicate minimal degradation of total RNA. For cDNA synthesis, the RNA has to have an optical density absorption ratio A$_{260/280}$ of more than 1.8. First-strand cDNA was synthesized using a cDNA amplification kit (TaKaRa) according to the user manual. Briefly, 1 μg RNA, 50 μM oligo$_{dT}$ (18) and 10 mM dNTP mix were added together to incubate at 65 °C for 5 min, then placed on ice for 5 min. After that, 4 μl 5 × RT buffer, 20 U RNase Inhibitor and 200 U Prime Script RTase were added and then incubated at 42 °C for 60 min. Finally, the RT reaction was terminated by incubating at 70 °C for 15 min. Each RNA sample was controlled for genomic DNA contamination without reverse transcriptase addition into cDNA synthesis mixture. All samples were diluted 1:10 with RNase-free water, prior to its use as a template in RT-qPCR. Samples were stored at −20 °C.

Primer design and RT-qPCR amplified product verification

18 candidate reference genes were chosen for further investigation to be able to find the most stably expressed reference genes to be used in RT-qPCR analysis (Table S1). This group of genes comprised several classical internal control genes which are most commonly used as reference for expression analysis and the others based on previous reports (Han et al. 2013; Tong et al. 2009). The nucleotide sequences of pear genes were found in the pear genome database (http://peargenome.njau.edu.cn:8004/), and the chosen pear genes were then used to query against the *Arabidopsis* protein database using BLASTX to obtain the homologous genes. The functional annotations of these pear reference genes were also based on *Arabidopsis thaliana*. Primers were designed using Beacon Designer 7.0 (Premier Biosoft International, USA) with the following criteria to ensure optimal polymerization efficiency and to reduce the impact of RNA integrity on gene expression in RT-qPCR: primers were selected with a melting temperature of 56–60 °C, optimal primer length of 17–24 bp, and GC content of 45–55 %. Primers were designed to amplify products within the range of 76–199 bp (Table S2). Specificity of the amplification product for each primer pair was verified by the presence of a single band at the expected size with agarose gel electrophoresis, and by the presence of a single peak in RT-qPCR melting curve products.

Quantitative RT-PCR

RT-qPCR was carried out in 96-well plates with an ABI7500 Real-Time PCR Detection System (Applied

Biosystems, USA) using SYBR Green Master ROX (Roche, Japan), following the manufacturer's instructions. Reaction mixtures and conditions used in this study according to the method reported by Xu et al. (2012). At the end of the reactions, a melting curve analysis was performed to evaluate primer specificity. Controls without template and a reverse transcription negative were included for each primer pair to detect possible reagent contamination and to ensure that there was no potential genomic DNA contamination. Each real-time PCR reaction was performed in triplicate (technical replicates) on three individual plants (biological replicates).

Data analysis

The expression level of each selected reference gene was determined by the number of amplification cycles (C_t) needed to reach a specific threshold level of the detection. The PCR efficiency showed in Table S2 was calculated for each gene with LinRegPCR program from raw fluorescence data taken from the ABI7500 detection system (Ramakers et al. 2003; Tong et al. 2009). Various analysis tools including geNorm, NormFinder and BestKeeper were used to estimate gene expression stability. The expression stability as well as the optimal combination of candidate genes to be used for normalization was determined by geNorm software. It provides a stability measure (M), excludes the least stable gene, and creates a line graph by a stepwise method to show stability of the selected genes (Vandesompele et al. 2002). NormFinder uses an ANOVA-based model to estimate intra- and inter-group variations and provides a direct measure for integrating all consistent results (Andersen et al. 2004). BestKeeper estimates the expression stability of reference genes by calculating the C_t value, and provides a comparative analysis based on pairwise correlations of all candidate reference genes (Pfaffl et al. 2004).

Expression of *PbCBL7* gene under different abiotic stresses

To evaluate the expression profiles of *PbCBL7* gene under various experimental conditions, its relative transcript levels were normalized by the geometric average of the three most reliable reference genes selected in all the sample pools in this study through the equation of relative ratio target gene/control = $(E_{\text{Target gene}}^{-C_t\,\text{Target gene}})/(E_{\text{Control}}^{-C_t\,\text{Control}})$ (Pfaffl 2001; Shi et al. 2012). Calculations were carried out with Microsoft Excel software. The data were statistically analyzed with DPS Version 7.55 software (DPS Institute, Hangzhou, China) using Duncan's multiple range test at the $P < 0.05$ level of significance.

Results

Verification of the amplified product

To select the best reference genes for pear gene expression profiling, the primers of the 18 common reference genes (Table S2) were designed for RT-qPCR analysis. Melting curves and agarose gel electrophoresis results revealed that there were no primer dimers or other non-specific amplification (Fig. 1a, b). In addition, no signals were detected from the controls of template lacking reverse transcriptase, indicating no genomic DNA contamination.

C_t value analysis

In an RT-qPCR assay, a positive reaction is measured by accumulation of a fluorescent signal. The number of cycles required for the fluorescent signal to cross the threshold is called the C_t value, which is used to evaluate the transcriptional levels of the reference genes. The results would provide an overview of the abundance of all the selected genes in the samples. In this study, the C_t values of the 18 reference genes showed a relative wide range, with the lowest (14.30) in *18SrRNA* to the highest (34.14) in *PPR2*, while the majority of these values ranged from 18.2 to 27.4 in all tested pear samples (Fig. 2). It took only 14.30 amplification cycles to reach the threshold fluorescence for *18SrRNA*, indicating that it was highly expressed compared with the other genes. The individual reference genes showed different expression levels across all samples used in this study. *18SrRNA* showed the smallest expression variation (below 7 cycles), while *PPR2* and *TUA* showed the highest gene expression variation (about 12 cycles) among all the selected genes. The wide expression ranges of the 18 tested reference genes indicated that no single reference gene had a constant expression under all experimental conditions in pear samples. Therefore, it is necessary to select a relatively reliable reference gene to normalize gene expression profiling under certain conditions.

