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Development of One Novel Multiple-Target Plasmid for Duplex Quantitative PCR Analysis of Roundup Ready Soybean

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To enforce the labeling regulations of genetically modified organisms (GMOs), the application of reference molecules as calibrators is becoming essential for practical quantification of GMOs. However, the reported reference molecules with tandem marker multiple targets have been proved not suitable for duplex PCR analysis. In this study, we developed one unique plasmid molecule based on one pMD-18T vector with three exogenous target DNA fragments of Roundup Ready soybean GTS 40-3-2 (RRS), that is, CaMV35S, NOS, and RRS event fragments, plus one fragment of soybean endogenous *Lectin* gene. This *Lectin* gene fragment was separated from the three exogenous target DNA fragments of RRS by inserting one 2.6 kb DNA fragment with no relatedness to RRS detection targets in this resultant plasmid. Then, we proved that this design allows the quantification of RRS using the three duplex real-time PCR assays targeting CaMV35S, NOS, and RRS events employing this reference molecule as the calibrator. In these duplex PCR assays, the limits of detection (LOD) and quantification (LOQ) were 10 and 50 copies, respectively. For the quantitative analysis of practical RRS samples, the results of accuracy and precision were similar to those of simplex PCR assays, for instance, the quantitative results were at the 1% level, the mean bias of the simplex and duplex PCR were 4.0% and 4.6%, respectively, and the statistic analysis (*t*-test) showed that the quantitative data from duplex and simplex PCR had no significant discrepancy for each soybean sample. Obviously, duplex PCR analysis has the advantages of saving the costs of PCR reaction and reducing the experimental errors in simplex PCR testing. The strategy reported in the present study will be helpful for the development of new reference molecules suitable for duplex PCR quantitative assays of GMOs.

KEYWORDS: Genetically modified organisms; plasmid with multiple targets; duplex PCR; Roundup Ready soybean

INTRODUCTION

Genetically modified (GM) soybean is one of the mainly planted biotech crops with the area of 58.6 million hectares in 2007, which has the ratio of 51% in global biotech area, exceeding the area of non-GM soy (*1*). With the rapid increase of biotech crops in the market, consumers are concerned about the risk of GM crops and their derivates, resulting in more than 40 countries and areas issuing genetically modified organism (GMO) labeling regulations (*2*). The labeling threshold was also defined in different countries and areas, such as 0.9% in the European Union (*3*), 3% in Korea (*4*), and 5% in Japan (*5*).

The implementation of labeling regulations is based on the reliable and precise techniques for the detection of GMOs. Quantitative real-time PCR technique targeting specific DNA fragment has become a powerful tool for determining the absolute or relative DNA amount in GM foods. Four different PCR strategies based on different target DNA fragments were developed in GMO detection, and the event-specific PCR strategy, which targets the unique junction sequence between the host genomic DNA and the introduced DNA, has been widely used for GMO identification and quantification because of its high specificity (*6*). Up to now, several event-specific PCR assays have been developed and applied in practical GM soybean and maize quantification (*7, 8*). Windels et al. described the soybean genomic DNA sequences flanking the functional insert of GM soybean event GTS 40-3-2 or RRS (*9*), and also several event-specific quantitative PCR methods have been established for RRS based on the 3′ integrated border between the genomic and inserted DNA of RRS (*6, 10–12*).

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Table 1. Primers and TaqMan Probes for Duplex Real-time PCR Analyses

target	primer name	sequences (5′−3′)	amplicon size (bp)	ref
CaMV35S	T-35S-1F	GCCTCTGCCGACAGTGGT	84	*20*
	T-35S-2R	AAGACGTGGTTGGAACGTCTTC		
	T-35S−P	FAM CAAAGATGGACCCCCACCCACG BHQ1		
NOS	T-nos-1F	TTGGCAATAAAGTTTCTTAAGATTGAAT	87	*21*
	T-nos-2R	ACATGCTTAACGTAATTCAACAGAAATT		
	T-nos-P	FAM CTGTTGCCGGTCTTGCGATGATTATCAT BHQ1		
RRS	T-rrs-1F	TAGCATCTACATATAGCTTC	85	*10*
	T-rrs-2R	GACCAGGCCATTCGCCTCA		
	T-rrs-P	FAM ACAAAACTATTTGGGATCGGAGAAGA BHQ1		
Lectin	T-lectin-1F	GCCCTCTACTCCACCCCCATC	117	*22*
	T-lectin-2R	GCCCATCTGCAAGCCTTTTTGTG		
	T-lectin-P	HEX TTCGCCGCTTCCTTCAACTTCACCT BHQ1		

