Validation of reference genes for expression analysis in three Bupleurum species

Ma Yu\textsuperscript{a,b}, Dan Liu\textsuperscript{b}, Yu-Chan Li\textsuperscript{b}, Chun Sui\textsuperscript{b}, Guang-Deng Chen\textsuperscript{c}, Zhi-Kang Tang\textsuperscript{b}, Cheng-Min Yang\textsuperscript{a}, Da-Bin Hou\textsuperscript{b} and Jian-He Wei\textsuperscript{a}

\textsuperscript{a}Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, PR China; \textsuperscript{b}School of Life Science and Engineering, Southwest University of Science and Technology, Mianyang, PR China; \textsuperscript{c}College of Resources, Sichuan Agriculture University, Chengdu, PR China

\textbf{ABSTRACT}
Radix Bupleuri (root of \textit{Bupleurum} spp.) is an important medicinal herb. Its lateral root number is one of the decisive factors that influence the content of a major bioactive component, saikosaponin. To identify genes associated with content and total yield of saikosaponin, it is of key importance to select stable references in gene expression analyses using quantitative real-time polymerase chain reaction (qRT-PCR). In this study, 18 candidate reference genes were selected and evaluated through their expression stability during the lateral root development in tissue samples from \textit{B. chinense} DC., \textit{B. falcatum} L. and \textit{B. scorzonerifolium} Willd. The GeNorm, NormFinder and Bestkeeper methods were used for selecting stably expressed internal controls in the three \textit{Bupleurum} species. These results revealed that, among these 18 candidate reference genes, ADF7 showed the best performance in all the experimental systems. ADF3 and ADF1b could also be proposed as suitable reference genes for gene expression studies. This study supplied more candidate reference genes to monitor the content and yield of saikosaponin during lateral roots growth in the \textit{Bupleurum} genus.

\textbf{Introduction}
In biological research, real time quantitative reverse transcription-PCR (qRT-PCR) is increasingly being used in gene expression analysis due to its technical ease, low reagent cost, less hands-on time, reproducibility and high throughput \cite{1}. However, multiple factors such as sample amount, RNA recovery, RNA integrity, cDNA quality and tissue or cell activities can affect the quantitative measurement of gene expression \cite{2}. To achieve accurate and stable results, normalization is required to correct for these variations. For normalization, one or several reference genes should serve as internal controls to normalize and monitor the expression variation between samples and reactions.

Theoretically, an ideal reference gene is stably expressed in various samples across different experimental conditions or treatments. However, no gene is universally stable among different plant species and differing experimental conditions. Hundreds of reference genes have been validated in plants including \textit{Oryza sativa}, \textit{Arabidopsis thaliana}, \textit{Zea mays}, \textit{Brassica juncea}, \textit{Brassica napus}, \textit{Solanum tuberosum}, \textit{Solanum lycopersicum} L., \textit{Setaria italic} L., \textit{Brachypodium} beav, \textit{Hordeum vulgare}, \textit{Sorghum bicolor}, \textit{Triticum} aestivum, \textit{Glycine} max, \textit{Vitis} vinifera, \textit{Cucumis} sativus, \textit{Nicotiana tabacum}, \textit{Phyllostachys} edulis and \textit{Bupleurum} chinense DC. \cite{3–5}. These genes have been validated across different tissues and different treatments by comparative delta Ct method, Bestkeeper, NormFinder and GeNorm \cite{2}.

Radix Bupleuri (root of \textit{Bupleurum} spp.) is one of the most important medicinal herbs in Eurasia and North Africa as treatment for fever, chronic hepatitis, nephrotic syndrome, inflammatory diseases, menstrual disorders and digestive ulcers \cite{6–9}. Among the 190 species of the \textit{Bupleurum} genus, only \textit{B. chinense} DC. and \textit{B. scorzonerifolium} Willd. are officially recorded as the source species of Radix Bupleuri in the Chinese Pharmacopoeia \cite{10–12}. In the Japanese Pharmacopoeia, the official botanical origin of Radix
Bupleuri is the root of *B. falcatum* L. [13]. It is believed that saikosaponins are responsible for the pharmaceutical properties of Bupleuri Radix, especially the oleane-saponins, saikosaponin a and saikosaponin d [14]. According to previous studies, the content and total yield of saikosaponin depend on the type of tissue, the growth period, the root structure and the environmental conditions such as drought, fertilizer treatment and light deficiency [15–17]. A previous study characterized 11 candidate genes for their suitability as reference genes in *B. chinense* DC.; however, it did not evaluate these genes in relation to saikosaponin content and yield [5].

