Identification and Testing of Reference Genes for qRT-PCR Analysis During Pear Fruit Development

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Research

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Abstract

Background: 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 was varied with cultivars, tissues and treatment. The reliable and stable reference genes were necessarily identified and evaluated according to different experimental requirements.

Result: In this study, 10 candidate reference genes were initially screened based on the transcriptome sequencing data of four pear fruit development stages of three different pear cultivars, including a candidate housekeeping gene *PbrTUB*. Furthermore, we ranked the expression stability of 10 candidate reference genes using algorithms GeNorm, NormFinder, BestKeeper and ReFinder. Finally, the result showed that *Pbr028511*, *Pbr038418* and *Pbr041114* were the most stable reference gene in Cuiguan, Housui and Xueqing fruit, respectively.

Concludion: Thee results provide a valuable resource that serve as significant reference for gene function explorations and molecular mechanism studies in fruit development and ripening of different pear cultivars.

Background

Gene expression analysis is used to verify mRNA transcription levels of target genes and to explore novel genes function in different biological processes, such as growth, development, signaling transduction, stress response and metabolic mechanism. Compared with other detect gene expression methods, quantitative real-time PCR (qRT-PCR) has become one of the most reliable and improved tools for studying gene transcript level on account of its high accuracy, sensitivity and specificity [1]. However, the accuracy and reliability of this technology are affected by a variety of other factors, such as RNA quality, the number of replicates, amplification efficiency of primers and suitable reference genes [2–4]. The most general approaches of qRT-PCR normalization that enhance the accuracy of the assay are the application of normalization step and internal reference genes or housekeeping genes [5].

Ideal reference genes should exhibit relatively stable and consistent level of expression in various cultivars, tissues and conditions. However, no absolute universal reference genes were reported until now. In previous studies, the traditional housekeeping genes were directly selected to standardize the results of the qRT-PCR assay, such as tubulin (*TUB*), actin (*ACT*), ubiquitin (*UBQ*), elongation factor 1-α (*EF1-α*), 18S ribosomal RNA (*18S rRNA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [6–8]. Nevertheless, these housekeeping genes do not always show constant expression in variable conditions. Invalidated or unstable reference genes can lead to erroneous conclusions in certain situations [5, 9]. For example, several reports have shown *EF1-α*, *ACT* and *TUB* was not consistently stably expressed [10–12]. Housekeeping genes may exhibit varied expression level in different developmental stage in *Solanum lycopersicum* [13]. In addition, some researches pointed out that a single reference gene cannot cope with the experimental requirements [14, 15]. Therefore, it is necessary to screen and validate stable reference

genes to accurately quantify target genes in diverse experimental background and cultivars 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 [16]. Multiple internal and external factors participate in the biological processes and characteristics of pear, including pollen growth [17, 18], self-incompatibility [19, 20], seed germination [21], fruit development and senescence [22, 23]. These factors also affect the expressions of related genes. Therefore, accurate and reliable analysis of expression patterns helps to reveal the gene functions and related molecular mechanisms. However, characterization of reference genes in pear has been only reported in limited tissues and cultivars[6, 24–29]. Accordingly, identification of appropriate reference genes in pear is in demand for obtaining 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 of four development stages of Cuiguan, Housui and Xueqing pear fruit. In addition, three software packages including geNorm, NormFinder, BestKeeper, along with an online tool RefFinder were performed to evaluate the expression stability of 10 candidate reference genes in three pear varieties at different developmental stages, respectively. Our results provide reliable reference genes for qRT-PCR normalization analysis in pear fruit of Cuiguan, Housui and Xueqing. It will contribute to the expression pattern analysis of targeted genes, and further to discover the breeding molecule mechanism.

