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ARTICLE

Simplex and Duplex Event-Specific Analytical Methods for Functional Biotech Maize

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Analytical methods are very important in the control of genetically modified organism (GMO) labeling systems or living modified organism (LMO) management for biotech crops. Event-specific primers and probes were developed for qualitative and quantitative analysis for biotech maize event 3272 and LY 038 on the basis of the 3′ flanking regions, respectively. The qualitative primers confirmed the specificity by a single PCR product and sensitivity to 0.05% as a limit of detection (LOD). Simplex and duplex quantitative methods were also developed using TaqMan real-time PCR. One synthetic plasmid was constructed from two taxon-specific DNA sequences of maize and two event-specific 3′ flanking DNA sequences of event 3272 and LY 038 as reference molecules. In-house validation of the quantitative methods was performed using six levels of mixing samples, from 0.1 to 10.0%. As a result, the biases from the true value and the relative deviations were all within the range of ±30%. Limits of quantitation (LOQs) of the quantitative methods were all 0.1% for simplex real-time PCRs of event 3272 and LY 038 and 0.5% for duplex real-time PCR of LY 038. This study reports that event-specific analytical methods were applicable for qualitative and quantitative analysis for biotech maize event 3272 and LY 038.

KEYWORDS: Genetically modified organism (GMO); living modified organism (LMO); event-specific; biotech maize; limit of detection (LOD); simplex; duplex; real-time PCR; reference molecule; limit of quantitation (LOQ)

INTRODUCTION

Biotech crops have been increasingly developed and commercialized since the first biotech tomato, Flavr Savr, in 1994. The global area for biotech crops has continuously increased from 1.7 million hectares in 1996 to 125 million hectares in 2008. The global adoption rates for major biotech crops were as follows: soybean, 70%; maize, 24%; cotton, 46%; and canola, 20% (*1*).

Maize (*Zea mays* L.) has been mainly used for human food and animal feed. Biotech maize was earlier developed to have herbicide tolerance and insect resistance for farmers. More recently, it is also expanded to functional traits for biofuels or the reinforcement of some useful amino acids. Event 3272 has been genetically modified to express a thermostable α-amylase enzyme (AMY797E) for use in dry-grind fuel ethanol production (*2*). LY 038 contains the *cordap*A gene from *Corynebacterium glutamicum*, which encodes the enzyme dihydrodipicolinate synthase (DHDPS). This enzyme is involved in lysine biosynthesis. The bacterial DHDPS enzyme, unlike the plant DHDPS enzyme, is not sensitive to lysine feedback inhibition, so lysine biosynthesis will continue in the presence of high levels of free lysine (*3*).

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Many consumers are concerned about the potential risk biotech crops may have for human health or ecological environment. A number of countries have made their own labeling systems for genetically modified products. The purpose of the genetically modified organism (GMO) labeling system is to inform consumers of the presence of the GM products in the crops or derived products and thereby help the consumers choose their preferred products. Many countries have established labeling systems based on their own criteria, with thresholds for the adventitious presence (AP) of biotech crops defined as 0.9% in the European Union (EU) (*4*), 3% in Korea (*5*), and 5% in Japan (*6*).

Analytical methods are very important in the control GMO labeling system or living modified organism (LMO) management for biotech crops. The Polymerase Chain Reaction (PCR) is the most efficient DNA-based analytical method to identify or quantify biotech crops. Qualitative PCR methods have been developed to detect the presence of biotech crops (*7*−*11*). Quantitative PCR methods have been also developed to analyze the content of biotech crops (*12*−*21*). In case of event 3272 and LY 038, although other event-specific quantitative detection methods have been validated in the EU, there is no event-specific method using one reference plasmid based on the 3′ flanking regions between the inserted DNA and the host genome. Especially, duplex PCR has the ability to analyze two targets in one PCR reaction at the same time, which could save working time and

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Table 1. Primers and Probes for Qualitative and Quantitative PCRs

