JOURNAL OF
AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Evaluation of Four Genes in Rice for Their Suitability As Endogenous Reference Standards in Quantitative PCR

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The genetically modified (GM) food/feed quantification depends on the reliable detection systems of endogenous reference genes. Currently, four endogenous reference genes including *sucrose phosphate synthase* (*SPS*), *GOS9*, *phospholipase D* (*PLD*), and *ppi phosphofructokinase* (*ppi-PPF*) of rice have been used in GM rice detection. To compare the applicability of these four rice reference genes in quantitative PCR systems, we analyzed the target nucleotide sequence variation in 58 conventional rice varieties from various geographic and phylogenic origins, also their quantification performances were evaluated using quantitative real-time PCR and GeNorm analysis via a series of statistical calculation to get a "*M* value" which is negative correlation with the stability of genes. The sequencing analysis results showed that the reported *GOS9* and *PLD* taqman probe regions had detectable single nucleotide polymorphisms (SNPs) among the tested rice cultivars, while no SNPs were observed for *SPS* and *ppi-PPF* amplicons. Also, poor quantitative performance was detectable in these cultivars with SNPs using *GOS9* and *PLD* quantitative PCR systems. Even though the PCR efficiency of *ppi-PPF* system was slightly lower, the *SPS* and *ppi-PPF* quantitative PCR systems were shown to be applicable for rice endogenous reference assay with less variation among the C_t values, good reproducibility in quantitative assays, and the low *M* values by the comprehensive quantitative PCR comparison and GeNorm analysis.

KEYWORDS: Real-time PCR; rice endogenous reference gene; GeNorm analysis; single nucleotide polymorphism

INTRODUCTION

As the recombinant DNA technique being widely used in modern agriculture, currently 155 genetically modified plant (GMP) events for 24 kinds of crops have been approved for commercialization and planting in the world (*1*). By the end of 2009, the global planting area of biotech crops has reached 134 million hectares in 25 countries (*1*). Meanwhile, the consumers' concerns on the safety of GMOs resulted in labeling regulations for GM foods and feeds in more than 40 countries and areas. For example, EU required the labeling of GM foods and feeds with a threshold of 0.9% (*2*, *3*), 3% in Korea (*4*), 5% in Japan (*5*), and zero in China (*6*). To implement the GMO labeling regulations, much effort was taken to establish sensitive, accurate, and reliable methods for GM event identification and quantification of each GMO individuals (*7*, *8*).

Real-time PCR is a most widely used approach for quantifying the GM contents of GMOs via comparing the copy numbers of endogenous reference gene with that of exogenous DNA

fragment. One ideal endogenous reference gene should display species specificity, stable and low copy numbers in the genome, and low heterogeneity among different cultivars (*9*, *10*). Furthermore, the reliable real-time PCR assay of the endogenous reference gene should have similar and high PCR amplification performance in different cultivars.

Rice is one of very important staple food crops in the world. Recently, some new rice varieties via transgenic improvement have been developed to increase the yield and improve nutritional content and better resistance to disease and pests (*11*). So far several GM rice varieties have been approved for commercialization, such as events CL121, CL141, and CFX51 from BASF, Inc., events LLRICE06 and LLRICE62 from Aventis Crop Science, and events LLRICE601 from Bayer CropScience (www.agbios. com). In China, one insect-resistant rice event TT51-1 expressing the fused Cry1Ab/Ac protein has been approved for commercialization in 2009 (*12*) (http://www.stee.agri.gov.cn/biosafety/spxx/P020091127591594596689.pdf), and also some transgenic rice events with various traits are in the pipeline for commercialization (*13*).

To meet the requirement of GMO labeling, establishment of a robust analysis system for the reference genes is a prerequisite for identification and quantification of GM products (*13−16*).

