Original

Development of Event-Specific Quantitation Method for GA21 Maize, Which Is a GM Event without CaMV35S Promoter

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A real-time PCR detection method was developed for event-specific quantitation of Roundup Ready® maize, GA21. The developed PCR method was designed to amplify an artificial junction site between the native maize genome DNA and the recombinant DNA of GA21 maize, which provides only one target sequence per haploid of GA21 genome. Thus, the amplification efficiency of the event-specific target for GA21 became closely similar to the amplification of *SSIIb*, and the conversion factor (Cf) for the quantitation method was similar to the theoretical value. The developed method demonstrated better performance than the existing construct-specific method that has been used as a Japanese official method. The developed method can easily be combined with the real-time PCR targeting of the CaMV35S promoter, and the multiplexed method should be an effective screening method for GM maize.

Key words: genetically modified (GM); GA21 maize; Roundup Ready®; event-specific; real-time PCR

Introduction

Biotechnology has been used in modern farming since the initial commercialization of a genetically modified (GM) tomato in the USA in 1994. In fact, the global area of GM crops exceeded 100 million hectares in 2006¹⁾, and is expected to continue to rise. GM crops have been authorized for food and/or feed by many countries based on their own criteria for safety assessment. However, consumers have been demanding appropriate information and labeling for foods derived from GM crops. Thus, labeling systems have been introduced for GM foods in the European Union (EU), Korea, Japan, Australia and other countries, and these systems are different from each other. For example, the threshold levels for the unintentional presence of GM materials in non-GM crops have been defined as 0.9% in the EU^{*1}, 3% in Korea^{*2}, and 5% in Japan²⁾.

^{*1} Regulation (EC) No. 1829/2003. Off. J. Eur. Union, L268, 1-23 (2003).

^{*2} Notification No. 2000-31; Ministry of Agriculture and Forestry of Korea. Seoul, Korea (2000).

For the monitoring of labeling systems, it has been necessary to develop methods for detecting GMOs in foods. Two approaches are available: detecting the protein produced by the introduced trait gene with a specific antibody, such as the enzyme-linked immunosorbent assay (ELISA) (reviewed in refs. 3 and 4); and detecting a specific DNA sequence used for gene modification by means of polymerase chain reaction (PCR) (reviewed in refs. 5-7). For the detection of GM maize in foods and food materials, PCR-based detection methods, which are able to detect even small amounts of transgenes in raw materials and processed foods, have been routinely used⁸⁾⁻¹⁷⁾.

On the other hand, the cost and the duration of GM analyses could constitute one of the major bottlenecks for providing consumers with cost-effective labeling. The conventional or non-GM food supply chains, however, required to be secured by affordable detection methods. Thus, the development of time- and cost-effective detection methods is important to ensure freedom of choice of consumers.

Maize is a major agricultural crop which GM-

Fig. 1. Schematic models of the recombinant DNA of GA21

(A) The mEPSPS expression cassette consists of the rice *actin1* promoter fragment including a part of non-coding region of rice *actin1* gene (indicated by a grey box), the artificial chloroplast transit peptide (CTP) sequence (filled box), the modified maize EPSPS gene (shaded box) and the nopaline synthase (NOS) terminator fragment (open box). White arrows above the diagram indicate sites for the construct specific detection primer pair used in the currently available screening method. (B) The scheme of the overall recombinant DNA (r-DNA) fragment of GA21 event. The r-DNA consists of a truncated mEPSPS cassette including a truncated promoter, three complete mEPSPS cassettes, an mEPSPS gene-truncated cassette, and a truncated promoter fragment. The small open boxes above the model of r-DNA sequence indicate the detection target regions for the construct-specific method. The small filled box below it indicates the detection target region for the event-specific method developed in this study. (C) The scheme of the 5' junction of maize genome (bar) and 5' flanking region of r-DNA inserted in GA21 maize. Black arrows below it indicate sites for the event-specific detection primer developed in this study. These schemes are based on the developer's report^{*5}.

