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Full Length Article

Validation of Internal Reference Genes for Accurate Gene Expression Analysis in Soybean Roots Interacted with *Heterodera glycines* and *Bacillus megaterium*

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Abstract

Soybean cyst nematode (SCN), *Heterodera glycines* and *Bacillus* induced gene expression alterations in plant host play a vital role in root invasion. The true differences in the level of genes of interest were accurately measured by real-time PCR (RT-qPCR). However, internal reference genes were indispensable for the namalization of transcription. Reference genes for the use of RT-qPCR in soybean under *H. glycines* and *Bacillus* interactions have been lacking in soybean. In the study, ten candidate reference genes (ELF1A, ELF1B, CYP2, UBC4, UBQ10, TUB4, G6PD, ACT2/7, ACT11, and CONS4) were evaluated and their expression stability was analyzed in *Bacillus megaterium* Sneb207 strain coated soybean roots and susceptible and resistant varieties of soybean roots infected by SCN. All the data were statistically analyzed using geNorm, NormFinder, BestKeeper and RefFinder. The results showed that *ELF1B* and *CONS4* were the most stable genes among soybean-Sneb207-nematode interactions. Furthermore, *ELF1A* and *TUB4* were the most stable among soybean-nematode interactions. Moreover, *ACT11*, *G6PD*, *ELF1B*, and *CONS4* could also be used as stable reference genes in different soybean varieties interactions with *H. glycines* and additional *Bacillus*. In addition, the relative expression of the *PR-2* gene was examined in susceptible soybean roots infected with SCN. This confirmed the results of the chosen reference genes. The results provide a basis for RT-qPCR gene expression analysis of soybean- *Bacillus*-nematode. © 2019 Friends Science Publishers

Keywords: *Bacillus megaterium*; *Heterodera glycines*; Reference genes; RT-qPCR; Soybean

Introduction

Gene expression analysis is an essential way to study cellular processes, complex biological processes, and molecular responses. Having features of sensitivity, specificity, reproducibility, and high throughput capacity, RT-qPCR is commonly applied for analysis of the expression of target gene (Walker, 2002; Bustin and Nolan, 2004; Gachon *et al.*, 2004). The true difference expression of genes of interest were accurately determined by RT-qPCR, and a normalization test for stably expressed reference genes is an essential pre-requisite (Chervoneva *et al.*, 2010). However, "housekeeping genes" which are always borrowed as reference genes might lead to the wrong expression model, suggesting no genes are stable under any treatment (Gronthos and Zannettino, 2011). Thus, it is particularly important to select stable reference genes as internal reference genes.

It is very common that housekeeping genes including tubulin (*TUB*), actin (*ACT*) and polyubiquitin (*UBQ*) are used as internal reference genes (Bustin, 2002; Czechowski *et al.*, 2005). However, recent research has suggested that there is no housekeeping gene that could be seen as a general reference (Suzuki *et al.*, 2000). In a long yellow daylily (*Hemerocallis citrina* Borani), *ACT* and *60S* were the most stable reference genes from different organs (Hou *et al.*, 2017), and EF1A was the most stable gene in all *Atlantic salmon* tissues (Olsvik *et al.*, 2005). In addition, a stably expressed internal reference that is stable under one condition may not be stable under another condition (Hu *et al.*, 2009; Jaramillo *et al.*, 2017). A former study showed that *TUB* is the most unreliable gene in soybean exposed to cadmium (Wang *et al.*, 2012), while *ACT11* and *TUA5* were the most stable reference genes in soybean roots under different photoperiods (Hu *et al.*, 2009). Therefore, a suitable stable reference gene should be selected before its use under different conditions.

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Soybean is among the most important economic crops due to industrial application and several nutritional benefits. The SCN is one of the most serious pests of soybean, causing an average of 30% of yield losses worldwide (Wrather and Koenning, 2006). Currently, biological control is one of the effective measures to control SCN (Zhou *et al.*, 2017). Thus, it is meaningful to study plant nematode diseases and biological controls. Previous studies have evaluated reference genes for normalization analysis in RT-qPCR experiments in soybean under drought stress, cadmium stress, and photoperiodic treatments (Jian *et al.*, 2008). However, there are no reference genes used for soybean roots infected with SCN or plant-bacteria interactions. Furthermore, there are no validating studies of reference genes for SCN-infected disease-resistant varieties and susceptible varieties to date.