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Table 1. Nucleotide Sequences of Primers and Probes

PCR type	gene name	primer name	sequence(5′-3′)	amplicon size (bp)	ref
sequence PCR	*SPS*	Sps-seq-1F	TTCCTTATGACGCTACCAACA	792	this paper
		Sps-seq-1R	ATTAGAGGAGTCCGTGGCTTG		
	GOS9	Gos-seq-1F	ATATTTCCGCACGTCCTT	444	this paper
		Gos-seq-1R	TCTTGTTCGGTTCATTCC		
	PLD	Pld-seq-1F	CTCATTATTCCGCCGTCTC	565	this paper
		Pld-seq-1R	CATTCAACTTTACTTCCCTGTT		
	ppi-PPF	PPF-seq-1F	CCTTTCGGAATTGACCTG	632	this paper
		PPF-seq-1R	TAGGTGGTGTTAGGTAAGGTTCTG		
real-time PCR	*SPS*	Sps-taq-1F	TTGCGCCCTGAACGGATAT	81	*16*
		Sps-taq-1R	CGGTTGATCTTTTCGGGATG		
		Sps-P	FAM-TCCGAGCCGTCCGTGCGTC-TAMRA		
	GOS9	Gos-taq-1F	TTAGCCTCCCGCTGCAGA	68	*17*
		Gos-taq-1R	AGAGTCCACAAGTGCTCCCG		
		Gos-P	FAM-CGGCAGTGTGGTTGGTTTCTTCGG-TAMRA		
	PLD	Pld-taq-1F	TGGTGAGCGTTTTGCAGTCT	64	*18*
		Pld-taq-1R	CTGATCCACTAGCAGGAGGTCC		
		Pld-P	FAM-TGTTGTGCTGCCAATGTGGCCTG-TAMRA		
	ppi-PPF	PPF-taq-1F	AATTCTGTCATGTATTTGAGCAGTTCA	79	*19*
		PPF-taq-1R	AATGACAACAAGCCCATCCAA		
		PPF-P	FAM-ACACTGTAAACAAAC-MGB		

Currently, four endogenous reference genes and their real-time PCR assays have been developed for rice DNA quantification. The *phospholipase D* (*PLD*) was used as rice endogenous reference gene for quantification of GM rice event LLRICE62 in EU (*17*) (http://www.crl-gmo.europa.eu). Another reference gene targeting to the specific region of the *sucrose phosphate synthase* (*SPS*) gene has been developed and validated at the global level (*15, 16*) and implemented as the national standard in Korea and China. Other two rice reference real-time PCR assays targeted at the *GOS9* (*18*) and *ppi phosphofructokinase* (*ppi-PPF*) (*19*). Although the species specificity of these four reference genes has been validated, the performances of these endogenous reference genes and their assays have not been systematically evaluated among different rice cultivars. Rice has a great genetic diversity with many single nucleotide polymorphisms (SNPs) in the genome among different cultivars (*20, 21*). The SNPs within the region of primers or probes may alter the quantification results. For instance, a SNP in the forward primer sequence of *Adh1* assay caused a decrease in the measurement of total DNA copy numbers (*22*).

The aim of this study is to evaluate the consistency of these four reported rice reference genes in quantitative PCR assays. We analyzed the target sequences and PCR performances of *GOS9*, *PLD*, *SPS*, and *ppi-PPF* using total 58 diverse rice varieties. Several SNPs and poor PCR performances were observed in the amplicons of *GOS9* and *PLD* in some rice varieties. The real-time PCR results and GeNorm analysis showed that the *SPS* and *ppi-PPF* genes and their assays are applicable for GM rice quantification.