technology has been applied, and in fact, eight events of authorized GM maize, *i.e.*, Bt11, Event176, GA21, MON 810, MON863, NK603, T25 and TC1506, are currently used for commercial purposes in Japan. Analysis of the recombinant DNA (r-DNA) constructions of these eight GM maize events elucidated that all the events except GA21 maize contain the same promoter fragment, which originated from the cauliflower mosaic virus (CaMV) and is designated as P35S. The Ministry of Health, Labour and Welfare of Japan (MHLW) announced a combinational method for the quantitation of P35S and a construct-specific quantitation for GA21 maize. This method has been officially used as a screening method for GM maize^{*3, 12)}, though it requires two independent quantitations. Thus, to improve the effi-

ciency of the GM analysis method, we have been trying to develop a multiplex screening method for GM maize. For the multiplexing of PCR methods, it is preferable that the amplification efficiencies should be as similar as possible. From this viewpoint, the PCR system used in the already established construct-specific quantitation method for GA21 maize¹²⁾ was unsuitable for multiplexing with the PCR system for P35S. The reason for this is that the PCR system for P35S has one or two amplification target(s) in a set of the relevant GM maize genome, while the PCR system has four or five amplification targets in a set of GA21 maize genome^{*4} (Fig. 1). Thus, in this study, we developed a new quantitation method for GA21 maize, which was event-specific and had one amplification target in a set of GA21 maize genome. In addition, we evaluated the developed method by means of a single-laboratory validation procedure.

Materials and Methods

Maize (*Zea mays*) and other cereal materials

Dry seeds of GM maize, *i.e.*, one progeny each of Bt11 and Event176 developed by Syngenta Seeds AG (Basel, Switzerland), a progeny of TC1507 developed by Dow Agrosciences LLC (Indianapolis, IN, USA), one progeny each of MON810, MON863, GA21, and NK603 developed by Monsanto Company (St. Louis, MO, USA), and a progeny of T25 developed by Bayer CropScience AG (Monheim am Rhein, Germany), were directly imported from the USA. Dry seeds of QC9651 maize (Quality Traders, Inc., Huntley, IL, USA) were also provided by the developer and used as a non-GM control. We used several other cereal materials as non-target controls, such as soy, rice, wheat and barley. Dry seeds of Roundup Ready® (RR) soy (Monsanto Company) were directly imported from the USA. Dry soybeans harvested in Ohio in 1998 were also directly imported and used as non-GM soy. Seeds of the rice (*Oryza sativa*) variety Kinuhikari, the wheat (*Triticum aestivum*) variety Haruyutaka and the barley (*Hordeum vulgare*) variety Harrington were obtained in Japan.

Preparation of test samples and DNA extraction

Dry seeds were ground with a P-14 speed rotor mill (Fritsch GmbH, Ibar-Oberstein, Germany). The ground materials were freeze-dried for 24 hr in an FDU-540 freeze drier (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and stored at -20°C until use. The four levels of simulated GM-mixture samples containing 0.5, 1, 5, and 10% (w/w) of each of MON810 and GA21 maize in non-GM maize were made from the ground materials. DNA extraction was performed with an DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) as described in our previous report¹²⁾. The DNA concentration of

^{*3} Notification No. 110 (Mar. 27, 2001), Department of Food Safety, MHLW, Japan (2001).

^{*4} Summary notification information format (SNIF) for products containing genetically modified higher plant (GMHP)—GA21 Roundup Ready maize—; based on Notifications for placing transgenic plants on the EU Market under Directive 2001/18/EC. Monsanto Europe S. A. (1998).

solutions was determined by measuring UV absorbance at 260 nm, and the quality was evaluated from the absorbance ratios at 260/280 nm and 260/230 nm. In the majority of maize varieties studied, the absorption ratio at 260/280 nm was between 1.7 and 2.0, and that at 260/230 nm was >1.7 . These DNA samples were used for the subsequent experiment.

Oligonucleotide primers and probes

To design primers, we used Oligo^{TM} primer analysis software (National Biosciences Inc., Plymouth, NM, USA) and Primer Express software (Applied Biosystems, Foster City, CA, USA). Primer Express software was also used for the design of GA21 esp-Taq probe. The sequences of oligonucleotides were checked using a public database (DDBJ), and it was confirmed that they did not match unintended DNA sequences. The primer pairs used in this study are listed in Table 1. These primers were synthesized by Fasmac Co., Ltd. (Kanagawa, Japan) and purified by HPLC. The TaqMan Probes were synthesized by Applied Biosystems, and labeled with 5-carboxyfluorescein (FAM) at the 5'-end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end. Each oligonucleotide was diluted to the appropriate volume of sterilized water.

