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Primers and Probes Development for Specific PCR Detection of Genetically Modified Common Bean (*Phaseolus vulgaris*) Embrapa 5.1

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ABSTRACT: The genetically modified common bean Embrapa 5.1, developed by Brazilian Agricultural Research Corporation (Embrapa), is the first commercial GM plant produced in Latin America. It presents high resistance to the *Bean golden mosaic virus*. In this work, primers and probes targeting a taxon-specific reference DNA sequence for the common bean (*Phaseolus vulgaris* L.) and a construct-specific DNA sequence of Embrapa 5.1 GM common bean were successfully developed. The primers and probes showed high specificity for the target detection. Both methods showed suitable efficiency and performance to be used as an endogenous target for detection of common bean DNA and for construct-specific detection of GM common bean Embrapa 5.1, respectively. Both real-time PCR assays proved to be valuable for future assessment of interlaboratory studies.

KEYWORDS: *common bean, GMO, genetically modified organism, real-time PCR, quantitative PCR*

INTRODUCTION

Brazil is the world's second largest producer of genetically modified (GM) crops, only behind the United States. The first GM crop approved for commercialization in Brazil was Roundup Ready soybean,[1] but several other GMO events (soybean, maize, and cotton) have been approved in Brazil since then (http://www.ctnbio.gov.br/index.php/content/view/12492.html). In 2011, Brazilian National Technical Commission on Biosafety (CTNBio) approved the first GM common bean (*Phaseolus vulgaris* L.), the Embrapa 5.1 (http://www.mct.gov.br/index.php/content/view/333614.html).

Embrapa 5.1 was developed by the Brazilian Agricultural Research Corporation (Embrapa), being the first commercial GM plant developed in Latin America.[2] This GM common bean is resistant to the *Bean golden mosaic virus* (BGMV), a geminivirus transmitted by the whitefly *Bemisia tabaci* (Gennadius) in a persistent, circulative manner, causing the golden mosaic of common bean. This disease is the main constraint to the bean production in Latin America. An RNA interference construct was used for developing the Embrapa 5.1 event, which produces a specific siRNA designed to induce post-transcriptional gene silencing of the *rep* viral gene, resulting in strong resistance to BGMV. Results showed that approximately 93% of the GM plants, both homozygous and heterozygous, were free of symptoms upon inoculation at high pressure.[3]

With the introduction of this novel GM common bean on the Brazilian market, the ability to detect this event has become a legal necessity. Current Brazilian regulation requires the labeling of food containing a 10 $g \cdot kg^{-1}$ threshold of GMO,[4] giving rise to the need in monitoring the GM common bean content in food commercialized in Brazil. In this sense, suitable and reliable methods for GMO detection and quantification are required for controlling the legal requirements. Quantitative real-time PCR (qPCR) is the most commonly used method, mainly because of the high specificity and sensibility.[5−8] The percentage of GMO should be expressed as GM DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of the haploid genome. Both methods for the GM event and the host genome should present a high degree of specificity and stability, as well as the ability to quantitatively determine the targets in complex samples, such as foodstuff.[9]

The GM event-specific PCR method has been the primary trend for GMO identification and quantification because of its high specificity based on the flanking sequence.[6] Additionally, GM construct-specific PCR method has been suitable for identifying the GMO origin even for different GM events containing the same GM construction. A plant endogenous reference DNA sequence must be nuclear, taxon-specific, present in low copy number, and exhibit high homogeneity among varieties.[7,10]

In this study, primers and TaqMan probes targeting a taxon-specific reference DNA sequence for common bean (*Phaseolus vulgaris* L.) and a construct-specific DNA sequence of Embrapa 5.1 GM common bean were successfully developed for real-time PCR detection.

