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Establishment of Quantitative Method for Five Events of Genetically Modified Maize (Bt11, Event176, GA21, MON810 and T25) by Real-Time QPCR

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ABSTRACT

In order to meet the implementated labeling requirement, this study focused on the development of real-time quantitative polymerase chain reaction (real-time QPCR) method to detect genetically modified maize (GM-maize). Primers and probes specific for inserted genes in Event176, Bt11 (Syngenta company), MON810, GA21 (Monsanto company) and T25 GM-maize (Aventis company) were designed and used to conduct the real-time QPCR assays. A plasmid containing both transgene and internal control gene targets on the same molecules for each GM-maize event was also constructed as quantitative reference molecules. Further, constructed plasmids and test samples were applied to validate this quantitative system. Results showed that the slope of the standard curve generated by serial dilution of constructed plasmids was in the range of -3.31~-3.45 with a correlation of 0.99~1.0. Test samples spiked 1%, 2% and 5% GM-maize were quantitated by the method developed in the study. The mean values of 1%, 2% and 5% test sample were between 1.01%~1.23%, 2.00%~2.31% and 4.46%~5.55%, respectively. The standard deviation (S.D.) ranged from 0.05 to 0.46 with coefficient variance (C.V.) of 5%~15%. The limit of quantitation was 0.1% (w/w) for these GM-maize crops. In addition, proficiency test samples were analyzed by the method developed in this study and good results were obtained.

Key words: PCR, genetically modified maize

INTRODUCTION

Genetically modified soybean, maize, cotton and rapeseed are currently the most common commercialized genetic modified crops on market[1,2,3]. Until 2003, the registered GM-maize are more than 20 events, mainly in improving its insect-resistant and herbicide-tolerant characteristics[1,2,3]. Genetically modified maize are mostly planted in the United States and Canada[1,2,3]. In 2002, the GM-maize in USA occupied 34% of maize planting area. Among them, 64% was herbicide-tolerant, 26% insect-resistant, and 6% both herbicide-tolerant and insect-resistant[1,2,3]. According to the statistical data from Taiwan's Council of Agriculture (COA), around 6 million tons of maize are imported to Taiwan annually. In which, about 30% is GM-maize[4]. Based on the differences of inserted insect-resistant proteins, the commercial insect-resistant maize can be classified into four categories: Cry1A (YieldGard), Cry9C (StarLink), Cry1F (Herculex I), and Cry3Bb1 (MON863)[1,2,3]. On the other hand, herbicide-tolerant maize can be classified into two categories: glyphosate, and glufosinate ammonium[1,2,3]. In addition, there were several stacked traits hybrid[5,6] commercialized, e.g. MON810 × GA21, MON810 × NK603 and MON863 × NK603.

In accordance with the implementation of labeling of genetically modified foods, several detection methods were published[7]. Among them, nucleic acids methods, e.g. PCR methods, and protein methods, e.g. ELISA, were the most popular[7,8]. The user of the PCR method is able to design primers based on the inserted genes to identify the corresponding products. The primers designed in PCR method have two major functions, product screening and product specific detection. Product-specific primers, designed based on the inserted genes, can be further classified as gene-specific, construct-specific, or event-specific[7,9]. In addition, PCR methods can be applied as quantitative identification. For instance, the so-called quantitative competitive PCR (QC-PCR) must have an internal standard competitor to calculate content of the target gene[7]. PCR-ELISA[7] and real-time PCR[7] were also developed. Real-time PCR can identify and quantify PCR products simultaneously. Based on the PCR monitoring materials that applied, the real-time PCR can be classified as non-specific or specific monitoring. SYBR Green I is used as the non-specific PCR monitoring materials. For specific PCR monitoring, such as TaqMan, molecular beacons and hybridization probes can be used as the specific probes[7]. Currently, the TaqMan real-time PCR methods are the most common quantitatively detection methods and commercially available kits for detecting GM-maize[7,10,11]. Since reference materials are not yet

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available for all GM events of maize, the critical point in developing methods for detecting GM-maize is how to obtain the reference material. Quantification method needs reference materials to generate the standard curves. Several methods were published for the generation of standard curves, e.g. from serial dilutions of 100% reference material[12], 1%, 2%, 5% of reference materials[13], serial dilutions of PCR products[14], or plasmids constructed by PCR products[15].

