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Validation of reference genes for quantitative gene expression analysis in *Ralstonia pseudosolanacearum* CQPS-1 under environment stress

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ABSTRACT

Quantitative real-time reverse transcriptase PCR (qRT-PCR) has become the method choice for quantification of gene expression changes, however, the accuracy of the method depends on the stability of reference genes. *Ralstonia pseudosolanacearum* (*R. pseudosolanacearum*) is an important plant pathogen, infecting > 450 plant species and causing bacterial wilt. In order to identify stable reference genes in *R. pseudosolanacearum* CQPS-1 under different environment stresses. We used five tools ($^{\triangle}$*Ct* method, *GeNorm*, *NormFinder*, *BestKeeper*, and *RefFinder*) to evaluate the stability of seven candidate reference genes including phosphoglycerate kinase (*PGK*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH),* 16S ribosomal RNA (*16S),* cell division protein *ftsZ* (*ftsZ*), DNA gyrase subunit A (*gyrA)*, Ribosomal protein L13 (*rplM*), and phosphoserine aminotransferase (*serC*) under biotic (growth phases) and abiotic stress (temperature, hydroxycoumarins, nutrition). Overall, *gyrA* and *serC* were the most stable genes under different growth phases, while *serC*, *gyrA* and *ftsZ* during temperature stress, *gyrA*, *ftsZ* and *16S* under hydroxycoumarins stress, and *serC* and *16S* under nutrition stress conditions. This study provides useful resources for normalizing expression changes of target genes in *R. pseudosolanacearum* subjected to environment stress.

1. Introduction

Ralstonia pseudosolanacearum (*R. pseudosolanacearum*) represents one of the most devastating plant bacterial pathogens among the top ten plant pathogens, heavily infecting > 450 plant species and causing seriously bacterial wilt worldwide (Genin, 2010; Mansfield et al., 2012). Recent studies showed that *R. pseudosolanacearum* would well adapt to the variously changing environmental conditions, including low PH, high temperature and high humidity (Cai et al., 2015; Jiang et al., 2017). In response to acidic conditions, the expression of virulence-associated genes (*popA*, *prhA* and *solR)* in *R. pseudosolanacearum* supplemented with PH 5.5 values was much higher than that other tested PH values (Li et al., 2017). Meanwhile, plant-derived compounds hydroxycoumarins were demonstrated suppressing the expression of virulence-associated genes (Yang et al., 2016). In order to decipher the processes triggered by *R. pseudosolanacearum* to adapt to different environment conditions, the molecular mechanisms involved in these processes can be elucidated by gene expression analysis. Quantitative real-time reverse transcriptase PCR (qRT-PCR) represents a specific, highly sensitive and reproducible technique for analyzing gene expression (Bustin, 2002; Bustin and Nolan, 2004). However, the accurate determination of relative expression of target gene requires normalization using a reference gene who should have ubiquitous expression, low variance, and reasonable stability under the experimental conditions.

The stability of reference genes in many bacteria under different growth phases and abiotic stress conditions has been validated. For example, 16S ribosomal RNA gene (*16S*) was the most stable candidate reference gene in *Clostridium botulinum* Group I strain ATCC 3502 under different growth phases (Kirk et al., 2014), the expression of three genes (*rho*, *23SrRNA* and *rpoD*) was stable throughout the growth of *Gluconacetobacter diazotrophicus* in different carbon sources (Galisa et al., 2012). Moreover, CTP synthetase (*pyrG*) had relatively stable in *Burkholderia pyrrocinia* JK-SH007 (Florindo et al., 2012). The stability of reference genes in some fungus also have been evaluated. The elongation factor 1 gene (*EF-1*) was identified as the most stably expressed reference gene in *Trichoderma afroharzianum* under oxalic acid stress (Lyu et al., 2017). While, ubiquitin (*UBQ*) reported as the most stably expressed in *Botrytis cinerea* under the same stress (Ren et al., 2017). From these studies, the stability of reference genes may greatly differ in various conditions or under the same conditions among different species. Recent studies showed that *serC* and *rplM* were used as the reference genes to normalize the transcription expression of *hrpB* and type III effector in *R. pseudosolanacearum* (Monteiro et al., 2012). However,

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the suitable reference genes used in *R. pseudosolanacearum* under different environment stresses were still largely unknown.