Results

Screening of stably expressed genes using transcriptome sequencing data

A total of 28,331 expressed genes were detected (RPKM > 0) at least one developmental stages in fruit tanscriptomes 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 [30]. 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 values in the four fruit developmental stages of three pear cultivars showed skewed distribution (Fig. 1A). The CV% was distributed between 3.3 and 172, including 2,142 genes with CV% < 20 (Fig. 1A), which had relatively stable expression in the developmental stages fruit of three pear cultivars. The value of CV% < 20 was the basic requirement for reference gene [25, 27]. Based on the transcript abundance and CV of gene expression, potential reference genes were selected to test, which had FPKM > 40 and CV% < 20. 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*) (Fig. 1B). The gene expression variability of the selected was analyzed in a $\log_2$RPKM box plot graph (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 using PCR. The candidate housekeeping gene *PbrTUB* was used here as a matter of comparison. The result showed that all the primer pairs had single bands and expected lengths, respectively (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, respectively (Fig. 3A). Moreover, *PbrTUB* transcript levels were the most variable with 5.83 Ct values, while *Pbr002841* showed the least variable levels with 1.54 Ct value (Fig. 3B). Meanwhile, since the gene transcript levels were negatively correlated to Ct values, *Pbr002841* had higher expression in pear fruit than other candidate reference genes.

geNorm analysis

Gene expression stability was verified by average expression stability M value in geNorm analysis, the lower the M value, the more stability it is, and vice versa [31]. M values of 10 candidate reference genes in all samples were lower than 1.5, the geNorm threshold recognized as stable (Fig. 4). In Cuiguan group, *Pbr028511* and *Pbr027964* had the most stable expressions through C1-10 developmental stages, while *PbrTUB* had the most unstable expression (Fig. 4A). In Housui group, *Pbr002841* and *Pbr027964* were the two most stable genes through H1-13 developmental stages, while and *Pbr016048* was the most unstable gene (Fig. 4B). Similarly, in Xueqing group, *Pbr041114* and *Pbr018827* genes showed more stability through X1-13 developmental stages (Fig. 4C).

NormFinder and BestKeeper analysis

NormFinder was used to determine the most suitable internal reference genes by the geNorm software. The ΔCt method was performed to directly evaluate the expression stability of candidate reference genes [32]. The smaller the value, the better the stability of the reference gene. *Pbr002841* showed the lowest stability value in Cuiguan group, which was the highest stability (Fig. 5A1). *Pbr038418* exhibited the lowest stability value in both Housui and Xueqing groups, which was the highest stability (Fig. 5A2 and A3). *PbrTUB* ranked the top in both Cuiguan and Xueqing groups, and was the most unreliable (Fig. 5A1 and A3).

The BestKeeper was used to estimate stability of candidate reference gene by standard deviation (SD) [33]. In Cuiguan, Housui and Xueqing group, the lowest SD values display was *Pbr002841* (0.49), *Pbr038418* (0.39) and *Pbr038418* (0.44), respectively (Fig. 5B1-3). The result showed that it was the most stable gene, respectively. Meanwhile, *PbrTUB*, *Pbr016048* and *PbrTUB* was the most variable reference gene with the highest SD value 0.79, 0.56 and 0.74 in Cuiguan, Housui and Xueqing group, respectively (Fig. 5B1-3). This results were consistent with the analysis of NormFinder.

RefFinder analysis

RefFinder was an online tool to comprehensively integrate the results of geNorm, NormFinder, BestKeeper and the ΔCt. It ranks basis of their geo-mean constancy. Consistent with the above three tools, the lowest ranking value exhibited the highest stability. Finally, the comprehensive ranking was displayed in Table 2. This integrated tool indicates that *Pbr028511*, *Pbr038418* and *Pbr041114* were the most stable reference gene in Cuiguan, Housui and Xueqing fruit, respectively.

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 wildly used to standardize the transcriptional accumulations of target genes such as *TUB*, *ACT*, *UBQ*, *EF1-α* and *GAPDH*. Nevertheless, the common reference genes are not consistently stably expressed and thus can't apply to all species [11, 12]. Based on previous studies, reference genes for fruit development are varying among different genera and cultivars, such as *TEF2*, *UBQ10* and *RP II* in peach [14], *EF1-α*, *CKL* and *WD40* in apple [34], *BPS1* and *ICDH1* in pear [26], *RPT6A* and *RPN5A* in strawberry [35]. 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 three pear cultivars of Cuiguan, Hosui and Xueqing.