In this study, 18 genes, including *Ubiquitin-protein ligase gene* (*UBC*), *Actin depolymerizing factor* (*ADF*), *Actin* (*ACT*), *Eukaryotic translation initiation factor* (*eIF*) and *Eukaryotic translational elongation factor* (*EF*), were selected as candidate reference genes for evaluation based on the analyses by three software programs (Bestkeeper, NormFinder and GeNorm) in *B. chinense* DC, *B. scorzonerifolium* Willd. and *B. falcatum* L. This research analyzed eight samples and aimed to select the well-founded gene which could potentially be used as a candidate reference gene in *Bupleurum* genus experiments in different tissues with various treatments.

**Materials and methods**

**Plant materials**

The three experimental materials, Zhongchai No. 2, Zhonghongchai No. 1 and B1, were from three species, *B. chinense* DC, *B. scorzonerifolium* Willd. and *B. falcatum* L., respectively. All of them were bred by systemic selection and purification selection from farm-holding populations. Zhongchai No. 2 and Zhonghongchai No. 1 were provided by Professor Jianhe Wei from the Institute of Medicinal Plant Development (IMPLAD), Chinese Academy of Medical Sciences & Peking Union Medical College. B1 was provided by Professor Da-Bin Hou from the School of Life Science and Engineering, Southwest University of Science and Technology.

For each genotype, 10 plants with similar growth vigour were selected to harvest as whole plants before lateral root germination. Tissue samples of the leaves and roots were taken after the first lateral root germination at the seedling stage. During the fruiting period, five plants of similar height and structure were selected for harvesting of their roots, stems, leaves, blossoms and fruit. Another replication of each tissue sample was collected from the same experimental plot at both the seedling and fruiting stage. All tissue samples were wrapped in tinfoil, immediately flash-frozen in liquid nitrogen and then kept at −80°C until RNA isolation.

**RNA isolation and cDNA synthesis**

RNAprep Pure Plant Kit (DP441) (TIANGEN BIOTECH (BEIJING) CO., Beijing, China) was used in the RNA isolation and genomic DNA elimination of 16 tissue samples following the manufacturer's instructions. The integrity of the RNA samples was checked by agarose gel electrophoresis, and the concentration and quality were examined by NanoDrop 2000 (Thermo, USA) at 230, 260 and 280 nm. Synthesis of cDNA was performed using the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Canada) following the manufacturer’s protocol [2].

**qRT-PCR**

A total of 18 candidate reference genes were selected for qRT-PCR (Table 1). Real-time PCR was carried out using the Trans-AQ111-02 Green qPCR SuperMix UDG (TransGen Biotech, Beijing, China) and the ABI CFX96 Touch™ Real Time PCR System (Applied Biosystems, Foster City, CA, USA). A reaction mixture of a total volume of 10 lL in each well in an optical 96-well plate was employed for qRT-PCR. This reaction mixture contained 5 lL of Trans-AQ111-02 Green qPCR SuperMix, 5 pmol/L of each primer, 5 ng of final cDNA and 3.4 lL of RNase-free water. The PCR procedures were described previously [5]. The ABI CFX Manager Software V3.1 was used for visualizing and analyzing the data, including the quantification cycle values, PCR efficiency and correlation coefficients.

