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ORIGINAL ARTICLE

Identification and testing of reference genes for qRT-PCR analysis during pear fruit development

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Abstract

Quantitative real-time PCR (qRT-PCR) is currently one of the most reliable and improved tools for analyzing gene expression. Various studies have shown that housekeeping genes vary with cultivars, tissues and treatment. Reliable and stable reference genes were necessarily identified and evaluated according to different experimental requirements. In this study, 10 candidate reference genes were initially screened based on transcriptome sequencing data of four pear fruit development stages for three pear cultivars, including a candidate housekeeping gene *PbrTUB*. Furthermore, we ranked the expression stability of 10 candidate reference genes using GeNorm, NormFinder, BestKeeper and ReFinder algorithms. The results showed that *Pbr028511*, *Pbr038418* and *Pbr041114* were the most stable reference genes in Cuiguan, Housui and Xueqing fruit, respectively. Taken together, these results serve as a useful reference for gene function investigations and molecular mechanism studies in fruit development and ripening for various pear cultivars.

Keywords Fruit development · Pear · qRT-PCR · Reference genes

Introduction

Gene expression analysis is used to verify mRNA transcription levels of target genes and to explore novel gene functions in various biological processes, such as growth, development, signal transduction, stress responses and metabolism. Compared with other gene expression detection methods, quantitative real-time PCR (qRT-PCR) has become one of the most reliable and improved upon for studying gene transcript levels on account of its high accuracy, sensitivity and specificity (Bustin et al. 2005). However, the accuracy and reliability of this technology are affected by a variety of other factors, such as RNA quality, number of replicates, primer amplification efficiency, and suitability of reference genes (Derveaux et al. 2010; Die and Roman 2012; Svec et al. 2015). The most general approaches of qRT-PCR normalization that enhance the assay accuracy include application of a normalization step and internal reference genes or housekeeping genes (Guenin et al. 2009).

Ideal reference genes should exhibit relatively stable and consistent expression levels in different cultivars, tissues and conditions. However, no absolute universal reference genes have been reported until now. In previous studies, the traditional housekeeping genes, such as tubulin (*TUB*), actin (*ACT*), ubiquitin (*UBQ*), elongation factor 1-α (*EF1-α*), 18S ribosomal RNA (*18S rRNA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were directly selected to standardize the results of the qRT-PCR assay (Chen et al. 2015; Sarwar et al. 2020; He et al. 2021). Nevertheless, these housekeeping genes do not always show constant expression in variable conditions. Invalid or unstable reference genes can lead to erroneous conclusions in certain situations (Jain et al. 2006; Guenin et al. 2009). For example, several reports have shown that *EF1-α*, *ACT* and *TUB* were not stably expressed consistently (Nicot et al. 2005; Gutierrez et al. 2008; Hong et al. 2008). Housekeeping genes may exhibit

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varied expression levels in different developmental stages in *Lycopersicon esculentum* Mill. (Exposito-Rodriguez et al. 2008). In addition, some studies pointed out that a single reference gene cannot satisfy with the experimental requirements (Tong et al. 2009; Chen et al. 2011). Therefore, it is necessary to screen and validate stable reference genes in order to accurately quantify target genes in diverse cultivars and experimental backgrounds during qRT-PCR normalization analysis.

Pear (*Pyrus*) is identified as one of the most important temperate fruit species with high economic value in the world (Wu et al. 2013). Multiple internal and external factors participate in the characteristics and biological processes of pear, including pollen growth (Wang et al. 2016; Tang et al. 2020), self-incompatibility (Wang et al. 2017; Chen et al. 2018), seed germination (Qi et al. 2019), fruit development, and senescence (Hao et al. 2018; Gu et al. 2020). These factors also affect the expressions of related genes. Therefore, accurate and reliable analysis of expression patterns helps to reveal gene functions and related molecular mechanisms. However, characterization of reference genes in pear has only been reported in limited tissues and cultivars (Wu et al. 2012; Chen et al. 2015; Liu et al. 2018; Wang et al. 2018; Wang et al. 2019; Chen et al. 2020a; Chen et al. 2020b). Accordingly, there is demand for identification of appropriate reference genes in pear to obtain reliable and accurate gene expression analysis data.

In this study, we measured the expression stability of 10 candidate reference genes, employing the RNA-seq data for four development stages of Cuiguan, Housui and Xueqing pear fruit. In addition, three software packages, including geNorm, NormFinder, BestKeeper, along with RefFinder, an online tool, were utilized to evaluate the expression stability of the 10 candidate reference genes in three pear varieties at different developmental stages. Our results provide reliable reference genes for qRT-PCR normalization analysis in Cuiguan, Housui and Xueqing pear fruit. This will contribute to both expression pattern analysis of targeted genes and discovery of the breeding molecular mechanism.

Materials and methods

Plant materials and experimental treatments

All 36 samples of the three pear cultivars were collected from the pear germplasm orchard of the Pear Engineering Technology Research Center of Nanjing Agricultural University in Nanjing, China. The fruits of pear cultivars Cuiguan (cg), Housui (hs) and Xueqing (xq) were collected from the fruitlet to ripening stages, including 10 cg (C1–10), 13 hs (H1–13) and 13 xq (X1–13). Four fruits at different stages (fruitlet, early enlargement, later enlargement, and ripening) were used for transcriptome sequencing. The flesh was ground into powder, then frozen in liquid nitrogen and stored at −80 °C until use.

Candidate reference gene selection and primer designing

Based on fruit transcriptome sequencing data of different pear cultivars from our laboratory, 10 candidate reference genes, including nine novel genes and one housekeeping gene, were applied to normalize and validate qRT-PCR experiments according to their RPKM and fold change values (Table 1). The primers of the candidate reference genes were designed using Primer Premier 5.0 software with the following parameters: annealing temperature (Tm) of 60–65 °C, GC percent of 45–60%, primer length of 18–28 bp and product length of 150–210 bp. Lin-RegPCR was used to calculate the qRT-PCR primer amplification efficiency of the 10 pairs of primers (Table 1) (Ramakers et al. 2003). All primers were synthesized by Sangon (Nanjin, China); the primer sequences are listed in Table 1. To assay the expression specificity and efficiency of all primers, PCR was performed and the products were analyzed on a 2.0% agarose gel.

Total RNA extraction and cDNA preparation

Total RNA was isolated using the RNAprep Pure Plant Kit (Tiangen, Beijing, China) according to instructions. The Nanodrop ND1000 spectrophotometer (Thermo Scientific) was used to determine total RNA concentration and purity, and RNA samples were then assessed with OD 260/280 > 2.0 and OD 260/230 > 1.8. For each sample, 500 ng of the extracted total RNA were reverse-transcribed with TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Beijing, China). RNA extraction and cDNA synthesis from all samples were performed with three biological replicates. Then, a reference gene was used to verify the quality of all cDNA samples by PCR before carrying out qRT-PCR. The results of 2.0% agarose gel electrophoresis showed that all cDNA templates had no genomic DNA contamination.