In this study, five candidate housekeeping genes including phosphoglycerate kinase (*PGK*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH),* 16S ribosomal RNA (*16S),* cell division protein *ftsZ* (*ftsZ*), DNA gyrase subunit A (*gyrA)*, and two reported reference gene Ribosomal protein L13 (*rplM*) and phosphoserine aminotransferase (*serC*) (Monteiro et al., 2012), were selected to validate gene expression stability under different biotic (growth phases) and abiotic stress (temperature, hydroxycoumarins, nutrition). Then, we used $^{\triangle}Ct$ method (Silver et al., 2006), *GeNorm* (Vandesompele et al., 2002), *NormFinder* (Andersen et al., 2004), *BestKeeper* (Pfaffl et al., 2004) to analyze the data. Finally, the most stable reference genes was validated by comprehensive ranking, which was carried out based on the Web-based analysis tool *RefFinder* (http://150.216.56.64/referencegene.php?type = reference) (Yang et al., 2014).

2. Materials and methods

2.1. Bacterial strains

The *R. pseudosolanacearum* wide-type strain CQPS-1 (phylotype I, race 1, biovar 3) used in this study were originally isolated from an infected tobacco plant in Chongqing, China in 2013 (Liu et al., 2017). *R. pseudosolanacearum* was incubated in rich B medium or M63 medium at 30 °C for shaking with 180 rpm/min.

2.2. Biotic factors (growth phases of bacteria)

To evaluate the stability of reference gene expression under different growth phases, the strain was incubated in rich B medium at 30 °C with shaking (180 rpm) for 4 h, 8 h, 10 h, 12 h, and 24 h. Bacterial cells were collected by centrifuged at 5000 rpm for 10 min at 4 °C and frozen in liquid nitrogen for RNA extraction.

2.3. Abiotic stress treatments

2.3.1. Temperature stress

The *R. pseudosolanacearum* was incubated in the B medium at 30 °C with 180 rpm for 7 h, exposed to high temperature (35 °C) or low temperature (4 °C) for 1 h in a temperature controlled incubator (LAC-250HPY-2, LongYue Instrument Equipment Co. Ltd., Shanghai, China). After treated with different temperature, the bacteria cells were collected and frozen immediately in liquid nitrogen for RNA extraction.

2.3.2. Plant-derived compounds stress

To investigate the reference genes expression under plant-derived compounds stress, *R. pseudosolanacearum* was inoculated in M63 medium supplemented with 100 mg L^{-1} hydroxycoumarins (umbelliferone, esculetin, and daphnetin) or DMSO, then incubated at 30 °C with shaking at 180 rpm for 6–7 h. The samples were centrifuged at 5000 rpm for 10 min at 4 °C and frozen immediately in liquid nitrogen for RNA extraction.

2.3.3. Nutrition stress

R. pseudosolanacearum was reported to be well adapted to plant xylem condition, considered as a nutrient-limiting, low-oxygen environment (Jacobs et al., 2012; Lowe-Power et al., 2017). Nutrition rich B medium and minimal medium M63 were used to evaluated the reference gene expression under nutrition stress. Briefly, *R. pseudosolanacearum* was incubated in rich B medium or M63 medium for 6–7 h. The bacteria cells were collected and frozen immediately in liquid nitrogen for RNA extraction.

2.4. RNA isolation and reverse transcription

Total RNA was isolated from the collected bacteria cells using the TRNzol reagent according to the manufacturer's instructions (Tiangen Biotech Co. Ltd., Beijing, China). RNA concentration and purity were quantified using the Thermo spectrophotometer (Thermo Fisher Scientific Oy, Vantaa, Finland), and the integrity of RNA was checked on 1% agarose gels by electrophoresis.