MATERIALS AND METHODS

Plant Material. Cowpea (*Vigna unguiculata* L.), GM common bean Embrapa 5.1, non-GM isogenic line (Olathe common bean variety), common bean varieties BRS Pontal, BRS Esplendor, and Pérola were provided by Embrapa Arroz e Feijão (Santo Antônio de Goiás, Goiás

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State, Brazil). Commercially available common bean (carioca, black, and red), soybean, maize, rice, pea, and lentil were purchased from local markets in Florianópolis, Santa Catarina State, Brazil. Certified Reference Materials (CRM) from RR soybean (5%, ERM-BF410), MON810 (5%, ERM-BF413f), Bt11 (4.89%, ERM-BF412f), Bt176 (5%, ERM-BF411f), and GA21 (1%, ERM-BF414d) were purchased from ERM (European Reference Materials, ERM, Geel, Belgium). MON88017 (>99.05%, AOCS 0406-D) and MON89034 (>99.42%, AOCS 0906-E) were purchased from AOCS (American Oil Chemists' Society, AOCS; Illinois, U.S.).

DNA Extraction Protocols. Plant genomic DNA was extracted and purified using a CTAB protocol [11] and the DNeasy Plant Mini Kit (Qiagen, CA, U.S.) according to the manufacturer's protocol with modifications as described in previous work:[5] a lysis treatment was carried out using CTAB buffer (20 g/L CTAB; 1.4 M NaCl; 0.1 M Tris buffer; 20 mM Na_2EDTA, pH 8.0) instead of AP1 buffer supplied in the kit. Also 20 μL of proteinase K (20 mg/mL) was added to each sample during the incubation at 60 °C for 15 min. DNA concentration was determined on a Thermo Scientific NanoDrop 2000 spectrophotometer (Wilmington, DE, USA) with measurements at 260 and 280 nm.

Primer Design. Primers and probes for real-time PCR detection were designed using Primer Express 1.0 (Applied Biosystems). For *Phaseolus vulgaris* L. taxon-specific detection, the PvSR2 detection method was developed by designing the PvSR2F03/PvSR2R04 primer pair and FEI probe targeting the sequence of PvSR2P [12] from *Phaseolus vulgaris* L. (Genbank Accession No. DQ109992.1). The P35S/SeqAC1 detection method, a construct-specific detection targeting the junction between P35S promoter and AC1 viral *rep* gene sequence [3] present in GM common bean Embrapa 5.1 (Figure 1) was developed by designing the P35SF03/SeqAC1R04 primer pair and OLA probe. PCR primers were synthesized by IDT (Coralville, IA, U.S.) and probes by Life Technologies (Foster City, CA, U.S.). Primers and probes sequences are presented in Table 1.

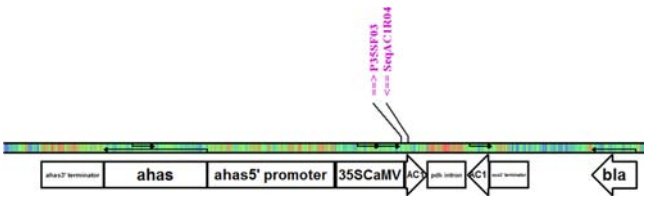

Figure 1. Schematic diagram of recombinant sequence present in EMBRAPA 5.1 GM common bean with indications for P35SF03 and SeqAC1R04 primer positions.

Real-Time PCR Analysis. Quantitative real-time PCR was performed in ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, U.S.). The amplification reactions were carried out in a final volume of 25 μL containing 12.5 μL of 2X SYBR Green Master Mix (Applied Biosystems), 200 nM of each primer, water and 50 ng of template DNA. After the optimization of primers and Taqman probes concentrations, the reactions were performed in a final volume of 25 μL with 12.5 μL 2X Master Mix (Applied Biosystems), 300 nM of PvsR2F03/PvsR2R04 primers and 200 nM FEI probe or 200 nM P35SF03/SeqAC1R04 and 150 nM OLA probe, water and template DNA. The amplification protocol consisted of the following: initial incubation at 50 °C for 2 min, a 95 °C incubation for 10 min, followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. All real-time PCR runs were evaluated separately by using the automatic settings for each run.

All samples were analyzed in duplicate by qPCR at a final concentration of 50 ng DNA per reaction, except for MON88017 and MON88034 GM maize samples, where 30 copies of DNA per reaction and GA21 5.4 ng DNA per reaction were used, respectively.