target	name	sequence (5′ → 3′)	specificity	length (bp)
taxon-specific (simplex PCR)				
SSIIb	SSIIb 3−5′	CCA ATC CTT TGA CAT CTG CTC C	zSSIIb	114
	SSIIb 3−3′	GAT CAG CTT TGG GTC CGG A	zSSIIb	
	SSIIb Taq	FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA	zSSIIb	
taxon-specific (duplex PCR)				
Adh1	zm Adh1-F	CGT CGT TTC CCA TCT CTT CCT CC	Adh1	135
	zm Adh1-R	CCA CTC CGA GAC CCT CAG TC	Adh1	
	zm Adh1-P	VIC-AAT CAG GGC TCA TTT TCT CGC TCC TCA-TAMRA	Adh1	
DNA walking				
event 3272	ES-1	GCC GGT CTT GCG ATG ATT	tNOS	
	ES-2	GAT TAG AGT CCC GCA ATT ATA CAT TTA A	tNOS	
	DW-ACP 2	ACP-TGGTC	in the kit	
	DW-ACPN	ACPN-GGTC	in the kit	
	uniprimer	TCACAGAAGTATGCCAAGCGA	in the kit	
LY 038	LS-1	CCC AAG CAA TGA TCT CGA AGT AT	Glb1	
	LS-2	TTC ATT GGC GCA CGA ACT T	Glb1	
	LS-3	GGG CGG CGC TTA CTT TCT	Glb1	
	DW2-ACP4	ACP-ACGTG	in the kit	
	DW2-ACPN	ACPN	in the kit	
	UniP2	GAGTTTAGGTCCAGCGTCCGT	in the kit	
event-specific				
event 3272	E 350-F	TAG CGC GCA AAC TAG GAT AAA TT	tNOS	350
	E 350-R	CAC ATA CAT CCA TCG ATC GAA CA	maize genome	
	E 101-F	GGC CAG CAT GGC CGT A	vector	112
	E 112-R	GAG GAG GGA CAA GAT GTT TAT TTC A	maize genome	
	E 101-Taq	FAM- CCG CAA TGT GTT ATT AAG TTG TCT AAG CGT CAA-TAMRA	vector	
LY 038	L 168-F	CCA GGT AGT AAT GCA CAG ATA TGC A	Glb1	168
	L 168-R	TGA AAG ACT GGG CAA TAA TTA TGG	maize genome	
	L 96-F	ACA TTA TAC GAA GTT ATT CTA GAG CGG C	vector	101
	L 196-R	TGT AAT ACC CTG AAA GAC TGG GC	maize genome	
	L 129-Taq	FAM-CCG CGG TGG AGC TCG GAT CC-TAMRA	vector	

load. The results from duplex PCR might be more accurate than those from simplex PCR because the pipetting error can be reduced in duplex PCR reaction.

In this study, we developed event-specific analytical methods for event 3272 and LY 038 based on the 3′ flanking regions. We report the qualitative and simplex quantitative PCR analytical methods for biotech maize event 3272. We also report the qualitative, simplex, and duplex quantitative PCR detection methods for LY 038. These methods were performed using the event-specific primer pairs, probes, and one standard plasmid, their applicability for practical use was confirmed by in-house validation test.

MATERIALS AND METHODS

Materials. Genuine seeds of biotech maize event 3272 and LY 038 were provided from Syngenta Seeds Co. (Research Triangle Park, NC) and Monsanto Co. (St. Louis, MO), respectively. Biotech maize MON 810, Bt 11, Bt 176, GA 21, T 25, NK 603, TC 1507, MON 863, MIR 604, and DAS-59122 were purchased from Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). T 25 and MON 88017 were also purchased from American Oil Chemists' Society (AOCS, Urbana, IL).

DNA Extraction and PCR Inhibitor Check. Maize seeds were ground by an electric mill (Fritsch pulverizette 14, Germany). According to the modified manufacturer's protocol (*22*), the genomic DNA was extracted from about 1 g of the ground sample by using the DNeasy Plant Maxi kit (Qiagen, Germany). The concentration of the extracted DNA was measured by UV spectrophotometer ND-1000 (Nano Drop Technologies) and confirmed on agarose gel by electrophoresis. As a good quality of DNA for PCR, the absorption ratios at 260/280 and 260/230 nm wavelengths should all be > 1.7.

The extracted DNA was checked to identify the presence of PCR inhibitor by quantitative PCR. Two levels of concentration (50 and 10 ng/μL) with event 3272 and LY 038 genomic DNAs were prepared and quantitated by SSIIb-3, E 101F/112R, and L 96F/196R primers/probe sets, respectively. The presence of PCR inhibitor was checked by the copy numbers between two levels of concentrations.

Junction Region Analysis by DNA Walking. The DNA Walking SpeedUp Premix Kits I and II (Seegene, Korea) were used to analyze the junction sites between the inserted DNAs and the maize genome of event 3272 and LY 038. DNA walking anneling control primer (ACP)-PCR reactions were performed according to the instructions of the manufacturer with gene-specific primers listed in **Table 1**.

Oligonucletide Primers and Probes. All primers and probes were designed by using Primer Express software 3.0 (Applied Biosystems,

