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Full Length Article

Establishment of Multi-Fluorescence Real-Time PCR Assay for GM Soybean Detection

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Abstract

In the past two decades, a growing number of genetically modified (GM) crops have been developed and commercialized worldwide. High-throughput methods for screening and identification of GM crops have become a requirement for food industry and government agencies. To effectively detect seven specific transgenic soybean lines named GTS40-3-2, MON89788, CV127, A5547-127, A2704-12, 305423, and 356043, seven different primer-probe sets (labeled with FAM, HEX, Taxas Red, Cy5, and Quasar 705) were designed for the multi-fluorescence real-time PCR (MF-RT-PCR) assay. Through a series of experiments and tests including of primer combination screening, system optimization, specificity test, sensitivity test, and applicability test, two GM soybean target specific systems, SOY-M1 and SOY-M2, were established. SOY-M1, a pentaplex PCR detection system, could efficiently identify Mon89788, A2704-12, 305423, and 356043, and SOY-M2, a tetraplex PCR detection system is able to detect GTS40-3-2 CV127, and A5547-127. The sensitivity of both systems is 0.1%. In summary, the MF-RT-PCR detection system established in this study is highly accurate, reliable, and efficient in detecting these seven soybean lines and their derivates. © 2020 Friends Science Publishers

Keywords: Detection; Genetically modified soybean; MF-RT-PCR; Event-specific

Introduction

The farming and commercialization of genetically modified (GM) crops has massively increased worldwide since the first commercialization of GM crop in 1996. In 2018, GM crops are grown in 26 countries on 191.7 million hectares, which has been increased by more than 113 folds (ISAAA 2018). Soybean is the first GM crop commercially grown by large scale and it is still the largest GM crop in cultivation now. As the production of GM crops is increasing year by year, the potential risks of GM crops to the environment and food safety have drawn more and more attention from the international community and the general public (Aqeel *et al.* 2019). International regulatory agencies are making efforts to supervise the release of GM crops and many GM labeling regulations have been established by different countries and groups to protect the public's right to information and the trade interests of agricultural products (Li *et al.* 2016). Therefore, the development of rapid and effective GM testing assays has become a requirement for implementation of the law and regulations (Zhang *et al.* 2015).

Up to now, detection of GM products is mainly based on PCR technology (Wang *et al.* 2018), including regular PCR, multiplex PCR (Mazur *et al.* 2017; Cottenet *et al.* 2019), qPCR (Guertler *et al.* 2019; Verginelli *et al.* 2019), and ddPCR (Niu *et al.* 2018; Bogožalec-Košir *et al.* 2019). Recently, isothermal amplification technology has also been employed in GMO detection, such as LAMP (Loop-mediated isothermal amplification) (Long *et al.* 2019) and RPA (Recombinase Polymerase Amplification) (Xu *et al.* 2014). The methods mentioned above have various limits in accuracy, cost and efficiency. Nowadays, with more and more GM events are continuously approved for commercial cultivation by countries each year (Simeon *et al.* 2005), the establishment of an accurate, rapid, high-throughput GM crop detection system is in high demand (Fu *et al*. 2017b). MF-RT-PCR (multi-fluorescence real-time PCR) has the advantage of harboring many pairs of primers and TaqMan probes labeled with different fluorescence in a single reaction to amplify multiple fragments in one tube. As this method has the benefits of saving time and cost, it has been widely used in the gene diagnosis field, for example, microbial detection (Wang *et al.* 2017; Zhang *et al.* 2020). MF-RT-PCR assays have also been reported for screening and detecting GM crops since 2002. Bhoge and associates used MF-RT-PCR assay to detect GM maize events, Bt11, Bt176, MON89034, MON810, and GA21 (Bhoge *et al.* 2016). As another example, 14 targets of transgenic maize,

including 7 common GM screening elements, 6 transgenic maize events, and a maize reference gene were identified by MF-RT-PCR (Wei *et al.* 2018). Multiplex droplet digital PCR protocols for quantification of GM maize events, Bt11, MON810, MON863, NK603, 59122, DAS1507, MIR604, GA21, MON89034, MIR162, MON8017, T25, and endogenous *hmgA* gene has been successfully established (Dobnik *et al*. 2018). Currently, MF-RT-PCR based methods have been widely used in screening elements or target genes in GM rice and maize but rarely in detecting GM soybean lines. The objectives of this study are to: 1), establish a multiplex real-time PCR assay for GM soybean detection; 2), utilize the assay to identify seven commercialized GM soybean lines. The method we established in this paper could serve as a novel method to detect GM soybean lines for the purpose of food safety supervision and GM soybean verification.

