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Comparison of the specificity, stability, and PCR efficiency of six rice endogenous sequences for detection analyses of genetically modified rice

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ABSTRACT

Species-specific endogenous reference sequences are indispensable in the development of methods to detect genetically modified (GM) crops and food/feed. We evaluated and compared the applicability of 6 rice (*Oryza sativa*) endogenous sequences, including 5 previously reported sequences; SPS1 derived from the *sucrose phosphate synthase* (SPS) gene, PLD1 and PLD2 derived from the *phospholipase D* (PLD) gene, GOS9 derived from the root-specific gene *gos9*, and ppi-PPF derived from the *ppi-phosphofructokinase* (ppi-PPF) gene, as well as a newly designed sequence, SPS2 in the rice *SPS* gene promoter region. PCR efficiency and stability were evaluated with 28 rice cultivars, and species specificity was evaluated using gDNAs isolated from major crops and rice-related species. SPS1 and GOS9 were less easy to be amplified and showed lower PCR amplification stabilities than the other sequences among rice cultivars. On the other hand, PLD1 showed high PCR efficiency and stability but low specificity against rice. Meanwhile, ppi-PPF was moderate in all evaluated characteristics. SPS2 and PLD2 showed higher PCR efficiencies and stabilities than those of other sequences, and also had acceptable species specificities. We conclude that SPS2 and PLD2 are ideal endogenous sequences for use in the development of methods to detect and quantify GM rice.

1. Introduction

Rice is one of the most important staple foods in the world, especially in Asian countries. Among grains, global production of rice is second only to maize (Faostat, 2011). A tremendous amount of time and effort has been devoted to cross breeding rice varieties in order to improve crop yield, pest resistance, and disease resistance. IR8 is a high-yielding, semi-dwarf variety known as miracle rice in the so-called rice “green revolution” (Sasaki et al., 2002). Genetically modified (GM) technology has also been applied to improve the production of rice. Several herbicide-tolerant GM rice have been developed, including LLRICE06, 62, and 601 (CERA, 2012). In China, insect-resistant rice events have been developed, and two, Huahui No.1 and Bt Shanyou 63, were approved for commercialization in 2009 (Lu, 2010). Furthermore, practical utilization of golden rice, which is engineered to accumulate beta-carotene (Hoa, Al-Babili, Schaub, Potrykus, & Beyer, 2003; Paine et al. 2005), is expected to help reduce vitamin A deficiency in the near future.

At present, however, many countries, including Japan, have not yet approved commercial cultivation and utilization of GM rice. Unauthorized GM rice contamination in food has often occurred and raised severe economic consequences. Specific, sensitive and reliable methods for detecting GM rice are required to monitor and control its distribution worldwide. Species-specific endogenous sequences are needed as internal positive controls for the development of detection methods for both authorized and unauthorized GM crops. Many countries legislate the labeling systems of authorized GM crops and their derived foods. To enforce such labeling systems, a practical threshold level of GM content and a

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Table 1
Primers and hydrolysis probes for real-time PCR systems.

Target	Name	Sequence	Length	Reference
SPS1	SPS 1-F	5′-TTG CGC CTG AAC GGA TAT-3′	81 bp	Jiang et al., 2009
	SPS 1-R	5′-CGG TTG ATC TTT TCG GGA TG-3′		
	SPS-P	5′FAM-TCC GAG CCG TCC GTG CGT C-TAMRA3′		
SPS2	SPS 2-F	5′-GGA TCA TCC CGA AAA GAT CAA C-3′	91 bp	This study
	SPS 2-R	5′-ATG GCA GTG GGA GAG ATT GTG-3′		
	SPS-P 2-3	5′FAM-ACG AGA CCA CCG TGG GCC CCA T-TAMRA3′		
PLD1	PLD F(KVM159)	5′-TGG TGA GCG TTT TGC AGT CT-3′	68 bp	JRC, 2006
	PLD R(KVM160)	5′-CTG ATC CAC TAG CAG GAG GTC C-3′		
	PLD P(TM013)	5′FAM-TGT TGT GCT GCC AAT GTG GCC TG-TAMRA3′		
PLD2	PLD3959F	5′-GCT TAG GGA ACA GGG AAG TAA AGT T-3′	80 bp	MHLW, 2012
	PLD4038R	5′-CTT AGC ATA GTC TGT GCC ATC CA-3′		
	PLD P	5′FAM-TGA GTA TGA ACC TGC AGG TCG C-TAMRA3′		
GOS9	GOS 1F	5′-TTA GCC TCC CGC TGC AGA-3′	68 bp	Hernández et al., 2005
	GOS 1R	5′-ATT CAG GCT GCG CAA CTG TT-3′		
	GOS P	5′FAM-AGA GTC CAC AAG TGC TCC CG-TAMRA3′		
ppi-PPF	PPF-1F	5′-AAT TCT GTC ATG TAT TTG AGC AGT TCA-3′	80 bp	Chaouachi et al., 2007
	PPF-1R	5′-AAT GAC AAC AAG CCC ATC CAA-3′		
	PPF-P	5′FAM-ACA CTG TAA ACA AAC-MGB3′		

validated quantitative method for estimating the unintentional commingling of GM crops are necessary. Thresholds for the unintentional commingling of approved GM events have been set at 0.9% (European Commission, 2003a, 2003b), 3% (Ministry of Agriculture and Forestry of Korea, 2000), and 5% (Consumer Affairs Agency, Government of Japan, 2011; Hino, 2002) in the European Union (EU), Korea, and Japan, respectively.