GeNorm analysis

Figure 3 shows the 18 candidate reference genes with M values that are less than 1.5. This is usually regarded as acceptable in terms of expression variability. Interestingly, the most stable reference gene was not identical among different sample pools. For all the 48 samples, *ACT2/7* and *UBI* were the most stably expressed genes, and *TUA* was the least stable gene (Fig. 3a). The least stable gene in the group under different $CaCl_2$ and hormonal treatments was also *TUA*, which was consistent with the findings above. The most stably expressed genes were *ACT11/UBQ10* and
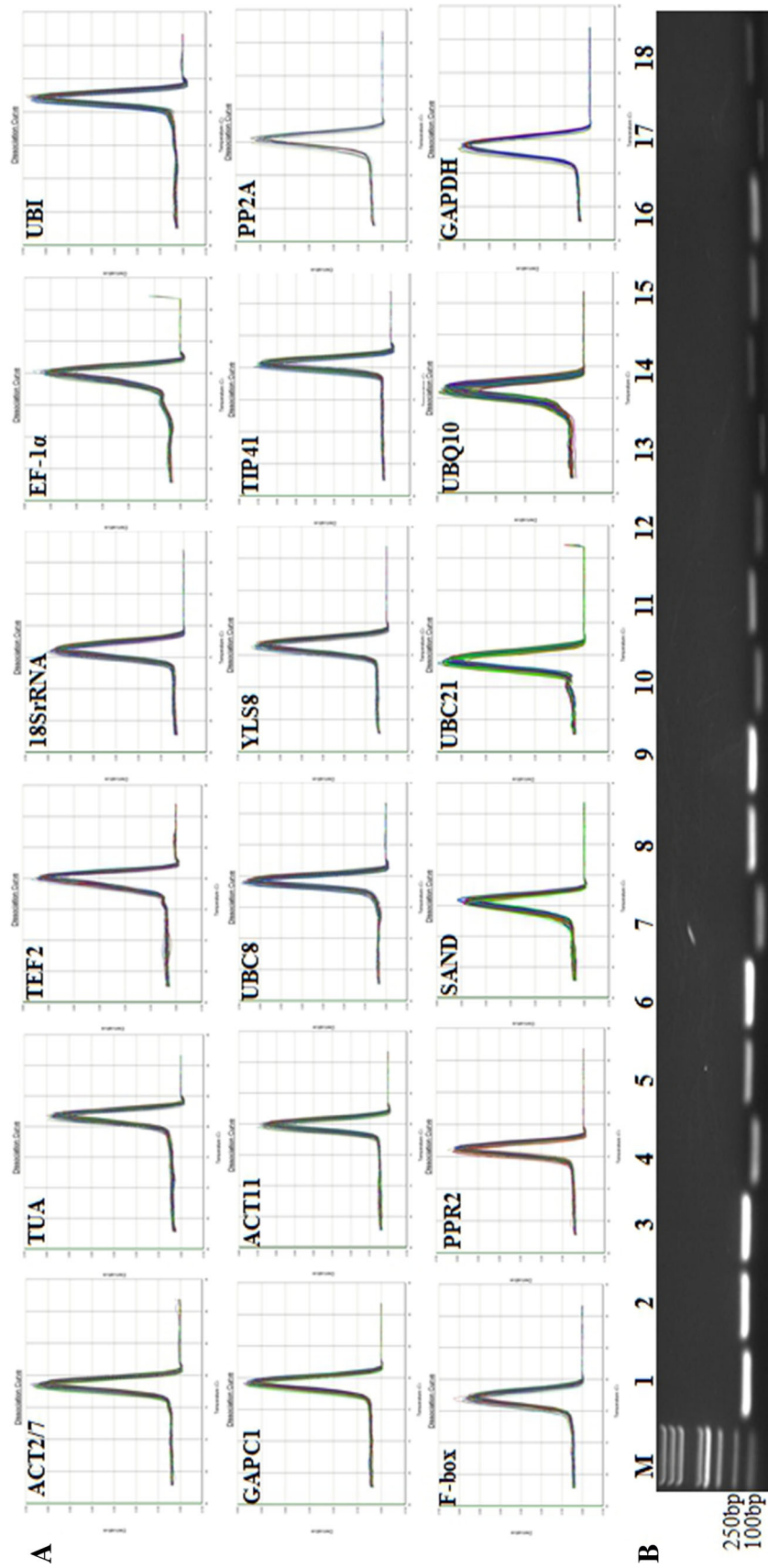
Fig. 1 Specificity of RT-qPCR and amplicon length. **a** Dissociation curve analysis generated for all amplicons. **b** *Lanes 1* to *18* are *ACT2/7, TUA, TEF2, 18SrRNA, EF-1α, UBI, GAPC1, ACT11, UBC8, YLS8, TIP41, PP2A, UBQ10, UBC21, F-box, PPR2, SAND, GAPDH*, respectively. *M* DL2000 plus DNA ladder

Fig. 2 Expression levels of candidate reference genes across all samples. A *line* across the *box* depicts the median. The *box* indicates the 25 % and 75 % percentiles. *Whiskers* represent the maximum and minimum values
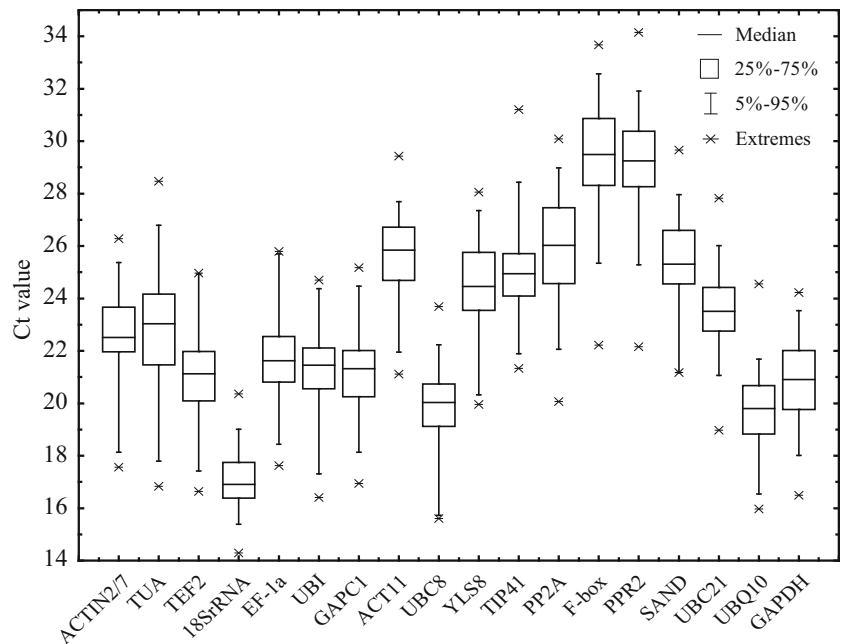
ACT2/7/EF-1α under different CaCl$_2$ and hormonal treatments, respectively (Fig. 3e, g). In the experimental group comprising different tissues, geNorm identified *TIP41* as the unstable gene, whereas *PP2A* and *GAPDH* were regarded as the best reference genes (Fig. 3b). In the experimental group comprising different cultivars, *UBI* and *UBC8* were considered as the best genes, while *PPR2* was thought to be the least stable (Fig. 3c). In the experimental group comprised of samples subjected to different concentrations of NaCl, *ACT2/7* and *UBQ10* were the most stable genes, and *GAPC1* was the least stable gene (Fig. 3d). In the experimental group subjected to different temperatures, *TEF2* and *UBC21* were the most stably expressed genes, and *18SrRNA* was the least stable gene (Fig. 3f). In the experimental group comprising different degrees of osmotic stress, *YLS8* and *PP2A* were the most stably expressed genes, and *UBQ10* was the least stable gene (Fig. 3h).

We considered the average expression stability (M) and the pairwise variation (V) when using geNorm to evaluate gene expression stability. To determine the necessity of adding the next internal control gene and the optimal number required for effective normalization, the pairwise variations Vn/Vn+1 between two sequential normalization factors NFs (NFn and NFn+1) were introduced. According to the cut-off value of 0.15 reported by Vandesompele et al. (2002), in the groups for all the samples analyzed together and different tissues and cultivars comparison, three reference genes should be selected (V3/4 < 0.15), while in the rest groups, two reference genes were sufficient (V2/3 ≤ 0.15) (Fig. 4).

NormFinder analysis

A more stable gene expression is indicated by lower average expression stability values. Table 1 shows the results calculated by NormFinder. The output of Norm-Finder with different sample pools was highly variable. When all 48 samples taken together, *ACT2/7, UBI* and *UBC8* showed remarkable stability in their expression levels and were always highly ranked; while *18SrRNA*, *TUA* and *TIP41* exhibited unstable expression profiles and were always included among the unstable genes.

When analyzed for the established groups with Norm-Finder separately, *YLS8* was calculated to be the most stable single gene in the group of different cultivars, whereas *PPR2* was the most variable one. NormFinder revealed *ACT2/7* to be the most stable in the experimental group of different tissues, which was consistent with the results obtained from the groups under different NaCl, CaCl$_2$ and osmotic stress treatment. In this context, the least stable genes were *TUA* in NaCl and CaCl$_2$ treatment groups and *UBQ10* in osmotic stress treatments groups. In groups involving exposure to various hormones and temperatures, *TIP41* was the most stable gene with the stability value of 0.174 and 0.034, while *TUA* and *18SrRNA* were the most unstable with the stability values of 0.709 and 1.399, respectively (Table 1).