The reverse transcription was performed using the iScript gDNA clear cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Briefly, all reaction volumes were 16 μL, contained 1 μg of RNA, 1.5 μL of reverse transcription buffer, 0.5 μL of iscript DNase, and 13 μL RNase-free H_2O. The mix was first processed on C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) at 25 °C for 5 min, 75 °C for 5 min to remove any genomic DNA contaminations. Then 4 μL of iScript Reverse Transcription Supermix was added, and the reverse transcription reaction was performed at 25 °C for 5 min, 46 °C for 20 min, 95 °C for 1 min. After reacting, the cDNA was diluted 1:4 to get a concentration of 200 ng/μL. All qRT-PCR experiments were performed with the same batch of cDNA.

2.5. Selection and validation of reference genes and primer design

Full-length sequences of the five candidate housekeeping genes from *R. pseudosolanacearum* CQPS-1 including phosphoglycerate kinase (*PGK*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 16S ribosomal RNA (*16S*), cell division protein *ftsZ* (*ftsZ*), DNA gyrase subunit A (*gyrA*), and two reported reference gene for *R. pseudosolanacearum* including Ribosomal protein L13 (*rplM*) and phosphoserine aminotransferase (*serC*), were selected to assess the expression stability (Monteiro et al., 2012). All primers for RT-PCR were designed using the Primer 3 software (http://bioinfo.ut.ee/primer3/, Table 1). The primers were checked with Primer 5 for their potential to form secondary structures, which allows to discard those forming these hairpins. PCR reactions were performed in technical triplicates.

3. qRT-PCR

All quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis of reference gene expression were performed using a CFX96 manager (Bio-Rad, Hercules, CA, USA). Each 20-μL qRT-PCR reaction system contained 10 μL of Sso Fast ™ EvaGreen Supermix (Bio-Rad), 7 μL of nuclease-free water, 0.2 mM of each primers and 1 μL of the cDNA. The amplification efficiency for each candidate reference gene was calculated by the slope of its standard curve (Table 1), which was generated using six 5-fold dilutions of the same cDNA samples (1, 1/5, 1/25, 1/125, 1/625, and 1/3125). The PCR parameters were as follows: 95 °C for 3 min, then 40 cycles at 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. After that, a melting curve from 60 to 95 °C was applied to test the specificity and consistency of the PCR products.

3.1. Analysis of reference gene expression stability

After qRT-PCR, Ct values of reference genes were exported from the CFX96 Manager (Strata gene) to Microsoft Excel. The stability of seven candidate reference genes was evaluated by the comparative $^{\triangle}Ct$ method (Silver et al., 2006), *GeNorm* (Vandesompele et al., 2002), *NormFinder* (Andersen et al., 2004), *BestKeeper* (Pfaffl et al., 2004), followed by a comprehensive ranking by *RefFinder*.

As stated by $^{\triangle}Ct$ method, the gene with the lowest ASD value (the average standard value) is considered as the most stable. *GeNorm* calculates the average expression value (M value) of all genes, and gene showing the lowest M value is presumed to be the most stably expressed reference gene. In addition, *GeNorm* calculates the pairwise variation $V_{n/n+1}$ between each combination of sequential normalization factors allowing the identification of the optimal number of reference genes for

Table 1
Primers for reference genes derived from quantitative Real-Time PCR (qRT-PCR) data analysis.

Gene name	Gene ID	Primer sequences (5′-3′)	Amplicon size (bp)	Amplification efficiency (%)	R^2
PGK	BC350_03905	F:TTCAAGCCGGAAGATTCGCT R:GCTGTTCTTCTTCTCGCCCT	165	113.5	0.997
GAPDH	BC350_10395	F:TGAAGTACGACTCGGTGCAC R:CGCCGACATGATCACCTTCT	230	118.2	0.993
16S	BC350_10325	F:TATCGATTCACCGCTTGCC R:GGTATCGGGTTGTGTGGCAT	199	119.8	0.998
ftsZ	BC350_09950	F:TCAAGCTGTCCGAGACCAAG R:GTCATGGTCTGCTGCTGTTG	181	117.7	0.998
gyrA	BC350_01710	F:ACCGAAGACCTCATTACGCC R:CCCACACCTTGAGCCAGTAG	229	112.5	0.995
rplM	BC350_04340	F:GCCAAGACGACCGACAAGAA R:GGCGTACACCTTCAGCTTCT	180	109.2	0.999
serC	BC350_01700	F:GTGCCCGACAACTACGAGAT R:CACTTCACCGTATTTGCGCG	174	113.5	0.997