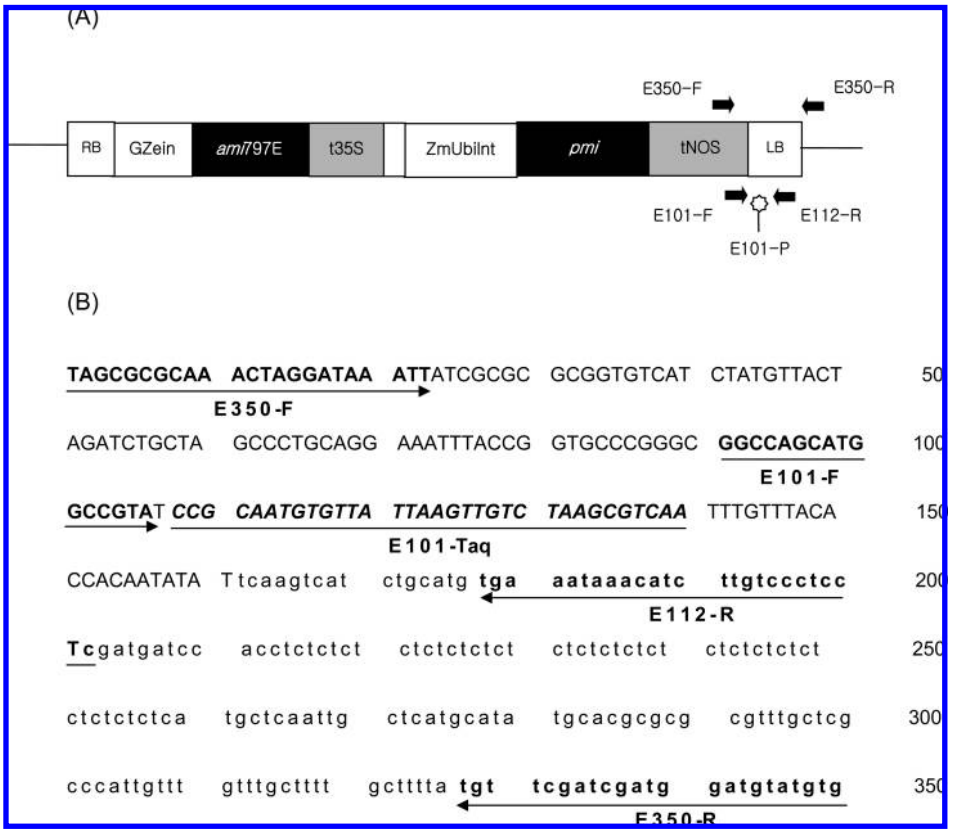

Figure 1. Schematic diagram of PCR strategy and nucleotide sequences for junction region of biotech maize event 3272. Arrows indicate specific primers and the star indicates specific probes for qualitative and quantitative detection for biotech maize event 3272 (**A**). Capital letters indicate the inserted DNA sequences, and lowercase letters indicate genomic DNA flanking sequences. Letters in bold express forward and reverse primers, and letters in italic express TaqMan probe (**B**).

Foster City, CA). They were synthesized and purified by Genotech Co. (Daejeon, Korea) and Applied Biosystems. The probes were labeled with fluorescent reporter dye FAM or VIC and fluorescent quencher dye TAMRA at the 5′ or 3′ end, respectively. The locations of primers and probes are shown in **Figures 1** and **2**, and their nucleotide sequences are listed in **Table 1**.

As the endogenous reference controls, SSIIb-3 primers/probe detected the maize starch synthase IIb (*zSSIIb*) gene for simplex PCR (*13*) and zmAdh1-F/R primers/probe detected the alcohol dehydrogenase 1 (*Adh1*) gene for duplex PCR (*23*). The former gene has been mainly used in Asia, and the latter gene has been also used in Europe. E 350-F/R and E 101F/112R primers and E 101-Taq probe were designed for qualitative or quantitative detection for event 3272 based on the 3′ flanking region between the nopaline synthetase (NOS) terminator (GenBank accession no. V00087) and maize genome. L 168-F/R and L 96F/196R primers and L 129-Taq probe were also designed for qualitative or quantitative detection for LY 038 based on the 3′ flanking region between the globulin 1 (Gbl1) terminator (GenBank accession no. X59084) and maize genome.

Qualitative PCR Analysis. Conventional PCR was performed to confirm the specificity of the designed primers on the genomic DNAs extracted from nonbiotech maize, 13 biotech maize events including event 3272 and LY 038. To determine the sensitivity of the qualitative PCR method, DNA mixtures were prepared from biotech maize event 3272 and LY 038 at the following levels: 0, 0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0% (w/w).

The conventional PCR was run with a 25 μL volume of reaction solution using a GeneAmp PCR System 9700 (Applied Biosystems). A reaction solution contained 50 ng of genomic DNA, 2.5 μL of 10× PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 1.25 unit of AmpliTaqGold DNA polymerase (Applied Biosystems). The PCR was performed according to the following program: 1 cycle of 10 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, and a final extension cycle of 7 min at 72 °C. The PCR product was resolved on 2% (w/v) agarose gel by electrophoresis.

Standard Plasmid as a Reference Molecule. As a reference molecule, a standard plasmid was constructed on the basis of a pTOP TA V2 vector (Enzynomics, Korea) integrated with four PCR products, which were amplified from the taxon-specific primers for maize SSII-b and Adh1 genes and from the event-specific primers for biotech maize event 3272 and LY 038 genes.

The standard plasmid was constructed according to the method of Kuribara et al. (*24*). Two kinds of PCRs were performed for connection and cloning of PCR products. First, to make a blunt-end PCR product for connection, a 50 μL volume of reaction solution was composed of 5 μL of 10× PCR buffer, 0.2 mM dNTP, 1 mM $MgSO_4$, 0.3 μM primer pair, 1 unit of KOD-Plus-DNA polymerase (Toyobo Co., Japan), and 2 ng of plasmid DNA as a reaction template. Second, to connect the first PCR products for cloning, a 25 μL reaction solution contained 2.5 μL of 10× PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 μM primer pair, 1.25 units of AmpliTaqGold DNA polymerase, and 2 μL of the two first PCR products as reaction templates. All PCRs were performed by a GeneAmp PCR system 9700 (Applied Biosystems) according to the following PCR program: 1 cycle of 10 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C, and a final extension cycle of 7 min at 72 °C.