To identify and quantify GMOs in the samples, the application of the reference materials or calibrators for constructing the calibration curve is critical. Previous reports revealed that several reference materials have been used as calibrators for GMO detection, such as the certified reference materials (CRMs) from IRMM (*7*). The limitations of CRMs are their confined quantitative range (0−5.0% GMO only), the inconvenient preparation procedures, high cost, and high price. To solve these problems, plasmid DNA (pDNA) as calibrator in GMO quantification has been demonstrated to be a good alternative to the genomic DNA (gDNA) extracted from conventional CRMs (*13, 14*). Until now, several quantitative PCR assays using pDNA as the calibrator have been developed (*8, 11, 15–19*). However, the reported pDNAs with single target or tandem multiple targets cannot be used for simultaneously quantifying endogenous and exogenous DNA targets of one GM event using duplex PCR. Duplex PCR has the ability to detect two targets in one PCR reaction, which could save considerable time and workload, and improve the efficiency and throughput of analyses. Also, the results from duplex PCR might be more accurate than those from simplex PCR since the pipetting error can be reduced in duplex PCR reactions.

In this study, we developed one novel plasmid with the exogenous DNA fragments (CaMV35S, NOS, and the RRS event-specific fragment) of RRS and the soybean endogenous *Lectin* fragment. This plasmid has been demonstrated to be applicable as calibrators for the three duplex quantitative PCR assays of CaMV35S, NOS, and RRS event with the soybean *Lectin* gene.

MATERIAL AND METHODS

Materials and DNA Extraction. Pure Roundup Ready soybean was developed and supplied by Monsanto Company. Non-GM soybean was purchased from a local market in Shanghai, China. A group of samples that contained different percentages of GM soybean GTS 40-3-2 (5.0%, 3.0% and 1.0%, W/W) in non-GM soybean was prepared gravimetrically in our laboratory by mixing dried non-GM and 100% GM soybean powder.

The genomic DNAs of the prepared samples were extracted and purified using the Plant DNA Mini-Prep kit (Ruifeng Agro-tech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The plasmid DNAs were isolated and purified using the Plasmid Mini Kit (Watson Biotechnologies, Inc., Shanghai, China). The quality and quantity of the extracted DNAs were evaluated and calculated using absorbance measurements at 260 and 280 nm wavelengths and further checked by 1% agarose gel electrophoresis. The copy number of the plasmid DNA was calculated on the basis of DNA quantity and the size of the constructed plasmid.

Oligonucleotide Primers and Probes. The oligonucleotide primers and TaqMan probes used in this study were synthesized by TAKARA Co. Ltd. (Dalian, China) and listed in **Table 1**. HEX (5-hexachlorofluorescein) was labeled on the 5′ end of the probe of *Lectin* as the fluorescent reporter, while FAM (6-carboxyfluorescein) was used as the fluorescent reporter for the probes of the three exogenous fragments of Roundup Ready Soybean (CaMV35S, NOS, and RRS event) on their 5′ ends. The fluorescent black-hole-quencher 1 (BHQ1) was linked to the 3′ end of all of the probes. The primer pair T-rrs-F/R combined with probe T-rrs-P targeted the 3′ junction fragment between soybean genomic DNA and rearranged DNA (*10*), and the primer pair T-35s-F/R and T-nos-F/R combined with probes T-35s-P and T-nos-P were used to quantify the amount of CaMV35S promoter and NOS terminator, respectively, in GM samples (*20, 21*). The *Lectin* gene was used to quantify the amount of soybean genomic DNA (*22*).