In this research, *ELF1A* (Glyma.05g114900), *ELF1B* (Glyma.02g276600), *CYP2* (Glyma.12g024700), *UBC4* (Glyma.18g216000), *UBQ10* (Glyma.11g135300), *TUB4* (Glyma.03g124400), *G6PD* (Glyma.19g082300), *ACT2/7* (Glyma.19g147900), *ACT11* (Glyma.18g290800), and *CONS4* (Glyma.12g020500) in the SCN-susceptible soybean variety Liaodou15 at 12, 24, 48, 72 h and 7 d after nematode infection and the SCN-resistant variety Kangxian12 at 12, 24, 48, and 72 h after SCN infection, were assessed by RT-qPCR. The expression stabilities of the ten candidate genes were comparatively estimated with statistical programs, namely, geNorm, NormFinder, BestKeeper and RefFinder. Additionally, the target gene (pathogen defense-related gene 2) PR2 was selected as checking the reliability of the ten reference genes.

Materials and Methods

Bacillus megaterium and Soybean Cyst Nematode

The *B. megaterium* strain Sneb 207 was preserved in the nematode laboratory in Liaoning Province, China, and the bacterial suspension was prepared (Zhou *et al.*, 2017).

Heterodera glycines race 3 was used in the experiment. SCN were originally collected from soybean field and were cultured in the greenhouse. The cysts were separated and hatched second-stage juveniles (J2s) as described by Zhou *et al.* (2017).

Plant Materials and Treatments

Two locally commercial soybean cultivars Liaodou15 and Kangxian12 were used throughout this study. Liaodou15 are susceptible to SCN, and Kangxian12 are resistant to SCN race 3. The soybean seeds were sterilized on the surface and dried. Half of Liaodou15 seeds were coated with a Sneb207 suspension and then air-dried (Zhou *et al.*, 2017); the other half of the sterilized Liaodou15 seeds were uncoated. The soybean seeds were planted in cone-tainers with a previously autoclaved soil/sand mixture (1:1).

Soybean at the two-leaf stage, half of the Sneb207-coated Liaodou15, half of the uncoated Liaodou15 plants, and half of the Kangxian12 plants were inoculated with a 5 mL suspension containing 2,000 J2 of SCN per plant, while the other pants were not inoculated. The roots were collected at 12, 24, 48 and 72 h after nematode inoculation in Kangxian12, and at 12, 24, 48, 72 h and 7 days after nematode inoculation in Liaodou15. All roots were frozen in liquid nitrogen and stored in a -80°C freezer until used. A total of 84 samples were obtained.

RNA Isolation and cDNA Synthesis

Total RNA was extracted with a Total RNA Kit (Tiangen, Beijing, China). The RNA purity and concentration were estimated with a NanoVue spectrophotometer (GE Healthcare, USA). A high-capacity cDNA reverse transcription kit (Promega, USA) in a 20μL reaction volume was used for RNA (1μg) reverse transcription.

Primer Design and RT-qPCR Analysis

Primers for *ACT11* and *ACT2/7* were used as reported by Jian *et al.* (2008) and *CONS4* was used as reported by Libault *et al.* (2008). Two pairs of primers (*TUB4* and *UBQ10*) were obtained from Wang *et al.* (2012) and five pairs of primers (*ELF1A*, *ELF1B*, *CYP2*, *G6PD* and *UBC4*) were obtained from De Jesus Miranda *et al.* (2013), respectively. All primers were commercially synthesized (Sangon Biotech, Shanghai, China) and verified by PCR. Standard curves of each primer pair, using a six-point 10-fold dilution serious of pooled cDNA were developed to compute the PCR amplification efficiency (E%) and the regression coefficient (Bustin, 2010). The sequences of primers are shown in Table 1.

RT-qPCR was tested with the Qtower^{3}G Real-time PCR System (Analytik Jena AG, Germany) and using the SYBR Green I Mix (Takara, China). Each reaction was run in a 25-μL volume including 1 μL of each forward and reverse primer (10 μM), 2 μL of diluted cDNA, 12.5 μL of SYBR Premix Ex TaqTM II and 8.5 μL of ddH_2O. The cDNA added corresponded to 20 ng of reverse-transcribed RNA for each sample. All the reactions were conducted using the following program: 95°C for 10 min, then 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Further, a melting curve analysis was generated from 60-95°C at the end of each PCR run. Each RT-qPCR analysis was executed with three technical repetitions and three biological repetitions.