The labeling of genetically modified soybean, maize and related products in Taiwan was promulgated in February 2001. The voluntary system was implemented since 2001, while the mandatory system was implemented in 3 years since year 2003 depending on the extent of processing of the products. Products containing more than 5% of genetically modified soybean or maize and the weight of substance exceeded 5% of final product are required to be labeled[16]. Until February 2003, there were 10 GM-maize events registered in DOH: Event176, Bt11, MON810, T25, GA21, NK603, TC1507, DBT418, DLL25 and MON863[16]. Among them, DBT418 and DLL25 were phased out. NK603, TC1507 and MON863 were marketed after 2000[2] and mass produced since 2003. The other five events were detected from domestic maize-grits[17,18]. In the present study, primers and probes specific for the inserted genes in the five GM maize events and CaMV 35S-promoter were designed and used to conduct the real-time QPCR assays. In addition, a plasmid containing both transgene and internal control gene targeting on the same molecules for each GM maize event was also constructed as quantitative reference molecules. The established methods were applied in the testing of international proficiency test samples, in order to evaluate its feasibility. Meanwhile, the method assessed in this study would be published as a reference method for the detection of GM-maize and to be referenced for food labeling.

MATERIALS AND METHODS

I. *Chemicals*

Chloroform was of reagent grade. Isopropanol, tris (hydroxymethyl) aminomethane (Tris-base) (Merck, Darmstadt, Germany), hexadecyltrimethyl-ammonium bromide (CTAB) (Sigma, St. Louis, Missouri, USA), and agarose (Amresco, Solon, Ohio, USA) were of biological grade.

II. *GM-maize References*

Five types of GM-maize were employed in the study as references: Event176 and Bt11 (Syngenta, Basel, Switzerland), MON810 and GA21 (Monsanto, St. Louis, MO, USA), T25 (Aventis, AgrEvo, Berlin, Germany). 1%, 2% and 5% of Event176 and MON810 reference GM-maize and 1%, 2% Bt11 reference GM-maize were purchased from Fluka Chemical Co. (Buchs, Switzerland). 1%, 2% and 5% of GA21 and T25 and 5% Bt11 were self-prepared by Bureau of Food and Drug Analysis (BFDA). Non-GM-maize, N79-P4, was from Syngenta (Basel, Switzerland).

III. *DNA Extraction, Purification Kits and Enzymes*

Wizard Minipreps DNA Purification Resin was purchased from Promega (Madison, WI, USA), DNeasy Plant Mini Kit and DNeasy Plant Maxi Kit were from Qiagen (Hilden, Germany). *Eco* RI, *Apa* I and T4 ligase were from New England Biolabs (Beverly, MA, USA).

IV. *Equipments*

PCR thermal controller model PTC-100 was purchased from MJ Research (Water Town, MA, USA). Real-time PCR (Model, ABI PRISM 7700 Sequence Detector) and automated DNA sequencer (Model, 3100-Avant Genetic Analyzer) were from Applied Biosystems (USA). UV-Visible Spectrophotometer (Model, UV-1601) was from Shimadzu (Japan).

V. *PCR Primers, Probes and Reagents*

In addition of references, the primers and probes used in this study were self-designed and synthesized by TIB Molbiol (Berlin, Germany) (Table 1). The 5'-end of probe was labeled with 6-carboxy-fluorescein and the 3'-end was labeled with 6-carboxytetramethyl-rhodamine. The DNA Polymerase Kit (PROtech Technologies, Inc., Taipei, Taiwan) was qualitative PCR kit. TaqMan Universal PCR Master Mix (Applied Biosystems, USA) was used as the quantitative PCR kit.

VI. *DNA Purification, Cloning Kits and Plasmid Extraction Kits*

The QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) was used for the recovery and purification of PCR-amplified DNA products. TOPO TA Cloning (Invitrogen Corporation, Carlsbad, CA, USA) or Promega pGEM-T vector (3000 bp) (Promega, Madison, WI, USA) was used for cloning. The QIAGEN Plasmid Mini Kit (Qiagen, Hilden, Germany) was used for the extraction of plasmid DNA.