ISO 21570 (2005) specifies that the copy number of an endogenous reference gene is used for normalizing the concentration of a GM-specific target gene in real-time PCR-based quantitative analysis. Several sequences targeting rice endogenous genes have been developed for this purpose, such as sucrose phosphate synthase (SPS) (Ding et al., 2004; Jiang et al., 2009; Wang et al., 2010), phospholipase D (PLD) (JRC, 2006), the rice-root specific gene (GOS9) (Hernández, Esteve, & Pla, 2005), ppi-phosphofructokinase (ppi-PPF) (Chaouachi et al., 2007), and starch branch enzyme (RBE4) (Zhang et al., 2012). The SPS target sequence, designated as SPS1, was validated by international collaborative studies (Ding et al., 2004; Jiang et al., 2009) and SPS1 was previously utilized in the Japanese standard method for detection of Bt rice (MHLW, 2007). The PLD target sequence, designated as PLD1, has been used in the detection of LLRICE 62 in both the EU (JRC, 2006) and Japan (MHLW, 2006). Since 2012, another PLD target sequence, designated as PLD2, has replaced SPS1 in the Japanese method for detecting 63Bt, NNBt and CpTI (MHLW, 2012).

In this study, we evaluated and compared the PCR efficiency and specificity of the rice endogenous sequences listed above (except for RBE4, as its target sequence was not identified in the Nipponbare whole genome sequence databases by our homology search, indicating that it is not conserved in a major rice cultivar). During the course of the evaluation, the performance of the respective PCR assays in each rice endogenous sequences was not necessarily sufficient. We then designed a novel rice-specific sequence, designated SPS2, in the *SPS* gene promoter region. Finally, we describe the comparison and evaluation of the rice endogenous sequences SPS1, SPS2, PLD1, PLD2, GOS9, and ppi-PPF.

2. Materials and methods

2.1. Plant materials

We mainly used 28 rice cultivars as follows: Aichinokaori, Akitakomachi, Balilla, Belle patna, Blue belle, Guangluai4, Haenuki, Hanaetizen, Hejiang19, Hinohikari, Hitomebore, Hoshinoyume, IR8, IR36, IR64, Kasalath, Kitaake, Kirara397, Koshihikari, Kusahonami, Massigura, Momiroman, Nipponbare, Nonghu6, Raffaello, Tachisugata, Tsugaruroman and Yumeakari (Table 2). We also used 2 ancient rice cultivars, black and red rice for sequence analysis of SPS2. Balilla, Belle patna, Blue belle, Guangluai4, Hejiang19, IR8, IR36, Kasalath, Kitaake, Nipponbare, Nonghu6, Raffaello were kindly provided by the National Institute of Agrobiological Sciences (Ibaraki, Japan); IR64 was kindly provided by the Japan International Research Center for Agricultural Sciences (JIRCAS) (Okinawa, Japan); Kusahonami, Momiroman and Tachisugata were kindly provided by the Japan Grassland Agriculture and Forage Seed Association (Hokkaido, Japan); and Aichinokaori, Akitakomachi, Haenuki, Hanaetizen, Hinohikari, Hitomebore, Hoshinoyume, Kirara397, Koshihikari, Massigura, Tsugaruroman, Yumeakari, red rice, black rice, foxtail millet (*Setaria italic*), and Japanese barnyard millet (*Echnochloa utilis*) were obtained from local markets in Japan.

Table 2
Comparison of Cq values of six endogenous rice sequences from 28 cultivars.

