International Collaborative Study of the Endogenous Reference Gene, *Sucrose Phosphate Synthase* (*SPS*), Used for Qualitative and Quantitative Analysis of Genetically Modified Rice

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One rice (*Oryza sativa*) gene, *sucrose phosphate synthase* (*SPS*), has been proven to be a suitable endogenous reference gene for genetically modified (GM) rice detection in a previous study. Herein are the reported results of an international collaborative ring trial for validation of the *SPS* gene as an endogenous reference gene and its optimized qualitative and quantitative polymerase chain reaction (PCR) systems. A total of 12 genetically modified organism (GMO) detection laboratories from seven countries participated in the ring trial and returned their results. The validated results confirmed the species specificity of the method through testing 10 plant genomic DNAs, low heterogeneity, and a stable single-copy number of the rice *SPS* gene among 7 *indica* varieties and 5 *japonica* varieties. The *SPS* qualitative PCR assay was validated with a limit of detection (LOD) of 0.1%, which corresponded to about 230 copies of haploid rice genomic DNA, while the limit of quantification (LOQ) for the quantitative PCR system was about 23 copies of haploid rice genomic DNA, with acceptable PCR efficiency and linearity. Furthermore, the bias between the test and true values of eight blind samples ranged from 5.22 to 26.53%. Thus, we believe that the *SPS* gene is suitable for use as an endogenous reference gene for the identification and quantification of GM rice and its derivates.

KEYWORDS: Collaborative trial; *sucrose phosphate synthase*; endogenous reference gene; *Oryza sativa*; qualitative and quantitative PCR

INTRODUCTION

As modern biotechnology developed quickly, more than 100 genetically modified plant (GMP) events have been approved for commercialization around the world. By 2007, the planting area of GMPs has reached 114.3 million ha (*1*). To protect the rights of consumers, labeling of genetically modified (GM) foods and feeds has become more and more important. In different countries, the labeling thresholds vary, for example, the labeling threshold defined as 0.9% in EU, 3% in Korea, 5% in Japan, and 0% in China (*2−5*). For the purpose of implementing the genetically modified organism (GMO) labeling regulations and accelerating the global harmonization of GMO detection methods, some organizations, such as the Chinese Ministry of Agriculture (MOA), the Community Reference Laboratory for GM Food and Feed (CRL-GMFF), and the International Organization for Standardization (ISO), have made significant efforts to initiate the validation and standardization of GMO analysis methods (*6−13*).

The international collaborative validation is the prerequisite step for proposing a method to be included as the ISO standard. CRL-GMFF and others have organized some deliberate collaborative ring trials for CaMV35S promoter and NOS terminator quantitative detection methods (*14−17*) and event-specific quantitative detection methods of TC1507, MON863, GA21, and MON810 maize events (*18*). Recently, we completed a ring trial to validate an event-specific qualitative detection method of RT73 canola among 12 invited laboratories (*19*).

For GMO detection, a crop endogenous reference gene is used as the golden standard for species identification and total genomic DNA amount quantification and several different endogenous reference genes have been reported and used for some GMP quantification, such as the high-mobility group protein (*hmga*), alcohol dehydrogenase (*Adh1*), *zein*, invertase A (*ivr*), and *zssIIb* genes for maize (*20, 21*), the *lectin* gene for soybean (*22*), the *LAT52* and metallo-carboxypeptidased (*Mcpi*) genes for tomato (*23, 24*), the *sucrose phosphate synthase* (*SPS*), *Oryzain β*, *Gos 9*, ppi phosphofructokinase

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(*ppi-PPF*) genes for rice (*25–27*), the high-mobility group protein (*HMG I/Y*) and acetyl-CoA carboxylase (ACC) *BnACCg8* genes for rapeseed (*28–30*), and the stearoyl-ACP desaturase (*Sad 1*) gene for cotton (*31*), etc. However, the various endogenous reference gene detection systems for a single plant species can cause confusion. Only a few reference genes have been systematically validated; thus, the validation and harmonization of endogenous reference gene detection systems at the international level are necessary.

Rice is a major staple food, and some GM rice varieties have been approved for commercialization, such as events CL121, CL141, and CFX51 from BASF, Inc. (*32*) and events LLRICE06 and LLRICE62 from Aventis Crop Science (*25*). Also, more and more transgenic rice varieties with various traits are in the pipeline for commercialization. Therefore, it is important to detect and monitor GM rice varieties and their derivates. The development of an endogenous reference gene is a prerequisite for specific detection and quantification of DNA of a given taxon (*33*). Although four endogenous reference genes were reported in rice (*25–27*), no validated rice endogenous reference gene at the international level was reported. Previously, we developed a module for the international validation of endogenous reference genes and performed the qualitative and quantitative polymerase chain reaction (PCR) validation of tomato endogenous reference gene *LAT52* by means of an international collaborative ring trial in 2007 (*34*). In this study, we organized another international collaborative ring trial with the assisstance of 12 laboratories from seven different countries and validated our previous reported rice endogenous reference gene, *SPS*, and its qualitative and quantitative PCR methods. This should greatly assist in the international harmonization of GM rice identification and quantification.