Materials and Methods

GM materials

The seeds of transgenic soybean events (GTS40-3-2, MON89788, CV127, A5547-127, A2704-12, 305423, 356043) were kindly provided by the Institute of Crop Science, Chinese Academy of Agricultural Sciences (Beijing, China). Non-transgenic soybean seeds were purchased from local market (Changchun, China) and validated to be GM-free by our laboratory.

To determine the specificity of the method, other GM crop events kindly provided by the corresponding developers were collected and used, including other 6 transgenic soybean events (MON87701, MON87708, MON87769, MON87705, FG72, SHZD32), 5 transgenic maize events (BT11, BT176, MON810, MON863, NK603), 3 transgenic rapeseed events (MS1, RF1, GT73), 4 transgenic rice events (KF-6, KMD, TT51, M12), and 4 transgenic cotton events (MON531, MON1445, MON15985, LLCOTTON25). Seeds of each pure line and non-GM crops were grinded into powder for DNA extraction.

DNA extraction

Genomic DNA was extracted and purified using the DNeasy Plant Mini Kit (QIAGEN) following the user's guide. The concentration and purity of extracted DNA were quantified using a NanoDrop ND-8000 spectrophotometer (Thermo Scientific Ltd., USA). DNA samples were diluted to a final concentration of 25 ng/μL and stored at -20°C before using.

Data analysis

All results were analyzed using the Ct values. The threshold of each element was set according to the final fluorescence of the negative samples. The reactions that have a Ct value no more than 38 are regarded as positive reactions. All results of multiplex fluorescence PCR detection systems were determined from data of sextuplicate reactions.

Primers and TaqMan probes

Seven GM soybean event specific genes and one soybean endogenous *Lectin* gene are the targets of our MF-RT-PCR assay. One system named SOY-M1 was developped for specific detection of GM soybeans MON89788, A2704-12, 3054423 and 356043, and the second system named SOY-M2 was for the detection of GM soybeans GTS40-3-2, CV127, A5547-127. The probe of the endogenous *Lectin* reference gene was labeled with 5-hexachloro-fluorescein (HEX) on the 5'-end as the fluorescent reporter in both systems. For the SOY-M1 system, the target of MON89788, A2704-12, 305423, the probes of them were labeled with FAM, CY5, Texas Red, and Quasar705, respectively on the 5'-end as the fluorescent reporters. For the SOY-M2 system, in which the targets of this assay are GTS40-3-2, CV127, and A5547, the probes of the target specific sequences were labeled with FAM, CY5, and TEX, respectively. TAMRA on the 3'-end as the fluorescent quencher dye was used in all probes in both systems. The primers and probes used in this research were synthesized by Sangon (Sangon, Shanghai, China) and the corresponding sequences are listed in Table 1 and 2. All primers and probes were diluted with an appropriate volume of distilled water, and stored at -20°C until use.

Optimization of MF-RT-PCR conditions

To optimize the MF-RT-PCR reaction systems, different primer concentrations were used to test the best condition for GM soybean detection. For SOY-M1 different concentrations of primers/probe to opitmize the PCR system were shown as A1 to A4 in Table 3, and for SOY-M2 were B1 to B4. In each system, we used four concentrations of primers, in which the amount of the corresponding probes were half of the primers. Other component of the MF-RT-PCR system were described as following.

Amplification systems and procedure

The real-time PCR assay for SOY-M1 and SOY-M2 were performed in a final volume of 25 μL that contains 1×HiTaq probe qPCR mastermix (Apexbio Biotechnology Co., Ltd., Beijing, China), template DNA, and primer/probe sets (final concentration shown in Table 1 and 2). The amount of DNA used in the reaction was 50 ng unless otherwise specified.