	Origin	SPS1	SPS2	PLD1	PLD2	GOS9	ppi-PPF
Aichinokaori	Japan	28.79	21.97	22.17	22.09	22.67	22.71
Akitakomachi	Japan	28.71	22.00	22.22	22.17	22.62	22.90
Balilla	Italy	29.04	22.77	23.02	22.93	23.59	23.50
Belle patna	USA	28.69	22.44	22.62	22.52	23.28	22.95
Blue belle	USA	29.05	22.40	22.61	22.49	23.35	22.96
Guangluai4	China	28.89	22.24	22.47	22.37	23.10	22.81
Haenuki	Japan	28.42	22.10	22.33	22.23	22.76	22.89
Hanaetizen	Japan	28.71	22.12	22.40	22.26	22.77	22.94
Hejiang19	China	28.67	22.41	22.63	22.51	23.18	23.02
Hinohikari	Japan	28.18	22.13	22.38	22.22	22.99	22.89
Hitomebore	Japan	28.44	22.11	22.30	22.20	22.83	22.84
Hoshinoyume	Japan	28.55	22.21	22.46	22.33	23.17	23.02
IR8	Philippine	28.85	22.10	22.44	22.34	23.03	22.70
IR36	Philippine	29.08	22.40	22.62	22.49	23.35	22.93
IR64	Philippine	27.98	21.98	22.05	22.10	22.64	22.25
Kasalath	India	28.92	22.19	22.41	22.31	22.77	22.92
Kitaake	Japan	28.33	22.31	22.60	22.48	23.26	23.08
Kirara397	Japan	28.78	22.03	22.27	22.17	22.65	22.93
Koshihikari	Japan	28.66	22.11	22.27	22.19	22.58	22.79
Kusahonami	Japan	28.89	22.50	22.74	22.62	23.33	23.25
Massigura	Japan	28.46	22.04	22.30	22.18	22.79	22.83
Momiroman	Japan	28.76	22.43	22.67	22.58	23.49	23.34
Nipponbare	Japan	28.68	22.21	22.44	22.42	23.06	23.02
Nonghu6	China	28.64	22.55	22.69	22.60	23.30	23.00
Raffaelo	Italy	28.62	22.35	22.57	22.50	23.08	22.97
Tachisugata	Japan	28.19	22.24	22.44	22.33	23.17	22.65
Tsugaruroman	Japan	28.18	22.12	22.29	22.22	22.97	23.00
Yumeakari	Japan	28.39	22.20	22.39	22.30	22.96	22.95

Wheat seeds (*Triticum aestivum*) of the Haruyutaka variety and barley seeds (*Hordeum vulgare*) of the Harrington variety were harvested in Japan, while soybean was purchased from Ryokokushoji (Hiroshima, Japan) and maize from Quality Technology International (Elgin, IL, USA).

2.2. Oligonucleotide primers and probes

The primers and hydrolysis probes (Bustin et al. 2009) used in this study are listed in Table 1. PLD1 and PLD2 have been used as rice endogenous taxon-specific sequences in the Japanese standard methods for LLRICE601 (MHLW, 2006) and Bt rice (MHLW, 2012), respectively. PLD1 has also been used in the European Commission's Joint Research Centre (JRC), Institute for Health and Consumer Protection, Ispra, Italy as a reference gene (JRC, 2006). The oligonucleotide primers and hydrolysis probes were synthesized by FASMAC (Kanagawa, Japan) and Life Technologies (Carlsbad, CA, USA), respectively. The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ ends and 6-carboxytetramethylrhodamine (TAMRA) or Minor Groove Binder-Non-Fluorescent Quencher (MGB) at the 3′ ends.

2.3. DNA extraction

Rice genomic DNAs (gDNAs) were extracted using GM Quicker 2 (NIPPON GENE, Tokyo, Japan) according to the manufacturer's manual. The concentration of extracted DNA solutions was estimated by measuring ultraviolet (UV) absorbance at 260 nm. gDNA solutions were adjusted at a concentration of 10 and 20 ng/μL for conventional and real-time PCR analyses, respectively.

2.4. Conventional PCR analysis

Conventional PCR was performed as a qualitative analysis using a thermal cycler, GeneAmp PCR system 9700 (Life Technologies). Conventional PCR and agarose gel electrophoresis were conducted as described previously by Kuribara et al. (2002). Briefly, a 25 μL volume reaction solution was prepared that consisted of 25 ng of sample DNA, 200 μM dNTP, 1.5 mM $MgCl_2$, 0.625 U AmpliTaq Gold DNA polymerase (Life Technologies), and 0.25 μM of each primer. The step-cycle program was as follows: 10 min at 95 °C, 40 cycles, 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, followed by a final extension at 72 °C for 7 min. Five micro-liters of PCR products were electrophoresed on 3.0% agarose gel supplemented with 0.5 μg/mL of ethidium bromide in TAE buffer.

2.5. Real-time PCR analysis

TaqMan real-time PCR assays were carried out using the ABI PRISM 7900HT (Life Technologies) in 25 μL final volume reactions, consisting of 50 ng sample DNA, 12.5 μL TaqMan Universal PCR Master Mix (Life Technologies), 0.5 μM primer pairs, and 0.2 μM probe. The thermal cycling condition was set as 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 30 s at 95 °C and 1 min at 59 °C under 9600 emulation mode. In the reaction plate, each sample was measured in triplicate, and the average values were used in each analysis. The data were analyzed using Cq (quantification cycle) values (Bustin et al. 2009) generally known as Ct (cycle of threshold) values, which were determined with Manual Ct mode (Threshold, 0.2).

2.6. Sequence analysis

A DNA fragment containing the corresponding DNA sequences of the target PCR region for SPS1 and SPS2 was amplified using following degenerate primer pair: 5′-GAAAACGAGTGAATTAAC-CAATGCCN-3′ and 5′-GCGCTTCTAGGCGTTCN-3′ (N = A or T or G or C) and a DNA fragment containing the target PCR region for PLD1 was amplified using following degenerate primer pair: 5′-GACAG-GAGCATCCAWGATGCN-3′ (W = A or T) and 5′-GACCTGMAGGTT-CATACTCACN-3′ (M = A or C). The amplified PCR products were analyzed with a DNA sequencing system, CEQ8000 (Beckman Coulter, Brea, CA, USA).