Construction of Multiple-Target Plasmids. In a duplex quantitative PCR, two fragments are coamplified using two different sets of primers and probes in one tube. We assumed that the plasmid with tandemly inserted multiple targets is not suitable for duplex PCR analysis because the forward primer of one set and the reverse primer of another set would produce an unexpected DNA fragment during duplex amplification. Thus, this unexpected amplification would result in quantification bias. To confirm this point, three exogenous gene fragments (RRS event, CaMV35S, and NOS) and one endogenous gene (*Lectin*) fragment of soybean were selected as multiple targets and constructed into two plasmids (pSOY and pSOY1). In pSOY, the three soybean exogenous DNA fragments were amplified separately and combined in tandem using overlapping PCR strategy, and then, the new synthetic fragment and the *Lectin* gene fragment were cloned into the pMD18-T vector at two separate sites with about 2.6 kb physical distance by the molecular manipulation method (*23*). In pSOY1, the three soybean exogenous DNA fragments and the soybean endogenous gene fragment were connected in tandem using overlapping PCR strategy and cloned into another pMD18-T vector without this 2.6 kb separation. The two obtained plasmids, pSOY and pSOY1, were confirmed by sequencing analysis using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems) by Shanghai BioAsia Biotechnology Co., Ltd. (Shanghai, China).

The constructed plasmids were then transformed into *E. coli* DH5α according to the routine molecular method, and the purified plasmids were linearized with *SnaB* I, which was located outside the integrated fragments. The resulting pDNAs were then diluted to 2×10^0, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies μL^{-1}.

Simplex and Duplex Real-Time PCR Conditions. Previous studies showed that the simplex quantitative PCR assay using plasmid DNA as calibrator gave comparable estimation of the expected GM content of soybean samples with that using genomic DNA as controls (*13, 15, 24*). Simplex and duplex PCR assays were carried out in 25 μL PCR volume containing the following ingredients: 1× PCR buffer, 400 μM each of dATP, dGTP, and dCTP, 800 μM dUTP, 1.5 U *Taq* DNA polymerase, 0.2 U amperase uracil *N*-glycosylase (UNG), and the optimized concentrations of $MgCl_2$, primer, and probe for each simplex and duplex PCR assays (**Table 2**).

PCR reactions were all performed according to the following program: 50 °C for 2 min, 95 °C for 10 min, and 50 cycles of 95 °C for 15 s and 60 °C for 30 s. The fluorescent signal was monitored

Table 2. Optimized Concentrations of $MgCl_2$, Primer, and Probe for Simplex and Duplex PCR Assays

Optimized Simplex PCR Assays				
	Lectin	RRS	CaMV35S	NOS
$MgCl_2$ (mM)	7	7	5	7
primer (pM)	320	240	240	240
probe (pM)	128	96	120	120

Optimized Duplex PCR Assays			
	Lectin and RRS	Lectin and CaMV35S	Lectin and NOS
$MgCl_2$ (mM)	7	7	7
primer for Lectin (pM)	240	160	160
probe for Lectin (pM)	96	48	48
primer (pM)	480	320	640
probe (pM)	192	96	192

during every PCR cycle at the annealing phase. The PCR reagents were purchased from Biocolor biotechnology Co., Ltd. (Shanghai, China) except for primers and probes.

Construction of Standard Curves. TaqMan real-time PCR assays were carried out in a fluorometric thermocycler Rotor-Gene 3000A, and the data was analyzed using the Rotor-Gene version 6.0 software (Corbett Research, Australia). Serially diluted pSOYs with the copy numbers of 1×10^1, 5×10^1, 5×10^2, 5×10^3, 5×10^4, 5×10^5, and 5×10^6 (absolute amount in 5 μL) were used as calibrators to construct the standard curves. Sonicated salmon testis DNA (10 ng/μL, Sigma D-9156) was used as the no-template control (NTC). All of the real-time PCR reactions were repeated three times and each time with triple replication. The threshold of the real-time PCR was determined using the Auto-Find Threshold bar of the software in the exponential phase of the amplification curve. The Ct value was defined as the cycle number that the amplification fluorescence exceeds the threshold. Then the obtained Ct values were plotted against the log of the absolute number of pDNA copies in each PCR tube to get the standard curve. PCR efficiency (E) was calculated using the equation $E = 10^{-1/\text{slope}} - 1$ (*25*).