Each reaction was initially denatured at 95°C for 10 min, followed by 45 cycles of 94°C for 15 sec (denaturation), and 60°C for 1 min (annealing and extension). The real-time quantitative PCR reactions of SOY-M1 and SOY-M2 were performed separately on a Bio-Rad CFX96 Real-Time thermal cycler. The real-time

quantitative PCR reactions of SOY-M1 for the endogenous *Lectin* gene and four specific transgenic soybean target events were performed together using the HEX, FAM, CY5, TexasRed, and Quasar705 channels. The real-time quantitative PCR reactions of SOY-M2 for the endogenous *Lectin* gene and the other 3 specific transgenic soybean events were performed together using the HEX, FAM, CY5, and Texas Red channels. Fluorescence signals were monitored and analyzed at the annealing and extension steps during every PCR cycle using CFX Manager Version 1.6 (Bio-Rad, Hercules, USA). Results were analyzed using the software Opticon Monitor_ 2 version 2.02 (MJ Research, Waltham, M.A., U.S.A.).

Specificity test of MF-RT-PCR

The specificity of the SOY-M1 and SOY-M2 systems were evaluated using GM soybean events and other GMOs events, including 13 GM soybean events, mixture of 5 GM maize events, mixture of 4 GM rice events, mixture of 4 GM cotton events, and mixture of 3 GM rapeseed events. The content of each GM event is 1%. Non-GM soybeans were used as a negative control.

Sensitivity test of MF-RT-PCR

The GM samples of SOY-M1 system were firstly prepared by mixing GM soybean powder of MON89788, A2704-12, 305423, 356043 in equal proportions, and the GM samples of SOY-M2 system were mixed with GTS40-3-2, CV-127, and A5547-127. The samples we used in sensitivity test containing target soybean events were formulated by the mass ratio of the mixed GM samples of SOY1 and SOY2 with non-transgenic soybean seed powders respectively to make 25, 6.25, 1.56, 0.4, 0.2, 0.1, 0.05, 0.025 and 0.01% of each transgenic soybean event. The preparation procedure was carried out in accordance with standard material candidate process (Chinese Ministry of Agriculture Bulletin No. 1782-Technical specifications of preparation of the GMO standard materials). DNA samples were extracted and diluted to a final concentration of 25 ng/μL and stored at -20℃ before using. The sensitivity of multiplex fluorescence PCR detection system was determined by SOY-M1 and SOY-M2 multi-PCR amplifications, 6 parallels reactions of each sample.

Applicability test of MF-RT-PCR

The DNA samples used in applicability test of MF-RT-PCR system was prepared by mixing one or some of the GM soybean DNA including MON89788, A2704-12, 305423, 356043, GTS40-3-2, CV-127, A5547-127 with the non-GM soybean DNA by the mass ratio. All the samples containing the target soybean DNA were 0.2%. The reaction system of SOY-M1 and SOY-M2 contains 50 ng DNA as template. 6 parallels of each sample were tested.