Table 1. Primers and Probes Designed for Construct-Specific Detection of EMBRAPA 5.1 GM Common Bean and for Taxon-Specific Detection of Common Bean (*Phaseolus vulgaris* L.)

primer/probe	sequence 5′–3′	amplicon size (bp)
EMBRAPA 5.1 GM common bean		
P35SF03	GTGACATCTCCACTGACGTAAG	183
SeqAC1R04	GTCACCTGGATTTCAACTCAAAGGTG	
OLA Probe	VIC-GCACTATCTCGAGCGTGTC-MGB	
Phaseolus vulgaris L.		
PvSR2F03	GTAGAGTTCACGAAAGAATATAATG	162
PvSR2R04	CAATTCTTAGAATGAAGGTTTTGCAC	
FEI Probe	FAM-AGAGTGTTCTCAAATCAACAATTAGAA-MGB	

Construction of Standard Curves. Genomic DNA isolated from leaves of GM common bean Embrapa 5.1 and non-GM isogenic line were serially diluted in water or in non-GM DNA solutions (25 ng/μL) of common bean, soybean, rice, pea, and lentil to final concentrations equivalent to 10^5 to 10^0 DNA copies. The copy numbers were calculated by using the 1C value of 637 Mbp for the *Phaseolus vulgaris* L. genome, which corresponds to 1.32 pg.[13] Therefore, the estimated copy numbers in the standard curves were calculated to be 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 copies of haploid genome. Concentrations from 67 to 6.7×10^{-4} ng DNA per PCR reaction were analyzed in five different PCR runs in duplicate. Amplification efficiencies were determined using the followed equation: Efficiency = $10^{(-1/\text{slope})} - 1$, where the slope is the value obtained from the standard curve. Coefficients of variation (CV) of qPCR assays were calculated by dividing the standard deviation by the mean Ct value and expressed in %.

RESULTS

Specificity of Real-Time PCR Assays. The specificity of PvSR2 and P35S/SeqAC1 methods was evaluated using the samples described in Table 2. The eight varieties of *Phaseolus vulgaris* were tested with the PvSR2 primers and FEI probe, designed for *P. vulgaris* taxon-specific detection. The DNA from common bean varieties presented the expected amplification signal, while DNA from all other nontarget plant varieties (maize, soybean, rice, lentil, pea, cowpea, one GM soybean, and six GM maize events) did not present amplification signals (Table 2).

When tested with the P35S/SeqAC1 primers and OLA probe, designed for construct-specific detection of the Embrapa 5.1 GM common bean, the DNA isolated from this GM common bean presented the expected amplification signal. As expected, all non-GM common beans as well as all other plant varieties tested in the present study, did not present any amplification signal (Table 2). Both methods showed high specificity and no cross-reaction with non-GM samples or other plant taxon samples were observed.

Limit of Detection of Real-Time PCR Assays. The limit of detection (LOD) for PvSR2 (FEI) and P35S/SeqAC1 (OLA) methods was determined through the standard curves containing 10^5 to 10^0 copies of GM common bean Embrapa 5.1. GM common bean DNA was diluted in water (Figure 2) or in non-GM DNA of common bean, soybean, rice, pea and lentil (Table 3).

The limit of detection for both methods was determined in the same PCR plate through 5 PCR runs using four different DNA isolations (A, B, C, and D) tested in duplicate. DNA isolations were prepared as previous described.[5] The results showed that Embrapa 5.1 GM common bean DNA diluted with

Table 2. Threshold Cycle (Ct) Values Generated by Real-Time PCR Assay with Taqman Probes Using FEI Probe for Taxon-Specific Detection of *Phaseolus vulgaris* L and OLA Probe for Construct-Specific Detection of EMBRAPA 5.1 GM Common Bean[a]