In the tests of the repeatability of these real-time PCR assays, six pSOY diluted concentrations of 5×10^1, 5×10^2, 5×10^3, 5×10^4, 5×10^5, and 5×10^6 copies (absolute amount in 5 μL) were used per reaction. Each reaction of one concentration was repeated three times and each time with triple replication.

Determination of GM Content of Blind Samples. According to the constructed standard curves, Ct values obtained from real-time PCR were used to determine the amount of transgenic and total plant genomic DNA of blind samples. For instance, the amount of soybean total DNA was calculated by Ct values of *Lectin* in the soybean endogenous PCR assay, and the amount of transgenic DNA was calculated by Ct values from CaMV35S, NOS, or RRS event PCR assays. The GM DNA content of the blind sample was calculated by the ratio of the amount of transgenic DNA (CaMV35S or NOS or RRS) and total soybean DNA with application of conversion factors (Cfs) determined by 100% RRS, according to the formula GM content (%) = (100% $\times$ transgenic DNA copy numbers)/(total soybean DNA copy numbers $\times$ Cfs) (*18, 19*).

RESULTS

Construction of pSOY and pSOY1. In this study, we designed and constructed two plasmids pSOY and pSOY1, and each contained four targets, that is, RRS event, CaMV35S, NOS, and *Lectin*. pSOY was supposed to be used as the duplex calibrator and pSOY1 as a control plasmid with tandem insertion of these four targets. In pSOY (**Figure 1A**), the soybean exogenous fragments and endogenous gene fragment were separated by inserting one 2.6 kb DNA fragment without obvious homology to the four targets, that is, RRS event, CaMV35S, NOS, and *Lectin*. While in pSOY1 (**Figure 1B**), the soybean exogenous gene fragments and endogenous gene

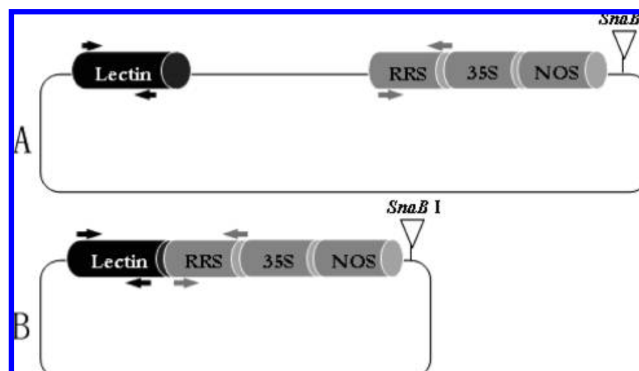

Figure 1. Schematic diagram of the integrated fragments in the two constructed plasmids, pSOY (**A**) and pSOY1 (**B**). Lectin, fragment of soybean endogenous reference gene *Lectin*; RRS, 3′ event fragment of GM soy line GTS 40-3-2; CaMV35S, fragment of CaMV35S promoter; NOS, fragment of NOS terminator. Arrowheads indicate the primers corresponding to the fragments.

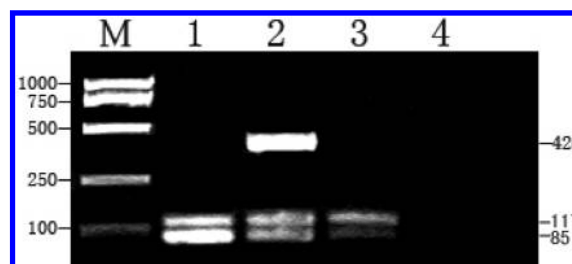

Figure 2. Gel image of PCR products amplified by the *Lectin* and RRS event specific duplex PCR assay. Lane 1, pSOY; lane 2, pSOY1; lane 3, gDNA of RRS; lane 4, NTC (no template control); lane M, DL2000 DNA marker. Short lines indicate the expected (85 bp *RRS* and 117 bp *Lectin*) and unexpected (420 bp) PCR products and the band sizes of the DNA marker.

fragment were tandemly connected by overlapping PCR and cloned into the vector. Sequencing results of the constructed plasmids (pSOY and pSOY1) showed that the expected plasmids were obtained, and the sizes of these two plasmids were 5.8 kb and 3.2 kb, respectively.