Results

Establishment of MF-RT-PCR

To establish the MF-RT-PCR system to detect the 7 soybean events, we used the specific primer/probe sets for each of these events as shown in Table 1 and 2. Due to the limit of the fluorescence signals channels, we divided the PCR test into two groups. One is named SOY-M1 specific to MON89788, A2704-12, 305423 and 356043 soybean events, and the other is named SOY-M2 specific to GTS40-3-2, CV127, and A5547-127. Both detection systems included endogenous soybean gene *Lectin*. Four different final concentrations of primer/probe sets A1 to A4 as shown in Table 3 left were used in the amplification to optimize the MF-RT-PCR system of SOY-M1. It showed that A1 to A4 reactions were generated with the samples mixed by 0.1% DNA of MON89788, A2704-12, 305423, and 356043 soybean events. In SOY-M1, with the A1 primer/probe concentration, the soybean events 356043, 305423, and MON89788 could not be amplified. Neither A3 nor A4 could amplify the soybean events 356043. Only A2 succeeded amplifying all these 4 target soybean events (MON89788, A2704-12, 305423, 356043) and the soybean endogenous *Lectin* gene. The entire test was repeated four times, and the mean Ct value of these four transgenic events under the A2 condition was no more than 38.0. So the final primer concentrations for *Lectin*, MON89788, A2704-12, 305423, and 356043 were determined to be 0.6, 0.8, 0.6, 0.4 and 0.4 μmol/L, respectively in SOY-M1 (Fig. 1A). Similarly, as it was shown in Fig. 1B (right), four primers/probe concentrations (B1, B2, B3 and B4) for the 3 transgenic soybean events (GTS40-3-2, CV127, and A5547-127) were tested in the SOY-M2 system. By the same way, we found the optimal primer/probe concentration for *Lectin*, GTS40-3-2, CV127, and A5547-127 was 0.4 μmol/L under the B2 condition. All these three target transgenic events (GTS40-3-2, CV127 and A5547-127) were amplified under the B2 condition. Correspondingly, the probe concentration in both SOY-M1 and M2 was half of the target primers.

Specificity of MF-RT-PCR

To investigate the specificity of the MF-RT-PCR assay the SOY-M1 and SOY-M2 were tested by amplifying the genomic DNA of the relevant GM plant events, including 13 GM soybean events (GTS40-3-2, MON89788, CV127, A5547-127, A2704-12, 305423, 356043, MON87701, MON87708, MON87769, MON87705, FG72, and SHZD32), a mixed sample of 5 GM maize events (BT11, BT176, MON810, MON863, and NK603), a mixture of 4 GM rice events (KF-6, KMD, TT51, and M12), a mixture of 4 GM cotton events (MON531, MON1445, MON15985, and LLCOTTON25), a mixture of 3 GM rapeseed events (MS1, RF1, and GT73). Genomic DNA of the 17 samples was diluted by non-transgenic soybean DNA to make the

Table 1: SOY-M1 Fluorescence Pentaplex PCR system primers information

Primer/Probe	Final concentration (μM)	Sequence (5'-3')	Amplicon length (bp)	Reference
Lectin-QF	0.6	TCCACCCCCATCCACATTT	81	Pauli *et al.* (2001)
Lectin-QR	0.6	GGCATAGAAGGTGAAGTTGAAGGA		
Lectin-QP	0.3	HEX-AACCGGTAGCGTTGCCAGCTTCG-TAMRA		
MON89788-QF	0.8	TCCCGCTCTAGCGCTTCAAT	139	Delobel *et al.* (2013)
MON89788-QR	0.8	TCGAGCAGGACCTGCAGAA		
MON89788-QP	0.4	FAM-CTGAAGGCGGGAAACGACAATCTG-TAMRA		
A2704-12-QF	0.6	GCAAAAAAGCGGTTAGCTCCT	64	Mazzara *et al.* (2007°)
A2704-12-QR	0.6	ATTCAGGCTGCGCAACTGTT		
A2704-12-QP	0.3	Cy5-CGGTCCTCCGATCGCCCTTCC-TAMRA		
305423-QF	0.4	CGTGTTCTCTTTTTGGCTAGC	93	Mazzara *et al.* (2013)
305423-QR	0.4	GTGACCAATGAATACATAACACAAACTA		
305423-QP	0.2	TexasRed-TGACACAAATGATTTTCATACAAAAGTCGAGA-TAMRA		
356043-QF	0.4	GTCGAATAGGCTAGGTTTACGAAAAA	99	Mazzara *et al.* (2010)
356043-QR	0.4	TTTGATATTCTTGGAGTAGACGAGAGTGT		
356043-QP	0.2	Quasar705-CTCTAGAGATCCGTCAACATGGTGGAGCAC-TAMRA		