3. Results

3.1. Conventional PCR analysis

To confirm whether sufficient PCR amplifications of SPS1, SPS2, PLD1, PLD2, GOS9 and ppi-PPF were obtained, we first conducted

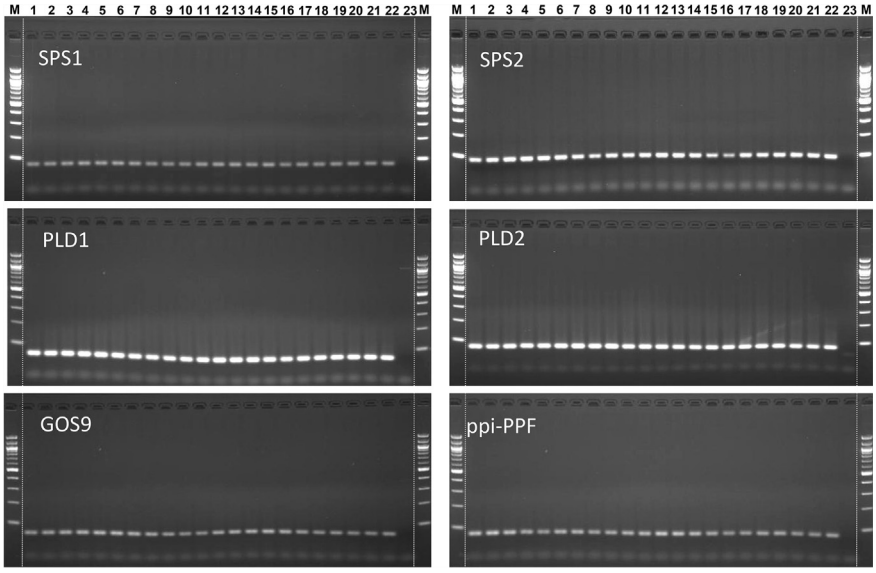

Fig. 1. Detection of PCR products of the 6 rice endogenous sequences. Lanes 1–22: amplifications from Akitakomachi, Kirara397, Koshihikari, Tsugaruroman, Hanaetizen, Hitomebore, Hinohikari, Hitomebore, Yumeakari, Nipponbare, Kasalath, Momiroman, Tachisugata, Kusahonami, IR8, IR64, Raffaello, Blue belle, Belle patna, Guangluai4, Hejiang19 and Nonghu6, respectively. Lane 23 is the negative control (no template). Lane M shows 100 bp ladder-size marker.

Table 3
Mean, SD, and Variance of Cq values of six endogenous rice sequences from 28 cultivars.

	SPS1	SPS2	PLD1	PLD2	GOS9	ppi-PPF
Mean	28.56	22.23	22.46	22.36	23.00	22.93
SD	0.55	0.20	0.21	0.20	0.35	0.22
Variance	0.304	0.040	0.043	0.040	0.122	0.048

Threshold, 0.2.

Table 4
Comparison of PCR efficiencies of six endogenous rice sequences using Nipponbare.

	SPS1	SPS2	PLD1	PLD2	GOS9	ppi-PPF
Slope	−3.458	−3.486	−3.496	−3.411	−3.729	−3.522
Linearity (R^2)	0.999	0.999	0.999	0.999	0.998	0.999
PCR efficiency	0.95	0.94	0.93	0.96	0.85	0.92

conventional PCR analysis using gDNA extracted from 22 rice cultivars as a template. Amplifications were clearly observed from all extracted DNAs with the 6 rice endogenous sequences (Fig. 1). Among them, it seemed that the PCR amplifications of SPS2, PLD1 and PLD2 were highly efficient, while those of SPS1, GOS9 and ppi-PPF were less efficient.

3.2. Comparison of PCR efficiency and stability of the 6 rice sequences with real-time PCR analysis

It is considered that the PCR amplification efficiency directly affects Cq values. To estimate PCR efficiencies of the 6 rice endogenous sequences as target using real-time PCR analysis, we first evaluated Cq values which were determined with a fixed threshold value at 0.2 using gDNA extracted from 28 rice cultivars (Table 2). The mean of Cq values of SPS2, PLD2, PLD1, ppi-PPF, GOS9 and SPS1 increased in that order (Table 3), and results corresponded with those obtained from the conventional PCR analysis. PCR efficiency was normally calculated using the slope of the standard curves as $10^{(-1/\text{slope})} - 1$(Dorak, 2006). We then prepared five serial dilutions of Nipponbare rice gDNA (200,000, 20,000, 2,000, 200, and 20 copy per reaction), and the real-time PCR analysis was conducted for the 6 rice endogenous sequences. As shown in Table 4, the obtained PCR efficiencies were almost consistent with the results of Cq values except for SPS1, meaning that the 5 rice endogenous sequences could be ranked as follows: PLD2 (highly efficient), SPS2 (highly efficient), PLD1 (highly or moderately efficient), ppi-PPF (moderately efficient), and GOS9 (less efficient). SPS1 seemed to be less efficient in the conventional PCR analysis (Fig. 1) and the Cq value was also remarkably higher than the others, but the PCR efficiency of SPS1 was high. For comparison, Cq values from 28 rice cultivars were determined using the threshold (0.2) in the linear phase (Fig. 2). When the threshold was set at 0.01, which looks like near the starting points of all amplification curves, the Cq values of SPS2, PLD1, PLD2, GOS9, and ppi-PPF were close each other ranging from 17 to 18. However, the Cq value of SPS1 was much higher than the others. These results suggested that there was certain retardation before the amplification curves of SPS1 appear, and the retardation but not PCR efficiency was responsible for the high Cq value. In fact, we found two mismatches between the forward primer and the corresponding rice genome sequences in the SPS1 target sequence. As shown in Table 1, the sequence of the forward primer of SPS1 was 5′-TTGCGCCTGAACGGATAT-3′ but the corresponding sequence of Nipponbare *SPS* gene (GenBank, EMBL, and DDBJ accession number, D45890) was 5′-TTGCGCCCTGA CGGATAT-3′, and the primer-template mismatches was conserved in all