The final-connected PCR product containing the *Hpa*I site was ligated into the pTOP TA V2 plasmid vector by a TOP Cloner PCR cloning kit (Enzynomics, Korea), and this recombinant plasmid transformed *Escherichia coli* strain DH5α cell (Enzynomics). The cloned plasmid was selected by *EcoR*I digestion (Roche Applied Science, Germany) and confirmed through DNA sequencing analysis by Solgent Co. (Daejeon, Korea).

The cloned plasmid was extracted by using the Qiagen Plasmid Midi kit (Qiagen), and the extracted plasmid DNA was cut by *Hpa*I restriction endonuclease (Enzynomics). The linearized plasmid DNA was purified from 2% agarose gel by the QIA Quick Gel Extraction kit (Qiagen). The concentration of the plasmid DNA was measured by the UV spectrophotometer ND-1000 (Nano Drop Technologies). As a calibrant for quantitation, the standard plasmid was serially diluted to

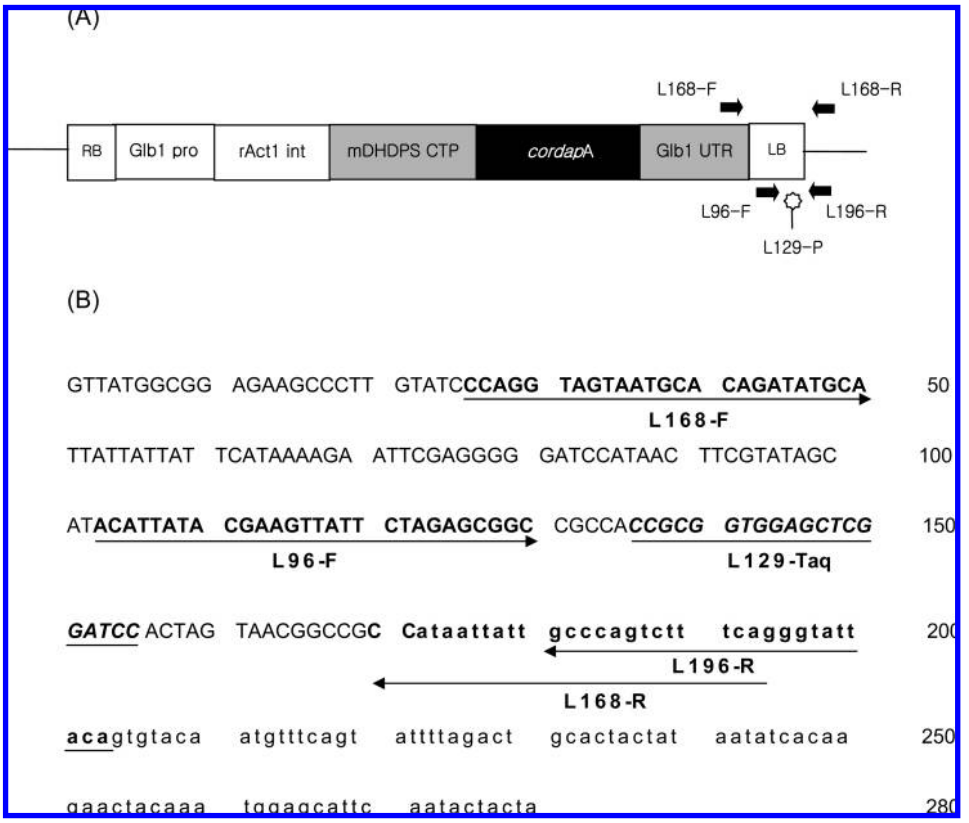

Figure 2. Schematic diagram of PCR strategy and nucleotide sequences for junction region of biotech maize LY 038. Arrows indicate specific primers and the star indicates the specific probes for qualitative and quantitative detection for biotech maize LY 038 (**A**). Capital letters indicate inserted DNA sequences, and the lower case letters indicate genomic DNA flanking sequences. Letters in bold express forward and reverse primers, and letters in italic express TaqMan probe (**B**).

1×10, 1.25×10^2, 1.5×10^3, 2×10^4, and 2.5×10^5 copies per 2.5 μL with ColE1 DNA (5 ng/μL, Wako, Japan).

Quantitative PCR Analysis. For quantitation of biotech maize DNA, a real-time PCR was performed in triplicates using three wells. In simplex real-time PCR, a 25 μL volume of one well contained 50 ng of sample DNA, 0.50 μM each of one forward and one reverse primer (1.0 μM each of one forward and one reverse primer for event 3272), 0.10 μM probe (0.2 μM probe for event 3272), and 12.5 μL of Universal Master Mix (Applied Biosystems). On the other hand, in duplex real-time PCR, a 25 μL volume of one well contained 100 ng of sample DNA, 0.20 μM primers, and 0.04 μM probe for Adh1, 1.00 μM primers and 0.20 μM probe for LY 038, and 12.5 μL of Universal Master Mix. The real-time PCR was carried out by ABI PRISM 7900 (Applied Biosystems). PCR amplification was run with the following program: uracil-*N*-glycosylate treatment at 50 °C for 2 min, denaturation at 95 °C for 10 min, 45 cycles (50 cycles for duplex) of denaturation at 95 °C for 30 s, and annealing or extension at 59 °C for 1 min.