Table 2: SOY-M2 Fluorescence Tetraplex PCR system primers information

Primer/Probe	Final concentration (μM)	Sequence (5'-3')	Amplicon length (bp)	Reference
Lectin-QF	0.4	TCCACCCCCATCCACATTT	81	Pauli *et al.* (2001)
Lectin-QR	0.4	GGCATAGAAGGTGAAGTTGAAGGA		
Lectin-QP	0.2	HEX-AACCGGTAGCGTTGCCAGCTTCG-TAMRA		
GTS40-3-2-QF	0.4	TTCATTCAAAATAAGATCATACATACAGGTT	84	Mazzara *et al.* (2007b)
GTS40-3-2-QR	0.4	GGCATTTGTAGGAGCCACCTT		
GTS40-3-2-QP	0.2	FAM-CCTTTTCCATTTGGG-TAMRA		
CV127-QF	0.4	AACAGAAGTTTCCGTTGAGCTTTAAGAC	88	Savini *et al.* (2011)
CV127-QR	0.4	CATTCGTAGCTCGGATCGTGTAC		
CV127-QP	0.2	CY5-TTTGGGGAAGCTGTCCCATGCCC-TAMRA		
A5547-QF	0.4	GCTATTTGGTGGCATTTTTCCA	75	Delobel *et al.* (2009)
A5547-QR	0.4	CACTGCGGCCAACTTACTTCT		
A5547-QP	0.2	TexasRed-TCCGCAATGTCATACCGTCATCGTTGT-TAMRA		

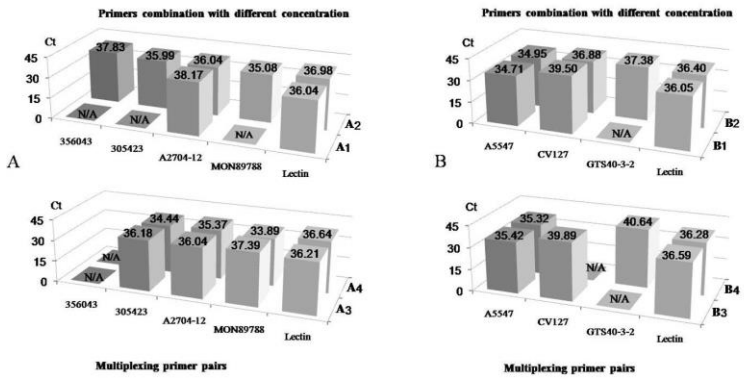

Fig. 1: Primer/Probes concentration optimization in MF-PCR systems using CFX96 System

A1-A4 represents the Ct values obtained by different primers/probe concentrations combinations in SOY-M1 real-time PCR system targeting specific sequences of the *Lectin*, MON89788, A2704-12, 305423, 356043 respectively; B1-B4 represents the Ct values obtained by different primers/probe concentrations combinations in SOY-M2 real-time PCR system targeting specific sequences of the *Lectin*, GTS40-3-2 CV127, A5547-127 respectively. (Concentrations of the primers/probe used in A1-A4 and B1-B4 are shown in Table 3)

content of each GM event 1%. Non-GM soybean was used as the negative control. The real-time PCR results from SOY-M1 and SOY-M2 showed that no positive amplification signal was detected from any of those samples except the target events and the endogenous Lectin gene in which the Ct value were no more than 40. These data indicated that the systems are specific to the 7 soybean events and appropriate to detect them (Table 4).

Sensitivity of MF-RT-PCR

The samples containing 25, 6.25, 1.56, 0.4, 0.2, 0.1, 0.05, 0.025 and 0.01% of the mixed GM soybeans were used in sensitivity test by SOY-M1 and SOY-M2 system (Table 5). The results showed that all the samples with soybean components had the *Lectin* gene amplified, while MON89788, A2704-12, 305423, 356043, GTS40-3-2, CV127, and A5547-127 could only be amplified when the transgenic target content was 0.1% or more. When the transgenic target content was lower than 0.05%, the two systems were unable to detect except the endogenous gene. The test was repeated 3 times and the results were consistent. The sensitivity tests indicate the limit of the detection by SOY-M1 and SOY-M2 is 0.1%.