Suitability of pSOY for Duplex Qualitative PCR. After obtaining pSOY and pSOY1, we further tested their applicability in duplex PCR assay by amplifying the specific DNA fragments of the *Lectin* gene and RRS event in one tube. We observed the expected amplicons of the *Lectin* gene and RRS event in all of these reactions using pSOY, pSOY1, and soybean genomic DNAs as templates (**Figure 2**), while one unexpected amplicon about 420 bp in length was observed in the PCR reaction using pSOY1 DNA as template. We presumed that this unexpected amplicon resulted from the amplification of the *Lectin* forward primer and RRS reverse primer, and further sequencing analysis results confirmed this (data not shown), suggesting that pSOY1 with tandem inserts was not suitable to be used as calibrator in duplex PCR assays for RRS detection. However, the amplified fragments of the duplex PCR assay using pSOY seemed to be comparable with the result of gDNA, suggesting that pSOY was suitable for use as calibrator in duplex PCR analysis.

Duplex and Simplex Real-Time PCR Assays Employing pSOY as a Calibrator. To further confirm whether the plasmid pSOY could be used as the calibrator in duplex PCR assay for GM soybean quantification, three duplex PCR assays (CaMV35S and *Lectin*, NOS and *Lectin*, and RRS event and *Lectin*) were established using the pDNA of pSOY as calibrators. In each duplex PCR assay, two standard curves were constructed, one