Abbreviations: *PGK*, phosphoglycerate kinase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *16S*, 16S ribosomal RNA; *ftsZ*, cell division protein *ftsZ* (alpha tubulin); *gyrA*, DNA gyrase subunit A; *rplM*, Ribosomal protein L13; *serC*, phosphoserine aminotransferase.

accurate normalization, with $V_{n/n+1}$ under 0.15 indicating that no additional genes are required for normalization. *NormFinder* uses a Microsoft based algorithm to estimate expression variation among all reference genes and calculates the stability values (SV). The gene of lowest SV is considered as the most stable reference gene. *BestKeeper* calculates the standard deviation (SD) of Ct values for the reference genes, and the lowest SD value is considered as the most stable reference genes. At last, *RefFinder* is a web-based interface that is used to deduce the most stable gene candidate among comprehensive results of the four commonly used programs: GeNorm, NormFinder, BestKeeper, $^{\triangle}Ct$ method.

4. Results

4.1. Primer specificity and amplification efficiency of seven reference genes

In this study, to evaluate gene expression stability of all seven reference genes in *R. pseudosolanacearum*, mRNA expression levels of all tests genes were measured by qRT-PCR. As shown in Table 1, the melt curve analysis confirmed that the primers amplified a unique product that ranged in size from 165 to 230 bp. The amplification efficiency ranged from 109.2% (*rplM*) to 119.8% (*16S*) and regression coefficient (R^2) ranged from 0.993 (*GAPDH*) to 0.999 (*rplM*).

4.2. General patterns of gene expression

The raw CT value was used to analysis the expression level of seven candidate reference genes. The mean CT value of seven genes ranged from 28.68 (*rplM*) to 32.33 (*16S*), which indicates that *rplM* was the most abundantly expressed gene, while *16S* was the lowest expressed one (Fig. 1). Seven reference genes ranging by increasing SDs was *ftsZ*, *gyrA*, *serC*, *rplM*, *GAPDH*, *16S*, *PGK*. Based on SD, the lowest variability in expression among all samples was observed for *ftsZ* (SD = 1.23), while the most variable expression was for *PGK* (SD = 2.76).

4.3. The gene expression stability of R. pseudosolanacearum under different growth phases

The expression stability of candidate reference genes under different growth phases was evaluated by four analysis tools. As stated by $^{\triangle}Ct$ methods, *GeNorm* and *NorFinder*, *GAPDH* and *gyrA* were the most stable genes. *16S* and *rplM* were identified as the least stable genes consistently by the four methods (Table. 2). However, the ranking of reference genes by *BestKeeper* revealed that *PGK* was the most stable gene, which differed from the results generated by the other methods. Based on the *RefFinder* rankings, the seven genes was ranked by decreasing stability as *GAPDH*, *gyrA*, *serC*, *PGK*, *ftsZ*, *16S*, and *rplM* (Fig. 2). Additionally, GeNorm analysis suggested that only pairwise variation values of $V_{2/3}$ was 0.144, which was below 0.15 (Fig. 3). As demonstrated above, the two stable reference genes (*gyrA* and *serC*) were required for optimal normalization under different growth phases.

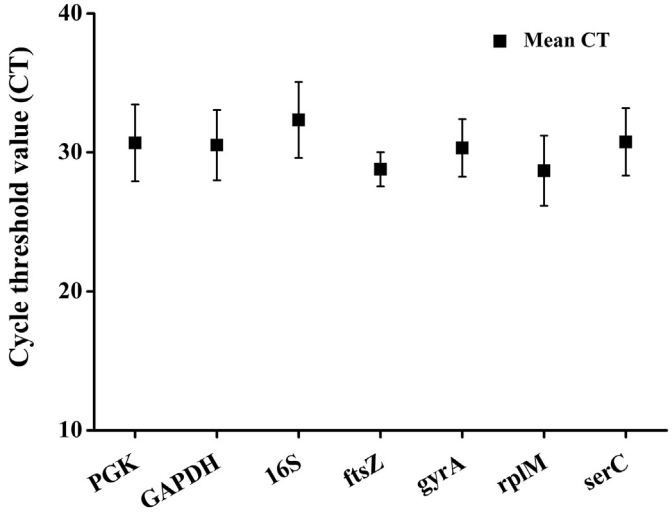

Fig. 1. Expression profiles of candidate reference genes in *R. pseudosolanacearum* CQPS-1 under different environment stresses. Black boxes indicate mean cycle threshold values of samples, and bars indicate SDs.