Statistical Analysis of the Reference Genes

For NormFinder and geNorm, raw Ct values were transformed into the relative quantity values using the formula, $2^{-\Delta Ct}$. For geNorm, the gene expression stability (M-value) was counted according to the average pairwise

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Table 1: Reference gene primer sequences and smplicon characteristics

Gene symbol	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'	Amplcon length (bp)	PCR efficiency (%)	Regression coefficient (R^2)
ELF1A	GACCTTCTTCGTTTCTCGCA	CGAACCTCTCAATCACACGC	195	105.02	0.993
ELF1B	GTTGAAAAGCCAGGGGACA	TCTTACCCCTTGAGCGTGG	118	101.52	0.998
CYP2	CGGGACCAGTGTGCTTCTTCA	CCCCTCCACTACAAAGGCTCG	154	104.75	0.997
UBC4	GAGCGAGCAGTTTCAGAC	CATAGGAGGGACGATACG	168	105.49	0.997
UBQ10	TCCCACCAGACCTCCCACCAGACC	CACGAAGACGCAACACAAGG	117	102.18	0.998
TUB4	GGCGTCCACATTCATTGGA	CCGGTGTACCAA TGCAAGAA	111	104.51	0.994
G6PD	ACTCCTTGATACCGTTGTCCAT	GTTTGTTATCCGCCTACAGCCT	126	110.83	0.998
ACT2/7	CTTCCCTCAGCACCTTCCAA	GGTCCAGCTTTCACACTCCAT	119	104.51	0.995
ACT11	CGGTGGTTCTATCTTGGCATC	GTCTTTCGCTTCAATAACCCTA	142	108.91	0.999
CONS4	GATCAGCAATTATGCACAACG	CCGCCACCATTCAGATTATGT	106	106.53	0.997

variation (V) for the gene of interest compared with all other candidate reference genes, and the reference gene with the lowest M-value is identified as the most stable gene. Additionally, geNorm estimates the pairwise variation (V_n/V_{n+1}) between two factors standards (FN/FN+1) to find the optimal number of reference genes in RT-qPCR normalization. Moreover, NormFinder uses an ANOVA-based model of each reference gene to evaluate gene variation, and the lowest values represent the most stable gene (Andersen *et al.*, 2004). BestKeeper calculates the standard deviation (SD) and the coefficient of variance (CV) with the Ct values to determine the expression stability of reference genes, and the lowest SD and CV values means the most stable expression. RefFinder combine the four statistical programs to comprehensively reorder the tested candidate reference genes.

Validation of Reference Gene Analysis

PR2 was used as a target gene to verify the stability of the reference genes for RT-qPCR. The RT-qPCR primer pairs for PR2 were 5'-TGAAATAAGGGCCACGAGTCCAAATG-3' (forward) and 5'-ATGGTACATGCAGCATTCAAGAATGCAGAT-3' (reverse).

Results

Verification of PCR Amplicons and Primer Specificity

The amplification efficiencies (E%) ranged from 101.52% for *ELF1B* to 110.83% for *G6PD* and the correlation coefficient (R^2) ranged from 0.993 for *ELF1A* to 0.999 for *ACT11* (Table 1). Melting curve analysis showed that all primer pairs matched one single soybean gene displayed without non-specific amplification, and these primer pairs were appropriate for application in all soybean samples.

Ct Value Analysis

The reference genes (*ELF1A*, *ELF1B*, *CYP2*, *UBC4*, *UBQ10*, *TUB4*, *G6PD*, *ACT2/7*, *ACT11*, and *CONS4*) expression levels were presented as Ct value (Fig. 1). For Sneb207 coated soybean, the variation range of *TUB4* gene expression was the smallest (1.87 cycles), and *UBC4* was the widest (6.36 cycles). For Liaodou15 soybean-nematode interactions, *UBQ10* showed the smallest gene expression variation (1.79 cycles), whereas the *ELF1A* showed the highest (3.58 cycles). Among the Liaodou15 soybean-Sneb207-nematode interactions, *TUB4* was the most stable (2.5 cycles) compared to *UBC4*, which was the least stable (7.67 cycles). Among the Kangxian12 soybean-nematode interactions, *ELF1A* was the most stable (0.98 cycles) while *ACT2/7* was the least stable (2.21 cycles). Similarly, the expression of *G6PD* and *ACT2/7* showed the lowest and highest variation values of 2.34 and 4.35 cycles, respectively, among the various-nematode interactions. In addition, the expression of *G6PD* and *UBC4* showed the lowest and the highest variation values of 5.96 and 8.56 cycles, respectively, among all soybean samples (Fig. 1).