MATERIALS AND METHODS

Plant Materials. A total of 10 different plants were collected by our laboratory: bamboo (*Phyllostachys*), green bristlegass (*Setaria viridis* Beauv.), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), foxtail millet (*Setaria italica*), rapeseed (*Brassica napus*), tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*), soybean (*Glycine max*), and *Arabidopsis* (*Arabidopsis thaliana*). A total of 12 different rice cultivars from different geographic and phylogenic origins were kindly provided by Prof. Zongxiu Sun from the Chinese National Rice Research Institute, in which seven varieties are *indica* cultivars, with four from southern China (Xiangwanxian, Jinbaoyin, Minghui78, and Guangluai4) and three from eastern China (Najing14, Zhe733, and Huke3), and five varieties are *japonica* cultivars, including Hejiang19 (from Heilongjiang province), Baizhehu (from Guizhou province), Taibei309 (from Taiwan Province), Nipponbare (from Japan), and Balilla (from Italy), respectively. Two rice varieties, such as 3M, an *indica* variety from U.S.A., and Shennong265, a *japonica* variety from eastern China, were also used in this study.

Five mixed rice samples were prepared in duplicate using rice powder and non-GM maize powder based on mass fractions, i.e., 10, 1, 0.1, 0.05, and 0.01% (w/w). These samples were mixed with a SPEX CertiPrep 6850 freezer/mill (SPEX CertiPrep, Inc., Metuchen, NJ). The dried rice (Guangluai4) seeds and non-GM maize seeds were first ground with the freezer/Mill to obtain the pure dried powder, respectively. Then, 5.0000, 0.5000, 0.0500, 0.0250, and 0.0050 g of pure dried rice powder and 45.0000, 49.5000, 49.9500, 49.9750, and 49.9950 g of pure dried non-GM maize powder were weighed with a Sartorius BS 224S Balance (readability is 0.0001 g), respectively. The rice powder and the corresponding non-GM maize powder were put into 50 mL grinding vials simultaneously (the total weight was 50.0000 g),

and the samples were mixed in liquid nitrogen in the freezer/mill for 10 min. After the vials were removed from the freezer/mill, they were kept at room temperature for 1 or 2 days without opening the vial caps. When the vials reached room temperature and the outside surfaces of the vials were free of condensation, these samples were put into small bottles.

Four rice varieties, such as 3M, Balilla, Guangluai4, and Shennong265, were selected and used for construction of the standard curves and preparation of the blind samples.

DNA Extraction. Plant genomic DNA samples were extracted using the CTAB method (ISO 21571) and purified with a silica-column DNA-purified kit (Ruifeng Agro-tech Co., Ltd., Shanghai, China) (*34*). The quality and quantity of purified DNAs were evaluated by an agarose gel electrophoresis analysis and ultraviolet spectrometric method according to ISO 21571 (*11*). Four calibration samples were prepared with a concentration of 50 ng/μL, and for the unknown samples, concentrations of 10, 5, 2, 1, and 0.5 ng/μL were prepared, whereas other DNA samples were diluted to a concentration of 20 ng/μL. Salmon sperm DNA (20 ng/μL) was chosen as the negative DNA target control, and the rice (Guangluai4) genomic DNA was used as the positive DNA target control.

Qualitative PCR Reaction. Our previous study revealed that the *SPS* gene is rice-specific with one copy number and low heterogeneity. Here, the previous qualitative PCR assay with an 81 bp amplicon was optimized (*25*). After the blasting analysis of the genomic DNA sequence of *SPS* (GenBank code U33175) in public gene database (EMBL) (*35*), we selected a rice-specific region of the *SPS* gene containing the genomic junction region between the promoter (−222) and the first exon (+57) with a consensus sequence of both *indica* and *japonica* subspecies. We designed the primer pair (SPS-1F/2R) based on this region for a qualitative PCR assay with 279 bp amplicon using Primer Premier 5.0 (primer sequences of SPS-1F/2R listed in **Table 1**). The sequences and the regions of the qualitative primers are shown in **Figure 1**. Then, we tested the applicability of the primer pair with an in-house validation. A total of 20 different plants species, such as wheat, maize, barley, tobacco, soybean, rapeseed, tomato, sunflower, carrot, pepper, eggplant, bamboo, peanut, sesame, cabbage, mung bean, shallot, garlic, millet, and *A. thaliana* and a total of 20 different rice cultivars with different geographic and phylogenic origin, such as 11 *japonica* rice varieties, Hanfeng, Nonghu6, 9520, Xiushui 04, Taihunuo, Taibei309, Shengnong265, Hejiang19, Baizhehu, Nipponbare, and Balilla, and 9 *indica* rice varieties, D5 Heiheaihui, Najing14, Jinyinbao, Minghui78, Huke3, Guangluai4, Zhe733, Xiangwanxian9, and 9311, were tested. The qualitative PCR reaction mixture contained the following reagents: 1× PCR buffer (10 mM KCl, 2 mM $MgSO_4$, 8 mM $(NH_4)_2SO_4$, and 10 mM Tris-HCl at pH 8.3−8.8), 200 nM each of dNTPs, 2.5 mM $MgCl_2$, 330 nM each primer, 100 ng of each DNA sample, and 1 unit of Taq DNA polymerase (TakaRa Biothchnology Co., Ltd.). The qualitative PCR profile was 95 °C for 15 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension step of 7 min at 72 °C. As expected, ideal results were obtained in the in-house validation.