4.4. Expression stability under temperature stress

Based on the calculation by three programs, including $^{\triangle}Ct$ methods, *GeNorm* and *NorFinder*, showed that *gyrA serC* and *ftsZ* were the most stable genes, while *GAPDH* and *rplM* was the least stable. *BestKeeper* identified *rplM* as the most stable gene and *ftsZ* as the second most stable gene (Table. 2). *RefFinder* showed that the order of stability ranking from most stable to the least stable gene was *gyrA*, *serC*, *ftsZ*, *PGK*, *rplM*, *16S*, *GAPDH*. According to the results of *GeNorm*, all pairwise variation values were < 0.15 and $V_{3/4}$ was the lowest with 0.073 (Fig. 3). As illustrated above, a combination of *serC*, *gyrA* and *ftsZ* should be used for optimal normalization of gene expression studies under various temperature treatments.

4.5. Expression stability under hydroxycoumarins stress

The expression stability under hydroxycoumarins stress was listed in Table 2, and all four analysis programs identified that *PGK* was the

Table 2
Ranks of the reference genes based on expression stability under different stress conditions.

Experimental conditions	Reference gene	△Ct methods		GeNorm		NorFinder		BestKeeper	
		Stability	Ranking	Stability	Ranking	Stability	Ranking	Stability	Ranking
Growth phases	*PGK*	1.011	5	0.788	5	0.664	4	0.823	1
	GAPDH	0.812	1	0.505	3	0.095	1	1.133	3
	16S	1.153	6	0.851	6	0.982	6	1.527	6
	ftsZ	1.009	4	0.73	4	0.679	5	0.872	2
	gyrA	0.825	2	0.386	1	0.35	2	1.342	4
	rplM	1.504	7	1.037	7	1.39	7	1.553	7
	serC	0.947	3	0.386	1	0.612	3	1.494	5
Temperature stress	*PGK*	0.613	4	0.319	3	0.327	3	0.621	3
	GAPDH	1.033	7	0.728	7	1.01	6	1.722	7
	16S	0.768	5	0.476	4	0.612	4	1.432	6
	ftsZ	0.609	3	0.306	2	0.326	2	0.595	2
	gyrA	0.524	1	0.103	1	0.051	1	0.932	4
	rplM	1.011	6	0.606	5	0.985	5	0.135	1
	serC	0.536	2	0.103	1	0.051	1	1	5
Hydroxycoumrins stress	*PGK*	0.979	6	0.649	5	0.773	6	1.155	6
	GAPDH	0.645	1	0.418	3	0.194	1	0.716	5
	16S	0.689	3	0.361	1	0.386	3	0.68	4
	ftsZ	0.781	4	0.361	1	0.602	5	0.563	3
	gyrA	0.652	2	0.397	2	0.23	2	0.452	1
	rplM	1.321	7	0.841	6	1.234	7	1.423	7
	serC	0.823	5	0.509	4	0.551	4	0.474	2
Nutrition stress	*PGK*	0.873	5	0.339	3	0.811	6	0.57	1
	GAPDH	0.788	4	0.615	5	0.444	4	1.635	6
	16S	0.688	3	0.189	1	0.488	5	0.81	2
	ftsZ	0.604	1	0.271	2	0.135	1	1.105	4
	gyrA	0.788	4	0.516	4	0.432	3	1.382	5
	rplM	1.313	6	0.814	6	1.28	7	2.242	7
	serC	0.646	2	0.189	1	0.38	2	0.868	3