geNorm Analysis

geNorm ranks the candidate reference genes on the basis of the M-value. M values below 1.5 indicated stable genes, and lower M values indicated increased stable expression (Vandesompele *et al.*, 2002). M values of all ten reference genes in all samples were less than 1.5, as displayed in Fig. 2. *TUB4* and *UBQ10*, respectively, were the most and least stable genes among the Liaodou15 soybean-nematode interactions and various-nematode interactions. Of the Sneb207-coated soybean and Kangxian12 soybean-nematode interaction samples, *ELF1A* was the most stable gene, while *UBC4* and *G6PD* were the least stable genes. Among the Liaodou15 soybean-Sneb207-nematode interactions, *ELF1B* and *UBC4* were the most and least stable genes, respectively. When all samples considered together, *ACT11* and *CONS4* were the most stable genes with a combined M value.

The demand of two or more reference genes is necessary for accurate normalization. The value of the pairwise variation (V_n/V_{n+1}) was no more than 0.15 (Gimeno *et al.*, 2014). The pairwise variation V_2/V_3 was lower than 0.15 among the Sneb207-coated soybean samples, Liaodou15 soybean-nematode interactions, Liaodou15 soybean-Sneb207-nematode interactions,

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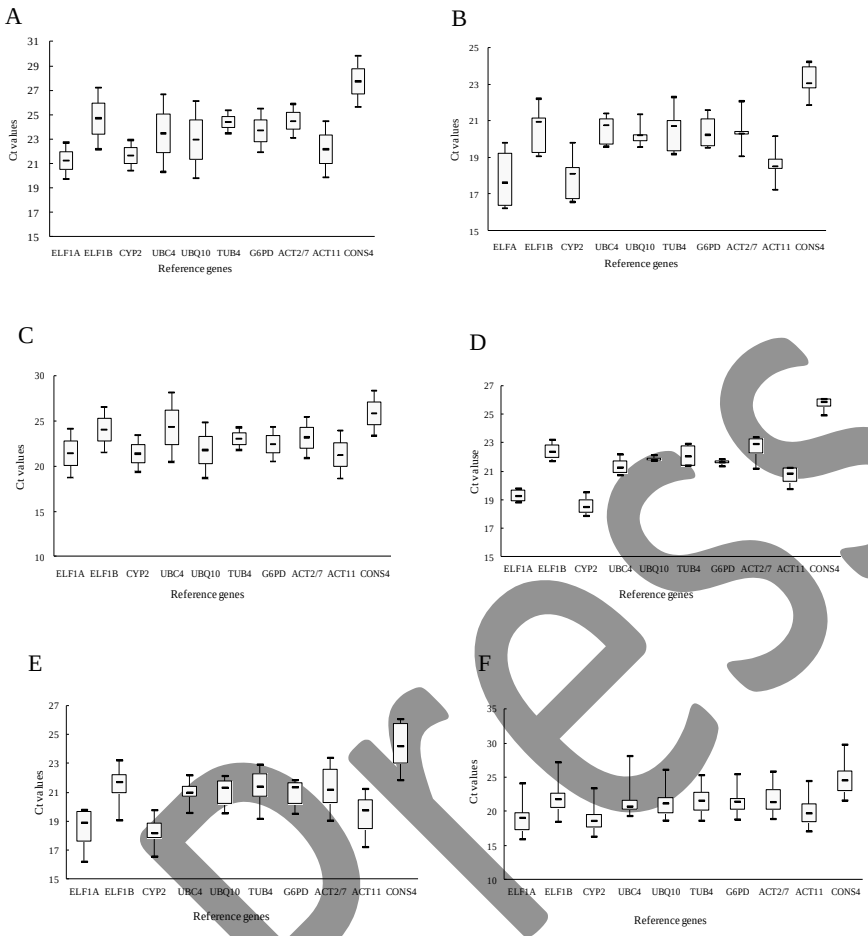

Fig. 1: Data statistics of Ct values of candidate reference genes in soybean. **A:** Sneb207 coated soybean. **B:** Liaodou15 soybean-nematode interactions samples. **C:** Liaodou15 soybean-Sneb207-nematode interactions samples. **D:** Kangxian12 soybean-nematode interactions samples. **E:** Various-nematode interactions samples. **F:** All conditions combined

Kangxian12 soybean-nematode interactions, and various-nematode interaction samples. Thus, the excellent reference gene combination was *ELF1A* and *ACT2/7*, *CYP2* and *TUB4*, *ELF1B* and *CONS4*, *ELF1A* and *UBC4*, and *ELF1B* and TUB4. When all samples were considered together, the pairwise variation V_2/V_3 was higher than 0.15 and V_3/V_4 was 0.15. Therefore, the three reference genes (*ACT2/7*, *ACT11* and *CONS4*) were sufficient for accurate normalization among all samples (Fig. 3).