VII. *DNA Preparation and Purification*

The CTAB method, published by Lipp[22] in 1999, was used for the extraction and purification of DNA. Two hundred milligram of sample was weighted and added with deionized water 100 μL, and incubated at 65°C for 1 hr. Then, 500 μL of CTAB buffer (CTAB 20 g/L, NaCl 1.4 M, Tris-base/HCl 0.1 M and Na2-EDTA 20 mM, adjusted to pH 8.0 with HCl) was added and incubated at 65°C for 30

Table 1. Primers and probes used in this study

Primer/Probe	Sequence 5'-3'	Specificity	Amplicon (bp)	Reference
Event176				
E176F	GTG GAC AGC CTG GAC GAG AT	CDPK-pro/sense		(19)
E176R	TGC TGA AGC CAC TGC GGA AC	*cryIA(b)*/anti-sense	105	(19)
E176P	(FAM)-AACAACAACGTGCCACCTCGACAG G-(TAMRA)			(19)
MON810				
M810F	TGA CAC TAT ATT GCT TCT CTT TAC ATA CGT	*hsp70*/sense		This study
M810R	GAT GTT TGG GTT GTT GTC CAT	*cryIA(b)*/anti-sense	139	(20)
M810P	(FAM)-CTCGATGCCTTCTCCCTAGTGTTGACCA-(TAMRA)			This study
T25				
T25F	TTA GGC CAG TTA CCC AGA TCT GA	*pat*/sense		This study
T25R	ATT CCC TTA TCT GGG AAC TAC TCA C	35S ter./anti-sense	119	This study
T25P	(FAM)-CATGCCCGCTGAAATCACCAGTCT CT-(TAMRA)			This study
Bt11				
Bt11F	CCA TTT TTC AGC TAG GAA GTT C	*adh1*-1S IVS6/sense		(21)
Bt11R	TCG TTG ATG TTK GGG TTG TTG TCC	*cryIA(b)*/anti-sense	110	(21)
Bt11P	(FAM)-TCCGCGGCTTGTTGTGGTCTT TTG-(TAMRA)			This study
GA21				
GA21F	TTC CAG GGG CTC AAG TCC A	OTP/sense		This study
GA21R	TCT CCT TGA TGG GCT GCA	*m-epsps*/anti-sense	136	This study
GA21P	(FAM)-TCGCCCGCCGCTCCTCCAGAA G-(TAMRA)			This study
35S				
35SF	CCG ACA GTG GTC CCA AAG AT	CaMV-35S/sense		This study
35SR	GCT TTG AAG ACG TGG TTG GAA	CaMV-35S/anti-sense	81	This study
35SP	(FAM)-CCCACCCACGAGGAGCAT CG-(TAMRA)			This study
HMG				
HMGF	GCT ACA TAG GGA GCC TTG TCC T	HMG/sense		(12)
HMGR	TTG GAC TAG AAA TCT CGT GCT GA	HMG/anti-sense	79	(12)
HMGP	(FAM)-CAATCCACACAAACGCACGCG TA-(TAMRA)			(12)

FAM: the 5'-end of probe was labeled with 6-carboxy-fluorescein.
TAMRA: the 3'-end of probe was labeled with 6-carboxytetramethyl-rhodamine.

min. After the supernatant was collected and centrifuged at 16,000 ×g for 10 min, 200 μL of chloroform was added. After vortex mixing for 30 sec, the supernatant was centrifuged at 12,000 ×g for 10 min. Twice volume of CTAB precipitating solution (CTAB 5 g/L and NaCl 0.04 M) was added and kept at room temperature for 60 min. Decant the supernatant after centrifuged at 14,500 ×g for 10 min, and then 350 μL of 1.2 M NaCl solution and 350 μL of chloroform were added. After vortex mixing for 30 sec, the supernatant was collected by centrifuging at 12,000 ×g for 10 min. The supernatant was mixed with 0.6-fold isopropanol, stood for 30 min to precipitate the DNA, and decanted after centrifugation at 15,000 ×g for 30 min. The pellet was washed with 500 μL of alcohol (70%, v/v), and decanted after centrifugation at 15,000 ×g for 10 min. The precipitated DNA was dissolved in 100 μL of deionized water.

Commercial kits, Wizard Minipreps DNA Purification Resin (Promega), DNeasy Plant Mini Kit or DNeasy Plant Maxi Kit (Qiagen) were also applied for the extraction and purification of DNA.