Table 3: Primers concentrations used to optimize the MF-PCR systems

Primers	Four sets candidate primers concentrations (μmol/L) used in SOY-M1 PCR reactions				Primers	Four sets candidate primers concentrations (μmol/L) used in SOY-M2 PCR reactions			
	A1	A2	A3	A4		B1	B2	B3	B4
Lectin gene F/R primers	0.4[a]	0.6	0.4	0.4	Lectin gene F/R primers	0.4	0.4	0.6	0.4
MON89788 F/R primers	0.4	0.8	0.8	0.8	GTS40-3-2 F/R primers	0.8	0.4	0.8	0.6
A2704-12 F/R primers	0.4	0.6	0.4	0.6	CV127 F/R primers	0.4	0.4	0.4	0.4
305423 F/R primers	0.4	0.4	0.4	0.4	A5547-127 F/R primers	0.4	0.4	0.4	0.4
356043 F/R primers	0.4	0.4	0.6	0.6	/	/	/	/	/

[a] Mean of final concentration for forward and reverse primers, The final probe concentration is half of the primer

Table 4: Specificity test results of the SOY-MI and SOY-M2 systems

Samples	SOY-M1 system					SOY-M2 system			
	Average Ct and SD (n=3) by Multiple Real-time PCR					Average Ct and SD (n=3) by Multiple Real-time PCR			
	Lectin	Mon89788	A2704-12	305423	356043	*Lectin*	GTS40-3-2	CV127	A5547-127
MON89788	23.60 ± 0.03	29.01 ± 0.12	—	—	—	23.45 ± 0.05	—	—	—
A2704-12	23.84 ± 0.11	—	29.96 ± 0.13	—	—	23.31 ± 0.04	—	—	—
305423	23.13 ± 0.07	—	—	30.54 ± 0.10	—	23.30 ± 0.01	—	—	—
356043	23.72 ± 0.16	—	—	—	31.30 ± 0.16	23.12 ± 0.05	—	—	—
GTS40-3-2	23.94 ± 0.04	—	—	—	—	23.20 ± 0.05	32.13 ± 0.09	—	—
CV127	23.11 ± 0.03	—	—	—	—	23.38 ± 0.08	—	29.37 ± 0.13	—
A5547-127	23.20 ± 0.16	—	—	—	—	23.27 ± 0.05	—	—	30.70 ± 0.10
MON87701	23.86 ± 0.04	—	—	—	—	23.19 ± 0.06	—	—	—
MON87708	23.32 ± 0.08	—	—	—	—	23.45 ± 0.04	—	—	—
MON87769	23.80 ± 0.14	—	—	—	—	23.58 ± 0.07	—	—	—
MON87705	23.46 ± 0.11	—	—	—	—	23.33 ± 0.16	—	—	—
FG72	23.66 ± 0.09	—	—	—	—	23.70 ± 0.02	—	—	—
SHZD32	23.41 ± 0.02	—	—	—	—	23.26 ± 0.10	—	—	—
GM corn mixes	—	—	—	—	—	—	—	—	—
GM rice mixes	—	—	—	—	—	—	—	—	—
GM cotton mixes	—	—	—	—	—	—	—	—	—
GM rapeseed mixes	—	—	—	—	—	—	—	—	—
Non-GM soybean	23.29 ± 0.09	—	—	—	—	23.47 ± 0.14	—	—	—

-, No amplification was detected.