NormFinder Analysis

To analyze the expression stability of the ten candidate reference genes using NormFinder showed that *CONS4* and *UBC4*, respectively, were the most and least stable reference genes among Sneb207-coated soybean (Table 2). The expression levels of *CYP2* (0.353) among Liaodou15 soybean-nematode interactions, and *ELF1B* (0.031) among Liaodou15 soybean-Sneb207-nematode interactions, *TUB4* (0.301) among various-nematode interactions were the most stable reference genes, consistent with the results of geNorm. Similarly, *ELF1A* (0.128) and *ACT2/7* (0.637) were the most and least stable genes among Kangxian12 soybean-nematode interactions. Additionally, we also discovered that *UBQ10* and *UBC4* were the least stable genes in most treatments according to NormFinder and consistent with the results obtained by geNorm. Furthermore, the expression of *ACT11* was the most stable reference gene across all soybean samples.

Best Keeper Analysis

The stability values of the ten reference genes were evaluated by BestKeeper in different situations. As shown in Table 3, *TUB4* was the most stable gene under Sneb207 coated soybean and Liaodou15 soybean-Sneb207-nematode interactions, *UBQ10* was the most stable under Liaodou15 soybean-nematode interactions, *G6PD* was the most stable under Kangxian12 soybean-nematode interactions and *UBC4* was the most stable under various-nematode interactions. *UBC4* was the least stable gene among Sneb207-coated soybean and Liaodou15

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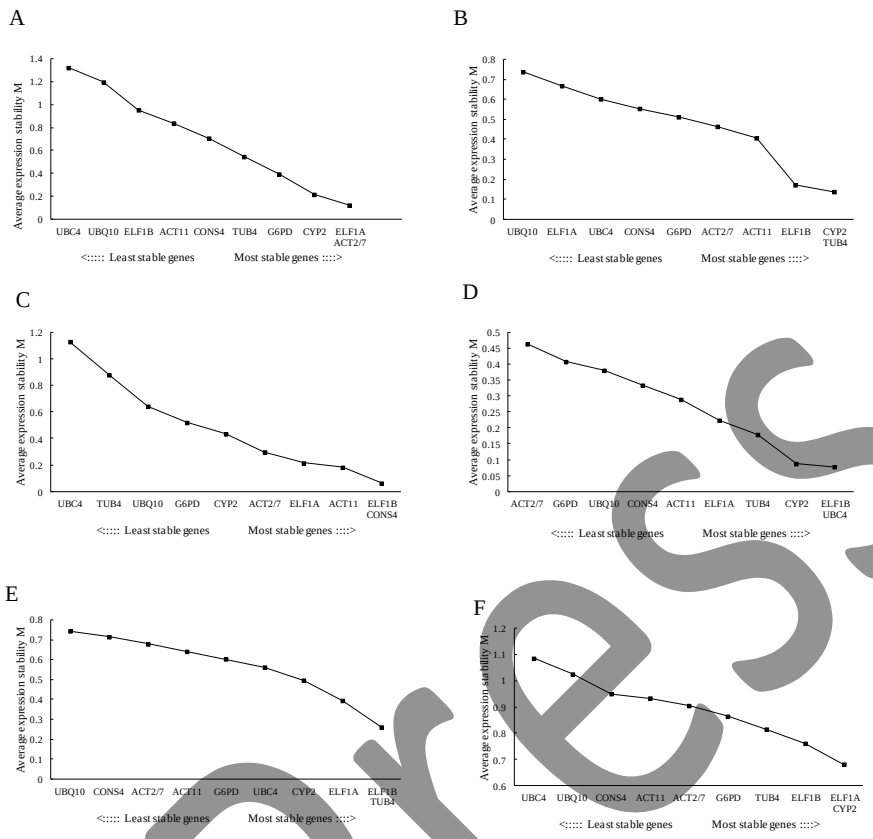

Fig. 2: Expression stability values (M) and ranking of the candidate reference genes as predicted by geNorm. Average expression stability values (M) were measured using stepwise exclusion of the least stable gene to organize candidate genes from the least to the most stable. **A:** Sneb207 coated soybean. **B:** Liaodou15 soybean-nematode interactions samples. **C:** Liaodou15 soybean-Sneb207-nematode interactions samples. **D:** Kangxian12 soybean-nematode interactions samples. **E:** Various-nematode interactions samples. **F:** All conditions combined

soybean-Sneb207-nematode interactions, consistent with the results produced by geNorm. In addition, *ACT2/7* were the least stable genes in various-nematode interactions and Kangxian12 soybean-nematode interactions. In all soybean samples, *G6PD* was the moste while *UBC4* was the least stable reference gene, respectively.