Quantitative real-time PCR

Quantitative real-time PCR amplification reactions were carried out by Light Cycler 480 (Roche, USA). 20 µL reaction mixtures contained 10 µL SYBR Green I Mix, 5 ng cDNA, ddH_2O, and a final primer concentration of 0.4 µM. Reaction mixtures were incubated for 10 min at 95 °C for preincubation, followed by 45 amplification cycles of 15 s at 95 °C, 15 s at 60 °C and 20s at 72 °C. After that, the specificity of the primer amplicons was checked by melting curve

Table 1 Primer sequences and amplification characteristics of 10 candidate reference genes

Gene ID	Gene annotation	Primer sequence	Amplicon size (bp)	PCR efficiency (%)	Correlation coefficient (R2)
Pbr041114	thiosulfate/3-mercaptopyruvate sulfurtransferase	F: 5'-ACTGGTGTGACAGCTTGCATTCTTG-3′ R: 5'-GTATTGGCAAACCCCAGGCTATTC-3'	196	100.3	0.996
Pbr018827	malate dehydrogenase	F: 5'-CCCCAAAAGAAGTTGATTACCTAACAGAT-3′ R: 5'-AAGTTCAGTCACCCCAGCATCTCC-3'	170	99.4	0.996
Pbr016048	endoplasmic reticulum-Golgi compartment protein	F: 5'-GAGGTGTTTTTACAGTTTCGGGGATAC-3′ R: 5'-CCTAGATCGATTCTGTCGCACAAAGT-3'	162	99.8	0.995
Pbr028511	retinal-binding protein	F: 5'-TCTCTCATTTTACCTTACCAACGCTAC-3′ R: 5'-GAAAACACTCATTCTTCAACCTCGT-3'	189	100.5	0.999
Pbr000214	DNA-binding protein transcriptional regulator	F: 5'-TTTGGGTCTGAATCCGTGCTCTT-3′ R: 5'-TCTCATTAACTCGCTCCGCTTCAC-3'	208	100.1	0.997
Pbr038418	SNF1-related protein kinase	F: 5'-GATGGTGCTATGAAGATGCCAAATGT-3′ R: 5'-TCCCGAGCATCACGATAGATTCAC-3'	210	98.5	0.999
Pbr016129	peroxisome biogenesis protein	F: 5'-GAGTACTGGTTGGGATGACCTTGC-3′ R: 5'-TGACTCAGTAACAGCATCGCACCAAT-3'	171	99.1	0.982
Pbr002841	V-type proton ATPase	F: 5'-ATGGGTATGCTCACTTGTCATCTGG-3′ R: 5'-ACGATGAGACCATACAAGGCAAGAG-3'	181	99.3	0.995
Pbr027964	UDP-D-apiose/UDP-D-xylose synthase	F: 5'-CCCAATCATCTGCAAGCTAAATAAGG-3′ R: 5'-GACTAACGACACCCGTAAGGCAAG-3'	168	98.3	0.997
PbrTUB (Pbr042345)	tubulin	F: 5'-TGGGCTTTGCTCCTCTTACTTCAC-3′ R: 5'-CTTCCTTGGTGCTCATCTTACCACG-3'	169	98.4	0.998

analysis. All samples had three independent biological replicates with three technical replicates each. Lin-RegPCR was used to estimate the amplification efficiency of the 10 pairs of primers in qRT-PCR (Ramakers et al. 2003). Expression levels of the 10 candidate genes in all samples were determined by their cycle threshold (Ct) values.

Statistical analysis

To rank the expression stability of the 10 candidate reference genes in pear fruit, four publicly available Microsoft Excel-based methods were used: geNorm analysis (Vandesompele et al. 2002), NormFinder analysis (Andersen et al. 2004), BestKeeper analysis (Pfaffl et al. 2004), and comparative Ct methods (Silver et al. 2006). Finally, to select the most stable reference genes according to different algorithms, we compared the candidates based on the web-based comprehensive tool *RefFinder* (http://www.leonxie.com/referencegene.php) (Xie et al. 2011).

The geNorm analysis enabled the selection of the optimal set of genes using a gene-stability measure (*M*). The two most stable genes, with the lowest M values, were ranked on the right. On the contrary, the least stable genes with the highest *M* values were ranked on the left. An

M value of no more than 1.5 for reference gene was the default limit (Vandesompele et al. 2002).

The Normfinder software, similar to geNorm, is another Visual Basic Application (VBA) for identifying and ranking the optimal normalization genes from the candidates (Andersen et al. 2004). NormFinder provides a stability value for each gene according to intergroup and intragroup expression variation. For the estimated expression, enabling variation evaluated the systematic error introduced when using a gene for normalization.

BestKeeper, another Excel-based tool, determines the most stable genes according to the coefficient of