The transcriptome sequencing analysis was a high-throughput sequencing technology, which provide unbiased test transcripts and increased test specificity [22, 36, 37]. Therefore, the transcriptome sequencing data provides a new resource for screening reference genes at the genome level. The candidate reference genes had been identified via transcriptome data in diverse plant species, such as *Oryza sativa* [38], *Fagopyrum esculentum* [39], *Brassica napus* [40], and *Euscaphis konishii* [41]. In this study, we selected 10 relatively stable candidate genes for reference genes based on transcriptome data of three pear cultivars in four different developmental stages (Table 1).

The studies on pear, which is identified as the third largest temperate fruit tree, are obtaining progresses with the release of the pear (*Pyrus bretschneideri*) genome datasets [16]. In previous studies, several traditional or novel reference genes were identified and evaluated under various biotic or abiotic stresses and at each stage of developmental in different tissues of diverse pear cultivars. Ubiquitous housekeeping genes may exhibit inconsistent stability in different conditions [24, 42, 43]. For instance,

EF1a and *TUB-b2* were the most stable in different pear cultivars, *GAPDH* and *EF1a* were the most suitable in different tissues, while *TUB-b2* and *GAPDH* were the most stable in developmental stage [24]. In addition, it has been reported that different groups of pear tissues have their own best reference genes for different experimental purposes. In pollen, the *PbrGAPDH*, *PbrPP2A* and *PbrUBI* were suitable reference genes for low temperature, NaCl treatment and CuCl2 treatment, respectively. And *PbrEF1a* was stable reference genes for all abiotic stresses [6]. In leaf, *SKD1*and *ARM* were the most apropos single reference genes for leave of the 'training_period' and 'training_space', respectively [29]. In pear peel, *ACT6/7/8/9* and *NAP1* were recommended as the optimal reference genes [27]. Reference genes in pear fruit were studied relatively more, *PbPDI.F1* presented the highest expression stability during pear fruit developmental [44], the housekeeping gene *EF1a* members showed an obviously unstable expression in pear fruits at different developmental stages [28], *SOX2* and *PP2A* showed most highly and stably expression in pear fruit [25], and *BPS1* and *ICDH1* were the high stable expressed genes [26]. In this study, we identified stably and novel reference genes in the fruits of three different pear varieties. Therefore, reliable and accurate reference genes were urgently needed to been 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 samples of 10 Cuiguan, 13 Hosui and 13 Xueqing, respectively. Although different statistical algorithms and analytical procedures may lead to divergent stability ranking, most results were consistent according to these three algorithms. Finally, an online tool RefFinder was used to comprehensively integrate all the ranking (Table 2). The result showed that *Pbr028511*, *Pbr038418* and *Pbr041114* were the most stable reference genes in Cuiguan, Housui and Xueqing fruit, respectively. In additiong, each of them possesses more stable expression than *PbrTUB* in pear fruits during all the stages of fruit development and ripening.

Conclusion

In this study, 3 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. In detail, *Pbr028511* was the most stable reference gene in Cuiguan, *Pbr038418* was the most stable reference gene in Cuiguan, and *Pbr041114* was the most stable reference gene in Xueqing fruit. In addition, each of them exhibits the more stable expression compared with *PbrTUB* in pear fruits during all the fruit developmental stages. Therefore, our study provides a series of stable and valuable reference genes that can be applied in exploring gene functions and molecular mechanisms during pear fruit development and ripening.

Materials And Methods

Plant materials and experimental treatments

All 36 different samples of three pear cultivars were collected from the pear germplasm orchard of 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 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 stage) were used for transcriptome sequencing, respectively. The flesh were ground into powder, than frozen in liquid nitrogen and stored at − 80°C until used.