Subsequently, we organized the collaborative ring trial. The qualitative primers (SPS-1F/2R) were synthesized and distributed into one tube with 1 OD by Invitrogen Co., Ltd. (Shanghai, China). All participating laboratories in this trial were requested to perform the qualitative PCR, employing the qualitative PCR reaction mixture and temperature−time program described above. The high-quality Taq DNA polymerase and PCR reagents were strongly recommended to be used. The qualitative PCR was suggested to be run with the following thermal cyclers, such as PTC-100 Thermal Cycler (MJ Research), ABI 2720 Thermal Cycler (Applied Biosystems), T1 Thermal Cycler (Biometra), etc. The PCR products were separated using 2% (w/v) agarose gel electrophoresis and visualized with ethidium bromide.

Table 1. List of Primers and Probe for the *SPS* Qualitative and Quantitative PCR

PCR system	primer name	sequence (5′ → 3′)	amplicon length (bp)
qualitative PCR	SPS-1F	5′-TTGCGCCTGAACGGGATAT-3′	279
	SPS-2R	5′- GGAGAAGCACTGGACGAGG-3′	
quantitative PCR	SPS-1F	5′-TTGCGCCTGAACGGGATAT-3′	81
	SPS-3R	5′- CGGTTGATCTTTTCGGGATG-3′	
	SPS-P	5′-HEX TCCGAGCCGTCCGTGCGTC TAMRA-3′	
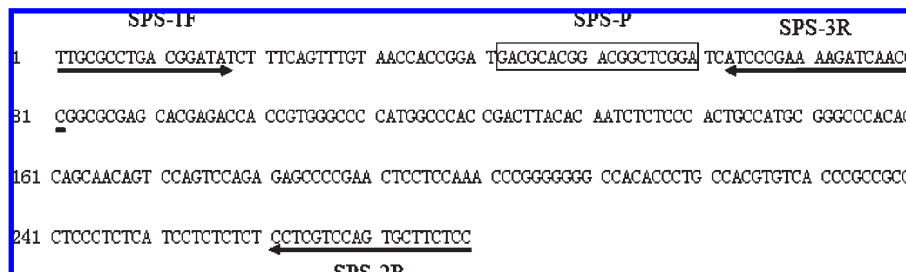
Figure 1. Nucleotide sequences and locations of the *SPS* qualitative and quantitative PCR primers in the amplified product. The arrows indicate the primers used in the qualitative and quantitative PCR detection, and the boxed sequence indicates the TaqMan probe.

Quantitative Real-Time PCR Reactions. In this collaborative ring trial, we adopted the quantitative real-time PCR primers reported in our previous study and employed a new TaqMan probe with complementary sequence as our previous reported probe because there was a G at the 5′ end of the previous probe (*25*), which could affect the signal intensity of fluorescence (dada not shown) (*36*). The sequences and locations of quantitative primers (SPS-1F/3R) together with the modified probe (SPS-P) are presented in **Table 1** and **Figure 1**. In this real-time PCR reaction, 25 μL of reaction mixture included 20 μL quantitative PCR reaction master mix and 5 μL of the sample DNA. The quantitative PCR reaction master mix contained the following: 1× quantitative PCR buffer (10 mM KCl, 5 mM $MgSO_4$, 8 mM $(NH_4)_2SO_4$, and 10 mM Tris-HCl at pH 8.3−8.8), 200 nM dNTPs, 400 nM each of the quantitative primers, and 1 unit of Hot star Taq DNA polymerase (Shanghai RuiCheng Co., Ltd., Shanghai, China). The temperature−time program of the real-time PCR was as follows: 95 °C for 15 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. The fluorescent data were collected in the elongation step of each cycle. The quantitative PCR amplification reactions were to be run in various fluorescence thermal cyclers, such as Rotor Gene 3000A (Corbett Research) and Prism ABI 7300 sequence detection system (Applied Biosystems). Each quantitative PCR plate required three performances, each time with three replicates.