BestKeeper analysis

Stable reference genes always showed a strong correlation with the BestKeeper Index. The results analyzed by this
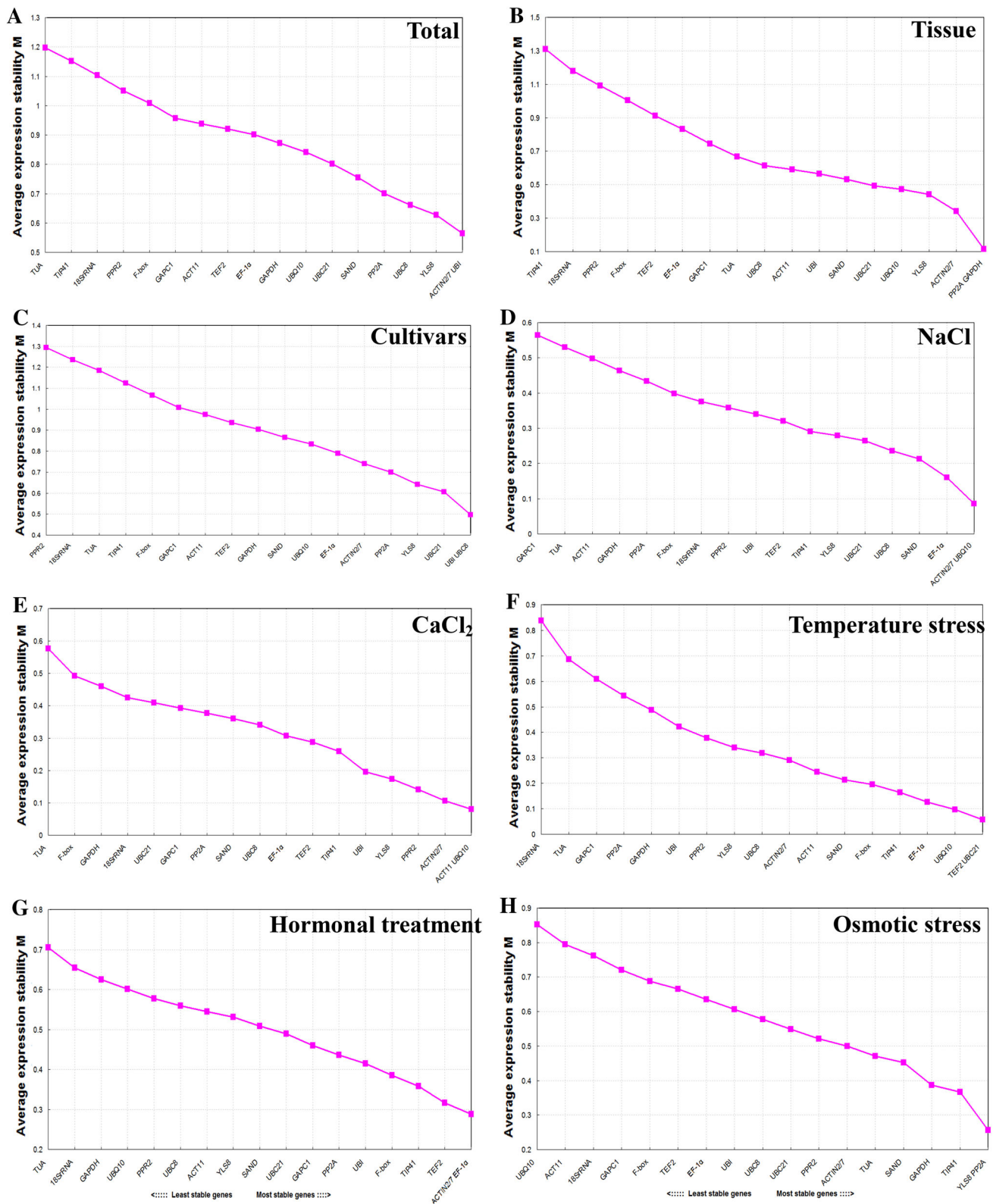
Fig. 3 Gene expression stability and ranking of the 18 reference genes as calculated by geNorm

method are presented in Table 2. High coefficients of correlation always indicate that these gene pairs exhibit similar expression patterns. When all samples were taken together for analysis, *ACT2/7*, *UBI*, *YLS8* and *SAND* showed strong correlation with the BestKeeper Index ($r > 0.96$), and ranked among the top four positions. These
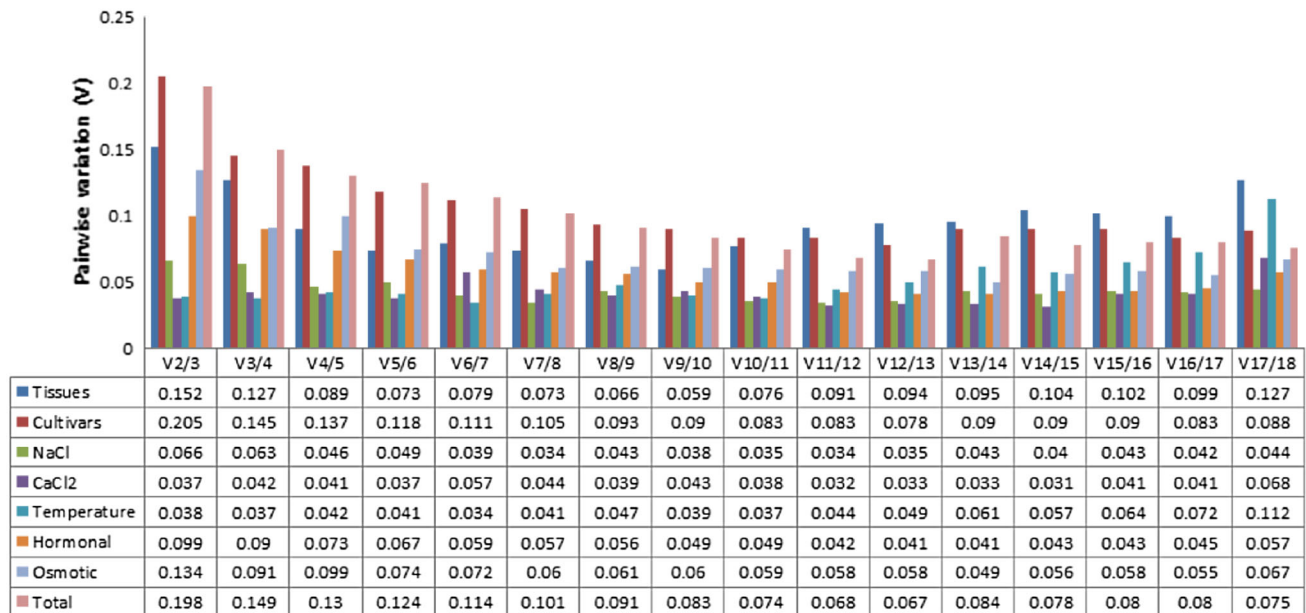
Fig. 4 Determination of the optimal number of reference genes. Every *bar* represents change in normalization accuracy upon stepwise addition of more reference genes according to the ranking in Fig. 3.

The pairwise variation (Vn/Vn+1) was calculated from the normalization factors NFn and NFn+1, with a recommended cut-off threshold of 0.150

results were consistent with that derived from geNorm and NormFinder. *ACT2/7* was consistently stably expressed, except in the groups involving different cultivars and temperature treatments wherein *YLS8* and *TEF2* were the most stable reference genes, respectively. *18SrRNA* consistently ranked poorly in almost all series, becoming the least reliable reference gene.