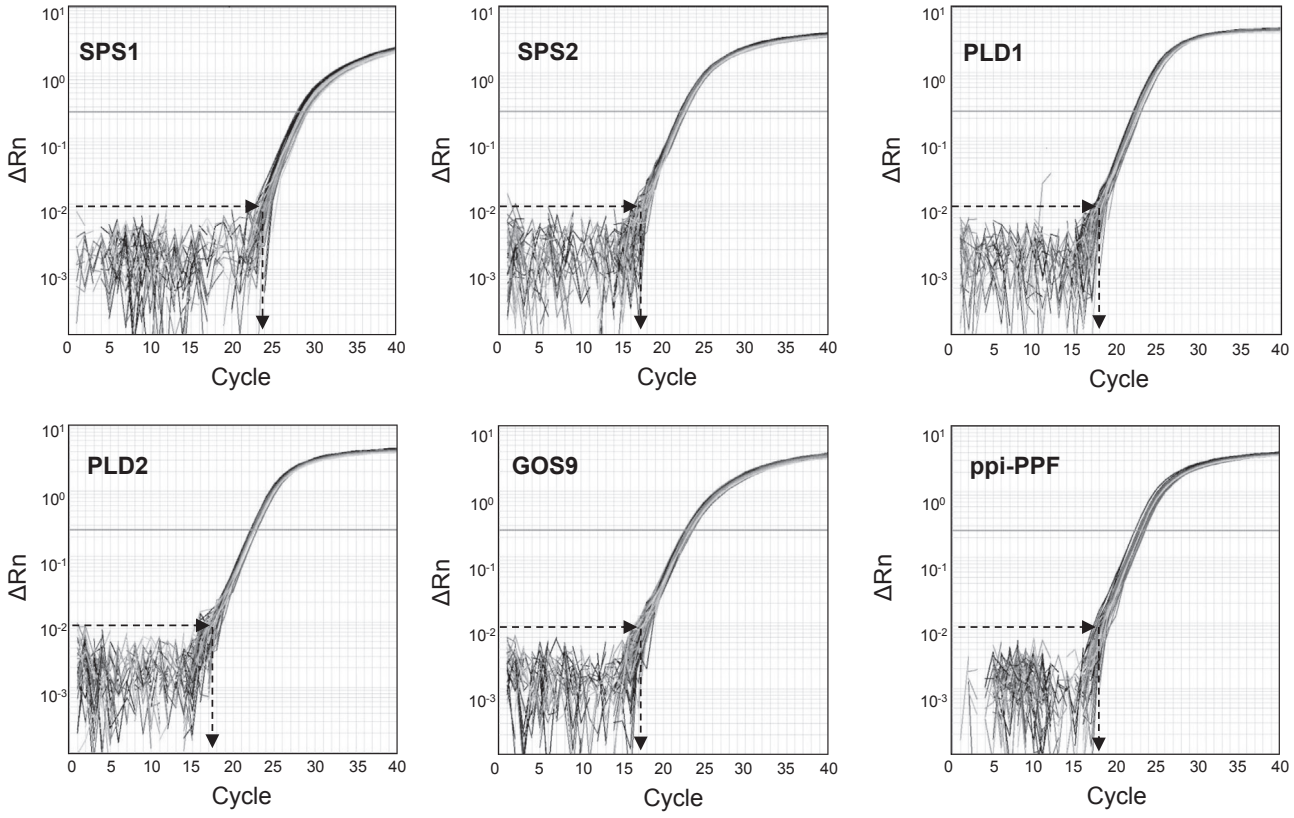

Fig. 2. Real-time PCR amplification profiles of 6 rice endogenous sequences from 28 rice cultivars were shown. Dotted arrows indicate the Cq values when the threshold lines were set at 0.01.

Table 5
Comparison of amplified stability of six endogenous rice sequences through *F* test.