VIII. *PCR*

ABI7700: Each reaction contains 25 μL of reagents, including 1.25 μL of 5 mM primers, 1.7 μL of 3.3 mM probe, 12.5 μL of Master mix, 5 μL of DNA (~100 ng), and 3.3 μL of H_2O. PCR condition: 50˚C for 2 min, 95˚C for 10 min, 45 reaction cycles at 95˚C for 15 sec, 60˚C for 1 min, and then cool down. The reagents were individually prepared, mixed, and 20 μL was relocated into each microtube and followed by adding 5 μL of DNA.

IX. *The Recovery Purification of PCR-amplified DNA and Extraction of Plasmid*

The PCR-amplified DNA in gel was sliced with a knife, recovered and purified according to the procedures of the kit. The plasmid was also extracted accordingly.

X. *Plasmid Transformation*

In this study, the plasmid transformation of the PCR product was in accordance to the procedures of the TOPO TA Cloning Kits (Invitrogen Corporation, Carlsbad, CA, USA) or Promega pGEM-T Vector Kits (Promega, Madison, WI, USA).

XI. *Construction and Verification of Reference Plasmids*

Six reference plasmids were constructed in this study, namely pE176 (GM-maize Event176), pBt11 (GM-maize Bt11), pM810 (GM-maize MON810), pT25 (GM-maize

T25), pGA21 (GM-maize GA21) and p35S (35S-promoter). The methods of construction and verification are described as follows. Taking pBt11 reference plasmid as an example, PCR reaction was first performed on Bt11 GM-maize by using the primers in Table 1 (Bt11F/Bt11R, HMGF/HMGR), in order to generate Bt11 product specificity and maize internal control gene (high mobility group, HMG), respectively. Two PCR fragments were recovered and purified, and then cloned into TOPO TA Cloning plasmid. Two plasmids with product-specific and HMG gene fragment were digested with *Eco* RI, electrophoreses analyzed. The fragment-specified gel sections were sliced and the DNA fragments were further purified. The product-specific DNA fragment and HMG DNA fragment were ligated with T4 ligase at 16˚C for 12-16 hr. The ligated DNA fragment was amplified by PCR, recovered and purified accordingly. The ligated DNA fragment was cloned into TOPO TA Cloning plasmid and transformed into *E. coli* JM109. After amplification, the plasmid was extracted and sequenced and analyzed to make sure that the two PCR-amplified fragments were in single copy in the plasmid. After further amplification, the plasmid was digested into linear form with *Apa* I, and the fragment was recovered, purified and diluted for later use. The other plasmids were constructed according to pBt11.

XII. *Plotting of Standard Curve*

After DNA concentration was identified, the linear constructed plasmid was serially diluted with distilled water to make concentrations of 20 copies, 80 copies, 1,280 copies, 20,480 copies and 1,310,720 copies in each 5.0 μL of solution. The corresponding threshold cycle value (Ct value) was 36 cycles, 34 cycles, 30 cycles, 26 cycles and 20 cycles, respectively.

XIII. *Determination of Coefficient Value for Each Event of GM-maize*

Each event of GM-maize was quantitatively determined, and its constructed reference plasmid was plotted as the standard curve. The coefficient value was calculated by below equation:

Coefficient Value = Target Gene in GM-Maize/Internal Control Gene in Maize

XIV. *Calculation of Quantitative Analysis*

After quantitative analysis, the following equation was used:

The amount of single event of GM-maize (%) = (Target Gene/Internal Control Gene in Maize) × (1/Coefficient Value) × 100

RESULTS AND DISCUSSION

There are currently as many as 20 events of GM-maize[1,2,5]. In Taiwan, by February 2003, the Department of Health (DOH) has accepted 10 registration submissions of GM-maize, including MON810, GA21, Bt11, Event176, T25, NK603, TC1507, DLL25, DBT418 and MON863. Some of the above mentioned events have already been approved[16]. The qualitative methods for the detection of six events of GM-maize have been established in our laboratory[17,18]. Also, in order to comply with the labeling requirement of genetically modified soybean and maize since 2003, our laboratory is responsible for establishing quantitative detection methods as well. The purpose of this study is to establish quantitative screening and product-specific detection methods for five events of GM-maize. This method can be provided as a reference for the industry and as a national detection method. According to a market survey, there are five common events of GM-maize in Taiwan and Japan[17,18]. Among them, all have 35S-promoter in the inserted gene (Event176, MON810, Bt11 and T25), except GA21. The quantitative detection in this study will use primer and probe of both 35S-promoter and GA21 as preliminary selection and quantitation method. That is to say, we verify the quantity of 35S-promoter and GA21 independently. For those with total amount over 4.5% (label criteria >5.0%), independent amount of the four single events will be detected. The total amount of the 5 independent events is the final test result.