Five levels of standard plasmid were used for the standard curve as calibrants, which are reference molecules. ColE1 DNA (5 ng/μL) was used as no-template control (NTC). To calculate the biotech maize content, the conversion factor (C_f) was required. It defines the ratio of the DNA copy numbers between an introduced gene and an endogenous gene, and it is used to change the experimental value into real value.

To validate the quantitative method, the test samples were prepared by mixing DNAs from conventional maize, event 3272, and LY 038 according to six levels of 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0%.

RESULTS AND DISCUSSION

Junction Region Analysis. We used the qualified genomic DNAs, which had no PCR inhibitor, extracted from event 3272 and LY 038. To design the event-specific primers for event 3272 and LY 038, analysis of the junction regions was performed by DNA walking ACP-PCR. We confirmed the sequences of the 3′ junction region between nopaline synthetase (NOS) terminator (GenBank accession no. V00087) and maize genome for event 3272 and those of the 3′ junction region between the globulin 1 (Gbl1) terminator (GenBank accession no. X59084) and maize genome for LY 038 (**Figures 1** and **2**).

Specificity of Qualitative PCR. The SSIIb-3 primer pair has been used as a taxon-specific primer for single-copy maize endogenous gene, z*SSIIb*. The newly designed primer pairs were E350-F/R and L168-F/R. When the DNAs of event 3272 and LY 038 were amplified as the templates, amplified fragments of 350 and 168 bp were observed, respectively. However, no amplification was observed from the nonbiotech maize and 11 biotech maize events (**Figure 3**). This specificity is basically attributable to the event-specific primer pairs designed to amplify the junction region containing the maize genome and insert DNAs of event 3272 and LY 038, respectively. These results showed that the developed qualitative PCR detection method could be applicable for the identification of event 3272 and LY 038.

Limit of Detection of Qualitative PCR. The sensitivity test was performed to determine the limit of detection (LOD) of the qualitative PCR method by three times repeat. The test DNA samples, mixed with nontransgenic maize, Event 3272 and LY 038, were prepared to nine levels; 0, 0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0 and 10.0% (w/w). As a result, the LODs for Event 3272 and LY 038 were all 0.05% (**Figure 4**). The lowest threshold value is 0.9% of EU in the world. Thus, this result indicated that the qualitative PCR methods developed from this study could be used in monitoring work for the GMO labeling systems in every country.

Standard Plasmid as a Reference Molecule for Quantitative PCR. A plasmid 3238 was constructed by the integration of four

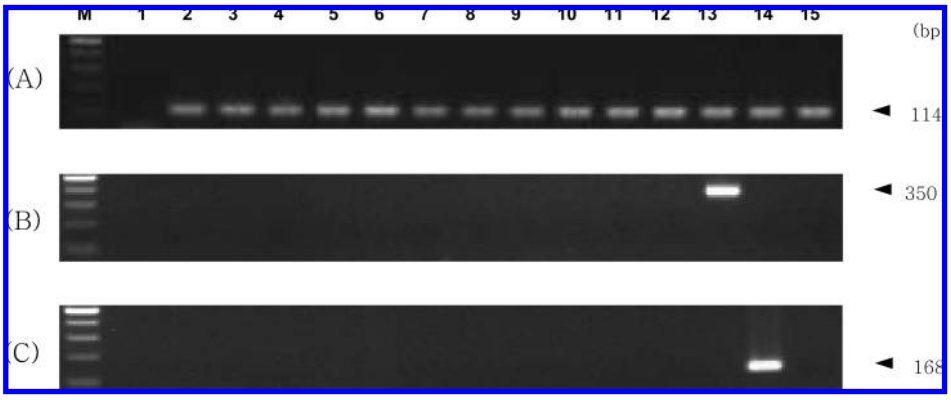

Figure 3. Specificity of the primer pairs designed for biotech maize event 3272 and LY 038. PCR products were electrophoresed on 2% agarose gel. Arrowheads indicate the expected PCR amplification products. The respective primer pairs for detecting SSIIb-3 (**A**), event 3272-qualitative (**B**), and LY 038-quantitative (**C**) were used. Template DNAs for each lane were as follows: lanes 1−15, no template control, non-biotech maize, MON 810, Bt 11, Bt 176, GA 21, T 25, NK 603, TC 1507, MON 863, MIR 604, MON 88017, event 3272, LY 038, and DAS-59122; M, 100 bp size ladder.

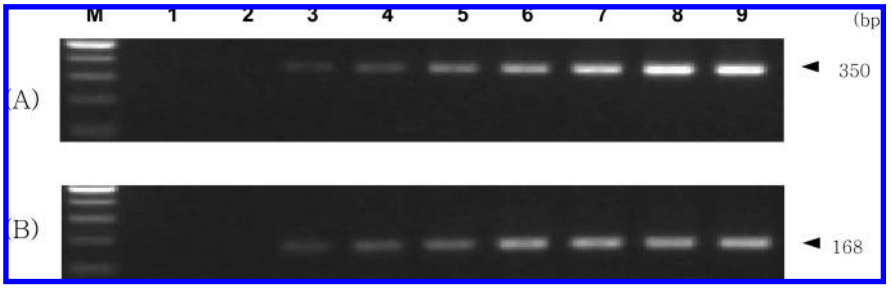

Figure 4. Sensitivity of the primer pairs designed for biotech maize event 3272 and LY 038. The PCR products were electrophoresed on 2% agarose gel. Arrowheads indicate the expected PCR amplification product. The primer pairs for detection of biotech maize event 3272 (**A**) and LY 038 (**B**) were used. Template mixing DNAs for each lane were as follows: lanes 1−9, 0, 0.01, 0.05, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0%, respectively; M, 100 bp size ladder.

