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Validation of reference genes for RT-qPCR analysis in *Burkholderia pyrrocinia* JK-SH007

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Abstract: *Burkholderia pyrrocinia* strain JK-SH007 isolated from poplar stems plays a highly significant role in the growth promotion and the biocontrol of poplar canker during colonization in poplar. In this research, the ideal reference gene was filtered and determined for the transcript normalization. Additionally, the expression of *pyrG* under all four conditions was relatively stable in *B. pyrrocinia* JK-SH007.

Key words: *Burkholderia pyrrocinia*; RT-qPCR; Reference genes; Expression stability

Currently, RT-qPCR is one of the most widely used methods for gene expression analysis in distinct biological systems, such as bacteria (Ritz et al., 2009) and plants (Doshi et al., 2007). It is critical to screen stable reference genes for the accuracy of this technology. An ideal reference gene should show similar expression levels in different experimental conditions. Selecting invalidate genes to normalize target gene expression will result in the incorrect interpretations of results.

In earlier studies, the endophytic bacterium *Burkholderia pyrrocinia* JK-SH007 was isolated from a poplar stem, and it showed strong growth-promoting and antifungal activities (Ren et al., 2011). In this article, the expression levels and stability of the candidate reference genes (*gyrB*, *ftsZ*, *pyrG*, *rpoB*, *atpD*, *thyA*, *recA* and 16S rDNA) (Florindo et al., 2012, Galisa et al., 2012, Liu et al., 2013, Metcalf et al., 2010, Nieto et al., 2009, Reiter et al., 2011, Thomas et al., 2011, Zhou et al., 2011) were studied with RT-qPCR under diverse cultural conditions (time, pH, temperature and different concentrations of saline).

According to published references, eight candidate reference genes were selected and primers were designed for RT-qPCR by Primer 3.0 (Table 1). The specific primers were selected with the PCR amplification. The PCR was performed as follows: pre-denaturation at 94 °C for 5 min, followed using 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s; and then final extension step at 72 °C for 7 min. In order to verify those specific reference gene primers, the cDNA of *B. pyrrocinia* JK-SH007 was used for PCR and RT-qPCR as a template. Results showed that the

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PCR products were with expected size and the primer specificity was proved (Fig. 1). The RT-qPCR was executed using a SYBR[®] Premix Ex Taq[™] kit (TaKaRa), with gene-specific primers (Table 1). Sequential 10-fold dilutions of cDNA with the Easy dilution (TaKaRa) were used to measure the standard curves to find out the relevant amplification efficiency (*E*) and nonspecific products. For each mRNA sample, the housekeeping gene and inducible gene content were analyzed using the comparative Ct method ($2^{-\Delta\Delta C_t}$).

To determine the optimal choice of reference genes, 12 samples were divided based on four factors and each factor has three treatments (pH: 4, 6, 8; Time: 12 h, 24 h, 36 h; Temperature: 25, 30, 37; Salt stress: 1 %, 0.5 %, 0.1 %). Each experiment only focused on one factor. All cultures were transferred on NA medium (Ren et al., 2011) and incubated at 30 °C with shaking (200 rpm) for 24 h. For pH stress, a pure culture of *B. pyrrocinia* JK-SH007 was incubated on NA medium with the pH adjusted to 4.0, 6.0 or 8.0. Temperature stress was applied by incubating the culture at 25 °C, 30 °C or 37 °C with shaking (200 rpm) for 24 h, respectively. For salt stress, the culture was incubated on NA medium supplemented with 0.1 %, 0.5 %, 1.0 % NaCl. To different developmental stages, the culture was incubated on NA medium at 30°C with shaking (200 rpm) for 12 h, 24 h, and 36 h, respectively. The broths from each experiment were collected, quickly frozen in liquid nitrogen and stored at -80 °C prior to RNA extraction. The total RNAs of different treatments for the JK-SH007 were extracted using the classical Trizol method with Trizol reagent (Ambion, Life Technologies[™], USA). The total RNA's concentrations and purities were assessed by Thermo Scientific NanoDrop 1000 spectrophotometer and visualized on 1 % agarose gel to verify their integrities. The cDNA for real-time PCR were synthesized with the TransScript[®] One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) according to the instructions of the producer.