	sample		mean Ct ± SD	
			FEI	OLA
common bean	Embrapa 5.1	leaf	25.75 ± 1.22 (n = 6)	24.91 ± 0.82 (n = 6)
	isogenic line	leaf	26.49 ± 0.85 (n = 14)	ND (n = 14)
	Carioca	grain	28.36 ± 0.98 (n = 4)	ND (n = 4)
	black	grain	34.40 ± 1.66 (n = 2)	ND (n = 2)
	red	grain	30.34 ± 0.85 (n = 2)	ND (n = 2)
	BRS Pontal	grain	29.30 ± 1.29 (n = 4)	ND (n = 4)
	BRS Esplendor	grain	29.78 ± 0.71 (n = 4)	ND (n = 4)
	Pérola	grain	26.74 ± 1.44 (n = 6)	ND (n = 6)
negative control	Cowpea bean	grain	ND (n = 2)	ND (n = 2)
	rice	grain	ND (n = 2)	ND (n = 2)
	maize	grain	ND (n = 4)	ND (n = 4)
	lentil	grain	ND (n = 2)	ND (n = 2)
	pea	grain	ND (n = 2)	ND (n = 2)
	soybean	grain	ND (n = 2)	ND (n = 2)
CRM	Roundup Ready soy	grain	ND (n = 2)	ND (n = 2)
	Bt 11	grain	ND (n = 2)	ND (n = 2)
	Bt 176	grain	ND (n = 2)	ND (n = 2)
	MON 810	grain	ND (n = 2)	ND (n = 2)
	MON 89034	DNA	ND (n = 2)	ND (n = 2)
	MON 89017	DNA	ND (n = 2)	ND (n = 2)
	GA 21	DNA	ND (n = 2)	ND (n = 2)

[a]Samples were analyzed at a final concentration of 50 ng DNA per reaction for all samples, except for CRMs MON88017 and MON88034 GM maize, 30 copies of DNA per reaction and GA21 5.4 ng DNA per reaction.

water was detected in 16 out of 16 reactions by both methods up to 10^2 DNA copies. Positive reactions were also observed in 10 out of 16 reactions and 8 out of 16 reactions for PvSR2 (FEI) and P35S/SeqAC1 (OLA) methods, respectively, at 10^1 copies. No amplification signals were observed for dilutions containing 10^0 copies of GM common bean Embrapa 5.1 DNA.

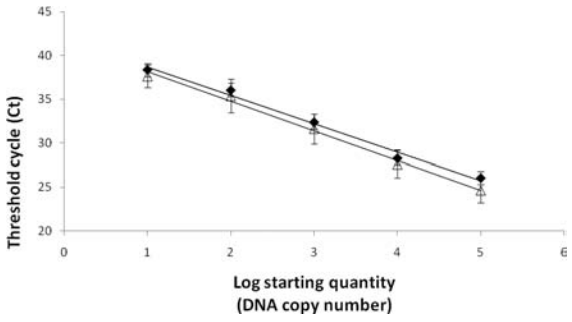

Figure 2. Standard curves resulting from 5 real-time PCR runs with Taqman probes. Curves were generated using 4 DNA extractions from EMBRAPA 5.1 GM common bean DNA as template DNA and PvSR2F03/PvsR2R04 primers and FEI probe for taxon-specific detection of *Phaseolus vulgaris* L. and P35SF03/SeqAC1R04 primers and OLA probe for construct−specific detection of EMBRAPA 5.1 GM common bean. Average Ct ± SD (n = 16). ♦ FEI probe and Δ OLA probe.

Embrapa 5.1 GM common bean DNA was then diluted with non-GM DNA (pea, soybean, and rice). The GM DNA was detected in all 16 tested samples until dilution 10^1 using both methods (Table 3) with the only exception for P35S/SeqAC1 (OLA) method, which presented no amplification signals for GM common bean diluted at 10^1 with DNA from non-GM common bean (isogenic line) and lentil (Table 3). These results showed a limit of detection of Embrapa 5.1 GM common bean between 10^1 and 10^2 copies of DNA per PCR reaction for both PCR assays.

Real-Time PCR Efficiency. The amplification efficiency and robustness of both qPCR methods were estimated by a serial dilution from 10^5 to 10^0 DNA copies of GM common bean Embrapa 5.1 in water (Figure 2). Calculated reaction parameters (efficiency, slope, and correlation coefficient) are presented in Table 4. The mean efficiencies were of 99% and 89% and correlation coefficient (R^2) were 0.98 and 0.99 for PvSR2 (FEI) and P35S/SeqAC1 (OLA) methods, respectively, when using DNA extraction protocol described in previous work.[5] Efficiencies ranged from 92 to 109% and from 90 to 95% for PvSR2 and P35S/SeqAC1 methods, respectively, when using CTAB protocol.[11] The R^2 values ranged from 0.976 to 0.988 and from 0.969 to 0.983, respectively.