**Data analysis**

Data are presented as mean values with standard deviation (±SD). After collecting and converting the quantification cycle (Cq) data, Cq average values were calculated statistically by SPSS 16.0 software (http://www.spss.com/). To obtain reliable results, the software programs Bestkeeper, NormFinder and GeNorm were used to analyse the expression stability of reference genes (RefFinder, http://150.216.56.64/reference-gene.php). Pearson correlation coefficients were generated for ranking results from four different algorithms using Minitab 15 software (http://www.minitab.com/).
Table 1. Oligonucleotide primers for amplification of 18 candidate reference genes.

| Primer | Sequencea | Primer | Sequence |
|--------|-----------|--------|----------|
| UBC6 (F) | ACCACCTTTTTTACCTTTTGCTAT | elf2a (F) | CAGACTGAGTTGGTGCTCGTCTGTA |
| UBC6 (R) | CACCAACTTTTTTCATATCCGATG | elf2a (R) | TGTGACTGTTGCTCTTATTCCT |
| UBC13 (F) | CGATGGTGGACATTAGTGGTGGGTTTA | elf2b | TAAATGGGCAGCAAATAGCCTG |
| UBC13 (R) | GCCTTCTCTCTGTTGGTTG | elf2b | TGGCCGACCAAATGATTGGAG |
| ACT7 (F) | TCTCTGTGCTTCTATTTCTTCTTTC | elf3 | ACTAGAGGGAGCTGAAAAGGTT |
| ACT7 (R) | TCTACACATGATTCCTGGTTG | elf3 | GTTTACCGAGGATTTGTTG |
| ADF1a (F) | TGAAGCAACTGATGACCAACA | UBC4 | ACCAGCAGCTCCCTTATTCAC |
| ADF1a (R) | TACAGAATACAAAAAGCAGCAGA | UBC4 (R) | CCCATATCGACAGGACCACCT |
| ADF1b (F) | CGGCATCTTGATGGCTG | elf4 | AGAACCTATTGAGACGATCC |
| ADF1b (R) | AGGAGAGGATTACATTAGGCT | elf4 | GAAATGAAATCTACACTAACCC |
| AD7 (F) | TGGGCGTAGGGCTAGCTG | AD5 | CGAGTCCCGCTGATACGTC |
| AD7 (R) | GACACATACATAAGCTGGCCCAT | AD5 | GACAGCTCAGCTGCTG |
| ACT1 (F) | GTAAGGACTGCTTCCACCCC | ACT2 | TGGCCGAGGTGCAAGATAT |
| ACT1 (R) | TGATGTTAATTAGATGCTGGTAAA | ACT2 | GACAGCTCAGCTCAGAT |
| ACT4 (F) | CCAGATCAAGCCACACTACACAG | elf6 | GTCAGGCGCAAGGCTTTT |
| ACT4 (R) | ACCAAAAAGCACAACAGAGAAA | elf6 | CTCAAAACGGGTCTCATA |
| EF1b (F) | TTCCTCTGTGCACAGCCTTTAAT | EF1a | CAACATGATTGAGAGTTCTAGCA |
| EF1b (R) | ACTGTTGATCTCAGGATGTTG | EF1a | GAAGAGGGAGCAAGGAGGTT |

aAll sequences are given in the 5'→3' direction.

Results and discussion

Expression profile of candidate reference genes

QRT-PCR has become a standard method for detection and quantification of RNA targets, because of its sensitivity, specificity and accuracy [18]. However, due to the potential systematic variation introduced by total RNA, first-strand cDNA synthesis and qRT-PCR assay, there is a need to normalize the raw expression data by expressing internal controls for accurate and reliable results [19,20]. Previous studies have shown that the expression of such controls could significantly change the stability in the tested plant tissues under differing experimental conditions. Therefore, no single control is appropriate for all experimental treatments [21,22]. In addition, with a variable reference gene, there could occur nearly 100-fold variations in the quantified expression of the target gene. This could eventually result in misinterpretation of the expression pattern and faulty understanding of the mechanisms under study [23]. Therefore, it is generally suggested to select suitable internal controls prior to use for normalization of specific experimental conditions.