Expression profile analysis of *PbCBL7*

To investigate the effect of different cultivars and tissues as well as abiotic stress on gene expression in pear, the transcript level of *PbCBL7* in 48 pear samples was assessed by RT-qPCR. The expression level of *PbCBL7* showed varied according to context after being normalized to *ACT2/7*, *UBI* and *YLS8*, which were the best reference gene combination selected by geNorm, NormFinder and Best-Keeper algorithm across all tested samples. The results showed that *PbCBL7* was expressed abundantly in petiole, leaf and peel and weakly in flesh (Fig. 5a). In the leaves of different cultivars, *PbCBL7* exhibited the highest expression level in 'CL', but in peel and flesh, *PbCBL7* was expressed the highest in 'YH' (Fig. 5b–d). *PbCBL7* level was down-regulated under salt and mannitol stress, exhibiting the lowest expression at 200 mM of NaCl (Fig. 5f, g). We speculated that the 100 or 200 mM NaCl stress treatment may not initiate the defense system in pear plant and at this time the expression of *PbCBL7* is negatively regulated by salt stress, but with the increase of

concentration, the defense system began to engage and this resulted in enhanced transcription of *PbCBL7* gene. The transcript level was higher under cold- or heat stress than at room temperature which indicates that *PbCBL7* gene was regulated by high or low temperatures (Fig. 5e). Since the CBL protein family is mediated by calcium ions, we measured the transcript level of *PbCBL7* upon exposure to $CaCl_2$. *PbCBL7* was up-regulated in response to $CaCl_2$, reached maximum at 12 h then decreased, similar to what was observed under PEG6000 treatment. Furthermore, different hormone treatments were also carried out, and interestingly, *PbCBL7* was consistently up-regulated under 6-BA treatment, while under ABA treatment, *PbCBL7* was induced in a transient way and reached a maximum at 2 h, then consistently decreased. With NAA treatment, *PbCBL7* level reached the maximum at 2 h, thereafter reached the minimum at 6 h then consistently increased after treatment for 12 h and 24 h (Fig. 6).

Discussion

RT-qPCR is verified to be a sensitive and accurate technique for gene expression analysis (Guénin et al. 2009; Li et al. 2010). Ideal reference genes whose transcription levels remained invariable under varying experimental conditions will greatly improve the reliability of RT-qPCR data when evaluating the expression levels of genes of interest (Klie and Debener 2011; Schmidt and Delaney

Table 1 Ranking of the 18 candidate reference genes according to the order of their expression stability as calculated by NormFinder

Rank	Total Gene	Total Stability	Cultivars Gene	Cultivars Stability	Tissues Gene	Tissues Stability	NaCl Gene	NaCl Stability	CaCl₂ Gene	CaCl₂ Stability	Hormone Gene	Hormone Stability	Temperature Gene	Temperature Stability	Osmotic stress Gene	Osmotic stress Stability
1	ACT2/7	0.306	YLS8	0.175	ACT2/7	0.120	ACT2/7	0.049	ACT2/7	0.066	TIP41	0.174	TIP41	0.034	ACT2/7	0.148
2	UBI	0.347	UBC8	0.316	YLS8	0.124	UBQ10	0.075	TIP41	0.123	UBI	0.175	TEF2	0.035	YLS8	0.201
3	UBC8	0.361	UBC21	0.348	UBQ10	0.201	TEF2	0.153	YLS8	0.136	ACT2/7	0.227	ACT2/7	0.048	TIP41	0.275
4	YLS8	0.404	ACT2/7	0.414	PP2A	0.233	SAND	0.164	TEF2	0.148	EF-1α	0.235	YLS8	0.093	GAPDH	0.296
5	SAND	0.411	UBQ10	0.427	SAND	0.254	UBC8	0.165	ACT11	0.165	YLS8	0.241	UBC8	0.169	PP2A	0.302
6	UBC21	0.461	UBI	0.488	UBC21	0.272	YLS8	0.165	PPR2	0.168	TEF2	0.260	F-box	0.173	UBC8	0.305
7	UBQ10	0.479	PP2A	0.492	GAPDH	0.290	EF-1α	0.170	UBQ10	0.173	GAPC1	0.279	EF-1α	0.193	PPR2	0.317
8	PP2A	0.485	SAND	0.514	ACT11	0.336	UBI	0.184	UBI	0.198	PP2A	0.283	UBQ10	0.231	UBC21	0.362
9	GAPDH	0.526	EF-1α	0.538	UBI	0.352	TIP41	0.219	PP2A	0.211	F-box	0.289	UBC21	0.235	SAND	0.365
10	ACT11	0.530	GAPDH	0.589	UBC8	0.422	PPR2	0.251	EF-1α	0.262	UBC21	0.307	SAND	0.282	UBI	0.369
11	EF-1α	0.585	ACT11	0.605	TUA	0.621	UBC21	0.262	SAND	0.262	SAND	0.320	ACT11	0.382	F-box	0.416
12	TEF2	0.653	TEF2	0.685	GAPC1	0.692	18SrRNA	0.283	UBC8	0.283	ACT11	0.333	UBI	0.385	TUA	0.432
13	GAPC1	0.765	F-box	0.799	EF-1α	0.837	F-box	0.331	GAPC1	0.289	UBC8	0.369	GAPDH	0.467	EF-1α	0.446
14	F-box	0.812	GAPC1	0.802	TEF2	0.994	PP2A	0.348	UBC21	0.324	PPR2	0.405	PPR2	0.502	TEF2	0.488
15	PPR2	0.885	18SrRNA	0.934	F-box	1.030	GAPDH	0.361	18SrRNA	0.373	UBQ10	0.432	PP2A	0.534	GAPC1	0.608
16	18SrRNA	0.943	TUA	0.962	PPR2	1.153	ACT11	0.406	F-box	0.431	GAPDH	0.498	GAPC1	0.658	18SrRNA	0.629
17	TUA	0.952	TIP41	1.001	18SrRNA	1.223	GAPC1	0.466	GAPDH	0.493	18SrRNA	0.544	TUA	0.907	ACT11	0.651
18	TIP41		PPR2	1.084	TIP41	1.580	TUA	0.542	TUA	0.843	TUA	0.709	18SrRNA	1.399	UBQ10	0.826

Table 2 Ranking of the 18 candidate reference genes according to correlations between reference genes and BestKeeper index