Collaborative Trials. The collaborative trial was organized by the GMO detection laboratory, Shanghai Jiao Tong University (GMDL−SJTU). A total of 12 laboratories were invited and participated in this trial. Each participant received 12 rice genomic DNA samples coded as U1−U12 (20 ng/μL), 10 other plant genomic DNA samples coded as W1−W10 (20 ng/μL), 10 mixed DNA samples with the five content levels of the mixed powder of maize and rice coded as S1−S10 (20 ng/μL), 4 purified rice cultivar genomic DNA samples (3M, Balilla, Guangluai4, and Shennong265) coded as A−D (50 ng/μL), respectively, and 8 blind DNA samples coded X1−X8. Participants also received one positive DNA target control consisting of the DNA solution of Guangluai4 rice (20 ng/μL) and one negative DNA control consisting of salmon sperm DNA solution (20 ng/μL). Moreover, all participants were provided with qualitative primers, quantitative PCR reaction master mix (1 mL × 6), and the DNA dilution solution [10 mM Tris-HCl and 1 mM ethylenediaminetetraacetic acid (EDTA), 1.2 mL × 2]. DNA samples A−D were used for the standard curve construction, respectively. Participants were requested to dilute each of these four DNA samples to the concentration of 10, 1, 0.1, 0.01, or 0.002 ng/μL

by using the DNA solution provided. A total of 5 μL of each DNA sample was used for the qualitative and quantitative PCR amplification using the PCR conditions described above. All of the DNA samples and reagents were stored in one insulated box filled with dry ice and ice bags. Within the 3 months after the samples were shipped, all of the participants sent back the validation result reports and the following analyses were performed. Statistical difference among samples and participating laboratories, such as least significant difference (LSD) test, was performed using SPSS 13.0 software.

Except for one participant, all of the participating laboratories performed the real-time PCR reaction condition described above. In this laboratory, 20 μL of PCR reaction volumes was used for the quantitative PCR reaction. Thus, the DNA samples A−D were diluted to the levels of concentration: 50, 5, 0.5, 0.05, and 0.01 ng/μL, and the quantitative PCR reaction consisted of 19 μL quantitative PCR reaction master mix and 1 μL of the diluted DNA samples. It was assured that the results from this laboratory were reasonable and used for final analysis.

RESULTS AND DISCUSSION

Applicability of the *SPS* Gene Used as the Rice Endogenous Reference Gene. The aim of this collaborative ring trial is to further validate the applicability of the *SPS* gene used as the rice-specific endogenous reference gene at the international level: species specificity, consistent stable low copy number, and low heterogeneity among cultivars of the *SPS* gene were tested.

Species Specificity of *SPS*. In our previous study, low heterogeneity of specific *SPS* sequence (GenBank code U33175) was analyzed in the GenBank website by the BLASTN program and further intralaboratory tested, employing 15 different plant species, such as wheat, maize, barley, tobacco, soybean, rapeseed, tomato, sunflower, carrot, pepper, eggplant, lupine, mung bean, plum, and *A. thaliana*, which were either evolutionarily related to rice or plant material frequently found in food and/or feed samples (*24*). Furthermore, we performed an in-house validation to test the qualitative PCR assay using the primer pair (SPS-1F/2R). A total of 20 different plant species were tested in the in-house validation, and except for the positive control, no amplified fragment with characteristic 279 bp in size was obtained with all of the 20 tested plant species. Then, we

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validated the species specificity of the *SPS* gene for rice in this collaborative trial. A total of 10 different plant genomic DNAs coded from W1 to W10 were tested, employing a qualitative PCR method.

We sent the prepared DNA samples extracted from 10 plant materials and the *SPS* qualitative primers to each participant. The advantage of sending DNA samples to the participants was to avoid the possible variation stemming from DNA extraction among the laboratories. The genomic DNA extraction approaches, such as the CTAB method, have been validated and accepted as the ISO standard (*12*). All participants were requested to perform the qualitative PCR reactions, employing their commonly used qualitative PCR systems available in their own laboratories, and each PCR was repeated 3 times. The results were expressed as positive or negative. Those results were acceptable when the positive control was amplified with the clear 279 bp fragment and no corresponding fragment in the negative and no template control (NTC).

The results from the 12 participated laboratories, as listed in **Table 2**, were statistically analyzed. Two false-positive results were obtained out of a total of 120 DNA samples of 10 plant materials in the 12 laboratories. Thus, the calculated false-positive rate for the ring trial was about 1.67%, and the accuracy for the *SPS* gene was above 98%. The two false-positive results in one participating laboratory were from potato and tomato DNA samples. We examined the returned pictures of gel electrophoresis from this participant, and the two false-positive results were only judged from the very faint gel electrophoresis band according to the picture of gel electrophoresis. Thus, we assume that these two false-positive results might be a result of the DNA contamination during the test, also considering the high sensitivity of PCR. This result of interlaboratory validation further proved that the detected sequence of *SPS* was rice species-specific.