Candidate reference gene selection and primer designing

Based on fruit transcriptome sequencing data of different pear cultivars from our laboratory, 10 candidate reference genes were applied to normalize and validate qRT-PCR experiments according to relatively stable expression of their RPKM and fold change values, including nine novel genes and one housekeeping gene (Table 1). The primers of 10 candidate reference genes were designed using Primer Premier 5.0 software with the following parameters: annealing temperature (Tm) of 60–66℃, GC percent of 45–60%, primer lengths of 18–28 bp and product length of 150–210 bp. All the primers were synthesized by Sangon (Nanjin, China) and the primer sequences were listed in Table 1. To assay the expression specificity and efficiency of all primers, PCR was performed and productions were analysis 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 instruction method. Nanodrop ND1000 spectrophotometer (Thermo Scientific) was used to determine total RNA concentration and purity. And RNA samples were assessed with OD 260/280 > 2.0 and OD 260/230 > 1.8. For each sample, 500 ng of total RNA extracted was reverse transcribed with TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Beijing, China). RNA extraction and cDNA synthesis from all samples was performed with three biological replicates. Then, a reference gene was used to verify all cDNA samples' quality by PCR before carrying out qRT-PCR. The result presented that all the cDNA templates no genomic DNA contamination in by 2.0% agarose gel electrophoresis.

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 20 s at 72°C. After that, the specificity of the primer amplicons was checked by the analysis of a melting curve. All samples were performed with three independent biological replicates, each with three technical replicates. Lin-RegPCR was used to estimate the amplification efficiency of the 10 pairs of primers in qRT-PCR [45]. 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, namely, geNorm analysis [31], NormFinder analysis [32],

BestKeeper analysis [33] and comparative Ct methods [46]. Finally, to select most stable reference genes from different algorithms, we compared the tested candidates based on a web-based comprehensive tool *RefFinder* (http://www.leonxie.com/referencegene.php) [47].

The geNorm analysis enables the selection of the optimal set of genes by a gene-stability measure (*M*). The most stable two genes with the lowest M values were ranked on the right, on the contrary the least stable with highest *M* value on the left. The *M* value of not more than 1.5 for reference gene is the default limit point [31].

The Normfinder software, Similar to geNorm, is another Visual Basic Application (VBA) for identifying and ranking the optimal normalization genes among the candidates [32]. NormFinder provides a stability value for each gene according to intergroup and intragroup expression variation, and for the estimated expression variation enabling evaluated the systematic error introduced when using the gene for normalization.

The BestKeeper, an anther Excel-based tool, determines the most stably genes according to the coefficient of correlation to the candidate reference gene's Ct values. Genes with the lowest standard deviation (SD) values are the most stable [33].

The *RefFinder* is the web-based comprehensive online program that calculates the geometric mean of their weights for the overall final ranking according to rankings from each program.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests.

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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. QKJ and GHR provided experimental materials. All authors have read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 and 2 are only available as a download in the Supplemental Files section.