MATERIALS AND METHODS

Sample Source. Seeds of 58 different conventional rice cultivars from different geographic and phylogenic origins were kindly provided by Shanghai Agro-biological Gene Center, China, and the detailed information of the 58 rice cultivars was listed in Supporting Information data set 1 in which 24 varieties are from Southern China (such as Fengaozhan, Shanlan no. 1, Xianghui 89, Ningbo no. 2, which is from Guangzhou, Hainan, Hunan, and Zhejiang Province, respectively), seven varieties are

from Northern China (such as Lulong, Guojing151, Zhandaozi, which if from Hebei, Liaoning, and Jilin Province, respectively), six cultivars were from East Asia, seven from South Asia, five from USA, five from South America, and other nine cultivars from several other countries and areas. A typical indica variety 9311 was used to establish the standard curves of four reference assays in this study. All of these seeds were germinated in the greenhouse to provide enough fresh leaves for genomic DNA extraction.

DNA Extraction and Purification. The plant genomic DNA samples used for sequencing PCR and real-time PCR analysis were extracted and purified using a mini-plant genomic DNA extraction kit (Shanghai Ruifeng Agro-tech Co. Ltd., Shanghai, China) according to the manufacturer's manual. The quality and quantity of purified DNA samples were evaluated by agarose gel electrophoresis and ultraviolent spectrometric method (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA). DNA samples of all rice cultivars and 9311 variety were first adjusted to final concentration of 20 ng/μL and then diluted to the required concentration gradients for further tests.

Primers and Probes. Four reported rice endogenous reference gene real-time PCR systems of *SPS* (U33175), *GOS9* (X51909), *PLD* (AB001919), and *ppi-PPF* (AP004027) were evaluated in this study (*16−19*). To determine whether there is sequence variation among the target regions in quantitative assays, the sequencing primers were designed to cover the quantitative PCR amplicons by Primer Premier 5.0 software. The sequences of real-time PCR primers and fluorescent dye-labeled probes of four endogenous reference gene assays were from previous reports and GMO detection database (GMDD, http://gmdd.shgmo.org) (**Table 1**) (*23*) . PCR primers and fluorescent probes were synthesized by Invitrogen Co., Ltd. (Shanghai, China) and TaKaRa biotechnology Co., Ltd. (Dalian, China), respectively. The TaqMan probes of SPS-P, GOS-P, and PLD-P were labeled with the fluorescent reporter dyes fluorescent 6-carboxyfluorescein (FAM) on the 5′ end and the fluorescent quencher dyes 6-carboxy-tetramethylrhodamine (TAMRA) on the 3′ end of probes. PPF-P, a minor groove binder (MGB) probe, was labeled with the fluorescent 6-carboxyfluorescein (FAM) on the 5′ end and with the MGB ligand attached to its 3′ end.

Sequencing and Alignment of Endogenous Reference Gene Target Regions. DNA fragments of four reference gene target regions in 58 rice varieties were obtained by PCR amplification employing the sequencing primers (**Table 1**) and a KOD-*plus*-DNA polymerase kit (TOYOBO Co.,

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Ltd. Shanghai, China). The PCR mixture contained the following reagents: 1 × PCR buffer for KOD-*plus*-, 0.2 mM dNTP, 1 mM Mg^{2+}, 0.2 μM each primer, 0.5 unit KOD-plus-DNA polymerase, and 20 ng each of DNA samples in the final volume of 50 μL. The PCR amplification was performed as follows: predenaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 58 °C for 30 s, elongation at 68 °C for 50 s, and a final extension at 68 °C for 5 min. Each PCR product was analyzed using 1.5% agarose gel electrophoresis to verify the success of the PCR amplification and the size of amplicons. Following cut off of the expected DNA fragments from agarose gel, DNA fragments were purified using a DNA gel extraction kit (Axygen Biotechnology, Hangzhou, China), and the purified DNA fragments were sequenced by Invitrogen Co., Ltd. (Shanghai, China). The obtained DNA sequences were aligned using Vector NTI Advance 10 software to analyze SNPs within the target regions in the quantitative real-time PCR assays.