The expression stabilities of the eight candidate reference genes were measured utilizing geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004). As stated by geNorm, the gene with the lowest stability expression measure (M value) is thought to be most stable, and the M value should be lower than the cut-off value of 1.5. In addition, in NormFinder, a set of candidate genes was ranked on the basis of their inter- and intra-group expression fluctuations (S value). The most stable gene possesses the lowest S value.

The result presented the most genes met the requirements of amplification efficiency, except *thyA* and *recA*. The descriptive statistics of all 32 samples' expression levels were worked out for every gene (Table 2). The average Ct values ranged from 9.45 to 26.08. The standard deviation (SD) was simultaneously calculated for each gene.

The stability of investigated genes' expression calculated using geNorm and NormFinder was shown in Table 3. According to the geNorm analysis, genes *pyrG* and *ftsZ* had the lowest average M values, which implied the high expression stability. The M values of all of the eight candidate genes were below the cut-off value of 1.5. Furthermore, NormFinder was used to analyze the stability of candidate genes (Table 3). In the eight candidate genes, *pyrG* (S=0.037) had the most stable expression levels

among all of the samples. By contrast, 16S rDNA ($S=0.295$) had the least stable levels.

Though the gene *ftsZ* had the highest expression stability in each individual sample, the total samples' comprehensive analysis indicated that gene *pyrG* was the most stable. In summary, the gene *pyrG* was considered as the applicable reference genes for the target gene expressions' normalization.

In the current research, the expression stability of eight genes under four distinct statuses during the full life cycle was assessed with RT-qPCR gene expression analysis of *B. pyrrocinia* JK-SH007. The combination of genes *pyrG* and *ftsZ* was recommended as a set of usable interior control genes. The *pyrG*'s stability was placed in the top three genes within all of screened samples. Though the *pyrG* is not widely applied as the reference gene, it is deemed to be a putative housekeeping gene. Additionally, the expression of *pyrG* under all four conditions was relatively stable in *B. pyrrocinia* JK-SH007.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with animals or human participants performed by any of the authors. The informed consent was obtained from all individual participants included in the study.

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Figures

Fig. 1. Agarose gel and melting curve analysis of primer specificity in amplification of genes from *B. pyrrocinia* JK-SH007

A: *pyrG*; B: *ftsZ*; C: 16S rDNA; D: *atpD*; E: *gyrB*; F: *recA*; G: *rpoB*; H: *thyA*

Tables

Table 1. Oligonucleotide primer sequences used for PCR and RT-qPCR in *B. pyrocinia* JK-SH007.

Target genes	Primer sequence	Product size (bp)	Function
RT-qPCR			
<i>ftsZ</i>	F: 5-GCGCACATGGTGTTTCATTAC-3 R: 5-GTCGACGTGATCCTCCAGTT-3	192	cell division protein FtsZ
<i>recA</i>	F:5-CGTGAAGGTCGTCAAGAACA-3 R:5-GATCTTCTCGCCGTTGTAGC-3	166	recA protein DNA replication, recombination, and repair
<i>gyrB</i>	F:5-TGCAGTGGAACGACAGCTAC-3 R:5-GCGATTTTCGTTATCGGTGAT-3	142	DNA gyrase, subunit B
<i>thyA</i>	F:5-CTCGACCTCGTTCGTACCAT-3 R:5-GATTTGAACGCGAGCTTCTT-3	149	thymidylate synthase
<i>rpoB</i>	F:5-CGTTTCGGTGAAATGGAAGT-3 R:5-CCCTTGACCAGGTTTTCGTA-3	128	DNA-directed RNA polymerase subunit beta
<i>pyrG</i>	F:5-AGTCACCCTCCTCAAACCTCG-3 R:5-TCGTGAAGTTGTTGGCCTTG-3	170	CTP synthetase
<i>atpD</i>	F:5-TCGAAACGGGTATCAAGGTC-3 R:5-TAACCGCCGTGTTTCCTTCGC-3	142	ATP synthase subunit beta
16S rDNA	F:5-GCTCGTGTCGTGAGATGTT-3 R:5-TGTGAAGCCCTACCCATAA-3	156	encoding the 16S rRNA

Table 2. Descriptive statistics of candidate reference genes.