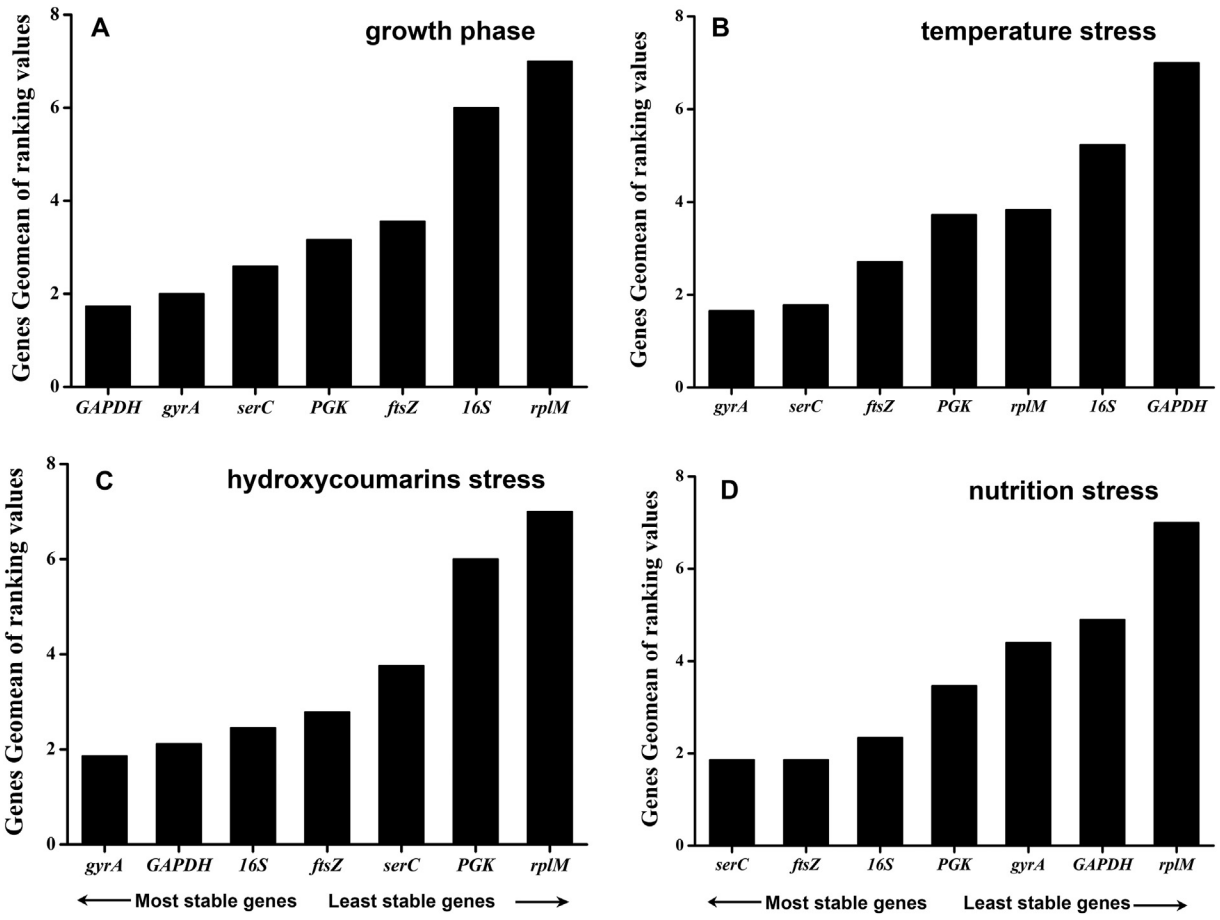

Fig. 2. Expression stability of seven candidate reference genes of *R. pseudosolanacearum* adapted to different environment stress calculated by *RefFinder*. (A) Different growth phases, (B) Temperature stress, (C) Hydroxycoumarins stress, (D) Nutrition stress.