Low Heterogeneity of *SPS* among Different Rice Cultivars. Low heterogeneity of the *SPS* gene among rice cultivars was in-house tested using 20 different rice cultivars from different geographic and phylogenic origin, such as 11 *japonica* rice varieties, Hanfeng, Nonghu6, 9520, Xiushui 04, Taihunuo, Taibei309, Shengnong265, Hejiang19, Baizhehu, Nipponbare, and Balilla, and 9 *indica* rice varieties, D5 Heiheaihui, Najing14, Jinyinbao, Minghui78, Huke3, Guangluai4, Zhe733, Xiangwanxian9, and 9311, in the in-house validation. Except the negative control and NTC, all 20 rice cultivar DNA samples amplified a 279 bp fragment. Then, we selected 12 different rice cultivars from different geographic origins to further validate the rice *SPS* gene with low heterogeneity, and these rice cultivars coded from U1 to U12 were sent to each laboratory. The returned results from the 12 laboratories showed that only 1 false-negative result was obtained out of a total of 144 DNA samples of the 12 rice cultivars (**Table 3**), with the false-negative result being from the rice variety Guangluai4. Because the rice variety Guangluai4 was used as a reference material for standard curve construction and a positive DNA target control, we proposed that the false-negative result might be a result from sample handling by accident. The calculated false-negative rate observed in the collaborative trial was 0.69%, and the positive rate was over 99%; therefore, we believe that the *SPS* gene has low heterogeneity among different rice cultivars.

Stable Single-Copy Number among Different Rice Cultivars. The rice *SPS* gene has been proven to be single-copy by means of Southern blotting and real-time PCR in our

Table 2. Results of the Collaborative Trial Validation of the Species Specificity of the *SPS* Gene

year of collaboration trail	2007
number of laboratories	12
number of laboratories submitting results	12
number of samples per laboratory	10
number of accepted results	120
number of positive results	2
number of negative results	118
false-positive rate (%)	1.67

Table 3. Allelic Variation Testing Results of the *SPS* Gene in the Collaborative Trial

year of collaboration trail	2007
number of laboratories	12
number of laboratories submitting results	12
number of samples per laboratory	12
number of accepted results	144
number of positive results	143
number of negative results	1
false-negative rate (%)	0.69

previous study (*25*). In this collaborative trial, the stable copy number of the *SPS* gene in different rice cultivars was further assessed by means of real-time PCR (*37*, *38*). All participants were requested to construct four separate standard curves using serially diluted genomic DNA samples from four different rice cultivars (3M, Balilla, Guangluai4, and Shennong265 labeled A, B, C, and D, respectively). The serially diluted genomic DNA samples contained DNA concentrations at 10, 1, 0.1, 0.01, and 0.002 ng/μL. The quantity of rice DNA used for each PCR reaction was 50, 5, 0.5, 0.05, and 0.01 ng (5 μL for each reaction), and the rice haploid genomic DNA copy numbers were about 115 500, 11 550, 1155, 115.5, and 23 per PCR reaction according to the haploid rice genomic DNA quantity (2310 haploid rice genome copies in 1 ng of DNA) (*39*).

The average PCR efficiencies of the four constructed standard curves deduced from the quantitative results from 12 laboratories ranged from 0.8463 to 1.2233. The square regression correlations (R^2) of the constructed standard curves of 12 laboratories were all above 0.99, with a standard deviation (SD) of less than 0.0037 (as shown in **Tables 4** and **5**).

Furthermore, the copy number of the serially diluted DNA samples from A–D were calculated on the basis of the four constructed standard curves, respectively, and the difference of the calculated values among the four rice cultivar samples and different laboratories were analyzed statistically. The mean copy numbers of all of the diluted DNA samples from A–D rice samples in 12 laboratories are listed in **Table 6**. The SD and relative standard deviation (RSD) values were calculated according to the mean copy numbers. Because the copy number of rice haploid genomic DNA ranged from 115 500 to 23 in the DNA dilutions of A–D samples, the SD values ranged from 11 716.90 to 1.32 and the RSD values ranged from 5.54 to 10.78%.