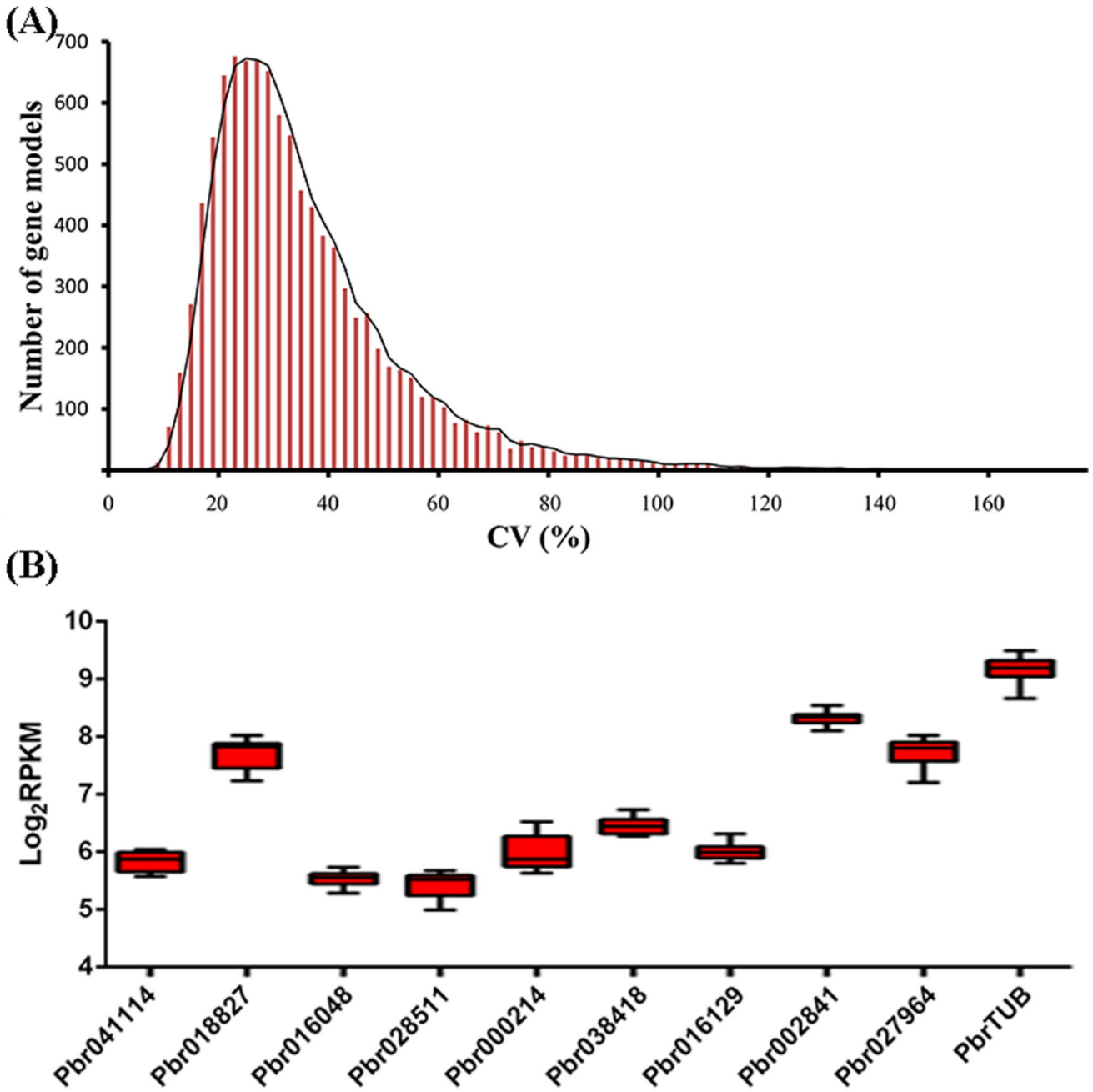

Fig. 1 Identification of candidate reference genes in pear fruit based on RNA sequencing data. (**A**) The distribution of CV% for the 10,236 genes with FPKM > 5 across the 12 fruit mRNA-seq experiments of three pear cultivars. (**B**) RPKM of 10 candidate genes. Box and whisker plot graph showing $\log_2$RPKM values of each candidate gene in all samples analyzed. The line across the boxes represent the medians, while the bottom and top box borders represent the 25/75 percentiles, respectively. Whisker caps represent the minimum and maximum values

correlation of the candidate reference gene's Ct values. Genes with the lowest standard deviation (SD) values are the most stable (Pfaffl et al. 2004).

RefFinder is a web-based comprehensive online program that calculates the geometric mean, taking into account the rankings from each aforementioned program to determine the overall final ranking.

Results

Screening of stably expressed genes using transcriptome sequencing data

A total of 28,331 expressed genes were detected (RPKM > 0) for at least one developmental stage in the fruit tanscriptome data of three pear cultivars. Genes with FPKM values lower than 5 were considered to be poor qRT-PCR references because of the difficulty in detecting and quantifying their expression (Stanton et al. 2017). After their removal, 10,236 genes in pear were evaluated in the subsequent studies. The coefficients of variation (CV) of the 10,236 gene expression showed a right-skewed distribution (Fig. 1A). The CV% was distributed between 3.3 and 172, including 2142 genes with CV% < 20 (Fig. 1A), which had relatively stable expression in all developmental stages. The value of CV% < 20 was the basic requirement for reference genes (Wang et al. 2019; Chen et al. 2020a). Based on transcript abundance and the CV values for gene expression, potential reference genes with FPKM > 40 and CV% < 20 were selected for testing. Finally, a set of 10 candidate genes were selected, including one common housekeeping gene *PbrTUB* (*Pbr042345*) and nine novel genes (*Pbr002841*, *Pbr028511*, *Pbr038418*, *Pbr016129*, *Pbr027964*, *Pbr041114*, *Pbr000214*, *Pbr016048* and *Pbr018827*) (Supplementary Table 1). The expression variability of the selected genes was analyzed in a $\log_2$RPKM box plot (Fig. 1B).

Identification and characterization of candidate reference genes

A total of 10 candidate reference genes were identified for qRT-PCR normalization from fruit transcriptome sequencing data of various pear cultivars. The details of gene ID, primer sequences, amplicon size, and annealing temperature were listed in Table 1. To identify amplification specificity of primers, agarose gel electrophoresis was performed following PCR. The candidate housekeeping gene *PbrTUB* was used for comparison. The result showed that all primer pairs had single bands at the expected positions (Fig. 2).

Ct values of candidate reference genes

The average cycle threshold (Ct) values were used to calculate transcript levels of the candidate reference genes in different stages of fruit development. The 10 candidate reference genes displayed a relatively wide range of Ct values, from 22.73 for *Pbr002841* to 31.83 for *Pbr016129* in the 36 tested samples. In addition, each candidate gene maintained a relatively consistent expression level in all samples (Fig. 3A). Moreover, *PbrTUB* transcript levels were the most variable with a CT value of 5.83, while *Pbr002841* showed the least variability with a 1.54 Ct value (Fig. 3B). Since gene transcript levels were negatively correlated with Ct values, *Pbr002841* had higher expression in pear fruit than the other candidate reference genes.

geNorm analysis

Gene expression stability was verified through geNorm analysis, with average expression stability represented by

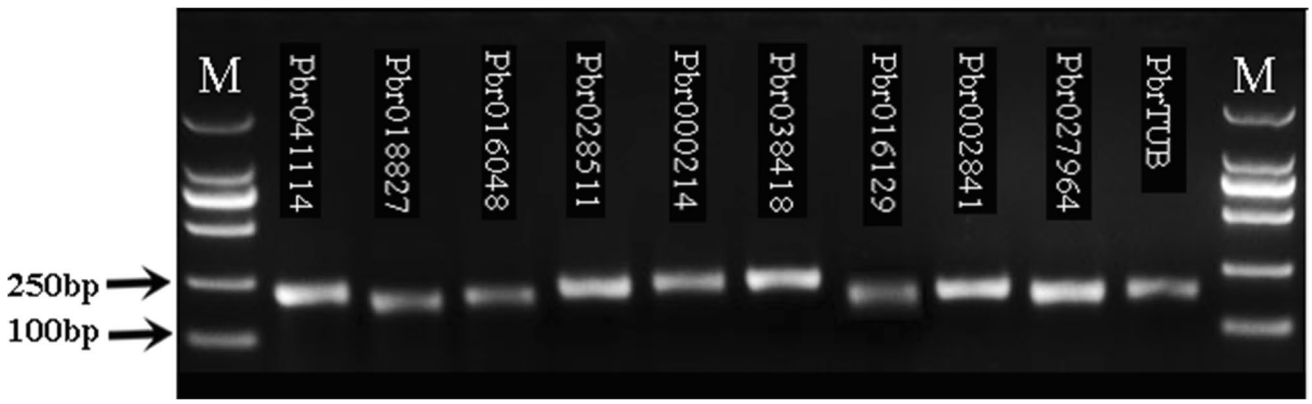

Fig. 2 Specificity of qRT-PCR and amplicon lengths of 10 reference genes. Amplified fragments were separated by 2% agarose gel electrophoresis. M: DL 2000 marker (in ascending order: 100, 250, 500, 750, 1000 and 2000 bp)