In Bupleurum genus, saikosaponins are the most important bioactive components due to their pharmacological properties [11]. Previous studies have reported the biosynthetic pathways of saikosaponins in B. falcatum L. B. kaoi, B. chinense DC. and B. scorzonerifolium Willd. [24–28]. Genes involved in the biosynthesis of saikosaponins such as squalene epoxidase, β-amylase, cytochrome P450 and uridine diphosphate glycosyltransferases were cloned and identified by their expression profiles in B. kaoi, B. falcatum L. and B. chinense DC [25,29–31]. However, only a few of these genes were associated with the content and total yield of saikosaponins, and none of them have been utilized in the metabolic pathway in saikosaponin production. Previous histochemical studies on B. chinense DC., B. falcatum L. and B. scorzonerifolium Willd. have demonstrated that saikosaponins are mainly found in the epidermal areas of the roots [15,32–34]. This was confirmed as plants with more lateral roots showed higher saikosaponin content than plants with less lateral roots. So the lateral root number is one of the decisive factors that influences the saikosaponin content.

To examine the genetic mechanism of lateral root development in B. chinense DC., B. falcatum L. and B. scorzonerifolium Willd., a total of 18 candidate reference genes were selected for determining the most stable one at various developmental stages and tissues. Amplification of each reference gene in 24 samples (2 replicates per sample) produced 48 Cq values, and samples with missing Cq values or inconsistencies between replicates (Cq differences >0.5 cycle) were removed from the analysis. Based on the standard curves using a serial dilution of cDNA samples, the efficiency of gene amplification ranged from 90.95% to 100.90%. The observed correlation coefficient $R^2$ values for most of the genes varied in the range of 0.989–1.000.

Over all, the Cq values of the 18 candidate reference genes varied over a wide range, and the mean Cq values of these genes varied in the samples from 16.82 to 30.17 (Table 2). Among these candidate reference genes, UBC13 was the most abundantly expressed gene (mean Cq ± SD = 16.82 ± 1.27) followed by AD7b (mean Cq ± SD = 16.84 ± 0.91), whereas elf6 was the least abundantly expressed gene (mean Cq ± SD = 30.17 ± 6.60). All candidate reference genes showed small standard deviations (SD) from 0.63 to 1.80, with the exception of elf6, which presented the...
largest variation among the Cq values (6.60). An individual value plot was used to evaluate and to compare all the samples. The results showed that all the genes had a similar distribution or trend except for eIF6 (Figure 1). eIF6 is an essential component of ribosome biogenesis, so its gene is ubiquitously expressed. This gene is involved both in ribosome biogenesis and protein synthesis, which might induce great expression changes in different organs and species. Similar results have been reported in Arabidopsis and Oryza sativa [35].

### Expression stability of the eighteen candidate reference genes

In order to analyse the expression of the candidate reference genes in greater detail, the 24 samples were divided into four experiment sets. Set 1 to 3 consisted of 8 samples from B. chinense DC., B. falcatum L. and B. scorzonerifolium Willd. individually. In set 4, all 3 sample sets were included. Three software packages, Bestkeeper, NormFinder and GeNorm, which use different algorithms were used to analyse and evaluate the stability of the candidate reference genes in the four experiment sets.

#### GeNorm analysis

Based on the expression stability analysis of the 18 candidate reference genes by GeNorm (Table 3), the five top ranked genes in B. chinense DC. were ADF1b = ADF5 > ADF7 > eIF2b > ACT2. The top ranked genes in B. falcatum L. were ACT2 > UBC4 > ADF5 > ADF1b > ADF7, and those in B. scorzonerifolium Willd. were ADF5 > ACT2 > ADF7 > eIF2b > eIF2a. The top five genes that were consistently expressed in set 4 were ADF7 = ADF1b > ADF5 > ACT2 > UBC4. In all four sets, ADF5, ADF7 and ACT2 were always determined as three of the top five most stable reference genes.