Figures

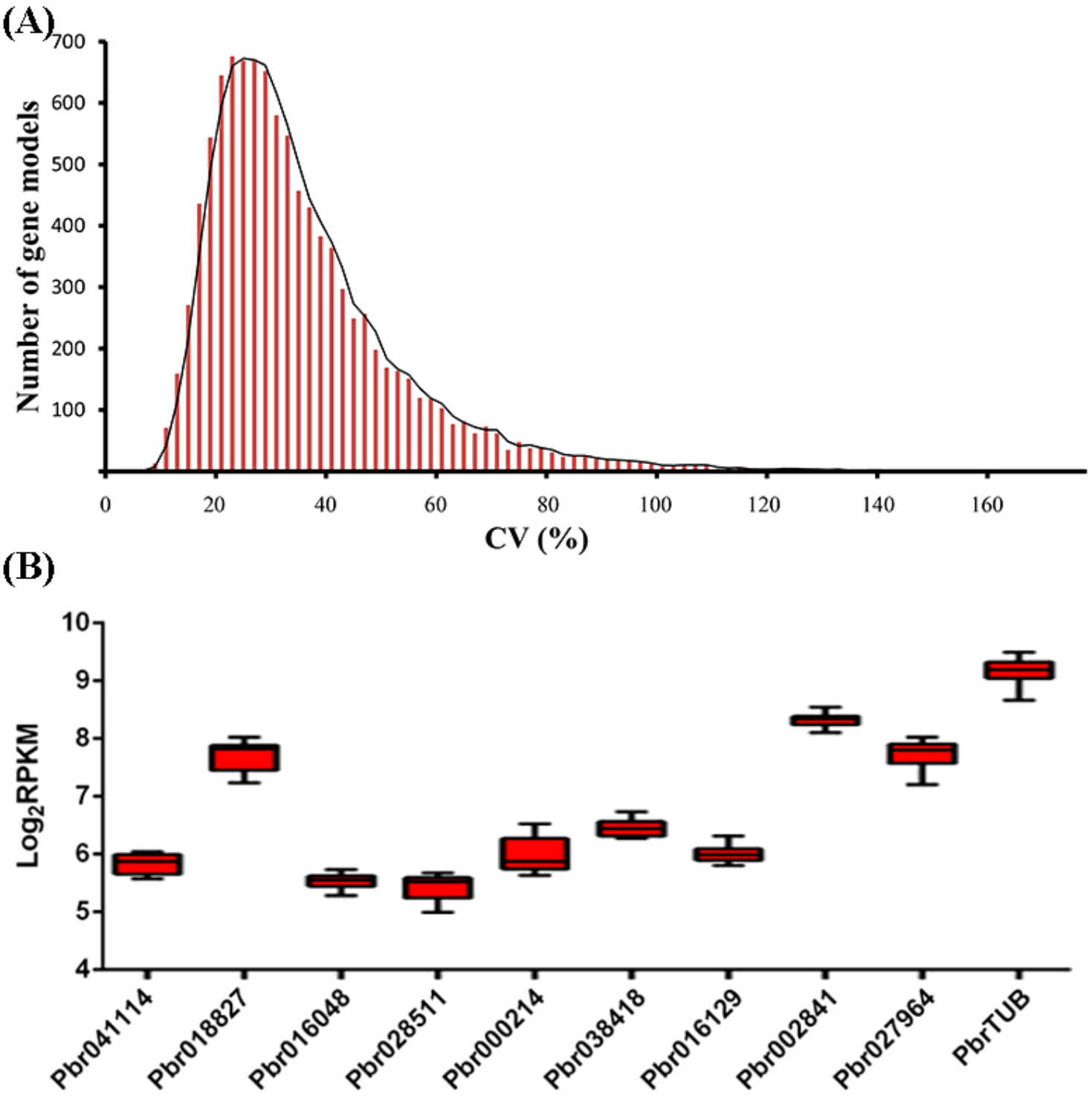

Figure 1

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 log2RPKM values of each candidate gene in all the samples analyzed. The line across the box and boxe represent the medians and 25/75 percentiles, respectively. Whisker caps represent the minimum and maximum values.

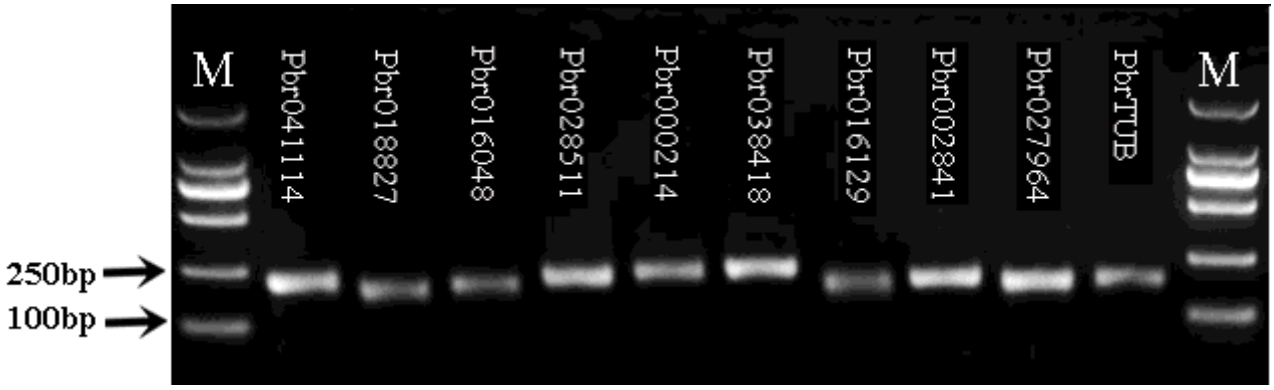

Figure 2

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).