Rank	Total		Cultivars		Tissues		NaCl		CaCl$_2$		Hormone		Temperature		Osmotic stress	
	Gene	r	Gene	r	Gene	r	Gene	r	Gene	r	Gene	r	Gene	r	Gene	r
1	ACT2/7	0.997	YLS8	0.974	ACT2/7	0.979	ACT2/7	0.997	ACT2/7	0.999	ACT2/7	0.972	TEF2	0.999	ACT2/7	0.984
2	UBI	0.994	SAND	0.967	YLS8	0.972	UBQ10	0.992	PP2A	0.994	EF1a	0.959	UBQ10	0.998	SAND	0.982
3	YLS8	0.990	UBC21	0.966	UBC21	0.969	UBI	0.992	TIP41	0.990	UBI	0.949	TIP41	0.998	PP2A	0.980
4	SAND	0.989	UBC8	0.966	SAND	0.967	TIP41	0.991	YLS8	0.984	TIP41	0.949	YLS8	0.997	GAPDH	0.973
5	UBC8	0.985	UBQ10	0.959	UBQ10	0.966	EF1a	0.991	UBC21	0.971	SAND	0.947	EF1a	0.996	UBC8	0.973
6	UBC21	0.982	UBI	0.955	PP2A	0.965	SAND	0.988	ACT11	0.967	UBC21	0.933	UBC8	0.995	PPR2	0.972
7	PP2A	0.978	ACT2/7	0.952	GAPDH	0.963	TEF2	0.986	TEF2	0.944	PP2A	0.928	UBC21	0.994	YLS8	0.969
8	UBQ10	0.976	EF1a	0.948	UBI	0.962	PP2A	0.975	GAPDH	0.939	TEF2	0.926	PPR2	0.993	UBC21	0.967
9	GAPDH	0.970	PP2A	0.926	ACT11	0.932	UBC8	0.965	SAND	0.932	GAPC1	0.897	SAND	0.993	UBI	0.966
10	EF1a	0.960	GAPDH	0.919	UBC8	0.925	YLS8	0.959	UBI	0.919	TUA	0.867	F-box	0.991	TUA	0.964
11	TUA	0.934	TEF2	0.913	TUA	0.896	UBC21	0.959	EF1a	0.906	F-box	0.832	TUA	0.989	TIP41	0.962
12	ACT11	0.920	F-box	0.903	GAPC1	0.885	GAPDH	0.935	PPR2	0.903	ACT11	0.829	UBI	0.984	UBQ10	0.959
13	F-box	0.915	TUA	0.896	EF1a	0.873	18SrRNA	0.920	UBQ10	0.889	YLS8	0.828	ACT2/7	0.983	EF1a	0.949
14	TEF2	0.890	ACT11	0.892	F-box	0.869	PPR2	0.912	GAPC1	0.877	UBQ10	0.801	GAPDH	0.978	GAPC1	0.942
15	PPR2	0.868	TIP41	0.871	TEF2	0.843	F-box	0.868	UBC8	0.825	UBC8	0.768	GAPC1	0.976	F-box	0.940
16	GAPC1	0.832	GAPC1	0.847	TIP41	0.842	TUA	0.857	F-box	0.782	PPR2	0.754	ACT11	0.976	TEF2	0.916
17	TIP41	0.812	PPR2	0.761	PPR2	0.783	GAPC1	0.854	TUA	0.763	GAPDH	0.740	PP2A	0.969	18SrRNA	0.850
18	18SrRNA	0.645	18SrRNA	0.715	18SrRNA	0.778	ACT11	0.851	18SrRNA	0.718	18SrRNA	0.583	18SrRNA	0.516	ACT11	0.841

r coefficient of correlation

2010; Zhu et al. 2013). Several common candidate reference genes are currently being used as internal controls regardless of their instability and variance. If not chosen properly, the internal control genes would not fulfill its role as a standard in gene expression analysis, leading to a wrong conclusion (Dekkers et al. 2012; Obrero et al. 2011). The present study is the first systematic survey on the expression stability of 18 genes used as internal controls for RT-qPCR analysis under different experimental conditions in 48 different pear samples. The expression stabilities of the 18 selected reference genes showed fluctuations and discrepancies across the different sample groups, indicating that none of the internal controls have invariable expression levels across varying conditions.

The expression levels of the reference genes for RT-qPCR are determined by C_t values (Klie and Debener 2011; Martin et al. 2008; Zhu et al. 2013). C_t values that are too high ($C_t > 30$) or too low ($C_t < 15$) are generally considered to be unsuitable as reference genes (Wan et al. 2011). Evaluation of Ct values, however, lacks sensitivity and reliability. Thus, to choose the best reference genes for RT-qPCR analysis, we used various statistical approaches including geNorm, NormFinder and BestKeeper to minimize bias in the quantification of gene expression (Tong et al. 2009; Wan et al. 2010; Zhu et al. 2013).

ACT2/7, *UBI* and *YLS8* consistently ranked as most stable by the three algorithms when all the samples were considered. With the different experimental conditions, different sets of samples had their own best reference genes. For example, *ACT2/7/UBQ10* is the best reference gene combination when studying samples exposed to NaCl, whereas *PP2A/GAPDH* did better than *ACT2/7/UBQ10* when studying samples of different tissues. *UBI/UBC8* was the best combination for different cultivars.

ACT2/7, the most frequently used reference gene, is one of the major components of cytoplasmic microfilaments in eukaryotic cells (Hu et al. 2009). *ACT2/7* expression is rather variable in peach and *A. thaliana*, but mostly stable in soybean and radish (Jian et al. 2008; McDowell et al. 1996; Tong et al. 2009; Xu et al. 2012). In this study, *ACT2/7* consistently ranked as most stable, and thus considered as a suitable reference gene. However, compared with *ACT2/7*, another candidate reference gene *ACT11* in the actin gene family showed most stability under CaCl₂ treatment, as determined by geNorm. *UBI* was evaluated as the most stable reference gene in the group of different cultivars by geNorm. These findings are consistent with the results obtained from cucumber (Wan et al. 2010). *UBQ10* was determined as an unsatisfactory reference gene in soybean and rice (Jain et al. 2006; Jian et al. 2008), but displayed stable expression in *A. thaliana* and tomato (Czechowski et al. 2005; Løvdal and Lillo 2009). However, in this study, *UBQ10* ranked well under both NaCl and

temperature treatment, but ranked poorly under different osmotic treatments. *TEF2* is needed for elongation and mRNA transcription in eukaryotes (Xu et al. 2012). In the group exposed to varying temperatures, *TEF2* exhibited stable expression patterns and was abundantly and constantly transcribed, consistent with previous studies in peach samples (Tong et al. 2009). In previous studies, *TIP41* showed stable expression level across tissues and during development in tomato, but performed poorly during development of grape berry and in the leaves and roots of *A. thaliana* under copper or cadmium exposure (Czechowski et al. 2005; Expósito-Rodríguez et al. 2008; Reid et al. 2006). In the present study, *TIP41* was the most unstable in groups involving different tissues. Among the members of the ubiquitin-conjugating enzyme gene family, it was reported in *Ammopiptanthus mongolicus* that *UBC5* and *UBC1* represent the best combination for normalization of differentially expressed genes under cold stress, *UBC2* under salt stress, and *UBC4* and *UBC5* under heat stress (Shi et al. 2012). In this study, *UBC8* showed the most stable expression in groups of different cultivars and *UBC21* was the most suitable gene in samples exposed to different temperatures, as analyzed by geNorm. *PP2A*, another novel reference gene, was reported to be stably expressed in rabbiteye blueberry (Vashisth et al. 2011), while in this study, *PP2A* showed the most stable expression in groups involving different tissues and samples exposed to osmotic stress, as assessed by geNorm. On the other hand, *EF-1α* was the least reliable control in *Arabidopsis*, *Salvia miltiorrhiza* and barley (Czechowski et al. 2005; Jarošová and Kundu 2010; Yang et al. 2010), but in this study, this gene was the most stable in groups exposed to different hormones, as assessed by geNorm. *TUA* is stably expressed during development in soybean and in different tissues in poplar (Brunner et al. 2004; Jian et al. 2008). In this study, *TUA* level was highly unstable under conditions involving different cultivars and exposure to NaCl, CaCl₂, temperature and hormones. *GAPDH*, involved in basic cellular functions, is one of the most stable genes in barley and grapevine (Jarošová and Kundu 2010; Reid et al. 2006), but unstable in tobacco (Schmidt and Delaney 2010), as evaluated by geNorm, this gene showed poor expression stability except for the group involving different tissues. *18SrRNA* has failed to be selected as an ideal reference gene because it displayed poor stability in most cases in this research, consistent with findings in broomrape tissues (Gonzalez-Verdejo et al. 2008).

