Selection and Validation of Reference Genes for Gene Expression Analysis in *Vigna angularis* Using Quantitative Real-Time RT-PCR

Chao Chi*, Yongqiang Shen*, Lihua Yin, Xiwang Ke*, Dong Han, Yuhu Zuo*

Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang, People’s Republic of China

* These authors contributed equally to this work.

Abstract

Adzuki bean (*Vigna angularis*) is one of the most important legume crops in Asian countries like China, Japan and Korea due to its nutritious protein and starch contents. In spite of its economic importance, gene expression analysis system for gene function verification of adzuki bean is still absent. Therefore, reference genes for gene expression analysis based on the quantitative real-time PCR (qRT-PCR) were screened in current study. A total of nine general housekeeping genes, including *ACT*, *Fbox*, *ZMPP*, *GAPDH*, *EF*, *PP2A*, *UBC*, *UBN* and *PTB* were evaluated for their expression stability by qRT-PCR in four adzuki bean cultivars, three different tissues, four abiotic stress and one biotic stress. The best group of candidates as reference genes were as follows: *PTB* and *ACT* for different cultivars; *EF* and *UBN* for different tissues; *ACT* and *ZMPP* for biotic stress and waterlogging stress; *Fbox* and *UBC* for salinity-alkalinity stress; *Fbox* and *PTB* for drought stress. Our results will provide a more accurate and reliable normalization of qRT-PCR data in adzuki bean.

Introduction

Adzuki bean (*Vigna angularis*) is one of the important legume crops of the Ceratotropis subgenus in Asian, under the papilionoid subfamily of the Fabaceae. Due to its sweet taste with high protein and starch content, adzuki bean is widely cultivated and used as traditional food material in East Asia and other areas [1]. To better understand the gene function of adzuki bean, gene expression analysis system of the crop is of significance. Quantitative real-time reverse transcription PCR (qRT-PCR) is an important technique in gene expression analysis, which relies on reference genes for data normalization. However, systematic evaluation of the reference genes used for gene expression analysis of adzuki bean is still lacking to date. In previous studies, 18S rRNA was used as reference gene to normalize the *VaXTHS4*, *VaXTH1* and *VaXTH2* expression level of adzuki bean treated with hypergravity [2]. All of these studies on adzuki bean gene expression surveyed have used only one single reference gene and no preliminary validations were performed. However, evidence showed that transcriptional levels of...
commonly used reference genes (e.g. GAPDH, ACT) may also vary considerably in response to changes in different experimental conditions and/or tissue types [3, 4]. Therefore, a suitable reference gene(s) that is (are) stable under specific experimental conditions needs to be screened, in order to make the results for adzuki bean gene expression accurate.

Quantitative real-time reverse transcription PCR represents a particularly suitable technology catering for this purpose, attributed to its sensitivity, specificity, dynamic range and high throughput capacity [5–8]. Commonly used reference genes include ribosomal RNA 18S rRNA and a number of housekeeping genes, such as those encoding actin (ACT), tubulin (TUB), polyubiquitin (UBQ) and elongation factor 1-α (EF1α) [9, 10]. The importance of choosing stable reference genes prompted the development of a set of software packages, such as geNorm, NormFinder and BestKeeper [11]. Different statistical algorithms in the software packages can result in inconsistent ranking of the optimal reference genes, thus the selection of reference genes requires the consideration of all statistical algorithms [12]. Validations of the stability of reference genes have been performed by the three different software packages in many plants, such as Arabidopsis thaliana [13], rice [14, 15], Brachypodium sp. [16], wheat [17], soybean [18], tomato [19], sugarcane [20] and grape [21]. Therefore all the three software packages were chosen for data analysis in the current study.

The whole genome sequence of recently reported adzuki bean [22] has facilitated genome-wide mining for reference genes. Nine typically used housekeeping genes were considered as candidate reference genes including actin-like protein (ACT), zinc-metallopeptidase (ZMPP), elongation factor 1-alpha (EF1-α), FBOX domain-containing protein (Fbox), polyubiquitin-C (UBC), ubiquitously expressed nuclear (UBN), branched-chain phosphotransacylase (PTB), serine/threonine protein phosphatase 2A (PP2A) and glyceraldehyde 3 phosphate-dehydrogenase (GADPH). To validate and develop a qRT-PCR method for adzuki bean gene expression analysis, different experimental conditions including four adzuki bean cultivars, three different tissues, four abiotic stress and a biotic stress were applied.