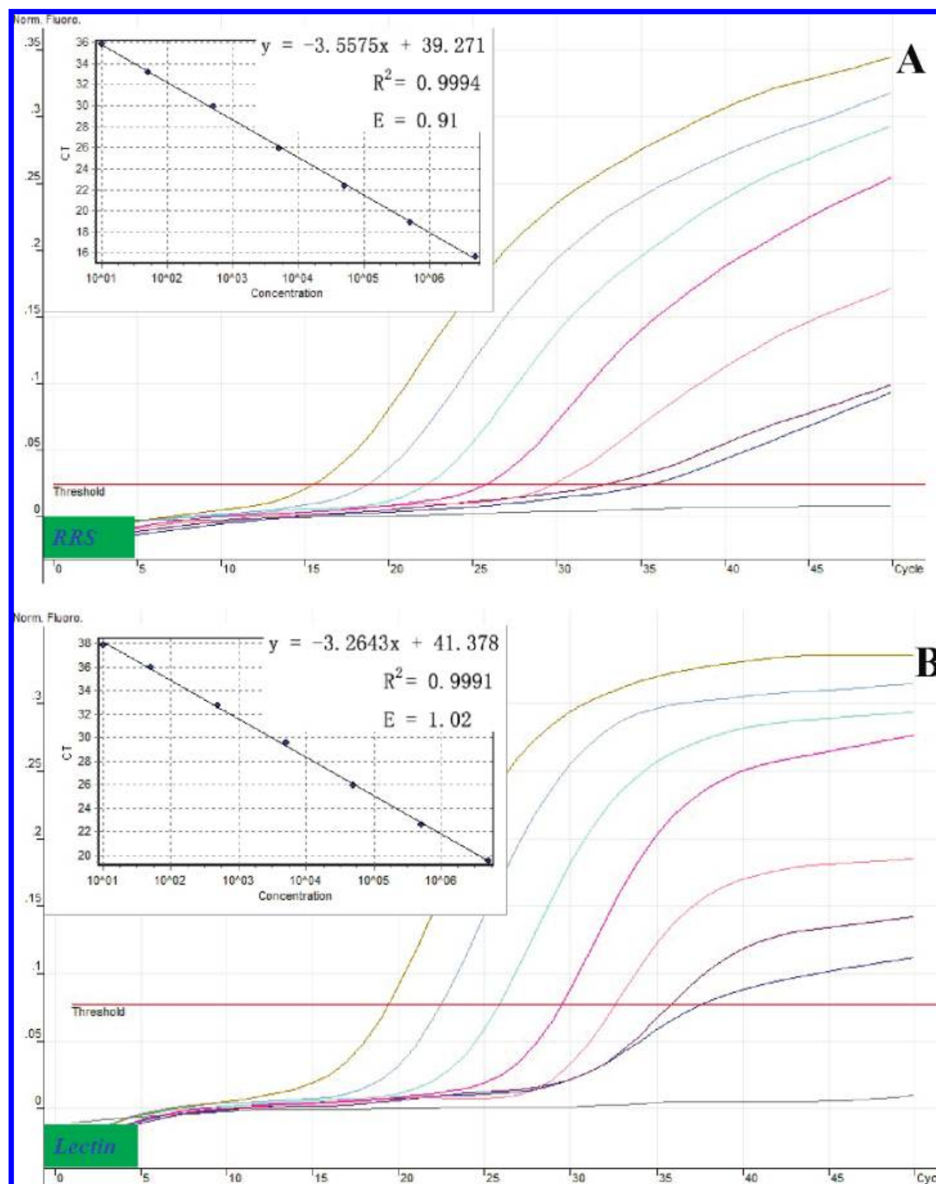

Figure 3. Amplification plots and standard curves for RRS event and *Lectin* duplex PCR assay. (**A**) RRS event PCR. (**B**) *Lectin* PCR. Seven serial dilutions of the pDNA from pSOY, corresponding to 1×10^1, 5×10^1, 5×10^2, 5×10^3, 5×10^4, 5×10^5, and 5×10^6 copies per reaction were used as the template for the duplex PCR assays.

Table 3. Characteristics of the Standard Curves Used for Duplex PCR Assays

duplex PCR assays		PCR efficiency	square regression correlations (R^2)	coefficient factor (Cf)
Lectin and CaMV35S	CaMV35S PCR	0.97	0.9968	1.31
	Lectin PCR	0.90	0.9997	
Lectin and NOS	NOS PCR	0.95	0.9986	1.43
	Lectin PCR	0.99	0.9975	
Lectin and RRS	RRS PCR	0.91	0.9994	1.02
	Lectin PCR	1.02	0.9991	

for the transgenic DNA quantification, and the other for total soybean DNA quantification (*Lectin*). A set of serially diluted pDNAs from pSOY (5×10^1, 5×10^2, 5×10^3, 5×10^4, 5×10^5, and 5×10^6 copies) were added to the duplex PCR reactions for standard curves construction. **Figure 3** shows the amplification plots and standard curves of duplex PCR assay for RRS event (**A**) and *Lectin* (**B**), and **Table 3** shows the PCR efficiencies and the square regression correlations (R^2) of duplex PCR assays. The PCR efficiencies were from 0.90 to 1.02, and the R^2 values were at least 0.996 for all of the standard curves for quantification. The high PCR efficiencies and the good linearity between copy numbers and Ct values visualized in the standard curves indicated that these duplex PCR assays established in this study were suitable for quantitative measurements. In the LOD and LOQ determination, the limit of detection (LOD) is the lowest copy number of initial template that can be detected. The limit of quantization (LOQ) is the lowest copies of initial template that can be quantified with a high degree of confidence (95%). In this study, 50 copies of pDNA from pSOY could be detected in all of the nine repeats for the three duplex PCR assays by GM targets and endogenous target, and 10 copies of this pDNA were detected occasionally (**Table 4**); therefore, we concluded that the LOD and LOQ of those duplex real-time PCR assays were 10 copies and 50 copies, respectively. On the basis of the plasmid size of pSOY (5 886 bp) and the soybean genome size (*6*), we deduced that the copy number ranging from 50 to 5×10^6 of the pDNA was sufficient to quantify GM soybean from 0.01% to 100% in 100 ng of genomic DNA for one reaction. Also, this range was supposed