#### NormFinder analysis

NormFinder analysis revealed that ADF7 and ADF1b were always two of the top five most stable reference genes in all four sets (Table 4). Gene ACT7 had the best stability of the 18 candidate reference genes in set 3, and occupied the top 6th and 3rd place in the 1st and 4th sets, respectively. However, this gene ranked 11th in set 2. ADF5 was one of the top five most stable reference genes in set 1, 2 and 4 but ranked 6th in set 3. ACT2 was rated as the 5th and 4th most stable reference gene in set 1 and 3, respectively.

#### BestKeeper analysis

Average Cq values were used to calculate the coefficient of variance (CV) and SD for each of the reference genes in BestKeeper analysis. Genes with higher variation were classified as less stable, whereas genes with lower variation were more stable. Based on this analysis, ADF7, UBC13, ADF1b and ADF5 always ranked as four of the top five most stably expressed genes across all the datasets (Table 5).

#### Comprehensive analysis of expression stability

For a comprehensive judgment of the suitable reference genes in the four sets, Pearson correlations were calculated using the ranks from the most stable to the least stable among the three methods (comparative GeNorm, NormFinder and Bestkeeper) used in this study. The Pearson correlations for the three stability tests showed a significant or extremely significant positive correlation in all of the four sets (Figure 2). This indicated that the ranking results from all of the three methods were nearly identical.

In previous studies, GAPDH, ACT, EF, eIF, UBC and 18S rRNA have already been used as reference genes.

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### Table 2. Expression levels of 18 reference genes in B. chinense DC., B. falcatum L. and B. scorzonerifolium Willd.

| Set  | Mean     | SD       | CV (%)   | SD     | CV (%)   | SD     | CV (%)   | SD     | CV (%)   | SD     | CV (%)   | SD     | CV (%)   |
|------|----------|----------|----------|--------|----------|--------|----------|--------|----------|--------|----------|--------|----------|
| 1st  | 16.97    | 1.11     | 4.94     | 0.88   | 4.04     | 0.79   | 4.04     | 0.79   | 4.04     | 0.79   | 4.04     | 0.79   | 4.04     |
| 2nd  | 17.78    | 1.16     | 6.53     | 1.13   | 5.05     | 1.36   | 5.05     | 1.36   | 5.05     | 1.36   | 5.05     | 1.36   | 5.05     |
| 3rd  | 17.86    | 0.88     | 4.94     | 0.79   | 5.20     | 1.18   | 5.20     | 1.18   | 5.20     | 1.18   | 5.20     | 1.18   | 5.20     |
| 4th  | 17.54    | 1.13     | 4.94     | 0.79   | 5.20     | 1.18   | 5.20     | 1.18   | 5.20     | 1.18   | 5.20     | 1.18   | 5.20     |

*The first set to third set consisted of 8 samples of B. chinense DC., B. falcatum L. and B. scorzonerifolium Willd. individually. In the fourth set, all three sample sets were included. SD: standard deviation; CV (%): variance coefficient expressed as percentage.*

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for expression studies in many plant species [2,36,37]. Different reference genes have been used in *Bupleurum* species. Actin has been used as the internal control in *B. kaoi* [26]; the genes for β-tubulin and actin, as reference genes in *B. chinense* DC; and β-actin, as the reference gene in *B. falcatum* L. [5,24,31]. In this study, we observed similar rankings of stability among three *Bupleurum* species. In GeNorm analysis, ADF5, ADF7 and ACT2 were three of the top five most stable reference genes in all three species. In NormFinder analysis, ADF7, ADF1b and eIF2b were three of the top five most stable reference genes in all three species. In BestKeeper analysis, ADF7, UBC13, ADF1b and ADF5 were ranked as four of the top five most stably expressed genes in all three species. The differences in these stability rankings could be attributed to the fact that the computational programs use different approaches and algorithms, rather than to species differences. Taking into account the results from all the three software analysis methods (Tables 3–5), the gene ADF7 was considered as one of the most suitable reference genes for normalization of all the samples of the three species of *Bupleurum* L. It is also worthy to note that all the three programs showed similar stability rankings on the least stable genes such as eIF6 in *B. falcatum* L. and ACT4 in *B. scorzonerifolium* Willd.