Table 5: Sensitivity test results of the SOY-MI and SOY-M2 systems

Sample names	Content of Mon89788 A2704-1 305423 356043	Average Ct and SD (n=6) by Multiple Real-time PCR					Sample names	Content of GTS40-3-2 CV127 A5547-127	Average Ct and SD (n=6) by Multiple Real-time PCR			
		Lectin	Mon89788	A2704-12	305423	356043			*Lectin*	GTS40-3-2	CV127	A5547-127
S1	25%	23.43±0.09	23.96±0.31	25.36±0.24	25.27±0.17	25.62±0.31	X1	25%	23.39±0.01	27.52±0.21	26.36±0.05	25.51±0.21
S2	6.25%	24.09±0.16	26.68±0.44	27.55±0.23	27.50±0.27	27.82±0.39	X2	6.25%	23.86±0.09	30.17±0.07	29.04±0.10	27.95±0.29
S3	1.56%	24.28±0.05	28.71±0.16	29.95±0.29	29.77±0.44	30.35±0.52	X3	1.56%	24.14±0.20	32.53±0.18	31.55±0.18	30.16±0.14
S4	0.4%	23.68±0.09	31.01±0.11	32.23±0.40	32.27±0.37	33.08±0.25	X4	0.4%	24.33±0.13	34.69±0.45	33.89±0.36	32.30±0.31
S5	0.2%	23.60±0.22	32.96±0.22	34.18±0.58	33.87±0.73	35.47±0.87	X5	0.2%	23.76±0.30	35.93±0.24	35.77±0.59	33.64±0.29
S6	0.1%	23.99±0.59	35.07±0.35	35.78±0.32	35.42±0.72	37.11±0.82	X6	0.1%	23.80±0.36	37.40±0.35	36.90±0.30	34.82±0.31
S7	0.05%	23.87±0.30	—	—	—	—	X7	0.05%	23.96±0.27	—	38.64±0.51	36.29±0.58
S8	0.025%	23.66±0.85	—	—	—	—	X8	0.025%	23.87±0.39	—	—	37.70±0.37
S9	0.01%	23.65±0.10	—	—	—	—	X9	0.01%	23.78±0.06	—	—	—

-, No amplification was detected.

Practical applicability of MF-RT-PCR

In order to test the practical applicability of these two reaction systems, we designed a series of sample combinations. First, samples with 1% of target soybeans including one, any two, any three, or all the four target soybean events (MON89788, A2704-12, 305423, 356043) were tested by SOY-M1, shown as Table 6. For SOY-M2 system, the samples containing one, any two, or all three target soybean events (GTS40-3-2, CV127, and A5547-127) were tested (Table 7). All the samples contained 0.2% of the target soybean DNA. The results showed all combinations of samples containing any or all 7 target soybean DNA were well detected by the two MF-RT-PCR systems. This indicates that these two MF-RT-PCR detection systems are able to detect the target transgenic soybean events in complex samples.

Discussion

GTS40-3-2, MON89788, CV127, A5547-127, A2704-12, 3054423, and 356043, the first batch of commercialized and cultivated GM soybean lines, used to occupy more than 80% of the GM soybean planting area of the world. With the acceleration of globalization and the rapid growth of international soybean trading, the supervision of GM

Table 6: Applicability assays of the SOY-M1 system

Sample	Average Ct and SD (n=6) by Multiple Realtime PCR				
	Lectin	Mon89788	A2704-12	305423	356043
Non-GM soybean	23.90 ± 0.17	—	—	—	—
MON89788	23.69 ± 0.13	32.93 ± 0.22	—	—	—
A2704-12	23.74 ± 0.25	—	34.31 ± 0.16	—	—
305423	23.65 ± 0.22	—	—	33.46 ± 0.13	—
356043	23.90 ± 0.17	—	—	—	35.38 ± 0.13
MON89788＋A2704-12	23.99 ± 0.19	32.71 ± 0.32	34.71 ± 0.35	—	—
MON89788＋305423	23.61 ± 0.18	32.62 ± 0.35	—	33.30 ± 0.22	—
MON89788＋356043	24.23 ± 0.15	32.63 ± 0.44	—	—	35.58 ± 0.18
A2704-12＋305423	23.92 ± 0.13	—	34.58 ± 0.38	33.09 ± 0.10	—
A2704-12＋356043	24.20 ± 0.25	—	34.50 ± 0.11	—	35.01 ± 0.12
305423＋356043	23.70 ± 0.33	—	—	33.38 ± 0.17	35.84 ± 0.37
MON89788＋A2704-12＋305423	23.91 ± 0.19	32.88 ± 0.28	35.89 ± 0.19	33.72 ± 0.29	—
MON89788＋A2704-12＋356043	24.07 ± 0.23	32.84 ± 0.35	35.26 ± 0.19	—	35.92 ± 0.17
MON89788＋305423＋356043	23.72 ± 0.23	32.91 ± 0.20	—	33.28 ± 0.10	35.26 ± 0.20
A2704-12＋305423＋356043	24.37 ± 0.05	—	35.22 ± 0.17	33.30 ± 0.10	35.23 ± 0.21
MON89788＋A2704-12＋305423 + 356043	24.39 ± 0.15	32.12 ±0.20	33.98 ± 0.33	32.55 ± 0.40	34.55 ± 0.40