Ref Finder Analysis

The ranking orders generated by geNorm, NormFinder, BestKeeper, and RefFinder were not completely consistent in different treatments (Table 4). The comprehensive ranking of the six groups showed that *G6PD* and *CONS4* were the most stable genes among Sneb207 coated soybean, *CYP2* and *ELF1B* showed good performances among Liaodou15 soybean-nematode interactions, *ELF1B* and *CONS4* were the most stable genes among Liaodou15 soybean-Sneb207-nematode interactions, *ELF1A* and *UBC4* exhibited the most stable expression among Kangxian12 soybean-nematode interactions, and *TUB4* and *ELF1B* showed the most stable genes among various-nematode interactions. However, *ELF1A* was the least stable gene among Liaodou15 soybean-nematode interactions, *UBC4* and *UBQ10* were the least stable genes under most experimental conditions. Among all experiment treatments, *ACT11* and *G6PD* can be used as the optimal reference genes for normalization under in different situations.

Reference Gene Validation

For validation of the screened reference genes, the level of *PR2* gene expression was evaluated by RT-qPCR under nematode-infected Liaodou15 conditions. The results showed that two reference genes were good for normalization of Liaodou15 soybean-nematode interactions. The most stable reference genes (*CYP2*, *ELF1B*, *CYP2+ELF1B*) and the most unstable reference genes (*ELF1A*) were selected to normalize *PR2* gene expression. Under nematode-infected Liaodou15 conditions, *PR2* showed similar response patterns when normalized by the most stable reference genes (*CYP2*, *ELF1B*, *CYP2+ELF1B*): the expression level initially decreased, increased at 48h, and finally decreased, but varied when normalized to the least stable gene *ELF1A* (Fig. 4).

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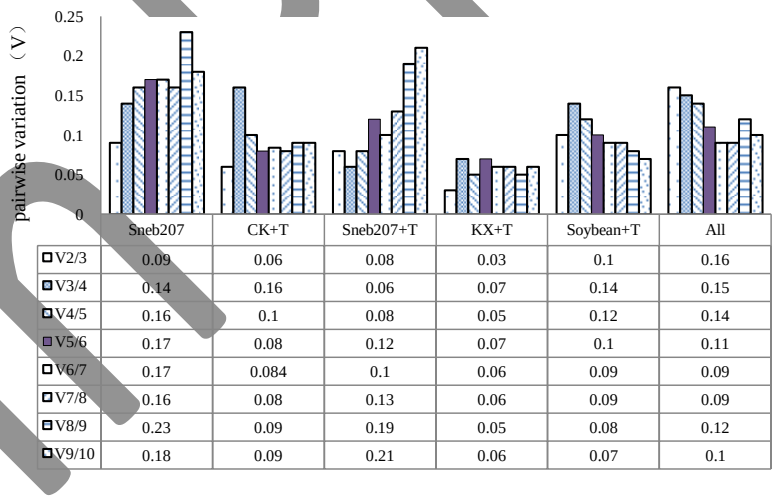
Table 2: Expression stability values for the ten candidate reference genes in the soybean samples as calculated using the NormFinder algorithm

Sneb207 coated soybean		Liaodou15 soybean-nematode interactions		Liaodou15 soybean-Sneb207-nematode interactions		Kangxian12 soybean-nematode interactions		various-nematode interactions		all soybean samples	
Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability
CONS4	0.156	*CYP2*	0.353	*ELF1B*	0.031	*ELF1A*	0.128	*TUB4*	0.301	*ACT11*	0.358
G6PD	0.212	*ELF1B*	0.355	*CONS4*	0.031	*ACT11*	0.237	*ELF1B*	0.331	*ELF1B*	0.396
ACT11	0.274	*G6PD*	0.358	*ACT11*	0.043	*CONS4*	0.269	*ACT11*	0.391	*G6PD*	0.478
ELF1B	0.714	*TUB4*	0.374	*ACT2/7*	0.125	*UBC4*	0.272	*G6PD*	0.426	*ACT2/7*	0.578
ELF1A	0.744	*ACT11*	0.399	*ELF1A*	0.161	*ELF1B*	0.274	*UBC4*	0.587	*CONS4*	0.632
ACT2/7	0.893	*ACT2/7*	0.493	*CYP2*	0.618	*TUB4*	0.295	*ACT2/7*	0.617	*ELF1A*	0.652
CYP2	1.132	*UBC4*	0.519	*G6PD*	0.798	*CYP2*	0.34	*ELFA*	0.63	*CYP2*	0.769
TUB4	1.665	*CONS4*	0.569	*UBQ10*	0.898	*UBQ10*	0.447	*CYP2*	0.634	*TUB4*	0.786
UBQ10	1.788	*ELFA*	0.897	*TUB4*	1.910	*G6PD*	0.449	*CONS4*	0.686	*UBQ10*	1.015
UBC4	1.796	*UBQ10*	0.926	*UBC4*	2.118	*ACT2/7*	0.637	*UBQ10*	0.702	*UBC4*	1.095