Materials and Methods

Varieties and stress treatments

The following varieties of adzuki bean were included in our experiments: Baoqinghong (BQH), Nonganhong (NAH), ZXC136 and ZXC143. Seeds were provided by the National Coarse Cereals Engineering Research Center (NCCERC, China). Plants were grown in a growth chamber under short daytime conditions (8 h light/16 h dark) at 24˚C–27˚C when fully expanded euphylla (about 10 d after sowing) were collected for gene expression test.

Only BQH were used to perform abiotic stress, biotic stress and different tissue treatment tests. Different tissues including root, stem and fully expanded euphylla (about 10 d after sowing) were then collected for gene expression assays.

Drought stress was induced by stopping watering while the euphylla were fully expanded. Subsequently, euphylla were sampled at 0, 6 and 12 d after drought treatment. For salinity-alkalinity stress, seedlings were treated with 100 mM complex neutral and alkali salts [NaCl: Na₂SO₄:NaHCO₃:Na₂CO₃ = 3:3:5:1], and euphylla sampled after treatments at 0, 6 and 12 d. For waterlogging stress, the pots were placed into large black tanks and the seedlings were submerged under the water which was 2 cm above the soil surface. Samples were collected after treatments at 0, 6 and 12 d. For biotic stress, the fully expanded euphylla were inoculated with uredospore of Uromyces vignae in a concentration of 10⁶ uredospore per milliliters as described previously [23]. The inoculated leaves were harvested at 0, 6 and 12 d post inoculation (dpi). Each sample with three replicates were collected and quickly frozen in liquid nitrogen and stored at -80˚C for RNA extraction.
RNA extraction and cDNA synthesis

Total RNA was extracted following the manufacturer instructions using Trizol™ Reagent (Invitrogen, Carlsbad, CA) and then treated with RNase-free DNasel at 37°C for 30 min. Purification with Trizol™ Reagent was repeated to remove DNasel. Only the RNA with A260/A280 ratio of 1.8–2.1 and A260/A230 ratio > 2.0 were used for subsequent analyses. The integrity of RNA samples was verified by 1.5% agarose gel electrophoresis with ethidium bromide staining. Following the manufacturer’s instructions, strand cDNA synthesis was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Shenzhen, China) and oligo-dT primers in a final volume of 20 μL. All cDNA samples were stored at -20°C and diluted 10-fold prior to being analyzed in qRT-PCR.

PCR primer design and qRT-PCR analysis

Primers of the nine candidate reference genes for qRT-PCR were designed using Primer Premier V5.0 (Premier Biosoft International, Palo Alto, CA) and the primer sequences are listed (Table 1). Specificity of the primers was tested by performing PCR using cDNA as template. PCR products were analyzed on 1.5% agarose gels. For each primer pair, PCR reaction efficiency estimates were derived from a standard curve generated from a serial of dilution pooled cDNA. Based on average cycle threshold (Ct) values of each five-fold dilution, a standard curve was generated using linear regression. PCR efficiency (E) was achieved by the equation: Efficiency % = (10^(-1/slope) - 1) × 100%. PCR conditions were as follows: 95°C for 1 min, 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Finally, dissociation curves were generated by increasing the temperature from 65 to 95°C, stepwise by 0.3°C every 10 s.

Statistical analysis

Ct values of all samples were transformed to relative quantities using the method of 2^−ΔΔCt [24], and then exported to geNorm v3.5 [25], NormFinder [26] and BestKeeper [27] for gene expression stability analysis. The geNorm statistical algorithm calculates gene stability (M value) with the average of pairwise variations, relying on the principle that the expression ratio of two ideal reference genes should be identical in all samples. By ranking gene stability via stepwise exclusion of the least stable gene, geNorm identified the genes with the lowest M values indicating the highest stability and the number of best reference genes. Additionally, Van desompele et al. [25] recommended that genes with lowest value of V_n/n+1 (V_n/n+1 < 0.15) are the most stable genes. NormFinder, another algorithm for identifying the optimal normalization gene(s) among a set of candidates, ranks the stability of candidate reference gene expression for all samples with no sub-group determination. According to the analysis, the lowest
stability value would be ranked the highest. Finally, the stability rankings of the reference genes from the three different algorithms were integrated, generating an overall ranking according to the geometric mean [28, 29].

Normalization of the target genes

For normalization experiments, catalase (CAT), chitinase (GLU) and β-1,3-glucanase (CHI) genes expression responsive to U. vignae infection of BQH was used. Sample was harvested at 0, 12, 24, 48 and 120 h after inoculation, respectively. RNA extraction and cDNA synthesis were performed as described above. Expression was normalized using four reference gene combinations: (1) the most stably expressed reference gene, (2) the two most stably expressed reference genes combination, (3) three most stably expressed reference genes combination and (4) the least stably expressed gene.