I. *Designation and Application of Primers and Probes in Real-time PCR*

Five events of GM-maize (Event176, Bt11, MON810, T25 and GA21) were studied in this work to develop the real-time QPCR method. TaqMan probe has been utilized in the QPCR method. Seven specific primers and five probes were designed for 35S-promoter and also for specific region in each of the five events. Also, seven primers designed for the internal control gene of maize: high mobility group protein (HMG) and Event176, MON810 and Bt11 were used[12,19-21]. Two probes were designed for HMG and events of Event176[12,19] (Table 1). In previous studies, zein gene, HMG, invertase gene[23] and maize starch synthase IIb gene[15] were used as the internal control genes of maize. It was essential that the selected gene possessed only one copy per genome for it to be selected as internal control gene in the quantitative assay. Previous studies have proven that zein gene is not necessarily one copy per genome in various maize varieties. Therefore, HMG was selected as the internal control gene in this study. We have designed seven primers and determined their specificity and detection limit. Firstly, primers specific for 35S-promoter were analyzed. All genetically modified samples containing 35S-promoter were detected, but non-GM-maize or those without 35S-promoter GM-maize, like GA21, cannot be detected with PCR products.

100% DNA of Event176 was serially diluted and the DNA fragments can be detected at 0.01%. When various percentage of Event176 samples were analyzed, the DNA fragments can be detected in samples containing 0.1% Event176 reference GM-maize. Other product-specific primers were also tested for MON810, T25 and G21 events, respectively. For instance, in the specificity test of GA21 primer, samples from Event176, Bt11, MON810, T25, G21 GM-maize and non-GM-maize were analyzed. Only GA21 had amplified fragment of PCR. Besides the primers, we also analyzed the probe in this study. Appropriate probes were selected according to the fluorescence curve of real-time PCR product. The selected primer and probe are listed in Table 1.

II. *Construction of Reference Plasmid for Quantitative Analysis*

One of the objectives in this study is to prepare reference plasmid as quantitative standard. The quantitative standard material has not been conclusively identified yet. Still, several methods could be used to plot the standard curve, including serial dilution from 100% reference material[12], preparing 1%, 2%, 5% standard solution[13], direct serial dilution from PCR-amplified product[14], and using constructed plasmid from of PCR fragments[15]. The advantages of preparing constructed plasmid are unlimited quantity and ability to serve as reference standard for both qualitative and quantitative studies. In addition, the constructed plasmid prevents influence factors like growing region, harvest time, varieties, and DNA extraction method. Similarly, problems like degradation of reference material and uneasy to obtain reference material can also be avoided.

In this study we have constructed a plasmid containing a quantitative PCR-amplified fragment (the target gene) of 35S-promoter, and a fragment of HMG gene (maize internal control gene). The constructed plasmid, p35S, was firstly tested by real-time QPCR, and then DNA was sequenced to confirm that both inserted fragments were in single copy in the plasmid. After the DNA sequenced plasmid was mass amplified, extracted, recovered, enzyme digested and purified, the DNA concentration was to be determined and serially diluted. Afterwards, appropriate concentration of linear plasmid DNA was selected in plotting a standard curve. Also, the DNA was extracted from 0.1% Event176 reference GM-maize (w/w) and quantified to determine the detection limit of the study method. Result showed that 0.1% Event176 could be detected, which meant the limits of quantitation (LOQ) was 0.1%. In the mean time, when 100% DNA of Event176 was serially diluted and analyzed, Event176 could be detected as low as 0.01%, suggesting the limits of detection (LOD) was less than 10 copies. Theoretically, standard curve between 20 to 1,310,720 copies can estimate 0.1% ~ 100% GM-maize in 100 ng of maize DNA samples[12,15,23]. In this study, the standard curve was plotted with 20, 80, 1,280, 20,480 and 1,310,720 copies in each 5 μL of DNA. The threshold cycle value (Ct) of these five points were 36, 34, 30, 26 and 20 cycles, respectively. As illustrated in Figure 1, the lowest concentration point in the standard curve of pBt11 plasmid is 20 copies, corresponding to cycle 36.