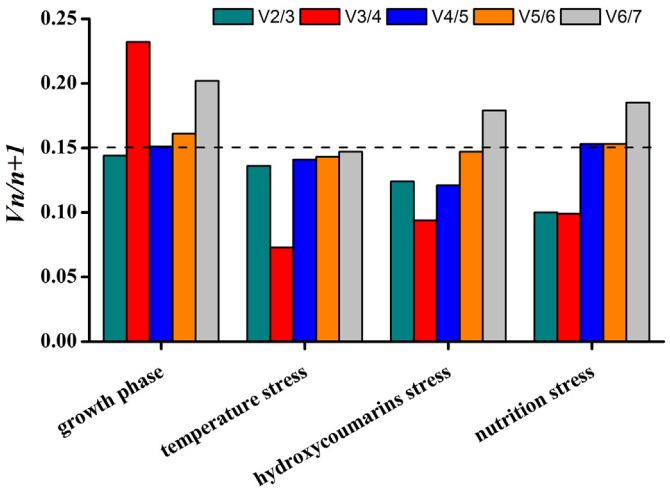

Fig. 3. The optimal number of reference genes required for accurate normalization in different sample groups was determined by calculating pairwise variation V_n/V_{n+1} using *GeNorm*. The value of V_n/V_{n+1} indicates the pairwise variation between two sequential normalization factors and determines the optimal number of reference genes required for accurate normalization.

least stable gene. *gyrA* was the most stable gene identified by the *BestKeeper*, and $^{\triangle}Ct$ methods, *GeNorm* and *NorFinder* identified it as the second most stable gene. *RefFinder* ranked the genes from the most to the least stable gene with *gyrA* being the most stable, followed by *GAPDH*, *16S*, *ftsZ*, *serC*, *PGK*, and *rplM*. *GeNorm* showed that the V3/4 was the lowest with 0.094, lower than 0.15 (Fig. 3). Thus, three candidate reference genes (*gyrA*, *ftsZ*, and *16S*) were required for suitable normalization of gene expression studies under hydroxycoumarins.

4.6. Expression stability under nutrition stress

As shown in Table 2, all four analysis tools identified the least stable genes *rplM* and *GAPDH*, while *serC* and *ftsZ* were the most stable genes identified by all methods, except *BestKeeper*, which identified *PGK* and *16S* as the most stable genes. The stability ranking from the most stable gene to the least stable gene calculated by *RefFinder* was *serC*, *ftsZ*, *16S*, *PGK*, *gyrA*, *GAPDH*, *rplM*. According to the results of *GeNorm*, V2/3 and V3/4 were similar near 0.10, lower than 0.15 (Fig. 3). Therefore,*serC* and *16S* would be required for optimal normalization of gene expression patterns under nutrition stress conditions.

5. Discussion

Identification of a suitable reference gene should be required to normalize the gene expression using the qRT-PCR. An increasing number of studies have focused on validating the stability of reference genes in bacteria under different experimental conditions (Kirk et al., 2014; Lyu et al., 2017; Metcalf et al., 2010). In this study, we used five tools ($^{\triangle}Ct$ method, *GeNorm*, *NormFinder*, *BestKeeper*, and *RefFinder*) to evaluate the stability of seven candidate reference genes from *R. pseudosolanacearum* strain CQPS-1 under different biotic and abiotic stress conditions. The results showed that *gyrA* and *serC* were the most stable reference genes under almost all experimental stress conditions. Many previous studies have demonstrated that *gyrA* had stable expression in some experimental conditions, such as different cultural growth conditions in plant pathogen *Pectobacterium atrosepticum* (Takle et al., 2007), different carbon fixation and solvent adaptation in *Clostridium ljungdahlii* DSM 13528 (Liu et al., 2013), different starvation or stress conditions in *Azospirillum brasilense* (McMillan and Pereg, 2014), different growth conditions in *Xanthomonas citri* Subsp. *citri* (Jacob et al., 2011). *SerC* was reported to be used as reference gene to normalize the expression of *hrpB* and type III effector transcripts in *R. pseudosolanacearum* (Monteiro et al., 2012). In agreement with these observations, our results showed that *SerC* and *gyrA* were the most stable genes across different growth phases, temperature and nutrition stress.