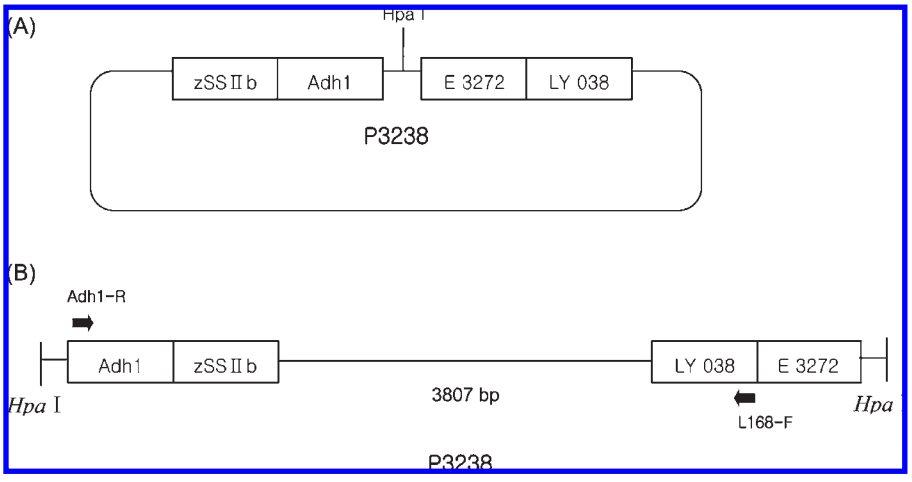

Figure 5. Standard plasmid p3238 as a reference molecule: schematic diagram of p3238 (**A**); linear standard plasmid p3238 cut with *Hpa*I (**B**).

PCR amplicons, which were from two endogenous genes, *SSIIb* and *Adh1*, and two introduced genes of event 3272 and LY 038 into a pTOP TA V2 vector (Enzynomics). The SSIIb-3 primer pair has been used for simplex PCR and the zmAdh1 primers for duplex PCR as taxon-specific primers. In the case of duplex PCR, we cannot observe any PCR product between zmAdh1 primers and LY 038 event-specific primers (data not shown). It is impossible to get any PCR product by the linear standard plasmid cut with *Hpa*I because the distance is about 4 kb between two primers (**Figure 5**).

As a reference molecule, five levels of standard plasmid were set to 1×10, 1.25×10^2, 1.5×10^3, 2×10^4, and 2.5×10^5 copies per reaction for the quantitative real-time PCR. These were sufficient to quantitate 0.1−100% of biotech maize using 50−100 ng of genomic DNA for one reaction ranging from 1×10 to 2.5×10^5 copies. The linearity of the standard curves for event 3272 and LY 038 was confirmed in the quantitative PCR using the designed primer pairs, probes, and the standard plasmid and calculated to above 0.990 of R^2 value (**Figure 6**).

As for the five levels of the standard plasmid (p3238), the repeatability of the copy number at each level of standard plasmid was confirmed from the data of triplicate reactions. The values of bias (mean vs true value) of the triplicate reactions of the plasmid copy numbers ranged from 0.1 to 23.0% (**Table 2**). All bias values