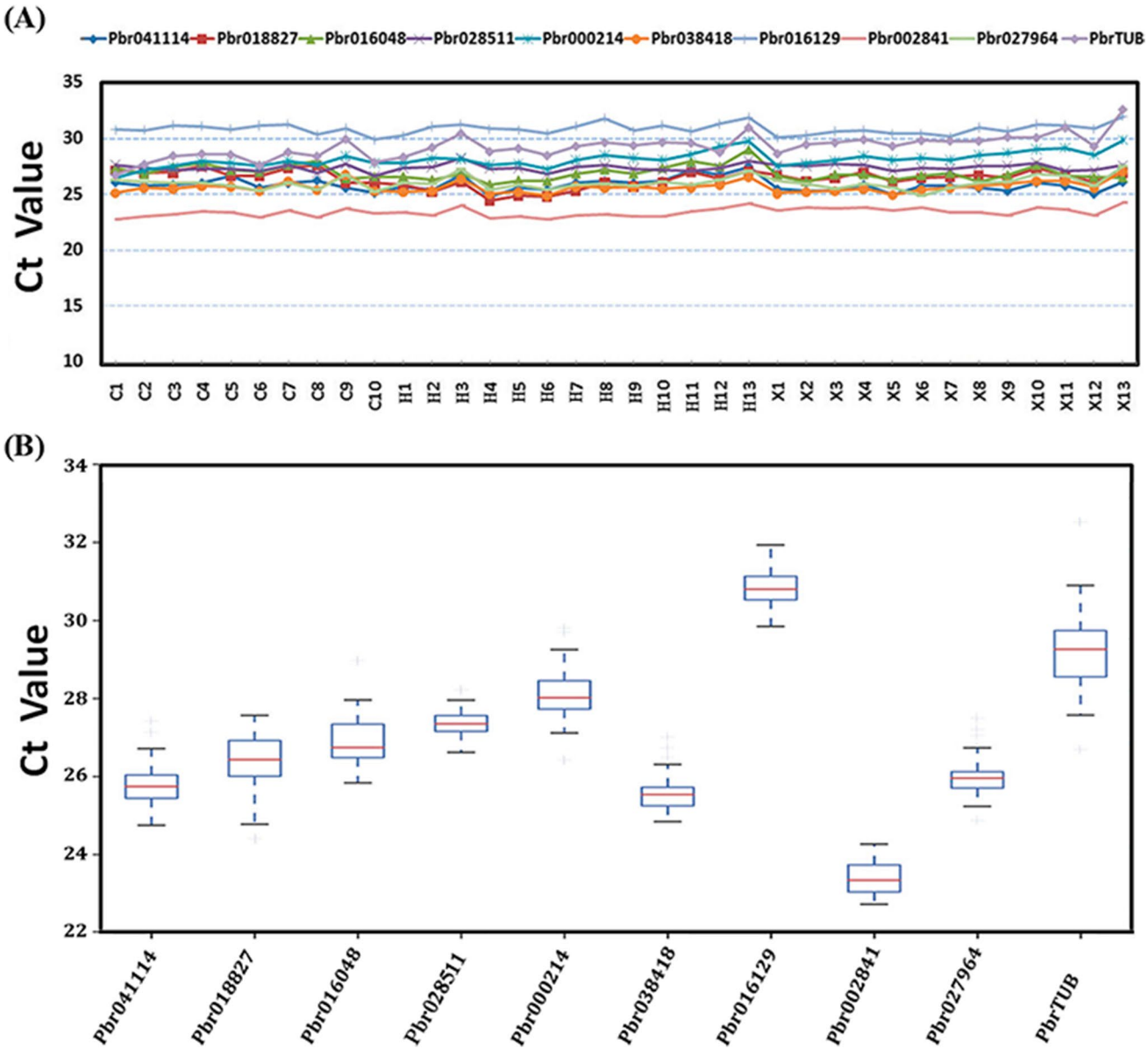

Fig. 3 Expression levels of 10 candidate reference genes tested in 36 samples of three cultivates. (**A**) Ct values of 10 candidate reference genes with three replicates. (**B**) The line across the box indicates the median. The box indicated the 25/75th percentiles. Whisker caps represent the minimum and maximum values

M value. The lower the M value, the more stable the gene, and vice versa (Vandesompele et al. 2002). The M values of the 10 candidate reference genes were lower than 1.5, the geNorm threshold considered stable, for all samples (Fig. 4). In the Cuiguan group, *Pbr028511* and *Pbr027964* had the most stable expressions through developmental stages C1–10, while *PbrTUB* had the most unstable expression (Fig. 4A). In the Housui group, *Pbr002841* and *Pbr027964* were the two most stable genes through developmental stages H1–13, while *Pbr016048* was the most unstable gene (Fig. 4B). Similarly, in the Xueqing group, *Pbr041114* and *Pbr018827* genes showed the most stability through developmental stages X1–13 (Fig. 4C).

NormFinder and BestKeeper analysis

NormFinder's geNorm software was used to determine the most suitable internal reference gene. The ΔCt method was performed to directly evaluate the expression stability of candidate reference genes (Andersen et al. 2004). The smaller the value, the better the stability of the reference gene. *Pbr002841* had the lowest Ct value in the Cuiguan group,