Results

Amplification specificity and efficiency

Total RNA extracted from all samples met the requirement for qRT-PCR analysis (S1 Table). Melting curve analysis and agarose gel electrophoresis showed that all primer pairs amplified a single fragment with expected size. No amplification was detectable in the absence of template. The amplification efficiencies for the nine candidate reference genes ranged from 90.23% (ZMPP) to 105.84% (Fbox), and regression coefficients from 0.9911 (Fbox and UBC) to 0.9995 (UBN) (Table 2).

Candidate reference genes expression stability analyses

To show transcriptional differences among various candidate genes, the average Ct value was calculated across all samples. The mean Ct values for nine genes ranged from 18.86 to 35.15 (Fig 1). Ct values were transformed to relative quantities using the ΔCt method. NormFinder, geNorm and BestKeeper were applied to calculate the expression stability of the set of candidate reference genes (S2, S3 and S4 Tables).

Average expression stability (M value) of all genes was calculated by geNorm (S2 Table). Most of the genes used in current study had an M value below the geNorm threshold of 1.5 (Fig 2). When challenged with various stresses, different cultivars responded with varying stabilities. Among the various cultivars, PP2A and ACT remained most stable, and GAPDH was least stable (Fig 2A). PP2A and UBN remained the most stable, and ACT the least stable among the various tissues (Fig 2B). In response to rust infection, UBC and UBN were the most stable, and PP2A had the least stability (Fig 2C). When challenged with drought stresses, ACT and

| Gene symbol | TM (˚C) | Amplification efficiency (%) | Regression coefficient (R²) |
|-------------|--------|-----------------------------|-----------------------------|
| ACT         | 53     | 94.45                       | 0.9913                      |
| Fbox        | 53     | 105.84                      | 0.9911                      |
| ZMPP        | 53     | 90.23                       | 0.9991                      |
| GAPDH       | 57     | 104.34                      | 0.9916                      |
| EF          | 55     | 97.24                       | 0.9976                      |
| PP2A        | 52     | 91.65                       | 0.9986                      |
| UBC         | 52     | 97.62                       | 0.9911                      |
| UBN         | 51     | 93.24                       | 0.9995                      |
| PTB         | 54     | 95.11                       | 0.9987                      |

doi:10.1371/journal.pone.0168479.t002
PTB showed the highest stability, and GAPDH the lowest (Fig 2D). Responding to salinity-alkalinity stress, UBC and UBN were the most stable, and GAPDH the least (Fig 2E); while in response to waterlogging stress, ACT and ZMPP were the most stable, and GAPDH the least (Fig 2F). To determine the optimal number of reference genes, geNorm was used to calculate the pairwise variation $\frac{V_n}{V_{n+1}}$ of two sequential normalization factors $NF_n$ and $NF_{n+1}$. In various cultivars, two genes were sufficient for normalization, since the $V_{2/3}$ value was $< 0.15$ (Fig 3). Differences in the expression stability of the candidate reference genes were less marked in the various cultivars treatment series, than in the other series (Fig 3). The $V_{2/3}$ value for the various tissues was 0.148, so that PP2A and UBN would be sufficient for normalization purposes. With the inoculation treatment, the combination of UBC, UBN and EF, as internal reference genes, produced a $V_{3/4}$ value of 0.134. With the drought stress, the combination of ACT, PTB and Fbox produced a $V_{3/4}$ value of 0.135. However, as for waterlogging and salinity-alkalinity stress, no $V_{3/4}$ values below the threshold were found; and the closest values were 0.200 and 0.186, respectively (Fig 3).

The stability ranking generated by NormFinder was slightly different from that determined by geNorm (Table 3). Fbox, PTB, ACT and PP2A were still ranked the highest for various cultivars, and EF and UBN the highest for different tissue samples, and ACT and ZMPP the highest for waterlogging stress. PTB, PP2A and EF was the most stable housekeeping genes for drought stress. However, among inoculation treatments, ACT and ZMPP emerged as the most stably expressed (ranked fourth and sixth by geNorm) and Fbox was the most stable for salinity-alkalinity stress.

Stability of expression was then re-analyzed using the program BestKeeper, which is mainly through comparing the standard deviation (SD) and coefficient of variation (CV) of Ct value...
to select the most stable genes. The smaller SD and CV of reflect more stable reference gene (Table 3). For various cultivars, tissue samples, drought stress, inoculation treatment and salinity-alkalinity stress, the ranking generated by BestKeeper was slightly different from that determined by geNorm and NormFinder (Table 3). For waterlogging stress, GAPDH emerged as the most stably expressed.