As for the other five events, the product-specific QPCR fragment and HMG gene fragment were also constructed to the same plasmid according to the method described as above. Five plasmids, pE176, pBt11, pM810, pT25 and pGA21, were constructed as the reference materials. The constructed plasmids were also tested, mass amplified and plotted into the standard curve. Also, 0.1% of each reference DNA extracted from the reference GM-maize (w/w) was detected. Meanwhile, test result from each 100% serially diluted 100% DNA event was detected as low as 0.01%, which meant the detection limit was less than 10 copies. Slope of each standard curve lied in the range of -3.31~-3.45, with correlation coefficient between 0.99 and 1.0. The concentration range of the standard curve was between 20 and 1,310,720 copies, which fulfilled the quantitative assay requirement for

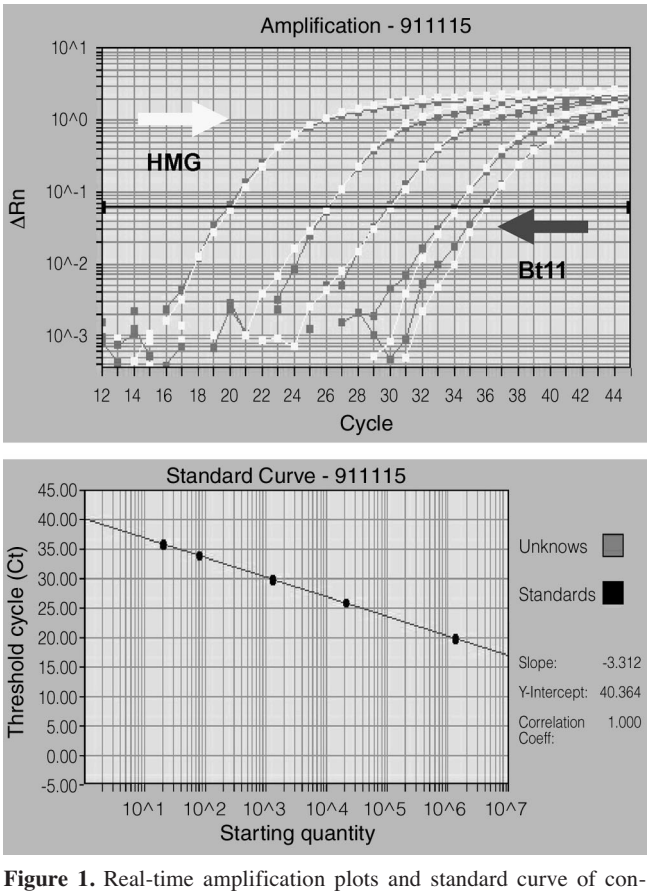

Figure 1. Real-time amplification plots and standard curve of constructed plasmid for event Bt11 GM-maize (pBt11)
HMG (white color): Real-time amplification plots of internal control gene (high mobility group) for maize.
Bt11(black color): Real-time amplification plots of specific gene for event Bt11 GM-maize.
36 threshold cycle value (Ct-value) = 20 copies; 34 Ct-value = 80 copies; 30 Ct-value = 1,280 copies; 26 Ct-value = 20,480 copies and 20 Ct-value = 1,310,720 copies.

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detecting 0.1%~100% GM-maize in 100 ng DNA of maize sample[12,15,24].