Real-Time PCR Conditions. In this study, real-time PCR mixture contained: 1× quantitative PCR buffer (10 mM Tris-HCl (pH 8.3) and 50 mM KCl), 6 mM MgCl$_2$, 200 μM dNTP, 1.25 units of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd. Dalian, China), 5 μL of DNA template, and appropriate concentration of primers and probes in the final value of 25 μL. The concentrations of primers and probes in PCR reaction of four different rice reference gene assays were optimized, and the final concentrations of primers were 400 nM, 300 nM, 200 nM, and 1 μM primers for *SPS*, *GOS9*, *PLD*, and *ppi-PPF* assays, respectively. The probe concentration of each assay was 200 nM per reaction. Quantitative PCRs were run on a fluorometric thermal cycler Rotor-Gene 3000 (Corbett Research, Sydney, Australia), and thermal cycling program was as follows: 94 °C for 5 min followed by 45 cycles of 94 °C for 15 s, 60 °C for 60 s. All real-time PCR reactions were performed three times and each time with three parallels in this study.

Validation of Quantitative PCR Efficiency and C_t Data. To evaluate the PCR systems of the four rice reference genes in 58 rice samples, standard curves were established using five gradient concentrations (10, 2, 0.4, 0.08, and 0.016 ng/μL) of the indica variety 9311 genomic DNA samples. The optimal threshold lines were automatically analyzed by Rotor-Gene software. The quantitative PCR efficiencies for each gene were calculated according to the following equation: $E = (10^{-1/m]} - 1) \times 100$ (*24*), where E is the PCR efficiency; m is the slope of the log-transformed of DNA quantities versus C_t values according to the equation $y = m \times \log(x) + b$. Using the same condition of developed standard curves, quantitative PCR amplifications for 58 rice DNA samples were performed at three concentrations (10, 2, and 0.4 ng/reaction). Measured C_t values under the optimal threshold of their respective standard curves were collected for all the three gradient assays of 58 rice samples.

Statistical Data Analysis Using GeNorm Software. For each endogenous reference gene, C_t values of three sets (10, 2, and 0.4 ng according to the amounts of initial DNA templates) were calculated and analyzed individually. The mean C_t value of each sample calculated according to nine repeats was used to statistically analyze the C_t range, mean value, and standard deviation value in each assay. The variation among different cultivar samples of these four assays was evaluated using the software of GeNorm version 3.5 (http://medgen.ugent.be/~jvdesomp/genorm/). GeNorm analysis is an algorithm widely used in evaluating and selecting suitable reference gene in expression studies. GeNorm is able to select two genes as the reliable genes among the candidates. In this study, C_t values were transformed to relative quantities by means of ΔC_t method, $Q = E^{-\Delta C_t}$, where E is the efficiency of a reference assay using rice cultivar 9311 as calibrators, and ΔC_t is the difference of C_t values between the individual sample from 9311 at the same DNA concentration. The relative quantities were used as input data to automatically calculate the M value which measured the corresponding average pairwise variation of a single reference gene to all other genes. The M value reflects the consistency of the interest genes, the higher of the M value, the less consistent of the particular gene (*25*). For the three tested DNA concentration levels, Q values of each reference genes were shown as average, median, 25th to 75th percentile, and the value ranges in a bar chart followed M values in a line chart according to the order of consistency of each gene.

RESULTS AND DISCUSSION

SNPs in Target Regions of Four Endogenous Genes among Different Rice Varieties. In this study, we sequenced the regions
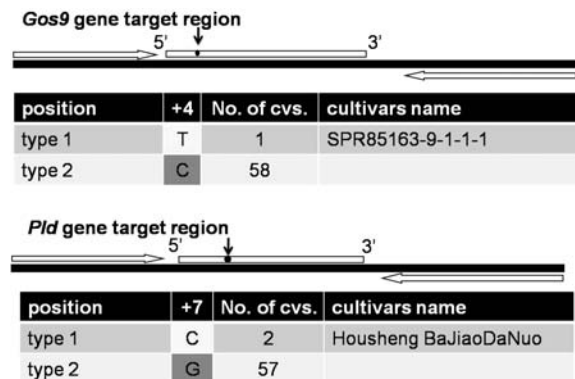