**Table 3. Expression stability of 18 candidate reference genes as calculated by GeNorm.**

| Rank | First set | Second set | Third set | Fourth set |
|------|-----------|------------|-----------|------------|
|      | Gene      | Gene       | Gene      | Gene       |
| 1    | ADF1b     | ACT2       | ACT2      | ACT2       |
| 2    | ACT5      | UBC4       | ACT2      | ACT2       |
| 3    | ADF7      | ADF5       | UBC4      | UBC4       |
| 4    | eIF2b     | UBC6       | ACT6      | ACT6       |
| 5    | ACT2      | ACT2       | ACT2      | ACT2       |
| 6    | eIF2a     | UBC13      | UBC13     | UBC13      |
| 7    | ADF7      | ADF7       | ADF7      | ADF7       |
| 8    | eIF2     | ACT1       | ACT1      | ACT1       |
| 9    | eIF3      | ACT7       | ACT7      | ACT7       |
| 10   | EF1a      | UBC4       | ACT4      | ACT4       |
| 11   | ACT1      | ACT1       | ACT1      | ACT1       |
| 12   | ACT6      | ADF1a      | ADF1a     | ADF1a      |
| 13   | eIF4      | ACT4       | ACT4      | ACT4       |
| 14   | ADF1a     | UBC6       | UBC6      | UBC6       |
| 15   | UBC6      | ADF1a      | ADF1a     | ADF1a      |
| 16   | UBC4      | UBC4       | UBC4      | UBC4       |
| 17   | ADF1a     | UBC4       | UBC4      | UBC4       |
| 18   | UBC6      | UBC6       | UBC6      | UBC6       |

The expression stability of the first set to the fourth set refer to Table 2.

**Table 4. Expression stability of 18 candidate reference genes as calculated by NormFinder.**

| Rank | First set | Second set | Third set | Fourth set |
|------|-----------|------------|-----------|------------|
| 1    | ADF7     | ADF1b     | ACT7      | ACT7       |
| 2    | ADF1b    | ADF7      | UBC4      | UBC4       |
| 3    | eIF2b    | ADF5      | ACT1      | ACT1       |
| 4    | ADF5     | ADF1a     | ACT1      | ACT1       |
| 5    | ACT2     | UBC13     | UBC13     | UBC13      |
| 6    | ACT7     | ADF1a     | ADF1a     | ADF1a      |
| 7    | eIF3     | ACT4      | ACT4      | ACT4       |
| 8    | eIF2a    | ACT4      | ACT4      | ACT4       |
| 9    | eIF4     | ACT4      | ACT4      | ACT4       |
| 10   | ACT1     | ADF1a     | ADF1a     | ADF1a      |
| 11   | EF1a     | UBC4      | UBC4      | UBC4       |
| 12   | UBC6     | ADF1a     | ADF1a     | ADF1a      |
| 13   | UBC4     | UBC4      | UBC4      | UBC4       |
| 14   | UBC6     | ADF1a     | ADF1a     | ADF1a      |
| 15   | UBC4     | UBC4      | UBC4      | UBC4       |
| 16   | ADF1a    | UBC4      | UBC4      | UBC4       |
| 17   | UBC6     | ADF1a     | ADF1a     | ADF1a      |
| 18   | EF1b     | ADF1a     | ADF1a     | ADF1a      |

*The expression stability of the first set to the fourth set refer to Table 2.*
Table 5. Expression stability of 18 candidate reference genes as calculated by BestKeeper.