Table 4. Repeatability of pSOY Used as the Calibrator in Duplex PCR Assays

duplex PCR assays	target PCR	standard	Ct value 1	Ct value 2	Ct value 3	Mean Ct	RSD%	target PCR	standard	Ct value 1	Ct value 2	Ct value 3	Mean Ct	RSD%
CaMV35SandLectin	CaMV35S	10	37.17	37.39	-	37.28	/	Lectin	10	37.97	37.96	-	37.96	/
		50	35.43	36.04	36.13	35.87	1.06		50	34.6	36.01	35.41	35.34	2.01
		500	32.58	32.95	33	32.84	0.7		500	32.02	32.18	32.13	32.11	0.25
		5,000	29.03	29.35	29.28	29.22	0.58		5,000	28.14	28.28	28.41	28.28	0.46
		50,000	25.37	25.29	25.56	25.41	0.55		50,000	24.42	24.33	24.5	24.42	0.37
		500,000	21.88	21.97	22.04	21.96	0.36		500,000	20.94	20.93	20.98	20.95	0.14
		5,000,000	18.53	18.65	18.69	18.62	0.43		5,000,000	17.65	17.64	17.72	17.67	0.28
NOS andLectin	NOS	10	38.07	39.62	-	39.01	/	Lectin	10	34.41	34.25	-	34.28	/
		50	36.15	36.21	36.09	36.15	0.17		50	32.79	32.86	32.71	32.79	0.23
		500	32.8	32.86	32.75	32.8	0.17		500	29.68	29.75	29.62	29.68	0.22
		5,000	29.51	29.46	29.6	29.52	0.24		5,000	26.43	26.5	26.36	26.43	0.26
		50,000	25.6	25.44	25.51	25.52	0.31		50,000	22.56	22.76	22.45	22.59	0.71
		500,000	22.12	22.06	21.79	21.99	0.82		500,000	19.03	19.05	19.14	19.07	0.31
		5,000,000	18.66	18.84	18.78	18.76	0.48		5,000,000	15.87	16.06	15.75	15.89	0.94
RRS andLectin	RRS	10	35.34	35.79	-	35.57	/	Lectin	10	37.81	-	-	37.81	/
		50	33.14	32.96	33.04	33.05	0.27		50	35.96	35.35	35.87	35.73	0.92
		500	28.99	29.92	29.45	29.45	1.58		500	32.77	32.71	32.73	32.74	0.09
		5,000	25.92	25.64	26.12	25.89	0.93		5,000	29.6	29.5	29.67	29.59	0.3
		50,000	22.13	22.13	22.4	22.22	0.68		50,000	25.99	25.81	25.95	25.92	0.35
		500,000	18.87	18.59	18.91	18.79	0.9		500,000	22.58	22.56	22.63	22.59	0.18
		5,000,000	15.77	15.96	15.67	15.8	0.95		5,000,000	19.61	19.56	19.53	19.57	0.20

Table 5. Accuracy and Precision Statistics for Simplex and Duplex Quantitative Methods

true value (%)		Cf	accuracy		precision		Cf	accuracy		precision	
			mean GMO (%)	bias (%)	SD	RSD (%)		mean GMO (%)	bias (%)	SD	RSD (%)
			Simplex					Duplex			
CaMV35S	5.00		5.15	2.93	0.06	1.17		4.75	5.00	0.10	2.11
	3.00	1.21	3.17	5.56	0.10	3.15	1.31	3.17	5.56	0.12	3.79
	1.00		0.93	6.67	0.17	18.28		0.99	0.67	0.17	17.17
NOS	5.00		4.95	0.93	0.09	1.82		5.03	0.60	0.21	4.17
	3.00	0.83	3.14	4.67	0.08	2.55	1.43	3.12	4.11	0.24	7.69
	1.00		0.98	2.00	0.05	5.10		1.06	5.67	0.20	18.90
RRS	5.00		4.88	2.40	0.07	1.43		5.13	2.67	0.17	3.31
	3.00	1.40	2.81	6.33	0.04	1.42	1.02	2.74	8.78	0.14	5.11
	1.00		1.03	3.33	0.11	10.68		1.07	7.33	0.13	12.15

to be sufficient to meet the needs of GMO labeling regulations. The RSD values of duplex PCR assays were calculated on the data of triplicate repeats of serial dilution levels of the pDNA, and the RSD values were between 0. 37% and 2.01% (**Table 4**).

Since simplex PCR is the generally used method for GM samples quantification, the duplex quantitative PCR results were comparable with those from simplex PCR in this study. The standard curves of simplex PCR assays for CaMV35S, NOS, RRS, and *Lectin* were constructed employing the pDNA from pSOY. The LOD and LOQ of the four simplex PCR assays were 10 copies and 50 copies, respectively. Compared with simplex PCR, duplex PCR assays had similar LODs and LOQs.