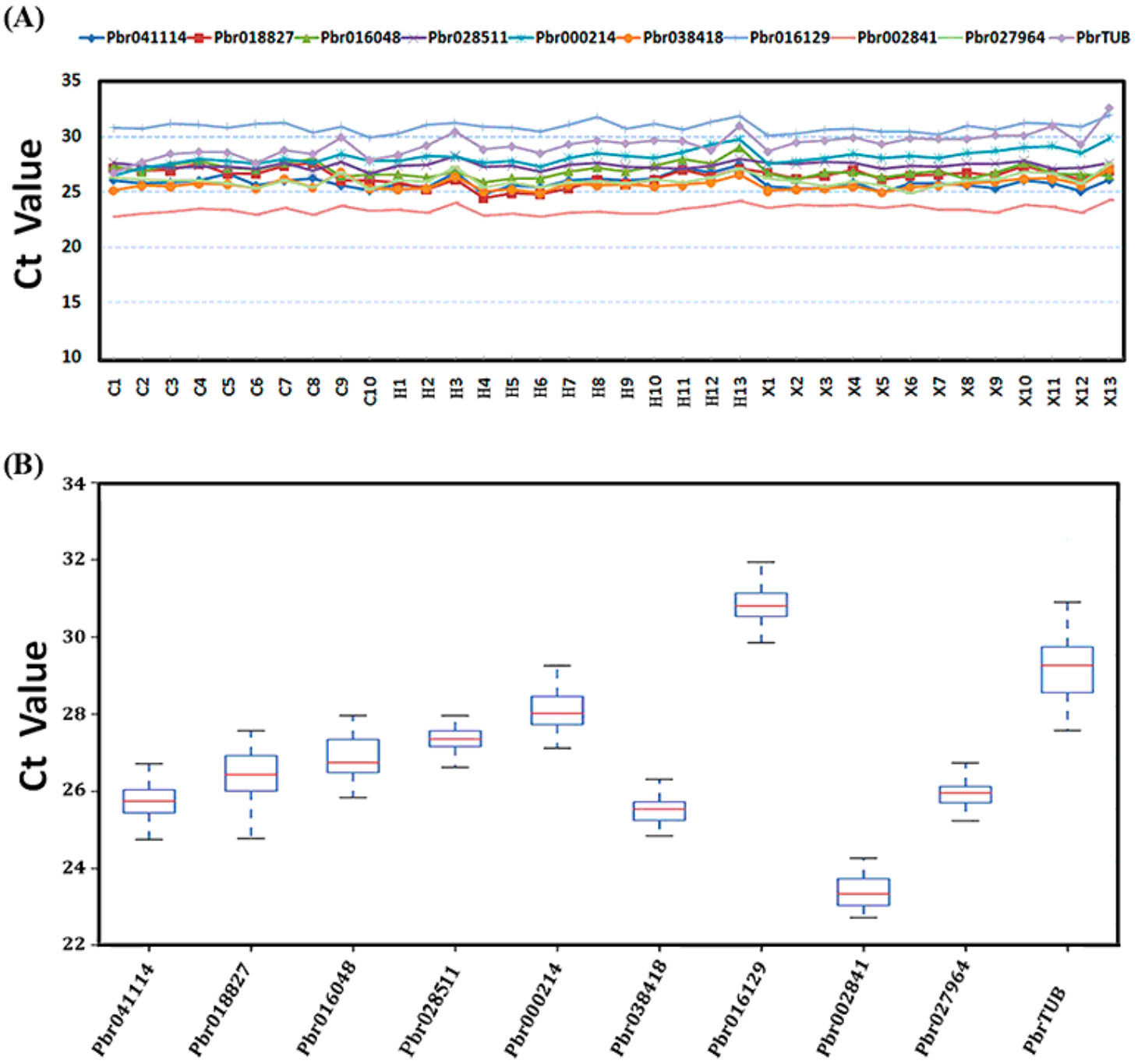

Figure 3

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 indicates the 25/75th percentiles. Whisker caps represent the minimum and maximum values.