*V*2		*V*1					
		SPS1	GOS9	ppi-PPF	PLD1	SPS2	PLD2
		0.304	0.122	0.048	0.043	0.040	0.040
SPS1	0.304	–	2.50	6.37	7.06	7.64	7.65
GOS9	0.122	–	–	2.55	2.55	3.05	3.06
ppi-PPF	0.048	–	–	–	1.11	1.20	1.20
PLD1	0.043	–	–	–	–	1.08	1.08
SPS2	0.040	–	–	–	–	–	1.00
PLD2	0.040	–	–	–	–	–	–

$F = V2/V1$
Critical Value of F is 1.90 ($\alpha = 0.05$). Underlined values mean significantly different.

analyzed 30 rice varieties in this study (data not shown). Therefore, these mismatches may reduce priming efficiency of PCR and attribute to the high Cq value. As the SPS1 sequence was less amplifiable, SPS1 is not considered to be preferable as an endogenous reference sequence for PCR detection analyses.

For the 28 rice cultivars listed above, precise genomic sequences were not determined, except for whole sequenced cultivars such as Nipponbare (International Rice Genome Sequencing Project, 2005) and Koshihikari (Yamamoto et al., 2010), suggesting that the targeted 6 endogenous sequences might not be completely conserved in some rice cultivars. It is also possible that the copy numbers of the 6 endogenous sequences in the haploid genome might be different among the rice cultivars. These differences would lead to variance of real-time PCR-based quantification among rice cultivars. To evaluate such intraspecific differences, amplification stabilities in the 6 endogenous sequences were evaluated as the variances of Cq values in the rice cultivars. According to the variance, the 6 rice endogenous sequences could be ranked as follows: PLD2 (0.040), SPS2 (0.040), PLD1 (0.043), ppi-PPF (0.048), SPS1 (0.122), and GOS9 (0.304) (Table 4). We further compared the amplified stabilities of the 6 endogenous sequences with an F test (Table 5). The stabilities of SPS1 and GOS9 were significantly different from those of PLD1, SPS2 and PLD2. These results indicate that SPS2, PLD1 and PLD2 are highly stable, SPS1 and GOS9 are less stable, and ppi-PPF are moderately stable sequences among the analyzed 28 rice cultivars.

3.3. Comparison of specificity of the 6 rice sequences

To evaluate the specificity of the 6 rice endogenous sequences, conventional and real-time PCR analyses were conducted. We prepared gDNAs from other major crops, such as maize, soybean, wheat and barley, and gramineous millets such as foxtail millet and Japanese barnyard millet, which are closely related to rice. In the conventional PCR analysis, the amplifications from SPS1, SPS2, PLD2, GOS9 and ppi-PPF were detected from rice cultivars, and with other crops there were no non-specific PCR amplifications observed to the vicinity of the target amplification products on the agarose gel. On the other hand, amplifications of similar-size target PCR product PLD1 were detected in maize, foxtail millet and Japanese barnyard millet, indicating that the target sequence of PLD1 was not rice specific (Fig. 3). In the real-time PCR analysis, several non-specific amplification curves from gramineous millets were observed in SPS1, SPS2, PLD2 and ppi-PPF (Fig. 4). Amplifications from foxtail millet were detected only once in triplicate in both SPS1 and SPS2. In contrast, amplifications from Japanese barnyard millet (but not foxtail millet) were observed in PLD2, and the amplification appeared three times in the triplicate analyses. In ppi-PPF, amplifications from both the foxtail millet and Japanese barnyard millet were observed. PLD1 showed the lowest specificity with real-time PCR analysis, because non-specific amplifications were observed not only from the gramineous millets but also from the maize. These results suggested that the specificity of the 6 rice endogenous genes against the related species could be ranked as follows: GOS9 (highly specific), SPS1, SPS2 and PLD2 (specific), ppi-PPF (moderately specific), and PLD1 (less specific).