rplM and *GAPDH* have previously been selected as promising reference genes in *R. pseudosolanacearum* (Monteiro et al., 2012) and *Trichoderma afroharzianum* gene expression (Lyu et al., 2017) respectively. Our results showed that *rplM* and *GAPDH* displayed unacceptable variations in response to certain treatment and were poorly stable in *R. pseudosolanacearum* under different growth phases, hydroxycoumarins stress and nutrition stress. *GAPDH* also was the least stable gene under many experimental conditions in plants or insects, such as various abiotic stresses in orchard grass (Huang et al., 2014), and different ages in *Bombus terrestris* (Hornakova et al., 2010). *rplM* was previously used as reference gene to quantified the virulence-associated expression in bacteria (Monteiro et al., 2012). Although *GAPDH* encodes the glyceraldehyde-3-phosphate dehydrogenase, a key enzyme involved in energy metabolism (Scharlaken et al., 2008), our results indicated that *GAPDH* and *rplM* were not suitable reference genes for *R. pseudosolanacearum*.

Recent studies have demonstrated that more than one reference gene is required normalize expression of target genes, due to one reference gene may not be stable enough to normalizing of target gene expression (Tricarico et al., 2002). Certain studies have shown that experimental conditions could significantly affect the expression stability of reference genes (Huang et al., 2016; Liu et al., 2013). In this study, the expression stabilities of seven candidate genes were ranked differently evaluated by *GeNorm*. *gyrA* (M = 0.386) and *serC* (M = 0.386) were the most stable genes under different growth phases, *serC* (M = 0.103), *gyrA* (M = 0.103) were most stable reference genes during temperature stress, *ftsZ* (M = 0.361) and *16S* (M = 0.361) were most stable reference genes under hydroxycoumarins stress, and *serC* (M = 0.189) and *16S* (M = 0.189) were most stable under nutrition stress conditions. Previous research proved that the use of too few or too many reference genes may be inaccurate for data normalization (Ling and Salvaterra, 2011). Our findings show that the number of reference genes best suited for gene expression studies differed under different environmental stress conditions (Fig. 3). This is the first study to validate reference genes for gene expression analysis under different environmental stress conditions in *R. pseudosolanacearum*.

The *Ralstonia pseudosolanacearum* species complex include thousands of strains and can be subdivided into four phylotypes with high genetic variation. Initial genomic studies revealed that *R. solanacearum* strains have a substantial backbone of common house-keeping genes and virulence functions. The pangenome is composed of the core-genome (genes present in all strains), the dispensable genome (genes present in some strains, but not all) and the specific genome (unique genes present in only one train) (Medini et al., 2005). Moreover, due to the last *R. solanacearum* comparative genomics research, the seven reference genes used in this study are part of the core-genome (Remenant et al., 2010; Remenant et al., 2011; Prior et al., 2016). It indicated that the most stable genes *SerC* and *gyrA* could be useful for the whole *R. solanacearum* species complex, and the stable of reference gene need further verification when placed in various metabolic and stress conditions. Further, the selected reference genes *SerC* and *gyrA* could use for target gene monitoring during plant pathogenesis or competition between strains.

6. Conclusion

In conclusion, we used five tools ($^{\triangle}Ct$ method, *GeNorm*, *NormFinder*, *BestKeeper*, and *RefFinder*) to evaluate the stability of seven candidate reference genes from *R. pseudosolanacearum* strain CQPS-1 under environmental stress. The results indicated that *gyrA* and *serC* were the most stable reference genes under almost all experimental stress conditions. *gyrA* and *serC* were the most stable genes under different

growth phases, *serC*, *gyrA* and *ftsZ* were most stable reference genes during temperature stress, *gyrA*, *ftsZ* and *16S* were most stable reference genes under hydroxycoumarins stress, and *serC* and *16S* most stable under nutrition stress conditions. *rplM* was the least stable reference gene in almost all the experimental stress, which indicated that it should not be used as a reference gene to study gene expression in *R. pseudosolanacearum*.

Authors and contributions

WD, LY conceived and designed the experiments. LY, YL, SZ performed the experiments. JC, SL, LY analyzed the data. LY, WD wrote and revised the paper.

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Interest statement

We declare that no competing interests exist.

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