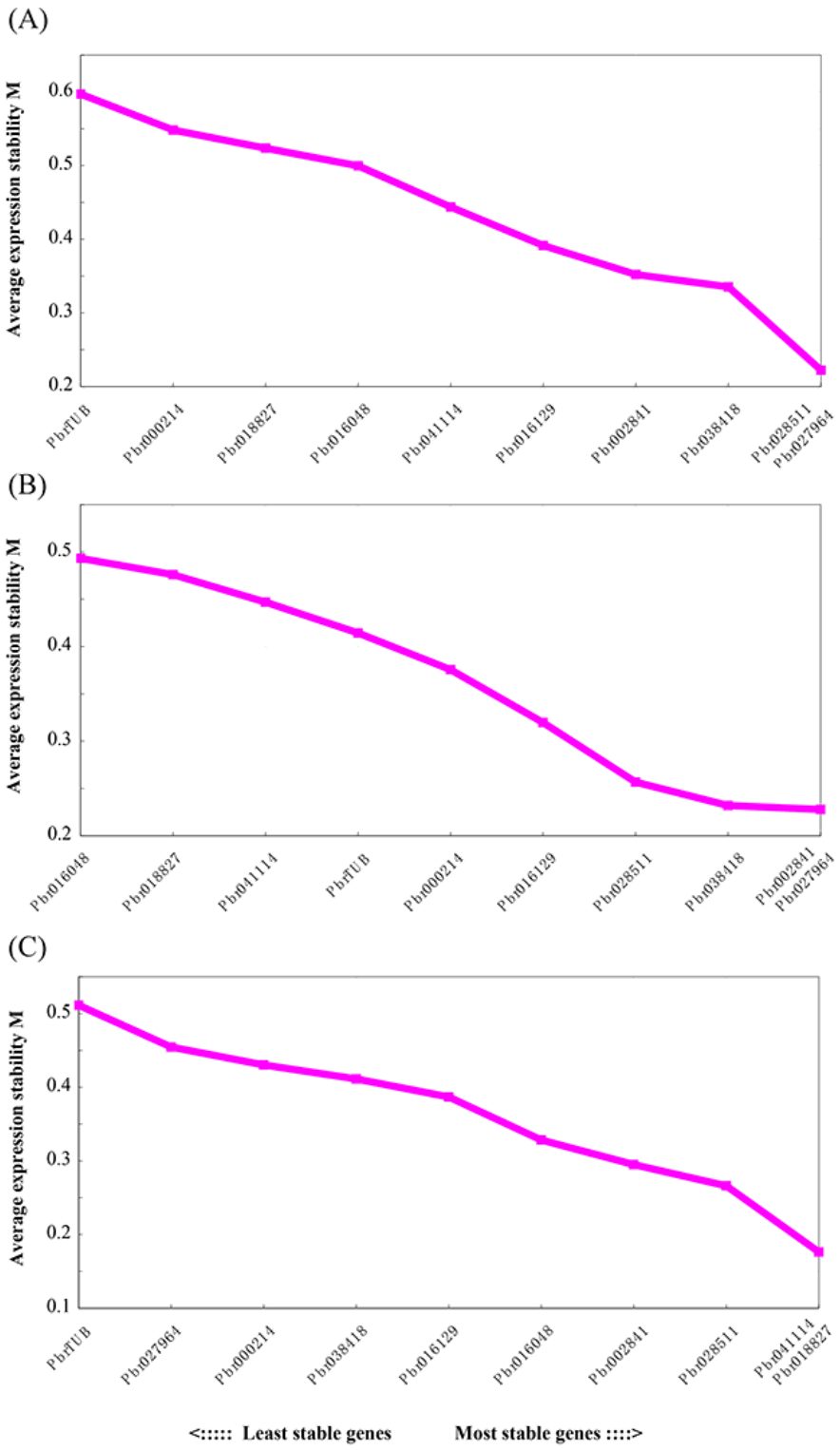

Figure 4

Expression stability values (M) of 10 genes in 3 sample groups indicated in the each figure by geNorm software. (A) the fruit of Cuiguan (C1-10). (B) the fruit of Housui (H1-13). (C) the fruit of Xueqing (X1-13).