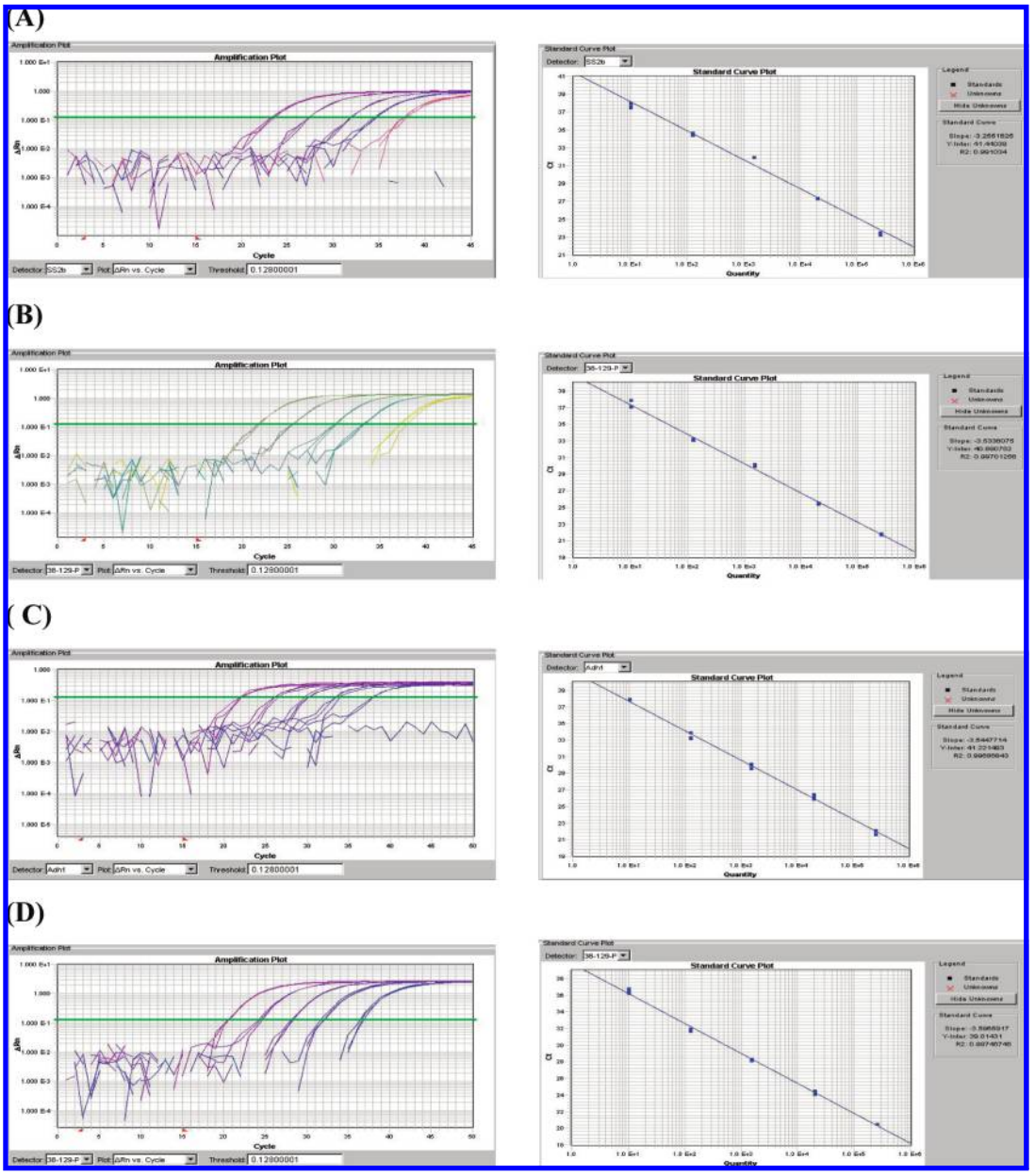

Figure 6. Amplification plots and standard curves of simplex (**A**, **B**) and duplex (**C**, **D**) real-time PCRs. Amplification plots and standard curves were generated from real-time PCR using taxon-specific primers and probes for endogenous SSIIb or Adh1 gene and L 96F/196R primers and L 129-Taq probe for the detection of biotech maize LY 038 by the five levels of p3238.

were below 25%, so the standard plasmid was confirmed to be stable and reliable reference material.

Compared with the genomic DNA reference material, the standard plasmid can be supplied as unlimited quantities with a consistent quality, and a single plasmid can be used as a single reference molecule for several biotech events. As a result of this study, we propose that this standard plasmid can be used as a good reference molecule for quantitating biotech maize event 3272 and LY 038.

Conversion Factor for Quantitation. The conversion factor (C_f) is a ratio of the copy numbers between a recombinant DNA and a taxon-specific DNA in a biotech maize event. The biotech maize content of a maize sample can be calculated from the following formula: (copy numbers of an event-specific recombinant DNA sequence/copy numbers of a taxon-specific DNA sequence) $\times$ $1/C_f \times 100$ (%). All experiments were repeated six times using two genomic DNAs, and the mean value was determined to be C_f for real content.

Theoretically, the C_f value of F_1 maize seed is either 0.4 or 0.6, depending on parental reproductive types of biotech maize if the biotech maize contains a single copy of transgene. The value is 0.4 if F_1 seed was derived from a male gamete of biotech maize and 0.6 if the seed was derived from a female gamete of biotech maize. The mean C_f values are 0.63 for event 3272 and 0.44 for LY 038 in simplex PCR and 0.47 for LY 038 in duplex PCR (**Table 3**). The result suggested that the F_1 seed of event 3272 provided by the developer originated from a female gamete of biotech maize and the male gamete of nonbiotech maize, and the F_1 seed of LY 038 provided by the developer originated from a male gamete of biotech maize and a female gamete of nonbiotech maize.

In-House Validation of the Quantitative Method. To evaluate the accuracy and precision, test DNA samples were prepared at six mixing levels: 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0%. The accuracy of the method was evaluated as the bias (%) of the experimental mean value from the theoretical value. The precision was evaluated by the relative standard deviation (RSD).