There is some discrepancy among the results obtained by geNorm, NormFinder and BestKeeper, which may be due to differences in algorithms and analytical procedures (Andersen et al. 2004; Pfaffl et al. 2004; Vandesompele et al. 2002). Taken together, these results indicate that a
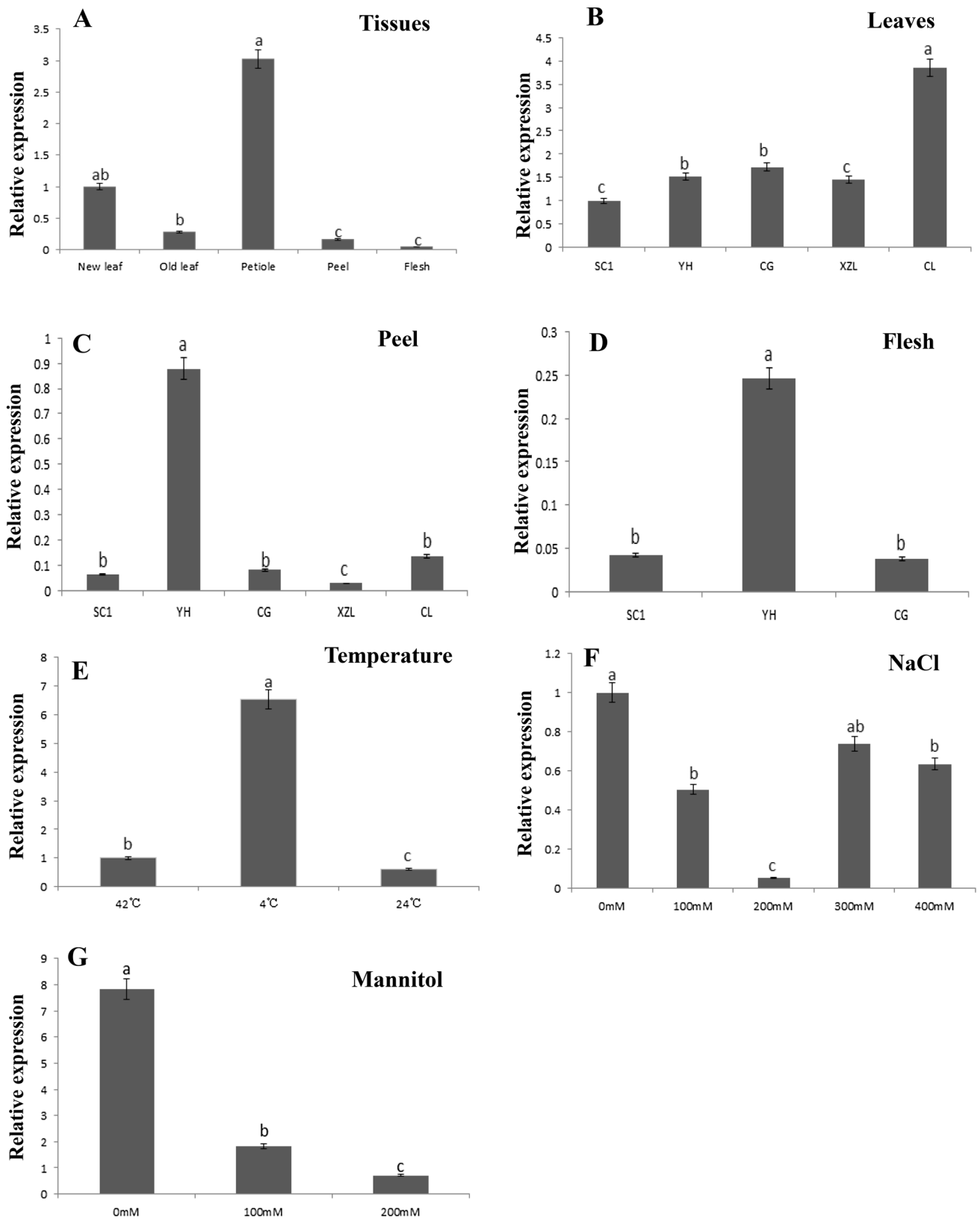
◄**Fig. 5** Expression analysis of *PbCBL7* by RT-qPCR. Different experimental conditions including different tissues (**a**) and cultivars (**b–d**) and varying treatments [temperature (**e**), NaCl (**f**) and Mannitol (**g**)]. The data show the relative transcript levels of the target gene as normalized to the best reference gene combination, *ACT2/7*, *UBI* and *YLS8*. Mean values and standard errors (*bars*) were obtained from three real-time quantitative PCR reactions. *Different letters* indicate significant differences at *P* < 0.05 according to Duncan's multiple range tests

reference gene with consistent expression under certain conditions may not be suitable for normalization of gene expression under other experimental conditions. Variability in the expression of reference genes may partly be explained by the fact they may not only involve in basal cell metabolism but also join in other cellular functions (Mafra et al. 2012; Wan et al. 2011).

The combination of two or more stable genes represents the ideal internal control for RT-qPCR analysis, which would improve the accuracy significantly (Silveira et al. 2009). Therefore, to get an accurate expression profiling among multiple samples, more than one reference genes should be used (Brunner et al. 2004; Coito et al. 2012; Nicot et al. 2005; Xu et al. 2012; Zhong et al. 2011).

The results obtained from the three algorithms were comprehensive in determining suitable reference genes for qPCR normalization (Chang et al. 2012a; Klie and Debener 2011). Thus, we can conclude that expression levels of all the 18 genes were variable to some extent, but that of *ACT2/7*, *UBI* and *UBC8* were in general the most stable. Thus, a combination of *ACT2/7*, *YLS8* and *PP2A* would be appropriate as a reference panel for normalizing gene expression data among different tissues, whereas the combination of *YLS8*, *UBC8* and *UBC21* would be most suitable for

different cultivars. Compared with others, *ACT2/7* and *UBQ10* represented the most suitable reference gene combination when the samples were treated with NaCl, and *ACT2/7* and *YLS8* represented the most suitable combination when samples were treated with $CaCl_2$. *TEF2* and *YLS8* represented the most suitable combination when the temperature was altered. *ACT2/7* and *EF-1α* were the most favorable genes in samples exposed to different hormones, and *ACT2/7* and *GAPDH* were stably expressed under osmotic stress. In contrast, *TUA*, *18SrRNA* and *TIP41* were the most unstable under several experimental conditions and should generally not be used as reference genes.

In addition, the three best reference genes, *ACT2/7*, *UBI* and *YLS8,* were used to normalize the expression level of *PbCBL7* in the 48 different pear samples. Calcineurin B-like proteins (CBLs) is a class of calcium-binding proteins which play an important role in the regulation of responses of plant to environmental stress (Gu et al. 2008; Kudla et al. 1999; Luan 2009; Zhang et al. 2008). Conditions that could adversely impact pear production include cold stress, heat stress, drought stress, flooding and salt stress. The expression profiles of *CBLs* in different cultivars and tissues as well as in samples exposed to various abiotic stresses would provide a preliminary understanding of the complicated stress tolerance mechanisms in pear.

In *Arabidopsis,* 10 *CBL* genes have been characterized, and several *CBLs* have been shown to function in an abiotic stress-specific signaling cascade (Pandey et al. 2004; Shi et al. 1999). While in *Populus, PeCBL1, 9, 2, 5* and *3* may play a role in response to specific abiotic stress (Zhang et al. 2008). Our previous study on *PbCBL7* has revealed that this gene can enhance *E. coli* tolerance towards a variety of stresses.