**Comprehensive Stability Analysis of Reference Genes**

To obtain a consensus result of the most stable reference genes as recommended by the three methods according to the RefFinder approach, the geometric mean of the three algorithms with the respective rankings for each candidate gene were calculated (Table 3). PTB was the most stable reference gene in different cultivars and drought stress. In the rust infection and waterlogging stress, ACT was most stable. Additionally, between different tissues, UBN was the best reference gene. And lastly Fbox was the most stably expressed under salinity-alkalinity stress.
Evaluation of the expression of **CAT**, **GLU** and **CHI** by qRT-PCR

To demonstrate the efficiency of the recognized reference genes in qRT-PCR, the expression of three genes (**CAT**, **GLU** and **CHI**) was analyzed during different times of **U. vignae** infection in **BQH** cultivar. The relative expression level of these genes were normalized by one or three most stable reference gene(s), and the least stable gene or two-gene combination. For **U. vignae** infection, the relative expression level of **CAT**, **GLU** and **CHI** showed no significant differences using either **ACT** alone or the combination **UNC**+**UBN** and **UBC**+**UBN**+**EF** as reference genes (Fig 4). However, the least stable reference gene(s) **PP2A** led to an underestimation of **CAT**, **GLU** and **CHI** transcript level. These results further confirmed the importance of the stability of reference gene, which could effectively reduce the occurrence of low precision or unreliable results.

**Discussion**

Molecular detection and quantification of transcript abundance using qRT-PCR under different conditions are important tasks for gene function verification. However, the deviation of
qRT-PCR are commonly introduced during RNA extraction, cDNA synthesis, and PCR process. Therefore, a suitable reference gene(s) for the specific set of chosen experimental conditions needs to be screened. An ideal reference gene should satisfy three norms: stable expression in test samples, similar amplification efficiency to target genes, and moderate level of expression.

Although \textit{ACT}, \textit{GAPDH}, \textit{EF}, or 18s rRNA have been reported as reference genes for gene expression analysis [30–32], adzuki bean is not involved in these studies. Therefore, nine genes were selected as candidate reference genes in current study and three excel-based approaches BestKeeper, \textit{geNorm} and \textit{NormFinder} were used to evaluate the expression stability. Although there were distinct features among statistical algorithms and analysis strategies, these three approaches provided similar results. However, in some cases the gene expression stability was inconsistent among different softwares, such as \textit{ACT}, \textit{EF} and \textit{Fbox} in waterlogging and salinity-alkalinity treatment, respectively. In this case, we selected a combination of two genes with most stable performances as reference. For example, both \textit{UBC} and \textit{UBN} were chosen as reference genes in salinity-alkalinity stress (Fig 3E), and both \textit{ACT} and \textit{ZMPP} as reference genes in