Figure 1. Detected SNPs within the amplified sequences of *GOS9* and *PLD* gene. A SNP of *C* > *T* at the fourth nucleotide position of *GOS9* gene probe in cultivar SPR85163-9-1-1-1 and a SNP of *G* > *C* at the seventh nucleotide position of *PLD* gene probe in HouSheng and BaJiaoDaNuo. No detectable SNP was found in *SPS* and *ppi-PPF* regions.

containing quantitative PCR targets of the reference genes individually using 58 rice cultivars. Sequence alignment against rice reference sequence in GenBank Database (http://www.ncbi.nlm.nih.gov/Genbank/) was performed (**Figure 1**), and results indicated that there was not any detectable SNP among all 58 rice samples for the amplicons of *SPS* and *ppi-PPF*, the nucleotide sequences of quantitative PCR primers, probes, and amplicons of both these two genes are consistent among all tested cultivars. For the *GOS9* gene, the alignment revealed that a SNP of *T* > *C* presented in one rice cultivar (SPR85163-9-1-1-1) at the position of fourth nucleotide from 5′ end of the probe, but no SNP was found in other samples. For the *PLD* gene, an identical SNP of *C* > *G* was found in two rice cultivars (Housheng and BaJiaoDaNuo) at the position of the seventh nucleotide from 5′ end of the probe.

Quantitative PCR Efficiency and C_t Values. To establish the real-time PCR systems of four rice reference genes, the indica rice cultivar 9311 was selected and used as the calibrator to evaluate the amplification efficiency. Five serial dilutions of 9311 rice genome DNA (50, 10, 2, 0.4, and 0.08 ng per reaction) were used in this analysis. As shown in **Table 2**, PCR efficiencies of these four reference gene assays were between 0.96 and 1.16, indicating that all these four reference assays have acceptable exponential efficiencies that are consistent with previous reported results with the exception of slight deviation of the *ppi-PPF* assay (*16*−*19*). The linear correlation (R^2) values of the four constructed standard curves were all above 0.998, and the threshold line with good linear correlation, indicating these four assays are workable with a wider dynamic range. Mean C_t values of each sample were obtained from triplicate reaction under the identical threshold. The detailed mean C_t values of each samples and systems were listed in Supporting Information data set 2 and used for evaluation of the consistency of each system.

The SNPs of Endogenous Reference Targets Affect the Quantitative PCR Assays. To evaluate the potential effect of these SNPs within TaqMan probes of *GOS9* and *PLD*, comparison of the C_t values in the varieties with the detectable SNPs was performed. The detailed C_t values of the cultivars containing SNPs were shown in **Table 3**. For the sample SPR85163-9-1-1-1, with a SNP of *T* > *C* in the *GOS9* gene, the observed C_t value was higher than those of other samples without this SNP. For the two rice cultivars (BaJiaoDaNuo and HouSheng) containing a SNP of *C* > *G* in the *PLD* target region, amplification cycles reaching the threshold were all delayed. We plotted the C_t data of these SNP cultivars and observed that the PCR efficiency of each reaction

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for these three cultivars were all below 90% (89% for *GOS9* assay using the template from SPR85163-9-1-1-1, 89% for *PLD* assay of BaJiaoDaNuo and 86% for *PLD* assay of HouSheng). We assumed that the worse efficiencies may be resulting from the mismatch of probe sequences to the genome sequence in the target regions. Similarly, the SNPs found in the primer region of maize *Adh1* real-time PCR assay greatly decreased the quantitative PCR analysis (*22*).