Table 3: Analysis of the ten reference genes by Bestkeeper algorithm

Sneb207 coated soybean		Liaodou15 soybean-nematode interactions		Liaodou15 soybean-Sneb207-nematode interactions		Kangxian12 soybean-nematode interactions		various-nematode interactions		all soybean samples	
Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability
TUB4	0.934	*UBQ10*	0.438	*TUB4*	1.25	*G6PD*	0.136	*UBC4*	0.619	*G6PD*	1.123
CYP2	1.258	*ACT2/7*	0.657	*G6PD*	1.927	*UBQ10*	0.137	*G6PD*	0.772	*TUB4*	1.396
ACT2/7	1.399	*UBC4*	0.685	*CYP2*	2.029	*CONS4*	0.38	*CYP2*	0.814	*ELF1A*	1.484
ELF1A	1.484	*ACT11*	0.714	*ACT2/7*	2.291	*ELF1A*	0.423	*UBQ10*	0.887	*CYP2*	1.531
G6PD	1.784	*CONS4*	0.730	*CONS4*	2.467	*UBC4*	0.505	*TUB4*	1.033	*UBQ10*	1.553
CONS4	2.098	*G6PD*	0.740	*ELF1B*	2.511	*ELF1B*	0.546	*ELF1B*	1.125	*ACT11*	1.584
ACT11	2.318	*TUB4*	0.997	*ACT11*	2.659	*ACT11*	0.558	*ACT11*	1.148	*ELF1B*	1.624
ELF1B	2.531	*CYP2*	1.015	*ELF1A*	2.72	*CYP2*	0.576	*ELF1A*	1.171	*ACT2/7*	1.667
UBQ10	3.173	*ELF1B*	1.089	*UBQ10*	3.087	*TUB4*	0.699	*CONS4*	1.251	*CONS4*	1.731
UBC4	3.179	*ELF1A*	1.335	*UBC4*	3.853	*ACT2/7*	0.714	*ACT2/7*	1.278	*UBC4*	1.778



	Sneb207	CK+T	Sneb207+T	KX+T	Soybean+T	All
V2/3	0.09	0.06	0.08	0.03	0.1	0.16
V3/4	0.14	0.16	0.06	0.07	0.14	0.15
V4/5	0.16	0.1	0.08	0.05	0.12	0.14
V5/6	0.17	0.08	0.12	0.07	0.1	0.11
V6/7	0.17	0.084	0.1	0.06	0.09	0.09
V7/8	0.16	0.08	0.13	0.06	0.09	0.09
V8/9	0.23	0.09	0.19	0.05	0.08	0.12
V9/10	0.18	0.09	0.21	0.06	0.07	0.1

Fig. 3: Pawise Variation (V) analysis of ten candidate reference genes in soybean samples. Pairwise variation (V_n/V_{n+1}) values were analyzed using the geNorm. When the pairwise variation (V_n/V_{n+1}) is less than 0.15, it is recommended that no additional genes are required for the normalization

Discussion

RT-qPCR is considered accurate molecular validation data techniques. The application of suitable reference genes to normalize RNA expression would make the results more reliable. Although some reference genes have been presented in potato nematodes (Castro-Quezada *et al.*, 2013), their usefulness in soybean under bacteria and SCN-infected plants remain less known to date. Analysis of these data showed that the expression stability of reference genes affected by bacteria, nematodes, and varieties. Therefore, a stable reference gene varies under different conditions, and more reliable test results can be obtained after verification before use.