To validate the allelic stability of the *SPS* gene, the Ct values of the four rice cultivars with the same dilution from 12 laboratories are presented in **Table 7** and analyzed using the LSD test in SPSS 13.0 software. The calculated LSD-test values showed that no significant difference was found among the four rice cultivars with the same dilution in each laboratory (LSD test, $p > 0.05$). Moreover, the largest

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Table 4. PCR Efficiency (*E*) of Each Standard Curve from 12 Laboratories

laboratories	A		B		C		D	
	E	SD	*E*	SD	*E*	SD	*E*	SD
1	0.9842		1.0009		0.9998		0.9726	
2	0.9608		1.0293		0.9769		0.9834	
3	0.9815		0.9706		1.0022		1.0263	
4	1.0303		1.0580		1.0093		0.9373	
5	1.2233		1.1434		0.9033		0.9943	
6	1.0285		1.0780		1.0032		1.0237	
7	1.0061	0.0903	1.0358	0.0740	1.0511	0.0587	1.0493	0.0354
8	1.0304		1.0595		1.0328		1.0313	
9	0.9472		0.9857		1.0778		1.0059	
10	1.0720		1.0720		1.1047		1.0370	
11	0.9400		0.8557		0.9258		0.9620	
12	0.8463		0.9521		0.9566		0.9643	

Table 5. Square Regression Correlations (*R*^{2}) of Each Standard Curve from 12 Laboratories

laboratories	A		B		C		D	
	R^2	SD	R^2	SD	R^2	SD	R^2	SD
1	0.9963		0.9988		0.9982		0.9970	
2	0.9883		0.9945		0.9874		0.9919	
3	0.9998		0.9991		0.9986		0.9986	
4	0.9990		0.9987		0.9970		0.9968	
5	0.9966		0.9859		0.9951		0.9977	
6	0.9975		0.9939		0.9987		0.9982	
7	0.9995	0.0031	0.9960	0.0037	0.9992	0.0034	0.9979	0.0020
8	0.9984		0.9977		0.9991		0.9991	
9	0.9987		0.9963		0.9994		0.9989	
10	0.9965		0.9973		0.9988		0.9994	
11	0.9982		0.9959		0.9987		0.9962	
12	0.9954		0.9993		0.9976		0.9989	

Table 6. Difference of the Quantified Copy Numbers among Four Rice Cultivar DNA Dilutions in This Trial

copy number	mean A	mean B	mean C	mean D	SD	RSD (%)
115500	126295.1	109311.2	98640.8	116933.5	11716.90	10.39
11550	13725.5	12224.8	10947.6	13820.2	1366.44	10.78
1155	1295.3	1181.4	1066.7	1307.9	124.25	9.30
115	128.1	115.5	110.2	121.8	7.04	6.63
23	23.4	23.1	22.4	25.4	1.32	5.54

difference between the mean and individual Ct values among the 12 laboratories was less than 1 (0.13−0.95). The variability of Ct values observed among the DNA samples of four rice cultivars in 12 laboratories might be attributed to unavoidable experimental errors, including DNA-quantity estimation, liquid handling, DNA solution stability, etc. The RSD values of the quantified results of each diluted sample from A to D among 12 laboratories are listed in **Table 8** and range from 13.24 to 34.97%. All calculated RSD values were acceptable when compared to the accepted value of RSD for GMO analysis (*40*). Therefore, all data from different laboratories are reliable and demonstrated that the copy number of the *SPS* gene in different rice cultivars is a stable single copy per haploid genome.

Validation of *SPS* Qualitative PCR Method. To assess the sensitivity of the qualitative PCR method of *SPS*, the limit of detection (LOD) was further validated. In this ring trial, 10 DNA samples with serially diluted samples coded from S1 to S10 were sent to each laboratory. The supplied DNAs were extracted from powder containing a mixture of maize and varying amounts of rice seeds at 10, 1, 0.1, 0.05, and 0.01% rice content with two repeated samples with different codes. The results showed that all 12 participants could detect the 279 bp fragment from the DNA samples with the rice contents of 10, 1, and 0.1%, respectively. A total of 4 of 12 laboratories obtained the positive results from the DNA samples with the rice content of 0.05%. A total of 2 of 12 laboratories could detect the 279 bp fragment in the DNA samples with the rice content of 0.01%.

From these results, we believe that the LOD of the *SPS* qualitative PCR assay is at least 0.1%, which corresponds to around 230 copies of haploid rice genomic DNA. The LOD of this qualitative PCR assay is considered sufficient for GMO detection (**Table 9**).