-, No amplification was detected

Table 7: Applicability assays of the SOY-M2 system

Sample	Average Ct and SD (n=6) by Multiple Realtime PCR			
	Lectin	GTS40-3-2	CV127	A5547-127
Non-GM soybean	24.04 ± 0.26	—	—	—
GTS40-3-2	23.99 ± 0.23	35.93 ± 0.20	—	—
CV127	23.97 ± 0.36	—	36.21 ± 0.26	—
A5547-127	23.67 ± 0.59	—	—	33.88 ± 0.36
GTS40-3-2＋CV127	24.34 ± 0.14	35.95 ± 0.28	36.14 ± 0.20	—
GTS40-3-2＋A5547-127	24.25 ± 0.22	36.16 ± 0.20	—	34.13 ± 0.41
CV127＋A5547-127	23.91 ± 0.22	—	36.01 ± 0.24	33.57 ± 0.17
GTS40-3-2＋CV127＋A5547-127	24.36 ± 0.19	36.14 ± 0.30	35.54 ± 0.47	32.69 ± 0.42

-, No amplification was detected.

soybean events becomes more and more challenging (Fraiture *et al.* 2015). At present, the commonly used molecular detection assays to identify the GM soybean traits are mainly based on the real-time PCR technique, but these assays suffer from some shortcomings, such as low detection throughput and high cost. In this study, SOY-M1 and SOY-M2 systems were established by combining the endogenous *Lectin* gene and seven GM soybean targets to achieve the simultaneous detection of multiple targets, which could greatly reduce the testing cost, facilitate the experimental operation, and improve the detection efficiency. In addition, soybean endogenous genes in both systems can effectively avoid false negative results caused by operation errors or PCR inhibitors.

It was found that the fluorescent value of the amplification curve of GTS40-3-2 in SOY-M1 and MON89788 in SOY-M2 were lower than those in other targets, and it could not be improved by further optimization. This may be relevant to the low efficiency of amplification of targets in a multiplex system. However, the specificity, sensitivity, and applicability test results all showed that the multiplex fluorescence PCR system is sufficient to detect these two targets accurately.

Compare to other high throughput screening methods, the assay we established is accurate, reliable, and easy to operate by research institutes, government agencies, and companies. So far, most of the published multiplex PCR based detection methods rely on the polymorphism of the product length to differentiate samples, since the PCR products need to be visualized through electrophoresis (Shang *et al*. 2017). Therefore, with those methods, it is very challenging to design PCR primers to generate products with appropriate length, and it is time-consuming and expensive to optimize the experiment. In contrast, the signal from the PCR products in our assay are collected and processed automatically; therefore, it is timely and cost efficient. Some techniques based on nested PCR with multi-fluoresce, such as ME-qPCR (Fu *et al.* 2017a) and MT-PCR (Wei *et al.* 2018), are able to detect as many as 26 targets simultaneously with the sensitivity of 0.001 g, which is 100 times more sensitive than our assay. However, these methods are vulnerable to contamination and false negative. In addition, a skilled operator is a key factor for the success of the assays due to the complicated protocols. From this aspect, the MF-RT-PCR developed in this study is more practical for research institutes, government agencies, and companies to use. Some researchers have established multi-fluorescence digital PCR method, which can detect multiple targets qualitatively and quantitatively. However, the digital PCR instrument is expensive and requires advanced operators. In summary, the MF-RT-PCR established in this study is easy to operate and practical to most users.

Conclusion

The MF-RT-PCR assay established in this study is accurate, reliable, and efficient, which could greatly enhance the efficiency of transgenic soybean detection. This assay could serve as a high throughput approach to detect the target among a large amount of GM soybean lines for the purpose of food safety supervision and GM soybean verification.

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