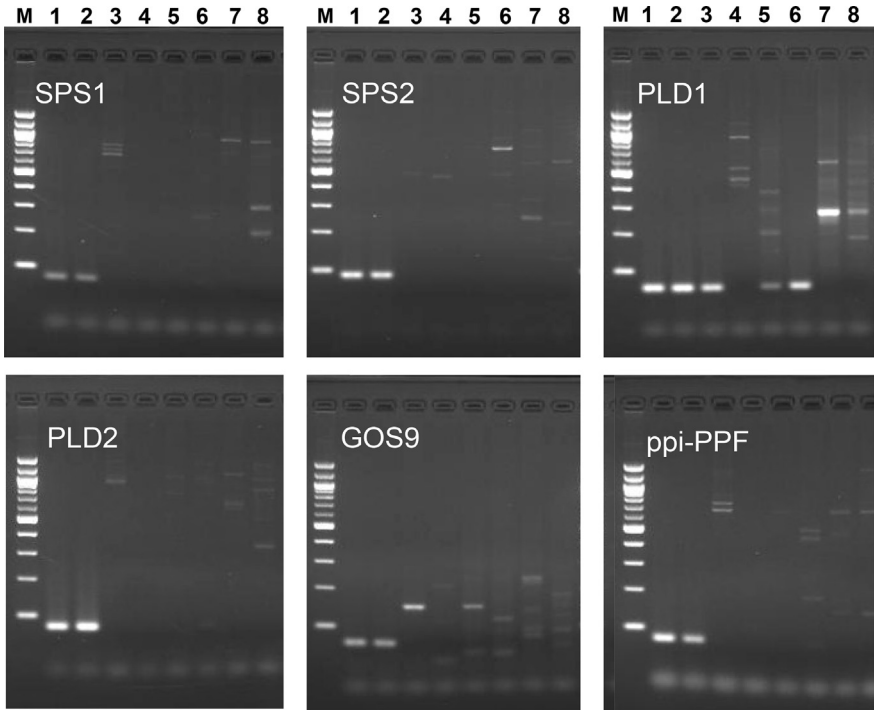

Fig. 3. Specificity test of primer pairs for the 6 rice endogenous sequences. Lanes 1–8 are amplifications from red rice, black rice, maize, soybean, foxtail millet, Japanese barnyard millet, wheat and barley, respectively. Lane M shows 100 bp ladder-size marker.

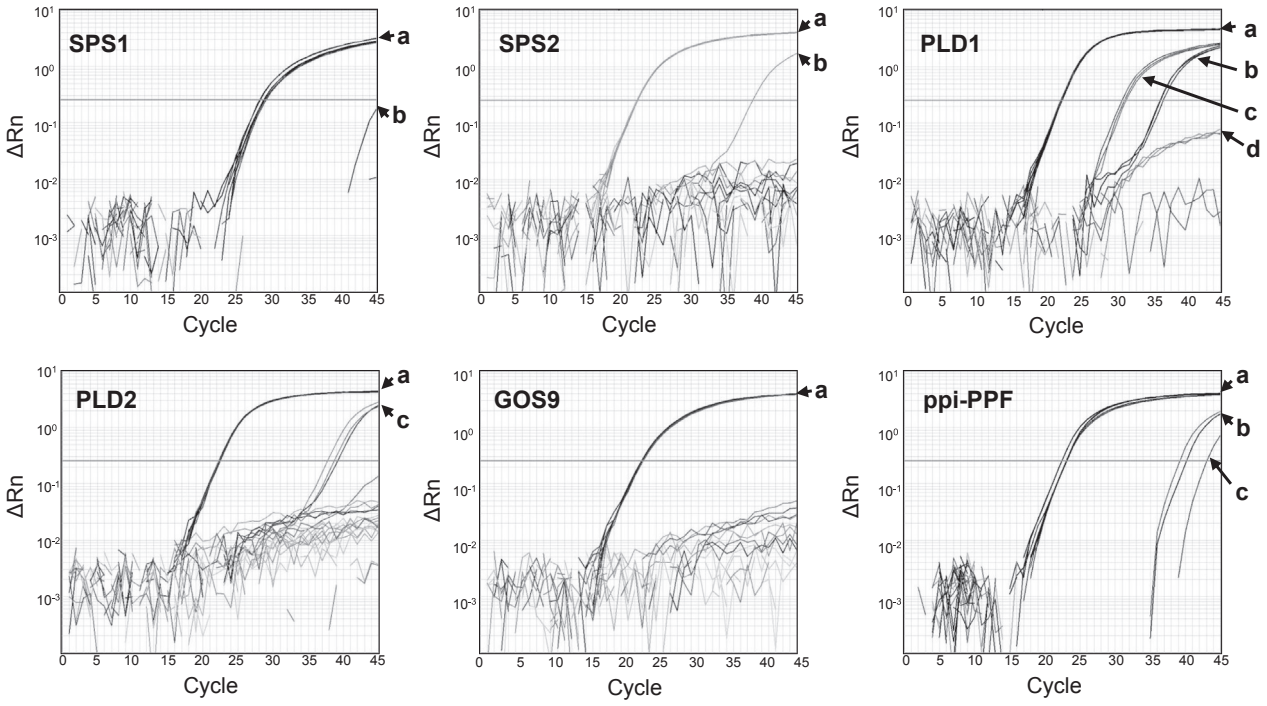

Fig. 4. Specificity test of primers and probes with real-time PCR analyses. Amplification profiles for 6 rice endogenous sequences were shown; a indicates amplifications from rice cultivars containing Nipponbare, red rice and black rice; b, c, and d indicate amplifications from foxtail millet, Japanese barnyard millet, and maize, respectively.