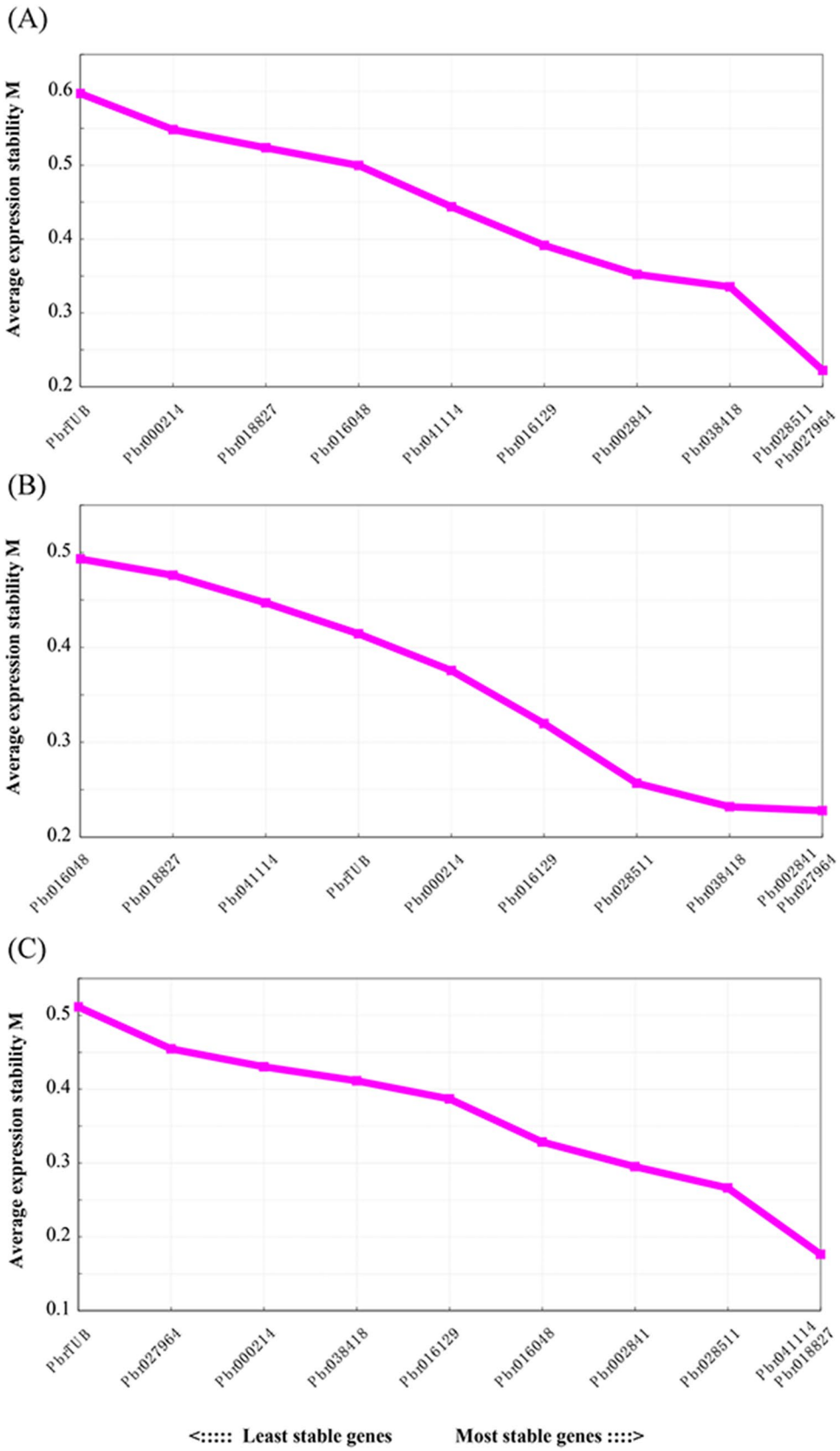

Fig. 4 Expression stability values (M) of 10 genes in three sample groups indicated in each figure by geNorm software. (**A**) The Cuiguan fruit (C1–10). (**B**) The Housui fruit (H1–13). (C) The Xueqing fruit (X1–13)

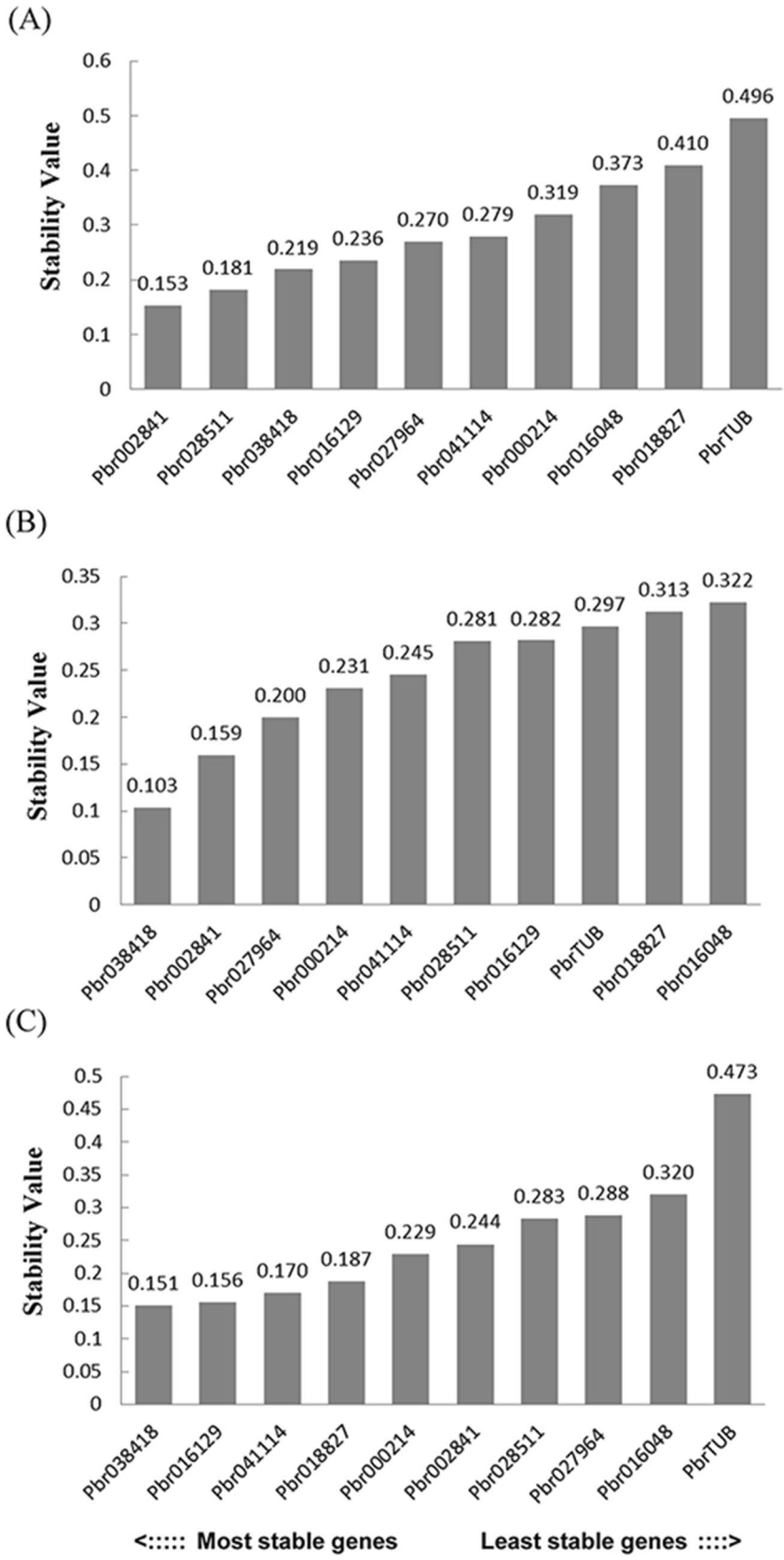

Fig. 5 Expression stability analysis of 10 candidate genes in three sample groups by NormFinder. (**A**) The Cuiguan fruit (C1–10); (**B**) The Housui fruit (H1–13); (**C**) The Xueqing fruit (X1–13). A lower average expression stability value indicates more stable expression