Table 2. Repeatability of the Copy Numbers of p3238

		accuracy			
		means	bias	precision	
target	true copy no.	exptl copy no.	true value (%)	SD[a]	RSD[b]
SSIIb-3	10	11.4	14.0	1.7	14.9
	125	107.2	−14.2	19.0	17.7
	1500	1299.5	−13.4	146.9	11.3
	20000	20342.9	1.7	1644.7	8.1
	250000	279701.4	11.9	29865.0	10.7
Adh1	10	10.1	1.0	0.6	5.9
	125	134.7	7.8	6.3	4.7
	1500	1155.5	23.0	54.8	4.7
	20000	19847.9	−0.8	1399.5	7.1
	250000	277781.5	11.1	19917.6	7.2
event 3272	10	10.9	9.0	2.1	19.3
	125	107.6	−13.9	13.7	12.7
	1500	1441.7	−3.9	102.8	7.1
	20000	21301.2	6.5	3376.3	15.9
	250000	232150.5	−7.1	12421.3	5.4
LY 038	10	11.4	14.0	11.9	22.8
	125	130.8	4.6	2.6	9.1
	1500	1177.0	21.5	92.4	7.9
	20000	22428.7	12.1	653.9	2.9
	250000	250263.7	0.1	12473.5	5.0

[a] SD, standard deviation. [b] RSD, relative standard deviations of the triplicate reactions in single experiment for SSIIb, Adh1, event 3272, and LY 038.

Table 3. Conversion Factors (C_f) of the Simplex and Duplex Quantitative PCRs for Event 3272 and LY 038

	target	mean	SD[a]	RSD[b]
simplex	event 3272	0.63	0.05	7.94
	LY 038	0.44	0.02	4.55
duplex	LY 038	0.47	0.05	8.47

[a] SD, standard deviation. [b] RSD, relative standard deviation calculated by dividing the standard deviation by the mean value, given in percent. Experiments were performed six times for each event.

In the case of event 3272, as a result at 1.0, 3.0, and 5.0% mixing levels, the biases (mean vs true value) were 16.0, 4.0, and 1.4%, and their RSDs were 12.1, 9.6, and 5.9%, respectively. In the case of LY 038, as a result at 1.0, 3.0, and 5.0% mixing levels, the biases (mean vs true value) were −1.0, −5.0, and −15.2% and their RSDs were 21.2, 8.1, and 10.4% in simplex PCR, respectively. On the other hand, in duplex PCR, as a result at 1.0, 3.0, and 5.0% mixing levels, the biases (mean vs true value) were −17.0, −13.0, and −5.4% and their RSDs were 7.2, 8.4, and 13.1%, respectively. Overall, the values of biases and RSDs of the three times repeated tests ranged from 0.0 to 30.0% at six levels (**Tables 4** and **5**). All of the variations were within 30%; therefore, the accuracy and precision of these simplex and duplex quantitative methods were credible for the method's practical application (*25*).

According to the GMO labeling systems of some countries, thresholds for the adventitious presence (AP) level of biotech products are 0.9% in the EU, 3.0% in Korea, and 5.0% in Japan. The limits of quantitation (LOQs) of the simplex PCR methods for biotech maize event 3272 and LY 038 were all 0.1%. The LOQ of duplex PCR for biotech maize LY 038 was 0.5%. Therefore, our simplex and duplex PCR methods can be applicable for GMO labeling systems around the world. In conclusion, we report that the qualitative and simplex or duplex quantitative

Table 4. Accuracy and Precision of the Simplex Quantitative PCR Methods

		accuracy				
		mean	bias	precision		
biotech event	true value (%)	exptl value (%)	true value (%)	SD[a]	RSD[b]	below 10 copies[c]
event 3272	0.1	0.11	10.0	0.03	27.3	0/3
	0.5	0.58	16.0	0.05	8.6	0/3
	1.0	1.16	16.0	0.14	12.1	0/3
	3.0	3.12	4.0	0.30	9.6	0/3
	5.0	5.07	1.4	0.30	5.9	0/3
	10.0	9.23	−7.7	0.48	5.2	0/3
LY 038	0.1	0.09	−10.0	0.02	22.2	0/3
	0.5	0.44	−12.0	0.06	13.6	0/3
	1.0	0.99	−1.0	0.21	21.2	0/3
	3.0	2.85	−5.0	0.23	8.1	0/3
	5.0	4.24	−15.2	0.44	10.4	0/3
	10.0	8.98	−10.2	0.52	5.8	0/3

[a] SD, standard deviation. [b] RSD, relative standard deviation. Experiments were repeated three times. [c] Below 10 copies, number of experiments below 10 copies/total number of experiments.

Table 5. Accuracy and Precision of the Duplex Quantitative PCR Method

		accuracy				
		mean	bias	precision		
biotech event	true value (%)	exptl value (%)	true value (%)	SD[a]	RSD[b]	below 10 copies[c]
LY 038	0.5	0.55	10.0	0.03	5.5	0/3
	1.0	0.83	−17.0	0.06	7.2	0/3
	3.0	2.61	−13.0	0.22	8.4	0/3
	5.0	4.73	−5.4	0.62	13.1	0/3
	10.0	10.73	7.3	1.05	9.8	0/3

[a] SD = Standard deviation. [b] RSD = Relative standard deviation. Experiments were repeated three times. [c] Below 10 copies = the number of experiments below 10 copies/total number of experiments.

PCR methods for biotech maize event 3272 and LY 038 were developed and validated in-house, and we confirmed that these methods could be used as practical monitoring methods for biotech maize event 3272 and LY 038. Subsequently, we will establish these methods with regard to international methods through an interlaboratory collaborative study.

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