Quantitative Analysis of the GM Soybean Samples Using pSOY as a Calibrator. To further analyze the GMO content of blind samples using pDNA as a calibrator in duplex PCR, the coefficient factor (Cf) was calculated by the copy number ratios of exogenous sequence to endogenous sequence in genuine RRS. Theoretically, the transgenic sequence (CaMV35S, NOS, or RRS) and the endogenous gene *Lectin* in haploid RRS genome are both one copy (*7*); therefore, the theoretical Cf should be 1.0 for each quantitative PCR assays. The experimental Cfs of CaMV35S, NOS, and RRS duplex quantification assays were 1.31, 1.43, and 1.02, respectively, while they were 1.21, 0.83, and 1.40, respectively, for simplex assays. Thus, these deduced Cf values should be 1.0, and the discrepancy between experimental and theoretical Cfs might be generated by different PCR efficiencies (*7, 8, 25*).

After determining the Cfs of the quantitative PCR assays, a group of soybean samples containing different percentage of RRS at 5.0%, 3.0%, and 1.0% were used to evaluate the accuracy and precision of the established duplex PCR methods. The determined content of the soybean sample was calculated by the defined formula, as described in the Material and Methods section. The accuracy of the quantitative results was measured by the bias between the mean GM content and the true percentage. The precision was measured by standard deviation (SD) and relative standard deviation (RSD). Simplex PCR was also performed to contrast the quantitative results of duplex PCR methods. As shown in **Table 5**, the bias ranged from 0.60% to 8.78%, and the RSD ranged from 1.17% to 18.90% for all of the PCR methods of different GM soybean percentage. The SD values were from 0.04 to 0.24 in the GM soybean samples (**Table 5**). Compared to the accuracy and precision of previously reported data from maize, soybean, cotton, and potato samples (*18, 19, 25, 26*), the quantitative results of these soy samples were acceptable.

Meanwhile, the quantitative data obtained from duplex and simplex PCR methods were compared by statistic analysis (*t*-

test). The quantified data of each soybean sample was divided into two groups (duplex PCR and simplex PCR), and each group contained the quantitative results from the three repeats each of RRS, CaMV35S, and NOS PCR assays. The *t*-test results ($P < 0.01$) showed that the quantitative data from duplex and simplex PCR had no significant discrepancy for each soybean sample (5.0%, 3.0%, and 1.0%), indicating that the established duplex PCR methods could possibly be used for GM soybean quantification instead of simplex PCR using pSOY as a calibrator. In addition, the quantitative results from these three duplex PCR assays (RRS, CaMV35S, and NOS assays) for each soybean sample were statistically analyzed by means of the *t*-test, and no significant difference was observed ($P < 0.01$), indicating that these three duplex PCR assays were all suitable for quantification of these RRS samples.

DISCUSSION

In this study, we first report a novel plasmid, pSOY, which could be used as a calibrator in duplex PCR instead of conventional CRMs for GM soybean quantification. This new constructed plasmid contained GM soybean line GTS 40-3-2 exogenous sequences (RRS event, CaMV35S, and NOS) and the soybean endogenous reference sequence (*Lecitn*) with a physical distance of 2.6 kb. The construction module for the previous preparation of reference plasmids is generally achieved by tandemly inserting the target fragments. In this study, we constructed the pSOY1 plasmid using this approach, and experimental evidence showed that unexpected amplification occurred by interaction of the two primer pairs in duplex PCR, which is not suitable for duplex PCR quantification. In addition, the accuracy and precision of the established duplex PCR assays using the new plasmid pSOY were similar to those of the simplex PCR assays. In a previous study (*8, 12, 13*), the bias and the RSD of the quantitative results of known samples were from 0.67 to 28.00% and from 0.83 to 26.20%. In this study, the bias ranged from 0.60% to 8.78%, and the RSD ranged from 1.17% to 18.90%, further proving that this plasmid-based duplex PCR assay could be used for GMO quantification with the advantages of saving time and workload, and improving efficiency.

In the established duplex quantitative PCR assays using the constructed plasmid, pSOY, RRS event and *Lectin* duplex PCR assay can be used for GTS 40-3-2 event specific quantification of RRS, and CaMV35S and NOS duplex PCR assays can be used for screening the quantification of GM soybean and other GMOs containing these elements.

In conclusion, this newly constructed multiple target plasmid and established duplex quantitative assays are suitable for the practical detection and quantification of GM soybean and their derivates.

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