| Method                              | Ranking order under different cultivars | Ranking order under different tissues | Ranking order of rust infection | Ranking order under waterlogging stress | Ranking order under salinity-alkalinity stress | Ranking order under drought stress |
|-------------------------------------|----------------------------------------|--------------------------------------|---------------------------------|----------------------------------------|-----------------------------------------------|----------------------------------|
| \textit{geNorm}                     | PTB/PP2A                               | ACT                                  | UBC                             | PP2A                                   | ZMPP                                          | UBC                             |
| \textit{NormFinder}                 | Fbox                                   | ACT                                  | UBC                             | PP2A                                   | ZMPP                                          | UBC                             |
| \textit{BestKeeper}                 | ACT                                    | PTB                                  | UBC                             | UBN                                    | ZMPP                                          | EF                              |
| \textit{Comprehensive}              | UBN                                    | EF                                   | Fbox                            | PP2A                                   | UBN                                           | ACT                             |
|                                     |                                        |                                       |                                 |                                        |                                               |                                 |
| \textit{geNorm}                     | UBN/PP2A                               | EF                                   | Fbox                            | ACT                                    | GAPDH                                         | PTB                             |
| \textit{NormFinder}                 | ACT                                    | ZMPP                                 | Fbox                            | PTB                                    | GAPDH                                         | UBC                             |
| \textit{BestKeeper}                 | ACT                                    | ZMPP                                 | PP2A                            | UBC                                    | GAPDH                                         | PTB                             |
| \textit{Comprehensive}              | ACT                                    | ZMPP                                 | PP2A                            | UBC                                    | GAPDH                                         | PTB                             |
|                                     |                                        |                                       |                                 |                                        |                                               |                                 |
| \textit{geNorm}                     | ACT/ZMPP                               | UBC                                  | PP2A                            | Fbox                                   | EF                                            | GAPDH                                         | PTB                             |
| \textit{NormFinder}                 | ACT                                    | ZMPP                                 | PP2A                            | UBC                                    | GAPDH                                         | PTB                             |
| \textit{BestKeeper}                 | ACT                                    | ZMPP                                 | ACT                             | UBN                                    | GAPDH                                         | PTB                             |
| \textit{Comprehensive}              | ACT                                    | ZMPP                                 | ACT                             | UBN                                    | GAPDH                                         | PTB                             |
|                                     |                                        |                                       |                                 |                                        |                                               |                                 |
| \textit{geNorm}                     | ACT/UBN                                | EF                                   | ACT                             | GAPDH                                  | ZMPP                                          | PTB                             |
| \textit{NormFinder}                 | ACT                                    | ZMPP                                 | PP2A                            | UBC                                    | GAPDH                                         | PTB                             |
| \textit{BestKeeper}                 | ACT                                    | ZMPP                                 | ACT                             | UBN                                    | GAPDH                                         | PTB                             |
| \textit{Comprehensive}              | ACT                                    | ZMPP                                 | ACT                             | UBN                                    | GAPDH                                         | PTB                             |
|                                     |                                        |                                       |                                 |                                        |                                               |                                 |
| \textit{geNorm}                     | ACT/PTB                                | Fbox                                 | ZMPP                            | UBN                                    | PP2A                                          | UBC                             |
| \textit{NormFinder}                 | PP2A                                   | EF                                   | PTB                             | Fbox                                   | ZMPP                                          | UBC                             |
| \textit{BestKeeper}                 | EF                                     | Fbox                                 | PTB                             | ACT                                    | ZMPP                                          | UBC                             |
| \textit{Comprehensive}              | PTB                                    | EF                                   | ACT                             | ZMPP                                    | PP2A                                          | UBC                             |

Table 3. Expression stability ranking of the 9 candidate genes.

Method | Ranking (better-good-average) |
|--------|-------------------------------|
|        | 1    | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    |

Ranking order under different cultivars
\textit{geNorm} | PTB/PP2A | ACT | UBC | Fbox | ZMPP | UBN | EF | GAPDH |
\textit{NormFinder} | Fbox | ACT | UBC | PP2A | UBN | ZMPP | UBN | EF | GAPDH |
\textit{BestKeeper} | ACT | PTB | UBC | UBN | ZMPP | Fbox | PP2A | EF | GAPDH |
\textit{Comprehensive} | PTB | ACT | UBC | Fbox | PP2A | UBN | ZMPP | EF | GAPDH |

Ranking order under different tissues
\textit{geNorm} | UBN/PP2A | EF | Fbox | ZMPP | PTB | GAPDH | UBC | ACT |
\textit{NormFinder} | ACT | UBN | Fbox | PP2A | ZMPP | UBC | ACT | GAPDH | PTB |
\textit{BestKeeper} | Fbox | EF | UBN | ZMPP | PP2A | UBC | GAPDH | PTB | ACT |
\textit{Comprehensive} | UBN | EF | Fbox | PP2A | ZMPP | UBC | GAPDH | PTB | ACT |

Ranking order of rust infection
\textit{geNorm} | UBN | PTB | EF | ACT | GAPDH | ZMPP | Fbox | PTB | PP2A |
\textit{NormFinder} | ACT | ZMPP | Fbox | PTB | UBC | GAPDH | UBN | EF | ACT |
\textit{BestKeeper} | ACT | ZMPP | UBC | UBN | EF | PTB | Fbox | GAPDH | PP2A |
\textit{Comprehensive} | ACT | ZMPP | UBC | UBN | EF | Fbox | PTB | GAPDH | PP2A |

Ranking order under waterlogging stress
\textit{geNorm} | ACT | ZMPP | UBC | PP2A | Fbox | EF | GAPDH | PTB | UBN |
\textit{NormFinder} | ACT | ZMPP | UBC | ACT | UBN | GAPDH | PTB | EF | UBN |
\textit{BestKeeper} | ACT | ZMPP | UBC | UBN | GAPDH | ACT | PTB | EF | UBN |
\textit{Comprehensive} | ACT | ZMPP | UBC | UBN | GAPDH | PTB | EF | UBN | |