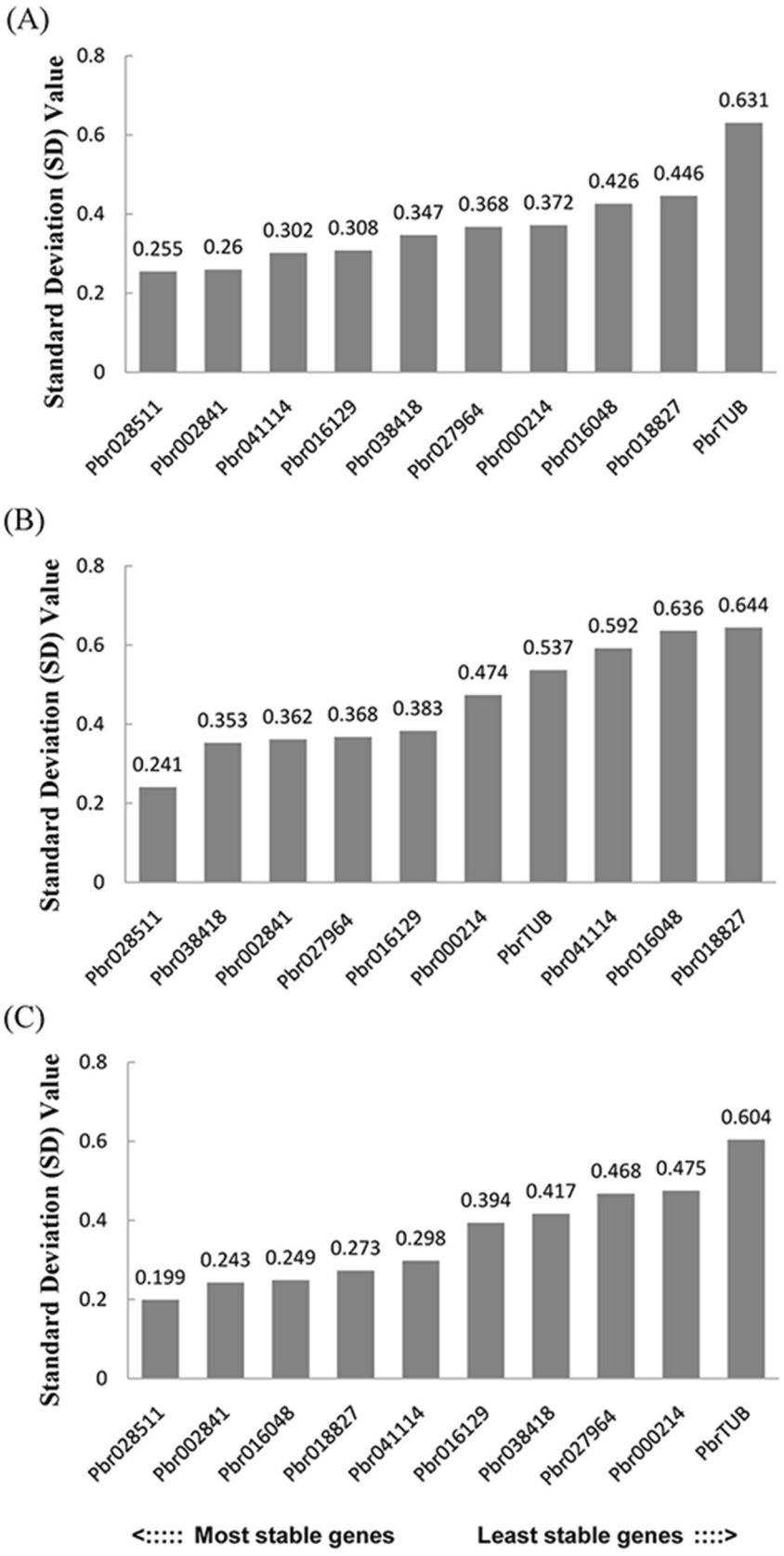

Fig. 6 Expression stability analysis of 10 candidate genes in three sample groups by BestKeeper. (**A**) The Cuiguan fruit (C1–10); (**B**) The Housui fruit (H1–13); (**C**) The Xueqing fruit (X1–13). A lower average expression stability value indicates more stable expression

indicating the highest stability (Fig. 5A). *Pbr038418* exhibited the lowest Ct value, indicating the highest stability, in both the Housui and Xueqing groups (Fig. 5B and C). *PbrTUB* had the highest Ct value in both Cuiguan and Xueqing groups, and was thus the most unreliable candidate (Fig. 5A and C).

BestKeeper was used to estimate the stability of candidate reference genes via standard deviation (SD) (Pfaffl et al. 2004). In the Cuiguan, Housui and Xueqing groups, the lowest SD values were 0.49, 0.39, and 0.44 for *Pbr002841*, *Pbr038418*, and *Pbr038418* respectively (Fig. 6). The results showed that these three genes were the most stable in their respective groups. Meanwhile, *PbrTUB*, *Pbr016048,* and *PbrTUB* were the most variable reference genes with the highest SD values of 0.79, 0.56 and 0.74 in the Cuiguan, Housui and Xueqing groups respectively (Fig. 6). These results were consistent with the analysis of NormFinder.

RefFinder analysis

RefFinder is an online tool used to comprehensively integrate the results of geNorm, NormFinder, BestKeeper, and the ΔCt. It ranks candidates on the basis of their geomean constancy. Consistent with the three tools discussed above, the lowest ranking value corresponded to the highest stability. The comprehensive ranking was displayed in Table 2. This integrated tool indicates that *Pbr028511*, *Pbr038418,* and *Pbr041114* were the most stable reference genes in Cuiguan, Housui and Xueqing fruit, respectively.

qRT-PCR validation

To test the applicability of candidate reference genes experimentally by qRT-PCR, eight genes were selected with contrasting expression patterns in the RNA-seq data (increased or decreased expression patterns) in the three cultivars (Supplementary Table 2). The specific primer pairs of eight selected genes for qRT-PCR validation are listed in Supplementary Table 3. The genes of *Pbr028511*, *Pbr038418,* and *Pbr041114* were used as reference genes in Cuiguan, Housui and Xueqing fruit, respectively. As expected, the eight genes had nearly identical expression profiles by qRT-PCR than by RNA-Seq analyses (Fig. 7), showing that *Pbr028511*, *Pbr038418,* and *Pbr041114* are reliable reference genes for gene expression studies.