4. Discussion

In this study, we evaluated and compared 6 rice endogenous sequences regarding PCR efficiency, amplification stability, and rice specificity. Although nothing has been commercialized yet, many kinds of GM rice have been developed to be practically used in Japan (Ito et al., 2006; Iwai et al., 2002; Komatsu, Ohtake, Hasegawa, Terakawa, & Wakasa, 2006; Shimono et al., 2007; Takagi et al., 2005). In the course of developing detection methods for GM rice, the evaluation of rice endogenous sequences has become necessary. According to our evaluation, the target sequences of SPS1 and GOS9 were not easy to be amplified compared to the other sequences. The Cq value obtained from SPS1 was remarkably high, and the PCR efficiency of GOS9 was less efficient. These features may influence analytical performance of GM detection methods. In addition, the PCR amplifications for SPS1 and GOS9 were less stable (Table 4), and low PCR stability increases the variance of obtained results among rice cultivars. The target sequence of PLD1 was not rice-specific, although the PCR efficiency and stability were good. We isolated homologous sequences of PLD1 from maize, foxtail millet and Japanese barnyard millet using a degenerate primer pair and sequenced the amplification products (Fig. 5A). Those sequences including Zea mays phospholipase D family protein (NCBI accession number, NM_001152770) and Setaria italica phospholipase D alpha 2-like (NCBI accession number, XM_004965886) showed high homology (approximately 90%)

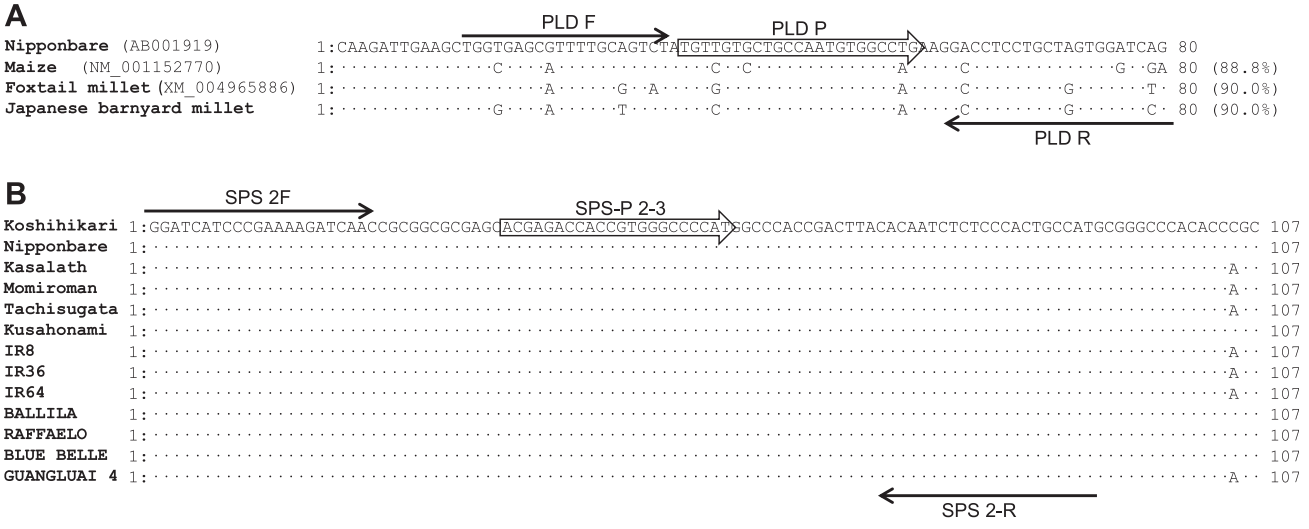

Fig. 5. Alignment of the target sequences. A, Sequences of amplified DNA fragments containing PLD1 or homologous sequences from Nipponbare, maize, and the gramineous millets were shown. B, Representative sequences of 13 rice cultivars of amplified DNA fragments containing SPS2 were shown. The primers and probes for the target sequences are boxed and underlined, respectively.

Table 6
Characterization of six endogenous rice sequences.

	SPS1	SPS2	PLD1	PLD2	GOS9	ppi-PPF
Cq value	Very high	Low	Low	Low	High	Moderate
PCR efficiency	Good	Good	Good	Good	Fair	Moderate
Stability	Fair	Good	Good	Good	Fair	Moderate
Specificity	Good	Good	Fair	Good	Excellent	Moderate

with rice Nipponbare PLD1 (GenBank, EMBL, and DDBJ accession number, AB001919), and explained its low rice specificity. In ppi-PPF, PCR efficiency, stability and specificity of the target sequence were moderate but not good. Low specificity in an endogenous sequence may jeopardize accurate assessments of GM rice detection methods. Particularly, in a qualitative analysis, a low rice-specific endogenous sequence should not be used as a positive control because one cannot be certain that observed amplifications were derived from the rice genome. High detection-sensitivity, species-specificity and the less variabilities among cultivars would be required for reference sequences for GMO monitoring. We therefore designed SPS2 as a rice endogenous sequence in the *SPS* gene promoter region. Amplification products containing the SPS2 target sequence were isolated using a degenerate primer pair, and were sequenced from 28 rice cultivars and two ancient rice cultivars (red rice and black rice). Sequence analysis revealed that the target sequence of SPS2 is 100% conserved in the 30 rice cultivars. Fig. 5B shows the representative results of 13 rice cultivars. The target sequence of SPS2 showed high efficiency, stability and specificity, although the obtained results were not always the best in all evaluated points. We summarized the characterization of the six rice sequences (Table 6), and SPS2 and PLD2 showed almost equally favorable results.

In conclusion, SPS2 and PLD2 have sufficient PCR efficiency, stability among cultivars, and specificity to be considered ideal-reference sequences for PCR-mediated GM rice detection methods.

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