Coefficients of variation (CV) of Ct values were calculated from five real-time PCR runs and presented below 4% for PvSR2 (FEI) method and below 5.6% for P35S/SeqAC1 (OLA) method (Table 5).

DISCUSSION

Both qPCR methods proved to be specific for the detection of the target sequences, showing no cross-reactions with any of the nontarget samples analyzed. As a consequence of such high specificity, the methods developed in the present study represent important tools for detecting the recently approved GM common bean Embrapa 5.1. The MGB technology used in this work allowed the design of shorter and more specific probes that can identify just a single nucleotide difference in probe regions and probably can outperform other PCR strategies in terms of specificity.[14]

The lowest amount that was reliably detected in both qPCR methods evaluated in the present study was about 10 copies of DNA per PCR reaction based on the haploid genome of common bean (*Phaseolus vulgaris* L.). This detection limit is in agreement with international criterion that establishes the lowest amount of an analyte in a sample should be less than 1/20th of the threshold relevant for legislative requirements, on at least 95% of the time.[15] However, the limit of detection using OLA probe was approximately 100 copies when GM common bean Embrapa 5.1 DNA was diluted with DNA from non-GM isogenic line and also with lentil DNA. These results showed a possible inhibition for detection of 10 copies of GM common bean Embrapa 5.1 DNA with P35S/SeqAC1 method, probably due to the matrix effect. Nevertheless, both qPCR methods were robust enough to detect 10 copies of the respective target even in samples containing DNA of soybean, rice, and pea. Results of GMO quantification depends crucially on sample matrix properties and extraction technique.[16]

Standard curves for the quantification of targets, endogenous reference DNA sequence and the Embrapa 5.1 event DNA

Table 3. Threshold Cycle (Ct) Values Generated by Real-Time PCR Analysis Using FEI Probe (*Phaseolus vulgaris* L Detection) or OLA Probe (EMBRAPA 5.1 GM Common Bean Detection) and EMBRAPA 5.1 GM Common Bean DNA (10^5 or 10^1 DNA Copies) in the Presence of Background DNA[a]

	FEI probe		OLA probe	
DNA copy number	10^5	10^1	10^5	10^1
background DNA	Ct	Ct	Ct	Ct
isogenic line	26.32 ± 0.66	26.26 ± 0.00	26.50 ± 0.28	ND
pea	24.93 ± 0.05	37.76 ± 0.06	22.80 ± 0.05	36.81 ± 0.24
lentil	25.68 ± 0.07	39.09 ± 0.31	25.81 ± 0.01	ND
soybean	24.93 ± 0.20	36.89 ± 0.85	22.68 ± 0.02	35.31 ± 0.53
rice	24.88 ± 0.18	37.03 ± 1.18	22.89 ± 0.05	36.44 ± 0.23

[a]Samples were analyzed in duplicate (n = 2). Mean Ct ± SD. ND means not detected.

Table 4. Parameters of PCR Standard Curves for *Phaseolus vulgaris* L. (FEI) and EMBRAPA 5.1 GM Common Bean (OLA) Detection Based on 8 Experiments Using EMBRAPA 5.1 GM Common Bean DNA Serial Dilution

		FEI			OLA		
PCR run	DNA sample	efficiency (%)	slope	R^2	efficiency (%)	slope	R^2
1	A	112	3.06	0.99	100	3.33	0.99
2	B	97	3.39	0.95	85	3.75	0.99
3	C	93	3.50	0.99	83	3.80	0.99
2	D	97	3.38	0.98	88	3.63	1.00
4	A	102	3.28	0.98	87	3.67	0.99
4	B	105	3.22	0.98	93	3.50	0.99
5	C	86	3.70	0.99	86	3.70	0.99
5	D	98	3.37	0.99	88	3.66	1.00
mean		99	3.36	0.98	89	3.63	0.99
SD		7.8	0.19	0.01	5.4	0.15	0.00