III. *Determination of Coefficient Value for Each Event of GM-maize*

The coefficient value of each event of constructed plasmid p35S, i.e. the ratio of target gene vs internal control gene of maize, was determined. In a constructed plasmid, those two genes were inserted as a single copy, whereas they may not exist as a single copy in reality. Therefore, the coefficient value shall be determined[15]. In this study, the coefficient value was determined as follows. The DNA of each events was extracted, and the target gene and internal control gene were determined by QPCR in triplicate. The ratio of target gene to internal control gene was then calculated. After determining six reference materials, in triplicate, 18 results were obtained. Mean of the 18 results was calculated as the coefficient value. The mean, standard deviation (S.D.), and coefficient of variance (C.V.) of events were also calculated. The coefficient values of Event176-35S, T25-35S, Bt11-35S and MON810-35S were 1.34, 0.86, 0.67 and 0.42, respectively (Table 2). Standard deviation was in the range of 0.01~0.08, while C.V. was less than 1%~9% (Table 2). Kuribara *et al*.[15] also used the constructed plasmid as the reference material and determined the respective coefficient value of each event. In Kuribara's report, the coefficient value of Event176-35S, Bt11-35S, MON810-35S and T25-35S were 0.88, 0.97, 0.45 and 0.34, respectively. Both our study and Kuribara's report suggested that the coefficient value did not simply reflect the ratio of target gene to internal control gene. Other factors, including target gene, internal control gene, PCR efficiency, PCR primer and probe, constructed plasmid selection, and the recovery of constructed plasmid, will all impact the test result. In comparison with Kuribara's report, our study used different plasmid construction method, different constructed fragments and number, and different primer and probe. The advantages of using single constructed plasmid in our study are easy construction and less test time needed after construction. When one of the events is not compatible, it can be easily replaced. The disadvantage of this method is each plasmid needs to be constructed and amplified individually. The advantage of using Kuribara's multiple reference plasmid method is only one mass amplification of plasmid is needed, but the disadvantage is the complication during construction. When one of the events is not compatible, it cannot be changed independently and so must be withdrawn. On the other hand, Kuribara's method takes longer time for conducting the post-construction test.

Coefficient values of the five constructed product-specific plasmids were also determined. Again, each event was tested by six reference materials in triplicate to generate 18 results. The mean, S.D. and C.V were calculated for each event. The calculated coefficient value of Event176, T25, Bt11, MON810 and GA21 were 2.97, 0.42, 0.72, 0.49 and 1.81, respectively (Table 2). The standard deviation was in the range of 0.02~0.12, and the coefficient of variance is less than 10% (Table 2). According to the

Table 2. Determination of coefficient value for each event of GM-maize

Event	Mean[a]	S.D.[b]	C.V.[c](%)
GA21	1.81	0.12	7
Event176	2.97	0.11	3
MON810	0.49	0.05	10
Bt11	0.72	0.06	8
T25	0.42	0.02	5
Event176-35S	1.34	0.08	6
MON810-35S	0.42	0.04	9
Bt11-35S	0.67	0.02	3
T25-35S	0.86	0.01	1

*Coefficient values are calculated by dividing the target gene by the internal control gene.

[a]Mean of six samples. Each sample was performed three times at Bureau of Food and Drug Analysis (BFDA).

[b]S.D.: standard deviation.

[c]C.V.: coefficient of variance are calculated by dividing the standard deviation by the calculated mean.

Table 3. Quantitative analysis of test samples by the method developed in this study

Event	GMO (%)	Mean[c]	S.D.[d]	C.V.[e] (%)
Event176	5.0[a]	4.54	0.29	6
	2.0[a]	2.16	0.14	6
	1.0[a]	1.23	0.12	10
MON810	5.0[a]	5.55	0.34	6
	2.0[a]	2.06	0.27	13
	1.0[a]	1.03	0.09	9
Bt11	5.0[b]	4.84	0.46	9
	2.0[a]	2.18	0.25	11
	1.0[a]	1.15	0.08	7
T25	5.0[b]	4.83	0.38	8
	2.0[b]	2.03	0.21	10
	1.0[b]	1.14	0.12	11
GA21	5.0[b]	4.63	0.42	9
	2.0[b]	2.08	0.32	15
	1.0[b]	1.01	0.05	5
Event176-35S	5.0	4.98	0.27	5
	2.0	2.00	0.14	7
	1.0	1.20	0.09	8
T25-35S	5.0	4.69	0.34	7
	2.0	2.31	0.21	9
	1.0	1.19	0.13	11
Bt11-35S	5.0	4.46	0.20	4
	2.0	2.24	0.12	5
	1.0	1.23	0.11	9
MON810-35S	5.0	5.47	0.40	7
	2.0	2.13	0.15	7
	1.0	1.07	0.06	6

[a]Reference samples were bought from Fluka Chemical Co.

[b]Test samples were prepared by BFDA.

[c]Mean of three samples. Each sample was performed three times at BFDA.

[d]S.D.: standard deviation.