Parameter	Gene							
	<i>pyrG</i>	<i>ftsZ</i>	<i>recA</i>	<i>gyrB</i>	<i>thyA</i>	<i>rpoB</i>	<i>atpD</i>	16S rDNA
GM[Ct]	21.01	26.08	25.78	25.73	26.83	24.91	23.18	9.45
AM[Ct]	19.26	25.02	24.19	23.57	24.19	23.60	20.80	7.87
Min[Ct]	20.96	25.84	25.75	25.70	26.76	24.88	23.13	9.29
Max[Ct]	21.07	26.29	25.81	25.80	26.86	24.94	23.24	9.55
Slope	-3.38	-3.53	-2.13	-3.41	-1.81	-3.27	-3.39	-3.10
R ²	0.994	0.991	0.932	0.984	0.893	0.994	0.991	0.999
E [%]	97.51	91.76	130.60	95.72	156.98	102.07	96.78	109.97
SD[±Ct]	0.699	0.850	0.808	0.456	0.866	0.873	1.195	3.151
CV[%Ct]	0.036	0.034	0.033	0.019	0.036	0.037	0.057	0.400

Note: GM[Ct], geometric mean of Ct value; AM[Ct], arithmetic mean of Ct value; Min[Ct] and Max[Ct], extreme values of Ct; R², correlation coefficients; E[%], amplification efficiency; SD[±Ct], standard deviation of Ct value and the number of mRNA molecules; CV[%Ct], coefficient of variance expressed as percentage of Ct value.

Table 3. Expression stability values (S) of the eight candidate reference genes in *B. pyrrocinia* JK-SH007 calculated using NormFinder.

Rank	Total		pH		Time		Temperature		Salt stress	
	Gene	Stability	Gene	Stability	Gene	Gene	Stability	Stability	Gene	Stability
1	<i>pyrG</i>	0.037	<i>recA</i>	0.061	<i>16s</i>	0.165	<i>rpoB</i>	0.224	<i>pyrG</i>	0.256
2	<i>rpoB</i>	0.047	<i>ftsZ</i>		<i>ftsZ</i>		<i>ftsZ</i>		<i>ftsZ</i>	
3	<i>gyrB</i>	0.050	<i>pyrG</i>	0.101	<i>atpD</i>	0.263	<i>pyrG</i>	0.343	<i>rpoB</i>	0.283
4	<i>recA</i>	0.052	<i>thyA</i>	0.170	<i>pyrG</i>	0.349	<i>gyrB</i>	0.536	<i>16s</i>	0.330
5	<i>ftsZ</i>	0.059	<i>16s</i>	0.191	<i>gyrB</i>	0.368	<i>recA</i>	0.642	<i>atpD</i>	0.423
6	<i>thyA</i>	0.060	<i>atpD</i>	0.253	<i>rpoB</i>	0.392	<i>thyA</i>	0.695	<i>thyA</i>	0.466
7	<i>atpD</i>	0.081	<i>gyrB</i>	0.303	<i>recA</i>	0.609	<i>16s</i>	0.732	<i>gyrB</i>	0.528
8	<i>16s</i>	0.295	<i>rpoB</i>	0.331	<i>thyA</i>	0.819	<i>atpD</i>	0.779	<i>recA</i>	0.580

Note: The S values are calculated following stepwise exclusion of the least stable gene across all treatment groups. Genes were then ranked according to their stability under different experimental conditions through calculation of the NormFinder.