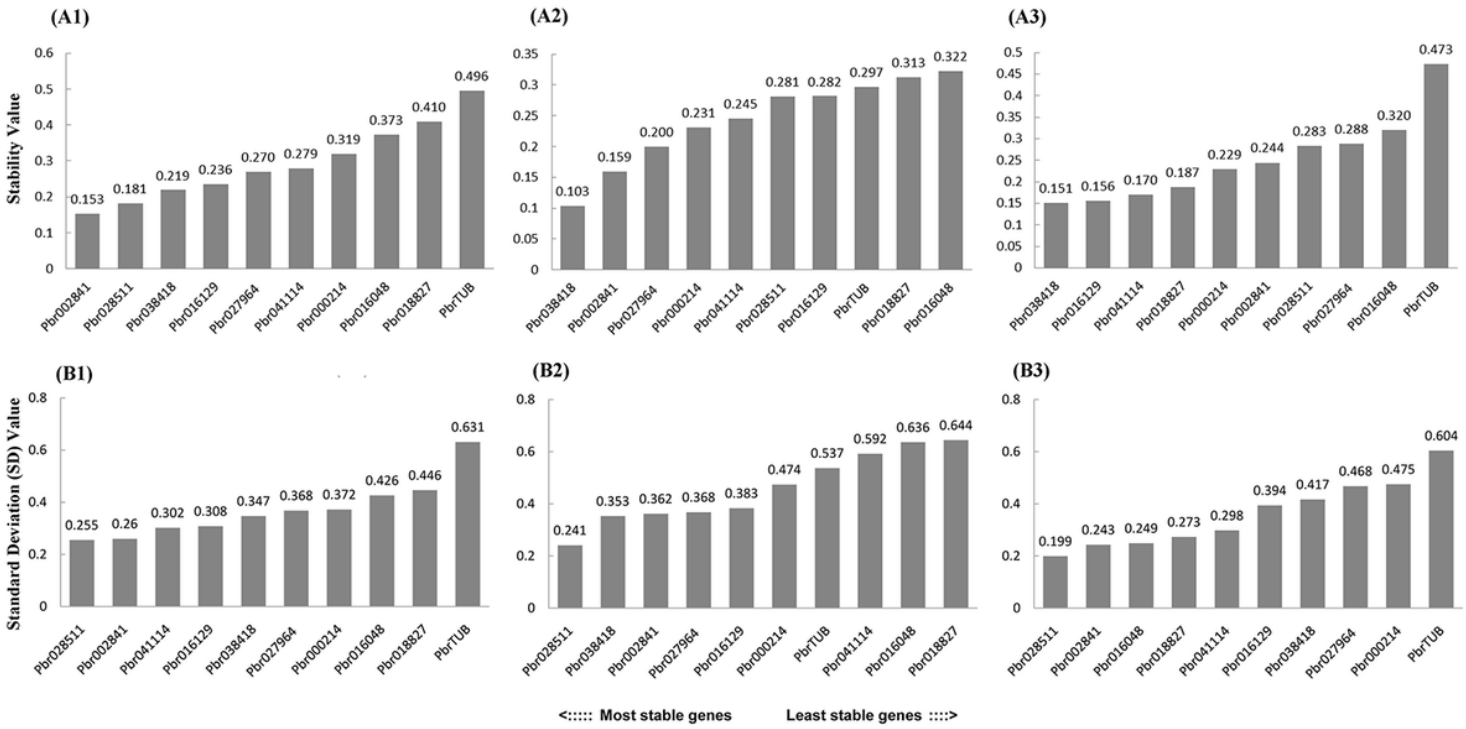

Figure 5

Expression stability analysis of ten reference genes in 4 sample groups by NormFinder (A1-3) and BestKeeper (B1-3). (A1, B1) the fruit of Cuiguan (C1-10); (A2, B2); the fruit of Housui (H1-13); (A3, B3) the fruit of Xueqing (X1-13). A lower average expression stability value indicates more stable expression.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.xlsx
- Table2.xlsx