[e]C.V.: coefficient of variance are calculated by dividing the standard deviation by the calculated mean.

report, coefficient value of Event176, T25, Bt11, MON810 and GA21 were 1.94, 0.35, 0.50, 0.42 and 1.54, respectively[15]. Different results in our study might be caused by the influence factors described above.

IV. *Quantitative Test of Commercial Reference Materials and Self-prepared Samples*

When the coefficient value of each event has been determined, the quantitation method is further tested by both commercial reference materials and self-prepared samples in 1%, 2% and 5% concentration. After three samples in each concentration groups were tested three times, nine results were obtained and their means calculated accordingly. The mean, S.D., and C.V. of 1% concentration samples were in the range of 1.01%~1.23%, 0.05~0.13, and 5%~11%, respectively. For 2% concentration samples, mean, S.D., and C.V. were in the range of 2.00%~2.31%, 0.12~0.32, and 5%~15%. For 5% concentration samples, mean, S.D., and C.V. were in the range of 4.46%~5.55%, 0.20~0.46, and 4%~9% (Table 3). In the testing of 35S screening and product-specificity of the five events, the S.D. and C.V. of all three concentrations of T25 and GA21, and of 5% Bt11 are generally higher than the other events. It is suspected that the results came from the sample preparation error. Due to no commercial reference materials were available for T25 and G21 in different concentrations, nor for 5% Bt11, they must be self-prepared. However, it can be concluded that the quantitation method in our study is applicable from the results in Table 3.

V. *International Proficiency Test of Genetically Modified Maize*

In order to confirm the applicability of the quantitative method developed in our laboratory, the international proficiency test samples has been tested. There were six test samples, and each sample contained seven events of GM-maize (namely Event176, Bt11, MON810, T25, NK603, CBH351 and GA21) in different concentrations. At the beginning of the proficiency test, we only received and informed with test samples of seven different event combinations. The test must be completed and the results must be reported within certain time frame, and then the actual events and their concentrations would be revealed. From the host organization, we were informed that six test samples were spiked with 0%, 0.5%, 1.5% or 5% of GM-maize. The test results of our method are listed in Table 4. For Bt11, MON810 and GA21, our method achieved 93%~100% accuracy. For 0% and 0.5% of T25, the accuracy was also approximately 100%. However, the test results of the two test samples in 5% concentration are 11%-20% deviated from the standard. Other quantitative methods have also obtained higher results for T25 event in these two test samples[25]. The deviation was suspected to be the consequence of sample preparation error. On the other hand, Event176 had the highest deviation. In its 0.1% and 1.5% samples, only 70%~80% accuracy could be achieved. We further tested it with commercial detection kits and obtained the similar results as the method developed in this study (data not shown). Only 70%~80% quantity can be detected in each Event176 spiked sample. We suspect that there is different copy number of HMG maize internal control gene between the non-GM maize test sample and Event176 GM maize. Actual reasons for the deviation must be further confirmed by coordination between each test laboratory and host organization. The test results of Event176 in this study were still within an acceptable range[25].

In conclusion, the quantitative PCR method developed in this study is confirmed to be able to detect five events of genetically modified maize. At the same time, the method can be applied successfully in the international proficiency test of genetically modified maize. The constructed plasmid can be used as either qualitative or quantitative reference standard to resolve the shortage of standard material.

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Table 4. Results of proficiency program samples analyzed by the method developed in this study

Sample no.	GM-maize (%)									
	T25		MON810		GA21		Event176		Bt11	
	S[a]	T[b]	S	T	S	T	S	T	S	T
1	0.50	0.50	N[c]	N	5.00	5.02	1.50	1.14	5.00	4.66
2	N	N	1.50	1.50	0.50	0.49	0.10	0.08	0.10	0.10
3	0.50	0.51	N	N	5.00	4.93	1.50	1.11	5.00	4.70
4	N	N	1.50	1.44	0.50	0.49	0.10	0.08	0.10	0.10
5	5.00	5.57	0.50	0.47	0.10	0.10	N	N	N	N
6	5.00	5.98	0.50	0.50	0.10	0.10	N	N	N	N

[a]S: Fortified samples containing combination of the events (T25, MON810, GA21, Event176 and Bt11). Concentration of the events will also vary among the samples.

[b]T: Analyzed by the method developed in this study, mean of three test results.

[c]N: Negative result.

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