Comparison of the Consistency of Quantitative PCR Systems of the Four Endogenous Reference Genes. To evaluate the consistency of the four reference quantitative PCR assays, 58 rice varieties were all tested in this study, and the C_t values obtained from all 58 varieties with the same threshold were used for statistical analysis and comparison (**Table 4**). First, the variability in gene quantitative assays was evaluated based on obtained C_t values and its variation among 58 tested samples. Less variation (smaller standard deviations) of the C_t values among different cultivars was observed for the *ppi-PPF* gene (24.79 ± 0.34, 26.64 ± 0.30, and 28.45 ± 0.34 according to three sets of assays) and *SPS* (27.70 ± 0.48, 30.10 ± 0.45 and 32.48 ± 0.51). While larger variation for *GOS9* (26.09 ± 0.51, 28.55 ± 0.52 and 31 ± 0.55) and *PLD* (27.99 ± 0.64, 30.47 ± 0.71, and 32.93 ± 0.76) was observed (**Table 4**). To compare the consistency of each assay among different reference genes, C_t values were converted to relative quantitative values. A boxplot (**Figure 2**) was constructed to show the middle value, mean value, and interquartile range of the relative quantity values (the details were shown in Supporting Information data set 2). In the tests with three different DNA concentrations (10, 2, and 0.4 ng per reaction), the analyzed data of *SPS* and *ppi-PPF* gene assays appeared relatively consistent among the 58 samples, however, the *ppi-PPF* gene assay displayed less reproducible compared with that of *SPS*. To further evaluate the consistency of these four genes, we performed the GeNorm analysis. First, we calculated the *M* values of each reference gene with all of the 58 cultivars, and the bigger *M* values were observed in the *PLD* gene (0.68, 0.66, and 0.70 for 0.4 ng, 2,

and 10 ng assays, respectively), while smaller values for *SPS* and *ppi-PPF* genes (0.58, 0.46, and 0.52 for 0.4, 2, and 10 ng assays, respectively). The *M* values of *GOS9* were 0.64, 0.58, and 0.61 for 0.4, 2, and 10 ng assays, respectively) (**Figure 3**). Furthermore, the analysis excluding the three cultivars with SNPs was done, and the result was similar to the former. The *M* values of *PLD* gene were still the biggest (0.65, 0.60, and 0.66 for 0.4, 2, and 10 ng assays, respectively), *SPS* and *ppi-PPF* genes had the smallest *M* values (0.52, 0.46, and 0.50 for 0.4, 2, and 10 ng assays, respectively). Both of the results suggested that the *SPS* and *ppi-PPF* assays were more consistent as the rice reference gene systems.

In short, we analyzed the nucleotide sequence variation of previous four reported quantitative PCR target regions of reference genes among 58 rice cultivars, and SNPs were found at the *GOS9* and *PLD* quantitative PCR probe regions in several
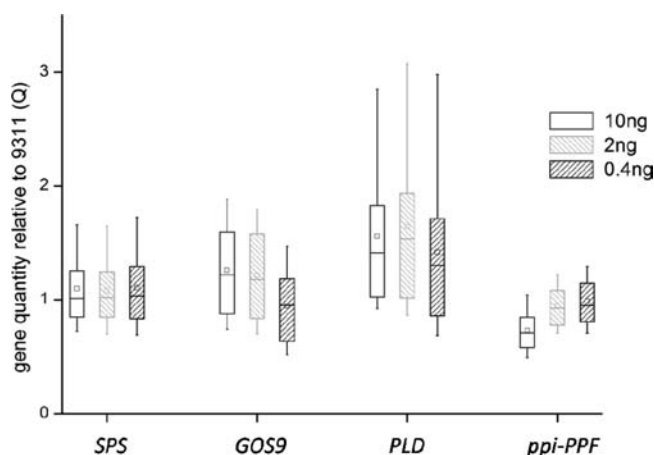
Figure 2. Quantity of rice reference genes in three sets of assays relative to calibrator 9311 ($Q = E^{\Delta C_t}$). Boxplot shows mean value, median value, and interquartile range box.
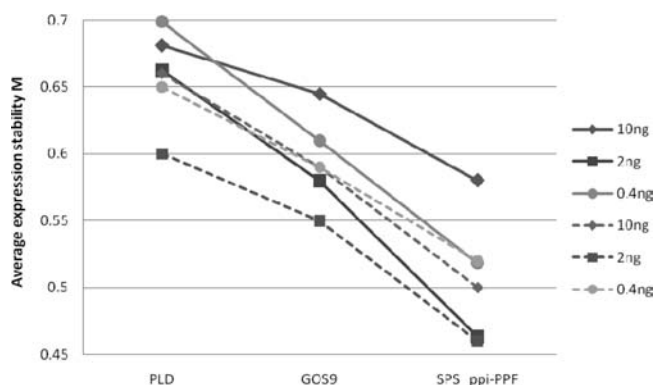
Figure 3. GeNorm analysis of four rice reference genes. The solid line diagram represents the result of analysis including all 58 cultivars, and the dotted line diagram represents the result for analysis excluding the three cultivars with SNPs, i.e. SPR85163-9-1-1-1, HouSheng, and BaJiaoDaNuo.