sequence, were prepared separately using serial dilutions of a standard reference material DNA. Thus, the difference in PCR efficiency between the reactions was evaluated. Such approach is robust since the amplification efficiency of different amplicons was taken into account.[16] The values of PCR efficiency for PvSR2 (FEI) method showed that 4 out of 5 PCR runs (80%) presented results within the acceptable range to be used for quantification by the European Network of Genetically Modified Organism Laboratories (efficiency ranging from 90% to 110% and a R^2 value equal to or higher than 0.98).[15] Therefore, the PvSR2 (FEI) method showed a suitable performance to be used as an endogenous target for common bean DNA quantification. The correlation coefficients for the standard curves of P35S/SeqAC1 (OLA) method were all equal to or higher than 0.99, and the overall efficiency was 89%, with only 1 out of 5 PCR runs presenting an efficiency lower than 85%. The PCR efficiencies and correlation coefficients of the standard curves indicate that these primers and probes are suitable for common bean and Embrapa 5.1 GM common bean PCR detection. The CV results indicate that both qPCR methods are reliable for Embrapa 5.1 GM common bean PCR detection.

Several authors describe the development of endogenous reference DNA sequence methods for GMO analysis[7,17−21] and traceability.[22,23] The endogenous reference DNA sequence method should be specific for the identification of a taxon and stable among different varieties. The endogenous reference DNA sequence method developed in the present study proved to be specific among tested plant taxa and GMO events and, according to the results, seems to be stable among the tested common bean varieties. However, this endogenous reference DNA sequence still needs to be tested regarding the stability of the common bean genome among a high number of different varieties. The requirement to carefully test reference gene methods for GMO detection was reported in a study presenting the bias caused by a single nucleotide polymorphism in the *adh*1 reference gene that can affect the quantification of GM maize events depending on the genotype of the sample.[24] Also, there is an urgent need for harmonizing the analytical methodology for GMO analysis [25] and to select reference genes methods to be used.[26]

Table 5. Comparison of Ct Values for *Phaseolus vulgaris* L. Taxon-Specific qPCR Assay (FEI) and Embrapa 5.1 Construct-Specific qPCR Assay (OLA)

	Ct					mean		
copy number	day 1	day 2	day 3	day 4	day 5	Ct	SD	CV
FEI								
100 000	27.34	26.27	26.00	25.52	25.45	26.00	0.77	2.94
10 000	29.78	29.69	27.94	27.16	28.16	28.16	1.15	4.08
1000	33.41	33.46	32.42	31.10	32.52	32.52	0.96	2.95
100	35.76	36.68	36.82	34.82	36.21	36.21	0.81	2.23
10	39.96	ND	38.20	38.15	38.22	38.21	0.89	2.32
OLA								
100 000	22.84	25.00	25.85	23.02	25.77	24.50	1.32	5.37
10 000	25.80	28.61	28.65	25.62	28.90	27.52	1.48	5.38
1000	29.64	33.09	32.80	29.47	32.68	31.54	1.62	5.15
100	32.46	36.86	36.76	33.20	36.81	35.22	1.97	5.58
10	36.63	39.44	ND	36.95	39.80	38.21	1.43	3.73

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The primers and probes developed in the present work showed high specificity for target detection. The limit of detection was approximately 10 copies. Both qPCR assays are efficient and reliable detection methods, being suitable to be evaluated in future in-house and interlaboratory performance studies. The present work reports, for the first time, the development of qPCR assays for the quantification of Embrapa 5.1 GM common bean, a GMO event recently developed by Embrapa and commercially approved in Brazil. The primers and probes developed will be useful for survey of compliance with labeling legislation in food containing GMO in Brazil by health regulatory authorities.[27] Moreover, they could be useful as routine analysis for GMO laboratories in other countries where GM common bean is not yet approved for commercialization. Detection of nonauthorized GMO is an analytical challenge[28,29] and providing analytical tools for new GM events detection is of major importance.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Ct: cycle threshold
CTAB: cetyltrimethylammonium bromide
CV: coefficient of variation
Embrapa: Brazilian Agricultural Research Corporation
GM: genetically modified
GMO: genetically modified organism
PCR: polymerase chain reaction
qPCR: quantitative polymerase chain reaction
SD: standard deviation
siRNA: small interfering ribonucleic acid

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