| Rank | Gene   | SD    | CV    | Gene   | SD    | CV    | Gene   | SD    | CV    | Gene   | SD    | CV    |
|------|--------|-------|-------|--------|-------|-------|--------|-------|-------|--------|-------|-------|
| 1    | ADF7   | 0.581 | 3.18  | ADF7   | 0.531 | 2.16  | ADF1b  | 0.489 | 3.48  | ADF5   | 0.479 | 3.72  |
| 2    | ADF1b  | 0.612 | 3.38  | ADF5   | 0.589 | 2.94  | ADF5   | 0.502 | 3.99  | ADF1b  | 0.512 | 3.71  |
| 3    | ADF5   | 0.691 | 4.2   | UBC13  | 0.618 | 4.33  | UBC13  | 0.511 | 4.03  | ADF7   | 0.531 | 2.31  |
| 4    | eIF2b  | 0.712 | 3.56  | ADF1b  | 0.616 | 4.04  | eIF2b  | 0.654 | 4.43  | UBC13  | 0.554 | 4.32  |
| 5    | UBC13  | 0.742 | 4.34  | ACT7   | 0.648 | 4.62  | ADF7   | 0.746 | 4.89  | ACT2   | 0.572 | 4.19  |
| 6    | ACT7   | 0.732 | 4.07  | eIF3   | 0.725 | 4.26  | ACT7   | 0.758 | 4.58  | ACT7   | 0.602 | 4.27  |
| 7    | eIF3   | 0.817 | 3.18  | ACT2   | 0.774 | 3.53  | eIF2a  | 0.784 | 4.68  | eIF3   | 0.616 | 4.27  |
| 8    | ACT2   | 0.816 | 5.45  | eIF2b  | 0.786 | 4.18  | UBC4   | 0.793 | 4.48  | eIF2a  | 0.625 | 5.43  |
| 9    | eIF1a  | 0.841 | 4.27  | EF1a   | 0.868 | 5.32  | eIF3   | 0.836 | 4.93  | eIF3   | 0.672 | 5.31  |
| 10   | eIF2a  | 0.853 | 4.71  | UBC4   | 0.937 | 3.72  | ACT1   | 0.862 | 4.97  | EF1a   | 0.737 | 5.16  |
| 11   | UBC4   | 0.892 | 3.98  | ADF1a  | 0.961 | 4.02  | ACT2   | 0.925 | 4.51  | eIF4   | 0.854 | 4.29  |
| 12   | ACT1   | 0.895 | 4.83  | eIF4   | 1.019 | 4.52  | ADF1a  | 0.965 | 5.09  | ACT1   | 0.922 | 4.45  |
| 13   | ADF1a  | 0.928 | 5.16  | ACT1   | 1.071 | 4.37  | eIF4   | 1.037 | 5.48  | UBC4   | 1.232 | 4.71  |
| 14   | eIF4   | 1.023 | 4.75  | ACT4   | 1.231 | 5.12  | eIF6   | 1.069 | 5.65  | ADF1a  | 1.321 | 5.29  |
| 15   | ACT4   | 1.041 | 5.04  | eIF2a  | 1.341 | 5.69  | UBC6   | 1.358 | 5.46  | ACT4   | 1.443 | 5.94  |
| 16   | UBC6   | 1.042 | 4.77  | EF1b   | 1.332 | 5.02  | EF1a   | 1.465 | 5.71  | UBC6   | 1.501 | 5.31  |
| 17   | eIF6   | 1.064 | 4.93  | UBC6   | 2.095 | 6.78  | EF1b   | 1.456 | 5.56  | eIF6   | 1.556 | 6.76  |
| 18   | EF1b   | 1.711 | 6.52  | eIF6   | 2.335 | 6.13  | ACT4   | 1.541 | 5.39  | EF1b   | 1.741 | 6.28  |

The expression stability of the first set to the fourth set refer to Table 2.

Figure 2. Comparison of the ranking results from BestKeeper, NormFinder and GeNorm. The correlation was evaluated for the ranking results of 18 candidate reference genes in all samples, by Bestkeeper, NormFinder and GenNorm. Correlation coefficient (r) values are shown (*p < 0.05, **p < 0.01).
Conclusions
In this study, the gene ADF7 performed optimally in all the experiments. Genes ADF5 and ADF1b could be also proposed as good starting points for gene expression studies. However, it is recommended to choose more than one reference gene for normalization, such that each of the chosen genes is involved in different biological functions and pathways.

Disclosure statement
The authors declare that they have no conflict of interest.

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