Fig. 6 Expression analysis of *PbCBL7* using qPCR in response to Ca^{2+} signal, PEG6000 and hormones stresses. The results were shown in relative transcript levels of the target genes normalized with the best reference gene combination, *ACT2/7*, *UBI* and *YLS8*. *CK* control, *h* hour. Significant up-regulation with respect to control was shown by **P*<0.05 or *** *P*<0.01. *Error bars* indicate standard errors
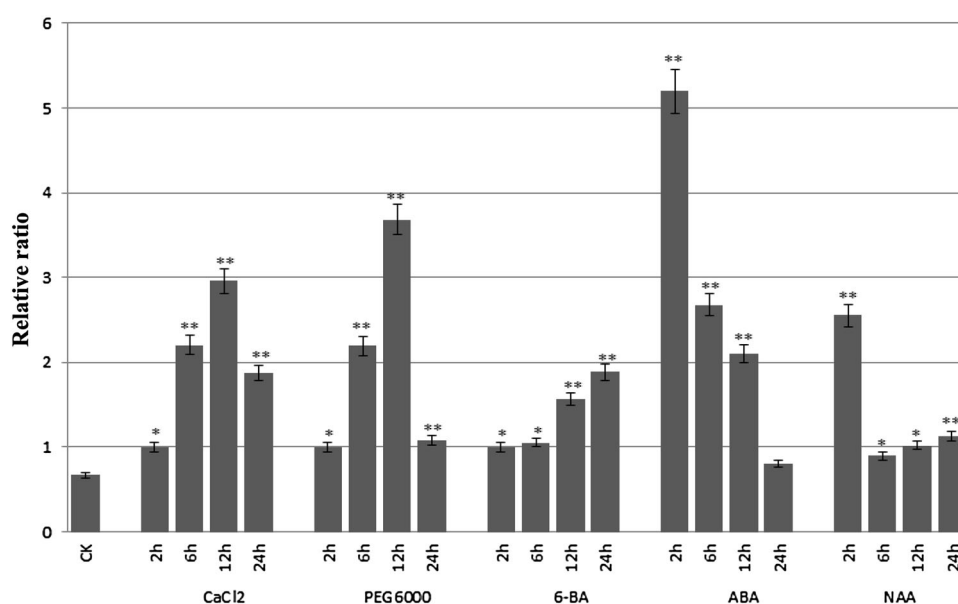
In this study, *PbCBL7* was expressed in different cultivars and tissues of pear as well as in samples exposed to various stress conditions. Interestingly, expression level varied in different tissues and cultivars, and also exhibited diverse up- or down-regulated expression patterns under various treatments, which further indicate that *PbCBL7* may also play a role in response to specific abiotic stress in pear.

In summary, this present study provides new evidence for the importance of appropriate reference genes in RT-qPCR analysis under given experimental conditions in pear. All 18 candidate reference genes selected showed different expression stabilities in each group, and among them, *ACT2/7*, *UBI* and *YLS8* exhibited the most stable expression across all the tested pear samples, while in the other experimental groups, different sets of samples had their own best reference genes. The results of the present study provide useful guidelines for further studies on gene expression analysis using RT-qPCR in pear.

Author contribution statement   XY prepared the plant materials, carried out the RT-qPCR experiments and drafted the manuscript. LH, LX, WZ and YQ participated in the data analysis by geNorm, NormFinder and Best-Keeper. CY and LJ conceived of the study, and participated in its design and coordination and revise the manuscript. The final manuscript was read and approved by all the authors.

Conflict of interest   All authors declare that they have no conflict of interest.

References

Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250

Brunner AM, Yakovlev IA, Strauss SH (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biol 4:14

Chang E, Shi S, Liu J, Cheng T, Xue L, Yang X, Yang W, Lan Q, Jiang Z (2012a) Selection of reference genes for quantitative gene expression studies in *Platycladus orientalis* (Cupressaceae) using real-time PCR. PLoS One 7:e33278

Chang YH, Li H, Cong Y, Lin J, Sheng BL (2012b) Characterization and expression of a phytochelatin synthase gene in birch-leaf pear (*Pyrus betulaefolia* Bunge). Plant Mol Biol Rep 30:1329–1337

Chi XY, Hu RB, Yang QL, Zhang XW, Pan LJ, Chen N, Chen MN (2012) Validation of reference genes for gene expression studies in peanut by quantitative real-time RT-PCR. Mol Genet Genomics 287:167–176

Coito JL, Rocheta M, Carvalho L, Amâncio S (2012) Microarray-based uncovering reference genes for quantitative real time PCR in grapevine under abiotic stress. BMC Res Notes 5:220

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. Plant Physiol 139:5–17

Dekkers BJW, Willems L, Bassel GW, van Bolderen-Veldkamp RP, Ligterink W, Hilhorst HWM, Bentsink L (2012) Identification of reference genes for RT-qPCR expression analysis in *Arabidopsis* and tomato seeds. Plant Cell Physiol 53:28–37

Expósito-Rodríguez M, Borges AA, Borges-Pérez A, Pérez JA (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol 8:131

Garson JA, Grant PR, Ayliffe U, Ferns RB, Tedder RS (2005) Real-time PCR quantitation of hepatitis B virus DNA using automated sample preparation and murine cytomegalovirus internal control. J Virol Methods 126:207–213

Gonzalez-Verdejo CL, Die JV, Nadal S, Jimenez-Marin A, Moreno MT, Roman B (2008) Selection of housekeeping genes for normalization by real-time RT-PCR: analysis of Or-MYB1 gene expression in *Orobanche ramosa* development. Anal Biochem 379:176–181

Gu Z, Ma B, Jiang Y, Chen Z, Su X, Zhang H (2008) Expression analysis of the calcineurin B-like gene family in rice (*Oryza sativa* L.) under environmental stresses. Gene 415:1–12

Guénin S, Mauriat M, Pelloux J, Van Wuytswinkel O, Bellini C, Gutierrez L (2009) Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. J Exp Bot 60:487–493

Gutierrez L, Mauriat M, Guénin S, Pelloux J, Lefebvre JF, Louvet R, Rusterucci C, Moritz T, Guerineau F, Bellini C, Van Wuytswinkel O (2008) The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnol J 6:609–618

Han B, Yang Z, Samma MK, Wang R, Shen W (2013) Systematic validation of candidate reference genes for qRT-PCR normalization under iron deficiency in *Arabidopsis*. Biometals 26:403–413

Hu RB, Fan CM, Li HY, Zhang QZ, Fu YF (2009) Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. BMC Mol Biol 10:93

Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 6:279–284

Huis R, Hawkins S, Neutelings G (2010) Selection of reference genes for quantitative gene expression normalization in flax (*Linum usitatissimum* L.). BMC Plant Biol 10:71

Jain M, Nijhawan A, Tyagi AK, Khurana JP (2006) Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun 345:646–651

Jakab G, Ton J, Flors V, Zimmerli L, Métraux JP, Mauch-Mani B (2005) Enhancing *Arabidopsis* salt and drought stress tolerance by Chemical priming for its abscisic acid responses. Plant Physiol 139:267–274

Jarošová J, Kundu J (2010) Validation of reference genes as internal control for studying viral infections in cereals by quantitative real-time RT-PCR. BMC Plant Biol 10:146

Jian B, Liu B, Bi YR, Hou WS, Wu CX, Han TF (2008) Validation of internal control for gene expression study in soybean by quantitative real-time PCR. BMC Mol Biol 9:59

Jiang Y, Liang G, Yu D (2012) Activated expression of WRKY57 confers drought tolerance in *Arabidopsis*. Mol Plant 5:1375–1388