Ranking order under salinity-alkalinity stress
\textit{geNorm} | UBN | Fbox | PP2A | ZMPP | UBC | ACT | GAPDH | PP2A | PTB |
\textit{NormFinder} | Fbox | PP2A | ZMPP | UBC | ACT | UBN | GAPDH | PTB | EF |
\textit{BestKeeper} | Fbox | ZMPP | PP2A | UBC | UBN | GAPDH | ACT | PTB | EF |
\textit{Comprehensive} | Fbox | ZMPP | UBC | UBN | GAPDH | PTB | EF | |

Ranking order under drought stress
\textit{geNorm} | ACT | PP2A | ZMPP | UBN | EF | GAPDH |
\textit{NormFinder} | ACT | UBN | PTB | Fbox | ZMPP | UBN | ACT | UBC | GAPDH |
\textit{BestKeeper} | EF | Fbox | PTB | ACT | ZMPP | UBN | UBC | PP2A | GAPDH |
\textit{Comprehensive} | PTB | EF | ACT | ZMPP | PP2A | UBN | UBC | GAPDH | |

doi:10.1371/journal.pone.0168479.t003
Fig 4. Relative quantification of CAT, GLU and CHI expression at different days after inoculation using validated reference genes for normalization. The validated reference gene(s), ACT, UBC+UBN+EF, UBC+UBN and PP2A, were used as normalization factors for analyzing catalase (CAT), chitinase (GLU) and β-1,3-glucanase (CHI) gene expression at different times of adzuki bean infected by U. vignae. ACT and UBC+UBN+EF were the most stable reference genes, while PP2A was the least stable. Results are presented as a mean fold change in relative expression compared to 0 h after inoculation.

doi:10.1371/journal.pone.0168479.g004
waterlogging stress (Fig 3F). In addition, BestKeeper [33] and a comprehensive stability analysis [29] were used to obtain a consensus result of the most stable reference genes. Therefore, in the current study, a comprehensive analysis was used to confirm the most stable reference gene(s). However, no single reference gene had a consistent expression level in different experimental conditions. Compared with a single reference gene, selecting two or three stable reference genes can obtain more accurate and reliable data.

In the present study, we normalized the expression of CAT, GLU and CHI with a total of four normalization factors using individual (ACT) and combination of three (UBC+UBN+EF) control genes acquired a similar expression patterns. In contrast, when we normalized the expression of CAT, GLU and CHI using the most unstable reference gene, we acquired inconsistent result. These results indicated that reference genes screened in current study are solid and useful.

Actins play vital roles in cell motility and cytoskeleton maintenance, which has been confirmed to be suitable for gene expression normalization in legume crops [11] but not suitable for rice, potato or sugarcane [34, 35]. In current study, VaACT was the most stable genes among different conditions including different cultivars, biotic stress and drought stress [11]. Compared with ACT, GAPDH has been considered unstable among the most commonly used reference genes [29, 36]. Similarly, GAPDH was confirmed to be the most unstable gene in present study among all of the sample pools by both geNorm, NormFinder and BestKeeper analysis.

In the present study, nine housekeeping genes selected based on the whole genome sequences of V. angularis and among which the most stable reference genes were validated under different experimental conditions. However, with the development of high-throughput sequencing technology, we can also select stable reference genes from transcriptome profiling. For example, Wei et al. [12] exploited the transcriptome data to search the candidate reference genes of P. massoniana and picea for gene expression analysis. Nevertheless, without the transcriptome data in adzuki bean, the reference genes selected in current study will be helpful for accurate normalization of qRT-PCR data and facilitate the future work on gene expression studies in V. angularis.

Conclusion

We have shown a set of stable housekeeping genes that are suitable to be used as reference genes in Vigna angularis. Different housekeeping genes responded with varying stabilities under various stresses. We found suitable reference genes among cultivars (ACT and PTB), various tissues (EF and UBN), and challenges including rust infection and drought stress (ACT and ZMPP), salinity-alkalinity stress (UBC and Fbox), and waterlogging stress (Fbox and PTB). We have proved that this is an accurate and reliable method for the normalization of qRT-PCR data in adzuki bean.

Supporting Information

S1 Table. Quality inspection of the total RNA using the Nanodrop 2000. (DOC)

S2 Table. Ranking of the candidate reference genes according to their stability value using GeNorm. (DOC)

S3 Table. Ranking of the candidate reference genes according to their stability value using NormFinde. (DOC)
S4 Table. Ranking of the candidate reference genes according to their stability value using BestKeeper. Notes: Descriptive statistics of 9 candidate genes based on the coefficient of variance (CV) and standard deviation (SD) of their Ct values were determined using the whole data set. Reference genes were identified as the most stable genes, those with the lowest coefficient of variance and standard deviation.