Discussion

Gene expression patterns were widely used to better analyze gene expression levels and understand their biological functions. Recognized as an effective tool for performing accurate and rapid quantification of target gene expression, qRT-PCR was commonly performed in bioresearch. Generally, traditional housekeeping genes were used to standardize the transcriptional accumulations of target genes such as *TUB*, *ACT*, *UBQ*, *EF1-α,* and *GAPDH*. Nevertheless, common reference genes are not consistently stably expressed and thus cannot be applied to all species (Hong et al. 2008; Gutierrez et al. 2008). Based on previous studies, reference genes for fruit development vary among different genera and cultivars, such as *TEF2*, *UBQ10,* and *RP II* in peach (Tong et al. 2009), *EF1-α*, *CKL,* and *WD40* in apple (Zhu et al. 2019), *BPS1* and *ICDH1* in pear (Chen et al. 2020b), and *RPT6A* and *RPN5A* in strawberry (Chen et al. 2021). Therefore, the selection of optimal reference genes for data normalization was critical in qRT-PCR assays. In this study, we identified the appropriate reference genes for fruit development and ripening in the pear cultivars Cuiguan, Housui, and Xueqing.

Transcriptome sequencing analysis is a high-throughput sequencing technology, which provides unbiased test transcripts and increased test specificity (Gu et al. 2018; Hao et al. 2018; Pei et al. 2020). Therefore, transcriptome sequencing data provides a new resource for screening reference genes at the genome level. Candidate reference genes have been identified via transcriptome data in diverse plant species, such as *Oryza sativa* L. (Narsai et al. 2010), *Fagopyrum esculentum* Moench (Demidenko et al. 2011), *Brassica napus* L. (Yang et al. 2014), and *Euscaphis konishii* Hayata (Liang et al. 2018). In this study, we selected 10 relatively stable candidate reference genes based on the transcriptome data of three pear cultivars in four different developmental stages (Table 1).

Studies on pear, which is identified as the third largest temperate fruit tree, have progressed with the release of pear (*Pyrus bretschneideri* Rehd.) genome datasets (Wu et al. 2013). In previous studies, several traditional or novel reference genes were identified and evaluated under various biotic or abiotic stresses and at each stage of development in different tissues of diverse pear cultivars. Ubiquitous housekeeping genes may exhibit inconsistent stability under different conditions (Wu et al. 2012; Imai et al. 2014; Li et al. 2015). For instance, *EF1α* and *TUB-b2* were the most stable in different pear cultivars, *GAPDH* and *EF1α* were the most suitable in different tissues, while *TUB-b2* and *GAPDH* were the most stable in different developmental stages (Wu et al. 2012). In addition, it has been reported that different groups of pear tissues have different optimal reference genes depending on the experimental purpose. In pollen, the *PbrGAPDH*, *PbrPP2A,* and *PbrUBI* were suitable reference genes for low temperature, NaCl treatment, and $CuCl_2$ treatment conditions, respectively. *PbrEF1α* was a stable reference gene for all abiotic stresses (Chen et al.

Table 2 The comprehensive ranking of 10 candidate reference genes in Cuiguan, Housui and Xueqing samples analysed by ReFinder

Groupe	Method	Ranking Order (Better--Good--Average)									
		1	2	3	4	5	6	7	8	9	10
cg	Delta CT	Pbr002841	Pbr028511	Pbr038418	Pbr016129	Pbr027964	Pbr041114	Pbr000214	Pbr016048	Pbr018827	PbrTUB
	BestKeeper	Pbr028511	Pbr002841	Pbr041114	Pbr016129	Pbr038418	Pbr027964	Pbr000214	Pbr016048	Pbr018827	PbrTUB
	Normfinder	Pbr002841	Pbr028511	Pbr038418	Pbr016129	Pbr027964	Pbr041114	Pbr000214	Pbr016048	Pbr018827	PbrTUB
	Genorm	Pbr028511| Pbr027964		Pbr038418	Pbr002841	Pbr016129	Pbr041114	Pbr016048	Pbr018827	Pbr000214	PbrTUB
	Comprehensive ranking (Geomean of ranking values)	Pbr028511 (1.41)	Pbr002841 (1.68)	Pbr038418 (3.41)	Pbr027964 (3.5)	Pbr016129 (4.23)	Pbr041114 (5.05)	Pbr000214 (7.45)	Pbr016048 (7.74)	Pbr018827 (8.74)	PbrTUB (10)
hs	Delta CT	Pbr038418	Pbr002841	Pbr027964	Pbr000214	Pbr041114	Pbr028511	Pbr016129	PbrTUB	Pbr018827	Pbr016048
	BestKeeper	Pbr028511	Pbr038418	Pbr002841	Pbr027964	Pbr016129	Pbr000214	PbrTUB	Pbr041114	Pbr016048	Pbr018827
	Normfinder	Pbr038418	Pbr002841	Pbr027964	Pbr000214	Pbr041114	Pbr028511	Pbr016129	PbrTUB	Pbr018827	Pbr016048
	Genorm	Pbr002841| Pbr027964		Pbr038418	Pbr028511	Pbr016129	Pbr000214	PbrTUB	Pbr041114	Pbr018827	Pbr016048
	Comprehensive ranking (Geomean of ranking values)	Pbr038418 (1.57)	Pbr002841 (1.86)	Pbr027964 (2.45)	Pbr028511 (3.46)	Pbr000214 (4.9)	Pbr016129 (5.92)	Pbr041114 (6.32)	PbrTUB (7.48)	Pbr018827 (9.24)	Pbr016048 (9.74)
xq	Delta CT	Pbr038418	Pbr041114	Pbr016129	Pbr018827	Pbr000214	Pbr002841	Pbr028511	Pbr027964	Pbr016048	PbrTUB
	BestKeeper	Pbr028511	Pbr002841	Pbr016048	Pbr018827	Pbr041114	Pbr016129	Pbr038418	Pbr027964	Pbr000214	PbrTUB
	Normfinder	Pbr038418	Pbr016129	Pbr041114	Pbr018827	Pbr000214	Pbr002841	Pbr028511	Pbr027964	Pbr016048	PbrTUB
	Genorm	Pbr041114 Pbr018827		Pbr028511	Pbr002841	Pbr016048	Pbr016129	Pbr038418	Pbr000214	Pbr027964	PbrTUB
	Comprehensive ranking (Geomean of ranking values)	Pbr041114 (2.34)	Pbr038418 (2.65)	Pbr018827 (2.83)	Pbr028511 (3.48)	Pbr016129 (3.83)	Pbr002841 (4.12)	Pbr016048 (5.9)	Pbr000214 (6.51)	Pbr027964 (8.24)	PbrTUB (10)