Validation of *SPS* Quantitative PCR Method. To validate the developed quantitative PCR method of *SPS*, four standard curves were constructed in each laboratory using rice genomic DNA serially diluted to 50, 5, 0.5, 0.05, and 0.01 ng (absolute amounts in each PCR reaction) from different rice varieties, such as 3M, Balilla, Guangluai4, and Shennong265. The PCR efficiency, linearity of the quantitative standard curve, limit of detection, repeatability, and reproducibility were used to assess the applicability of quantitative real-time PCR. All of these parameters were tested in this trial. Furthermore, eight practical blind rice samples from four different cultivars with different quantities were

Table 7. Amplification Data in Real-Time PCR and Allelic Stability Determination of the *SPS* Gene for Four Different Rice Cultivars

laboratory code	mean Ct values[a]				mean	SD of the mean	difference[b]	difference[c]
	A	B	C	D				
1	23.01	23.08	23.80	23.08	23.24	0.37	0.23	0.56
2	22.87	23.19	23.48	22.66	23.05	0.36	0.39	0.43
3	23.63	23.59	23.38	23.21	23.45	0.19	0.24	0.18
4	22.73	23.54	24.42	23.18	23.47	0.72	0.74	0.95
5	24.45	24.62	25.16	24.55	24.70	0.32	0.25	0.46
6	23.69	23.60	23.63	23.08	23.50	0.28	0.42	0.19
7	23.33	23.55	23.53	23.77	23.55	0.18	0.22	0.22
8	22.44	23.16	22.24	22.59	22.61	0.39	0.37	0.55
9	22.97	23.12	23.21	23.32	23.15	0.15	0.18	0.17
10	22.77	23.14	23.42	22.27	22.90	0.50	0.63	0.52
11	22.81	23.38	23.96	23.61	23.44	0.48	0.63	0.52
12	20.67	20.64	20.62	21.07	20.75	0.21	0.13	0.32

[a] A total of 115 500 rice haploid genomic copies used. [b] The difference between the mean and the smallest Ct value. [c] The difference between the mean and the largest Ct value.

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Table 8. Difference of the Quantified Results of Rice DNA Dilutions among 12 Laboratories in This Trial

	A			B			C			D		
copy number	mean	SD	RSD (%)	mean	SD	RSD (%)	mean	SD	RSD (%)	mean	SD	RSD (%)
115500	126295.1	30894.78	24.46	109311.2	29678.23	27.15	98640.8	32388.37	32.83	116933.5	39613.14	33.88
11550	13725.5	4758.64	34.67	12224.8	3783.28	30.95	10947.6	3465.87	31.66	13820.2	4420.14	31.98
1155	1259.3	388.13	30.02	1181.4	364.39	30.85	1066.7	319.19	29.92	1307.9	393.29	13.24
115	128.1	38.02	29.67	115.5	30.91	26.76	110.2	38.55	34.97	121.8	40.42	33.17
23	23.4	8.11	34.72	23.1	7.91	22.35	22.4	7.13	31.91	25.4	8.71	34.30

Table 9. LOD of the Qualitative PCR Assay of *SPS*

year of collaboration	2007				
relative concentration ($W_{rice/rice+maize}$) (%)	10	1	0.1	0.05	0.01
number of laboratories	12	12	12	12	12
number of laboratories submitting results	12	12	12	12	12
number of samples per laboratory	2	2	2	2	2
number of laboratories accepted results	12	12	12	12	12
number of laboratories with the detection of positive results	12 (100%)	12 (100%)	12 (100%)	4 (33.33%)	2 (16.67%)

quantified using the *SPS* quantitative real-time PCR assay. The bias between the quantified value and true value was calculated and used to assess the suitability of this quantitative PCR assay for practical rice sample quantification.

Efficiency and Linearity of Quantitative Standard Curves. Each participant was requested to dilute the DNA standards, run the quantitative PCR, and submit the Ct values. These Ct values were plotted against the log of the absolute amount of the rice genomic DNA (ng) to obtain the standard curves. PCR efficiency (E) was calculated using the equation $E = 10^{(-1/\text{slope})} - 1$. The PCR reaction efficiencies ranged from 0.8463 to 1.2233 (**Table 4**). The square regression correlation (R^2) values were all above 0.99 (**Table 5**). In addition, the difference of E and R^2 among four rice cultivars in each laboratory was statistically analyzed in **Table 10**. The values of SD and RSD were acceptable for a standard real-time PCR assay. The high PCR efficiency and linearity of the standard curves indicated that the *SPS* gene was suitable for the quantitative analysis of the rice samples.

Limit of Quantification (LOQ). To determine the LOQ of the quantitative PCR assay of *SPS*, five serial dilutions of the DNA samples of four different rice cultivars were employed for construction of standard curves. The dynamic range of the constructed standard curves was 0.01−50 ng in a 25 μL volume of the PCR reaction. The high PCR efficiency and good linearity of the four standard curves at this dynamic range were revealed from the reports of these 12 laboratories, and the quantified result of each DNA dilution from 0.01−50 ng was within the 95% confidence interval. Thus, the deduced LOQ of this quantitative PCR was as low as 0.01 ng, which is about 23 copies of haploid rice genomic DNA, showing that the rice *SPS* gene quantitative PCR has sufficient sensitivity for rice sample quantification.