Acknowledgments
We are grateful to Dr. Yichao Huang for editing as well as critical inputs of the manuscript. We also thank Dr. Zhiyuan Yin for technical assistance.

Author Contributions
Conceptualization: XK YZ.
Data curation: CC YS XK YZ.
Formal analysis: CC YS LY.
Funding acquisition: XK LY YZ.
Investigation: CC YS DH.
Project administration: LY XK.
Resources: LY XK.
Supervision: XK YZ.
Validation: XK LY CC YS DH YZ.
Visualization: CC YS DH.
Writing – original draft: CC YS.
Writing – review & editing: CC YS XK YZ.

References
1. Yamaguchi M, Rubatzky VE. Word vegetable: principles, production and nutritive values. Plant Science Textbook 1997.
2. Nakamura T, Yokoyama R, Tomita E, Nishitani K. Two azuki bean XTH genes, VaXTH1 and VaXTH2, with similar tissue-specific expression profiles, are differently regulated by auxin. Plant Cell Physiol 2003; 44(1): p. 16–24. PMID: 12552143
3. Ruan W, Lai M. Actin, a reliable marker of internal control? Clin Chim Acta 2007; 385(1–2): p. 1–5. doi: 10.1016/j.cca.2007.07.003 PMID: 17698053
4. Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, et al. Housekeeping genes as internal standards: use and limits. J Biotechnol 1999; 75(2–3): p. 291–5. PMID: 10617337
5. Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR—a perspective. J Mol Endocrinol 2005; 34(3): p. 597–601. doi: 10.1677/jme.1.01755 PMID: 15956331
6. Gachon C, Mingam A, Charrier B. Real-time PCR: what relevance to plant studies? J Exp Bot 2004; 55(402): p. 1445–54. doi: 10.1093/jxb/ehr181 PMID: 15208338
7. Walker NJ, Tech S. A technique whose time has come. Science 2002; 296(5567): p. 557–9.
8. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002; 29(1): p. 23–39. PMID: 12200227
9. Czechowsk i T, Stitt M, Altmann T, Udvardi MK, Scheible W. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol 2005; 139(1): p. 5–17. doi: 10.1104/pp.105.063743 PMID: 16166256

10. Gutierrez L, Mauriat M, Pwlloux J, Bellini C, Wuytswinkel V. Towards a systematic validation of references in real-time rt-PCR. Plant Cell 2008; 20(7): p. 1734–5. doi: 10.1105/tpc.108.059774 PMID: 18664615

11. Hu R, Chengming F, Hongyu L, Qingzhu Z, Yong F. Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. BMC Mol Biol 2009; 10: p. 93. doi: 10.1186/1471-2199-10-93 PMID: 19785741

12. Wei Y, Liu Q, Hongyu D, Zhichun Z, Yanping H, Xuelian C, et al. Normalisation of real-time RT-PCR gene expression measurements in Arabidopsis thaliana exposed to increased metal concentrations. Planta 2008; 227(6): p. 1343–9. doi: 10.1007/s00425-008-0706-4 PMID: 18273637

13. Hong SY, Seo P, Yang M, Xiang F, Park C. Exploring valid reference genes for gene expression studies in Brachypodium distachyon by real-time PCR. BMC Plant Biol 2008; 8: p. 112. doi: 10.1186/1471-2229-8-112 PMID: 18992143

14. Jain M., Nijhawan A, Akhilesh K, Khurana J P. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun 2006; 345(2): p. 646–51. doi: 10.1016/j.bbrc.2006.04.140 PMID: 16690022

15. Remans T, Smeets K, Opdenakker K, Mathijsen D, Vangronsveld J, Cuypers A. Normalisation of real-time RT-PCR gene expression measurements in Arabidopsis thaliana exposed to increased metal concentrations. Planta 2008; 227(6): p. 1343–9. doi: 10.1007/s00425-008-0706-4 PMID: 18273637

16. Hong SY, Seo P, Yang M, Xiang F, Park C. Exploring valid reference genes for gene expression studies in Brachypodium distachyon by real-time PCR. BMC Plant Biol 2008; 8: p. 112. doi: 10.1186/1471-2229-8-112 PMID: 18992143

17. Paolacci AR, Tanzarella OA, Porceddu E, Ciaffi M. Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. BMC Mol Biol 2009; 10: p. 11. doi: 10.1186/1471-2199-10-11 PMID: 19232096

18. Libault M, Thibivilliers S, Bilgin DD, Radwan O, Benitez M, Clough SJ, et al. Identification of Four Soybean Reference Genes for Gene Expression Normalization. Plant Genome 2008; 1(1):44–54.