Table 2. PCR Amplification Efficiencies of Four Reference Genes in Real-Time PCR Assays Using Indica Rice 9311

gene	efficiency	linearity (R^2)	standard curve
SPS	0.96	0.999	$y = -3.422 \times \log \chi + 31.146$
GOS9	1.00	0.999	$y = -3.311 \times \log \chi + 29.623$
PLD	0.97	0.999	$y = -3.398 \times \log \chi + 31.931$
ppi-PPF	1.16	0.998	$y = -2.983 \times \log \chi + 27.316$

Table 3. A Summary of the C_t Values of the Three SNPs Contained Cultivars; The Range of C_t Values among the Other Non-SNPs Contained Cultivars Are in Parentheses

	GOS9	*PLD*	
	SPR85163-9-1-1-1 ($C > T$)	BaJiaoDaNuo ($G > C$)	Housheng ($G > C$)
10 ng	27.05 [25.11−27.02]	30.55 [26.64−28.71]	28.78
2 ng	29.55 [27.52−29.47]	33.24 [29.00−31.43]	31.48
0.4 ng	32.08 [30.09−32.01]	35.60 [31.43−33.97]	33.99

Table 4. Summary of Cycle Threshold (C_t) Values of Four Rice Reference Genes

	10 ng				2 ng				0.4 ng			
gene name	$C_{t\,range}$	$C_{t\,min}$	$C_{t\,max}$	mean C_t ± SD	$C_{t\,range}$	$C_{t\,min}$	$C_{t\,max}$	mean C_t ± SD	$C_{t\,range}$	$C_{t\,min}$	$C_{t\,max}$	mean C_t ± SD
SPS	2.26	26.78	29.04	27.70 ± 0.48	2.29	29.12	31.42	30.10 ± 0.45	2.40	31.36	33.78	32.48 ± 0.51
GOS9	1.94	25.11	27.05	26.09 ± 0.51	2.03	27.52	29.55	28.55 ± 0.52	1.99	30.09	32.08	31.00 ± 0.55
PLD	3.91	26.64	30.55	27.99 ± 0.64	4.24	29.00	33.24	30.47 ± 0.71	4.17	31.43	35.60	32.93 ± 0.76
ppi-PPF	1.30	24.17	25.47	24.79 ± 0.34	1.75	26.04	27.78	26.64 ± 0.30	1.93	27.77	29.70	28.45 ± 0.34

cultivars, causing the bias of PCR analysis wehile the analyzed regions of *SPS* and *ppi-PPF* have no sequence polymorphism. Although the PCR efficiency of *ppi-PPF* system was slightly lower than others, the PCR analysis and GeNorm test indicated that the established *SPS* and *ppi-PPF* quantitative PCR systems are applicable for being used as rice endogenous reference assays due to their better quantification consistency.

Supporting Information Available: Sample names and origin of the 58 rice cultivars in this study; C_t values of four reference genes in all cultivars. This material is available free of charge via the Internet at http://pubs.acs.org.

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