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Table 4: Analysis of the ten candidate reference genes in the soybean samples as calculated using the RefFinder software program

Sneb207 coated soybean		Liaodou15 soybean-nematode interactions		Liaodou15 soybean-Sneb207-nematode interactions		Kangxian12 soybean-nematode interactions		various-nematode interactions		all soybean samples	
Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability
G6PD	2.51	*CYP2*	1.68	*ELF1B*	1.57	*ELF1A*	2.11	*TUB4*	1.5	*ACT11*	1.57
CONS4	2.91	*ELF1B*	3.03	*CONS4*	2.11	*UBC4*	2.78	*ELF1B*	2.21	*G6PD*	2.45
ELF1A	2.99	*TUB4*	3.22	*ACT11*	3.71	*ELF1B*	2.78	*UBC4*	3.34	*ELF1B*	3.44
ACT2/7	3.08	*ACT11*	4.23	*ACT2/7*	4.23	*ACT11*	4.28	*G6PD*	3.72	*CONS4*	3.87
CYP2	4.14	*ACT2/7*	4.36	*CYP2*	5.05	*CONS4*	4.58	*ACT11*	4.58	*ACT2/7*	3.22
TUB4	4.23	*G6PD*	4.82	*G6PD*	5.12	*G6PD*	5.2	*CYP2*	5.26	*TUB4*	5.09
ACT11	4.58	*UBQ10*	5.62	*TUB4*	5.2	*CYP2*	5.38	*ELF1A*	5.86	*ELF1A*	5.24
ELF1B	6.26	*UBC4*	6.05	*ELF1A*	5.32	*UBQ10*	5.66	*ACT2/7*	7.33	*CYP2*	6.51
UBQ10	9	*CONS4*	6.65	*UBQ10*	8.24	*TUB4*	6	*UBQ10*	7.95	*UBQ10*	7.77
UBC4	10	*ELF1A*	9.24	*UBC4*	10	*ACT2/7*	10	*CONS4*	9	*UBC4*	10.00

The ranking of selected ten genes was consistent between these programs, with slight differences. These results were consistent with similar findings (Choudhary *et al.*, 2016; Hou *et al.*, 2017). This variation could be because for geNorm, NormFinder, and BestKeeper analysis, the potential algorithms employed are different (Andersen *et al.*, 2004). It is better to estimate reference genes with multiple methods.

RefFinder produced an overall credible final ranking. *ACT11* and *G6PD* were observed as the most useful in all samples. *ELF1B* was demonstrated as a reliable reference gene for soybean-Sneb207-nematode and various nematode interactions, and our outcome, confirmed by other researchers (Hu *et al.*, 2009). Further, the present results showed that *UBQ10* was not good as a reference gene for soybean under single or multiple stresses. *UBQ10* was the least stable gene in cell types and different tissues at variant development stages in rice. Thus, in order to truly study the expression level of the target gene, it is necessary to determine which stable reference genes are used according to the actual situation.

PR2 has β-1,3-glucanase activity. The expression level of *PR2* was assessed in nematode-infected Liaodou15 plants to validate the selected reference genes. The expression levels of the target genes were consistent when the genes CYP2, ELF1B and CYP2+ELF1B were used. However, some divergence was observed in the expression level, which were normalized by the most unstable candidate reference gene *ELF1A*. Therefore, the results indicated that unstable reference gene used for normalization might lead to biased results.

Conclusion

Validation of reference genes in soybean hereby demonstrated that *ACT11*, *G6PD*, *ELF1B*, and *CONS4* could be used as stable reference genes in the interactions of different soybean varieties with *H. glycines* and additional *Bacillus*. This study opens the first door to evaluate the reference genes in soybean roots infected with SCN and *Bacillus*. Moreover, the results in the present study provided a more maximum choice in gene analysis and functional studies in nematode-infected soybean.

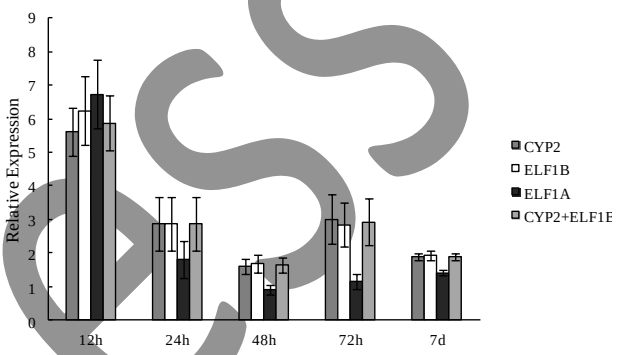

Fig. 4: The expression level of the *PR2* gene in nematode-infected Liaodou15 plants by using different reference genes for normalization. The error bars represent standard errors

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