Klie M, Debener T (2011) Identification of superior reference genes for data normalisation of expression studies via quantitative PCR in hybrid roses (*Rosa hybrida*). BMC Res Notes 4:518

Kudla J, Xu Q, Harter K, Gruissem W, Luan S (1999) Genes for calcineurin B-like proteins in *Arabidopsis* are differentially regulated by stress signals. Proc Natl Acad Sci USA 96:4718–4723

Li QF, Sun SSM, Yuan DY, Yu HX, Gu MH, Liu QQ (2010) Validation of candidate reference genes for the accurate normalization of real-time quantitative RT-PCR data in rice during seed development. Plant Mol Biol Rep 28:49–57

Løvdal T, Lillo C (2009) Reference gene selection for quantitative real-time PCR normalization intomato subjected to nitrogen, cold, and light stress. Anal Biochem 387:238–242

Luan S (2009) The CBL-CIPK network in plant calcium signaling. Trends Plant Sci 14:37–42

Mafra V, Kubo KS, Alves-Ferreira M, Ribeiro-Alves M, Stuart RM, Boava LP, Rodrigues CM, Machado MA (2012) Reference genes for accurate transcript normalization in citrus genotypes under different experimental conditions. PLoS One 7:e31263

Marino ER, Borges AA, Perez AB, Perez JA (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol 8:131

Maroufi A, Bockstaele EV, Loose MD (2010) Validation of reference genes for gene expression analysis in chicory (*Cichorium intybus*) using quantitative real-time PCR. BMC Mol Biol 11:15

Martin RC, Hollenbeck VG, Dombrowski JE (2008) Evaluation of reference genes for quantitative RT-PCR in *Lolium perenne*. Crop Sci 48:1881–1887

Marum L, Miguel A, Ricardo CP, Miguel C (2012) Reference gene selection for quantitative real-time PCR normalization in *Quercus suber*. PLoS One 7:e35113

McDowell JM, Huang S, McKinney EC, An YQ, Meagher RB (1996) Structure and evolution of the actin gene family in *Arabidopsis thaliana*. Genet 142:587–602

Morgante CV, Guimarães PM, Martins ACQ, Araújo ACG, Leal-Bertioli SCM, Bertioli DJ, Brasileiro ACM (2011) Reference genes for quantitative reverse transcription-polymerase chain reaction expression studies in wild and cultivated peanut. BMC Res Notes 4:339

Nicot N, Hausman JF, Hoffmann L, Evers D (2005) Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J Exp Bot 56:2907–2914

Obrero Á, Die JV, Romén B, Gómez P, Nadal S, Gonzalez-Verdejo CL (2011) Selection of reference genes for gene expression studies in zucchini (*Cucurbita pepo*) using qPCR. J Agric Food Chem 59:5402–5411

Pandey GK, Cheong YH, Kim KN, Luan S (2004) The calcium sensor calcineurin B-like 9 modulates abscisic acid sensitivity and biosynthesis in *Arabidopsis*. Plant Cell 16:1912–1924

Pandey S, Nookaraju A, Upadhyaya CP, Gururani MA, Venkatesh J, Kim DH, Park SW (2011) An update on biotechnological approaches for improving abiotic stress tolerance in tomato. Crop Sci 51:2303–2324

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes, and sample integrity: bestKeeper-Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515

Ramakers C, Ruijter JM, Deprez RH, Moorman AF (2003) Assumption free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 339:62–66

Reid KE, Olsson N, Schlosser J, Peng F, Lund ST (2006) An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol 6:27

Remans T, Smeets K, Opdenakker K, Mathijsen D, Vangronsveld J, Cuypers A (2008) Normalisation of real-time RT-PCR gene expression measurements in *Arabidopsis thaliana* exposed to increased metal concentrations. Planta 227:1343–1349

Schmidt GW, Delaney SK (2010) Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. Mol Genet Genomics 283:233–241

Shi J, Kim KN, Ritz O, Albrecht V, Gupta R, Harter K, Luan S, Kudla J (1999) Novel protein kinases associated with calcineurin B-like calcium sensors in *Arabidopsis*. Plant Cell 11:2393–2406

Shi J, Liu M, Shi J, Zheng G, Wang Y, Wang J, Chen Y, Lu C, Yin W (2012) Reference gene selection for qPCR in *Ammopiptanthus mongolicus* under abiotic stresses and expression analysis of seven ROS-scavenging enzyme genes. Plant Cell Rep 31:1245–1254

Silveira ÉD, Alves-Ferreira M, Guimarães LA, da Silva FR, Carneiro VT (2009) Selection of reference genes for quantitative real-time PCR expression studies in the apomictic and sexual grass *Brachiaria brizantha*. BMC Plant Biol 9:84

Tong ZG, Gao ZH, Wang F, Zhou J, Zhang Z (2009) Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 10:71

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034

Vashisth T, Johnson LK, Malladi A (2011) An efficient RNA isolation procedure and identification of reference genes for normalization of gene expression in blueberry. Plant Cell Rep 30:2167–2176

Visioni A, Tondelli A, Francia E, Pswarayi A, Malosetti M, Russell J, Thomas W, Waugh R, Pecchioni N, Romagosa I, Comadran J (2013) Genome-wide association mapping of frost tolerance in barley (*Hordeum vulgare* L.). Plant. Genomics 14:424

Wan HJ, Zhao ZG, Qian CT, Sui YH, Malik AA, Chen JF (2010) Selection of appropriate reference genes for gene expression studies by quantitative real-time polymerase chain reaction in cucumber. Anal Biochem 399:257–261

Wan H, Yuan W, Ruan M, Ye Q, Wang R, Li Z, Zhou G, Yao Z, Zhao J, Liu S, Yang Y (2011) Identification of reference genes for reverse transcription quantitative real-time PCR normalization in pepper (*Capsicum annuum* L.). Biochem Biophys Res Commun 416:24–30

Wu J, Wang Z, Shi Z (2013) The genome of the pear (*Pyrus bretschneideri* Rehd.). Genome Res 23:396–408

Xu Y, Zhu X, Gong Y, Xu L, Wang Y, Liu L (2012) Evaluation of reference genes for gene expression studies in radish (*Raphanus sativus* L.) using quantitative real-time PCR. Biochem Biophys Res Commun 424:398–403

Yang Y, Hou S, Cui G, Chen S, Wei J, Huang L (2010) Characterization of reference genes for quantitative real-time PCR analysis in various tissues of *Salvia miltiorrhiza*. Mol Biol Rep 37:507–513

Zhang H, Yin W, Xia X (2008) Calcineurin B-Like family in *Populus*: comparative genome analysis and expression pattern under cold, drought and salt stress treatment. Plant Growth Regul 56:129–140

Zhang X, Allan AC, Yi Q, Chen L, Li K, Shu Q, Su J (2011) Differential Gene expression analysis of Yunnan red pear, *Pyrus Pyrifolia*, during fruit skin coloration. Plant Mol Biol Rep 29:305–314

Zhang H, Zhang S, Qin G, Wang L, Wu T, Qi K, Zhang S (2013) Molecular cloning and expression analysis of a gene for sucrose transporter from pear (*Pyrus bretschneideri* Rehd.) fruit. Plant Physiol Biochem 73:63–69

Zhong HY, Chen JW, Li CQ, Chen L, Wu JY, Chen JY, Lu WJ, Li JG (2011) Selection of reliable reference genes for expression studies by reverse transcription quantitative realtime PCR in litchi under different experimental conditions. Plant Cell Rep 30:641–653

Zhu J, Zhang L, Li W, Han S, Yang W, Qi L (2013) Reference gene selection for quantitative real-time PCR normalization in *caragana intermedia* under different abiotic stress conditions. PLoS One 8:e53196