Repeatability and Reproducibility. Repeatability and reproducibility were both calculated using the standard rice genomic DNA dilutions. The standard deviation (SD^r) and relative standard deviation (RSD^r) of repeatability values are reported, as estimated from ring trial results for each DNA dilution level. The standard deviation (SD^R) and relative standard deviation (RSD^R) of reproducibility describe the interlaboratory variation. All of those values were calculated from the data 3 times and each time with three replicates according to ISO 5725. The RSD^r of the quantitative PCR of the *SPS* gene ranged from 0.16 to 9.76%, and

Table 10. Efficiency and Square Regression Correlation of the Quantitative PCR of *SPS*

laboratory code	efficiency	SD	RSD (%)	R^2	SD	RSD (%)
1	0.99	0.0136	1.37	0.9976	0.0011	0.11
2	0.99	0.0294	2.97	0.9905	0.0033	0.33
3	1.00	0.245	2.47	0.9990	0.0006	0.06
4	1.01	0.0516	5.11	0.9979	0.0012	0.12
5	1.07	0.1442	13.52	0.9938	0.0054	0.54
6	1.03	0.0317	3.07	0.9971	0.0022	0.22
7	1.04	0.0208	2.01	0.9981	0.0016	0.16
8	1.04	0.0140	1.35	0.9986	0.0007	0.07
9	1.00	0.0548	5.46	0.9983	0.0014	0.14
10	1.07	0.0276	2.58	0.9980	0.0013	0.13
11	0.92	0.0459	4.99	0.9973	0.0014	0.14
12	0.93	0.0559	6.01	0.9978	0.0018	0.18

Table 11. Repeatability and Reproducibility of the *SPS* Gene Quantitative PCR

copy number	SD^r	RSD^r (%)	SD^R	RSD^R (%)
115500	169.89	0.16	10005.64	9.26
11550	53.93	0.43	16.62	0.13
1155	16.96	1.39	134.75	11.08
115	4.98	4.18	24.42	20.51
23	2.32	9.76	2.98	12.56

the RSD^R ranged from 9.26 to 20.51% (**Table 11**), indicating that this established quantitative PCR system is stable and reliable in rice genomic DNA quantification.

Quantification of Blind Samples. Eight blind rice DNA samples with varying rice DNA concentrations were provided to each participating laboratory. Each participant was requested to quantify the blind sample using the four constructed standard curves. The calculated data are presented in **Table 12**. During the statistical analysis, 14 quantitative data points of a total of 864 (1.6%) were excluded on the basis of Cochran's test. The average bias between the quantified values and true values of blind samples among 12 laboratories ranged from 5.22 to 26.53%. One slight higher bias value (26.53%) was obtained in the blind samples with the concentration of 1 ng/μL. For the blind sample quantification, the repeatability and reproducibility of the results were also calculated. The RSDs of repeatability ranged from 14.29 to 20.98%, and the RSDs of reproducibility ranged

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Table 12. Results of Blind DNA Samples Using Quantitative *SPS* Real-Time PCR

unknown samples (ng)	0.5	0.5	1	1	2	5	5	10
laboratories returning results	12	12	12	12	12	12	12	12
samples per laboratory	1	1	1	1	1	1	1	1
total data number	108	108	108	108	108	108	108	108
data excluded	4	2	0	8	0	0	0	0
reason for exclusion	Cochran's test	Cochran's test		Cochran's test				
mean value	0.4078	0.4121	0.8146	0.7344	1.8575	5.2610	5.4457	10.8896
repeatability SD	0.0583	0.0719	0.1435	0.111	0.3362	0.9364	1.1427	1.8677
repeatability RSD (%)	14.29	17.44	17.62	15.11	18.10	17.80	20.98	17.15
reproducibility SD	0.1302	0.0618	0.2496	0.0927	0.2013	0.8109	0.9896	1.6175
reproducibility RSD (%)	31.92	14.99	30.65	12.63	10.84	15.41	18.17	14.85
bias (absolute value)	0.0922	0.0878	0.1853	0.2655	0.1424	−0.2610	−0.4458	−0.8896
bias (%)	−18.44	−17.57	−18.54	−26.53	−7.12	5.22	8.92	8.90

from 10.84 to 31.92%. All of the results suggest that the quantified results of blind samples are reliable, and the *SPS* quantitative PCR system is suitable for the quantification of rice samples.

In conclusion, with the contribution of the participants of the collaborative trial, we have validated the rice *SPS* gene with three main characteristics necessary for an endogenous reference gene: species specificity, low heterogeneity, and stable single-copy number in rice cultivars. Furthermore, the *SPS* qualitative and quantitative PCR methods were validated with high efficiency, high sensitivity, reliable repeatability and reproducibility, and acceptable accuracy in blind sample quantification, which qualifies the *SPS* gene as a reliable endogenous reference gene in transgenic rice identification and quantification.

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