19. Exposito-Rodriguez M, Borges AA, Borfes A, Perez JA. Selection of internal control genes for quantitative real-time RT-PCR during tomato development process. BMC Plant Biol 2008; 8: p. 131. doi: 10.1186/1471-2229-8-131 PMID: 19102748

20. Iskandar HM, Simpson RS, Casu RE, Bonnett GD, Maclean DJ, Manners JM. Comparison of reference genes for quantitative real-time polymerase chain reaction analysis of gene expression in sugarcane. Plant Molecular Biology Reporter 2004; 22(4):325–337.

21. Reid KE, Olsson N, Schlosser J, Peng F, Lund ST. An optimized grapevine RNA isolation procedure and statistical determination of reference genes for real-time RT-PCR during berry development. BMC Plant Biol 2006; 6: p. 27. doi: 10.1186/1471-2229-6-27 PMID: 17105665

22. Yang M, Xiang F, Park C. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem Biophys Res Commun 2006; 345(2): p. 646–51. doi: 10.1016/j.bbr.2006.04.140 PMID: 16690022

23. Zheng S, Ke X, Huang D, Gao X, Voegele Ralf T, Kang Z, et al. Validation of reference genes for gene expression analysis in Valsa mali var. mali using real-time quantitative PCR. World J Microbiol Biotechnol 2013; 29(9): p. 1563–71. doi: 10.1007/s11274-013-1320-6 PMID: 23508400

24. Vandesonompele J, Preter KD, Pattyn F, Poppe B, Roy NV, Paepe AD, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3(7): p. RESEARCH0034.

25. Andersen CL, Jensen JL, Omtoft TF. 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 2004; 64(15): p. 5245–50. doi: 10.1158/0008-5472.CAN-04-0496 PMID: 15289330

26. Pfaffl MW, Tichopad A, Prgemet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity BestKeeper—Excel-based tool using pair-wise correlations. Biotechnol Lett 2004; 26(6): p. 509–15. PMID: 15127793

27. Ma R, Xu S, Zhao Y, Xia X, Wang R. Selection and Validation of Appropriate Reference Genes for Quantitative Real-Time PCR Analysis of Gene Expression in Lycoris aurea. Frontiers in Plant Science, 2016, 7(651).
29. Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Molecular Biology, 2012, 80(1):75–84.

30. Vasquez-Robinet C, Watkinson JI, Sioson AA, Ramakrishnan N, Lenwood S, Grene R. Differential expression of heat shock protein genes in preconditioning for photosynthetic acclimation in water-stressed loblolly pine. Plant Physiol Biochem 2010; 48(4): p. 256–64. doi: 10.1016/j.plaphy.2009.12.005 PMID: 20171112

31. Saint-Marcoux D, Proust H, Dolan L, Langdale JA. Identification of reference genes for real-time quantitative PCR experiments in the liverwort Marchantia polymorpha. PLoS One 2015; 10(3):e118678.

32. Reddy DS, Bhatnagar-Mathur P, Reddy PS, Cindhuri S, Ganesh AS, Sharma KK. Identification and Validation of Reference Genes and Their Impact on Normalized Gene Expression Studies across Cultivated and Wild Cicer Species. PLoS One 2016; 11(2):e148451.

33. de Vega-Bartol JJ, Santos RR, Simoes M, Miguel CM. Normalizing gene expression by quantitative PCR during somatic embryogenesis in two representative conifer species: Pinus pinaster and Picea abies. Plant Cell Rep 2013; 32(5): p. 715–29. doi: 10.1007/s00299-013-1407-4 PMID: 23529547

34. Zhu J, He F, Song S, Wang J, Yu J. How many human genes can be defined as housekeeping with current expression data? BMC Genomics 2008; 9: p. 172. 2

35. Thorrez L, Deun KV, Tranchevent L, Lommel LV, Engelen K, Moreau Y, et al. Using ribosomal protein genes as reference: a tale of caution. PLoS One 2008; 3(3): p. e1854. doi: 10.1371/journal.pone.0001854 PMID: 18365009

36. Tong Z, Gao Z, Wang F, Zhou J, Zhang Z. Selection of reliable reference genes for gene expression studies in peach using real-time PCR. BMC Mol Biol 2009; 10: p. 71. doi: 10.1186/1471-2199-10-71 PMID: 19619301