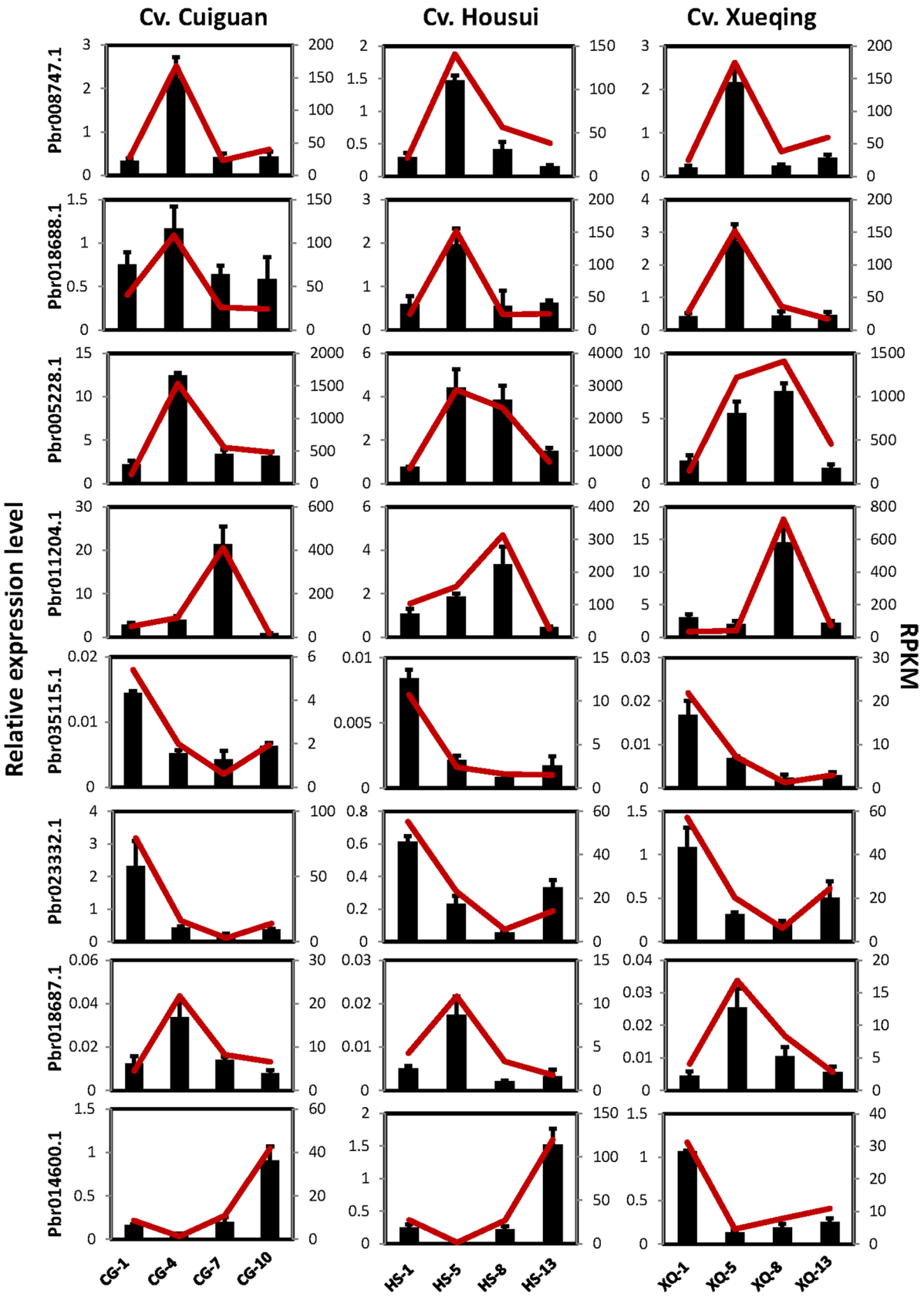

Fig. 7 qRT-PCR validation by expression profiles of eight genes. *Pbr028511*, *Pbr038418,* and *Pbr041114* were used as reference genes in Cuiguan, Housui and Xueqing, respectively. Left and right Y axes represent the relative expression levels and RPKM of target gene, respectively

2015). In leaf, *SKD1* and *ARM* were the most apropos single reference genes (Liu et al. 2018). In pear peel, *ACT6/7/8/9* and *NAP1* were recommended as the optimal reference genes (Chen et al. 2020a). Of the more well-researched reference genes in pear fruit, *PbPDI.F1* displayed the highest expression stability during pear fruit development (Ke et al. 2018), the housekeeping gene *EF1α* members displayed a clearly unstable expression in pear fruit at different developmental stages (Wang et al. 2018), *SOX2* and *PP2A* displayed stable expression in pear fruit (Wang et al. 2019), and *BPS1* and *ICDH1* were the high and stable expressed genes (Chen et al. 2020b). In this study, we identified stable and novel reference genes in the fruits of three different pear varieties. Therefore, reliable and accurate reference genes were identified according to different experimental requirements.

We used three analysis algorithms (geNorm, NormFinder and BestKeeper) to evaluate the expression stability of 10 candidate genes in different stages of 10 Cuiguan, 13 Housui, and 13 Xueqing pear fruit samples. Although different statistical algorithms and analytical procedures may lead to different stability rankings, most results were consistent according to these three algorithms. Finally, the online tool RefFinder was used to comprehensively integrate all of the rankings (Table 2). The result showed that *Pbr028511*, *Pbr038418,* and *Pbr041114* were the most stable reference genes in Cuiguan, Housui, and Xueqing fruit, respectively. In addition, each of the above reference genes possesses more stable expression than *PbrTUB* in pear fruit in all stages of fruit development and ripening.

Conclusion

In this study, three genes were screened and identified as the most reliable and stable reference genes based on a series of stability analyses in Cuiguan, Housui, and Xueqing pear fruit. *Pbr028511* was the most stable reference gene in Cuiguan, *Pbr038418* was the most stable reference gene in Housui, and *Pbr041114* was the most stable reference gene in Xueqing pear fruit. Therefore, our study provides a series of stable and valuable reference genes that can be applied to exploring gene functions and molecular mechanisms in pear fruit development and ripening.

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Authors' contributions WGM carried out the experimental design, data analysis. GC and ZSL designed the experiment and revising the manuscript. WGM and GZH performed the experiments. SLG revised the manuscript and language. QKJ and GHR provided experimental materials. All authors have read and approved the final manuscript.

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Data availability The datasets supporting the conclusions are included within the article.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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