DNA sequencing

The fragment was amplified with GA21 genome 1 primer and GA21 3'-m primer, and subcloned into the pGEM®-T vector (Promega Co., Madison, WI, USA). The subcloned fragment was sequenced with a CEQ^{TM}2000 DNA analysis system (Beckman Coulter, Fullerton, CA, USA) by the dideoxy sequencing method using a DTSC quick start kit (Beckman Coulter).

Qualitative PCR conditions

The reaction volume of 25 μ L contained 25 ng genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L MgCl₂, 0.5 μ mol/L of the 5' and 3' primers, and 0.625 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). The reactions were buffered with the PCR buffer II (Applied Biosystems) and amplified in a thermal cycler, the Silver 96-Well GeneAmp PCR System 9700 (Applied Biosystems) in Max mode, according to the following step-cycle program: pre-incubation at 95°C for 10 min; 40 cycles consisting of denaturation at 95°C for 0.5 min, annealing at 60°C for 1 min and extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min.

Quantitative PCR conditions and calculation of GM contents

In general, the experimental procedures of quantitative PCRs followed the established methods we reported previously¹²⁾, that were also employed in Japanese official methods for the detection of GM food^{*3}. For quantitative PCR, the reaction volume of 25 μ L contained 12.5 μ L of Universal Master Mix (Applied Biosystems), 50 ng genomic DNA as template, 0.5 μ mol/L of the 5' and 3' primers, and 0.2 μ mol/L of TaqMan® probe. Sample DNA was diluted to 20 ng/ μ L in TE buffer (pH 8.0), and 2.5 μ L of the dilution was added to the reaction mixture. The real-time PCR reactions were performed with an ABI PRISM 7700 (Applied Biosystems) according to the following step-cycle program; pre-incubation at 50°C for 2 min and 90°C for 10 min, 40 cycles consisting of denaturation at 95°C for 0.5 min, annealing and extension at 59°C for 1 min. For the event-specific quantitation method, we constructed a plasmid molecule that is a pUC19 plasmid vector with two inserted fragments, *i.e.*, the fragment amplified from pMul5 plasmid¹²⁾ with SSIIb01-5' primer and P35 S 1-5' and the fragment amplified from GA21 maize genome with GA21esp5'-2 and GA21esp3'-1, and used it for experimental calibration. The plasmid was extracted from bacterial bulk culture using the QIAGEN® plasmid Mega kit (QIAGEN) and linearized by restriction digestion at sites located outside the inserted fragments. The linearized plasmid DNA was purified by CsCl density gradient centrifugation and the purified DNA solution was diluted with TE buffer including 5 ng/ μ L of ColE1 plasmid to five different concentrations, *i.e.*, 20, 125, 1,500, 20,000, and 250,000 copies per 2.5 μ L, based on the quantitative copy numbers of SSIIb fragments.

Results and Discussion

Confirmation of genomic DNA sequence of GA21 maize

To design event-specific primers for GA21 maize, we first confirmed the DNA sequence of the artificial junction region between the native maize genome DNA and r-DNA of GA21 maize. A 112 bp part of the junction region was amplified by a primer pair, GA21 5'-m and GA21 3'-m^{*5} (Table 1) and sequenced. Using this sequence, the genomic clone sequence (Accession# CG 765412) was found by BLAST search of the DDBJ database. Then, we designed a primer, GA21 genome1,

Table 1. List of primers

Name	Sequence	Specificity
GA21 5'-m (GA21 primer1)	5'-CTTCTCGTTATGCTATTTGCAACTTTAGA-3'	Maize genome
GA21 3'-m (GA21 primer2)	5'-TGGCTCGCGATCCTCCT-3'	Rice ACT1 5'-UTR ^a
GA21 genome1	5'-TCCCAGTCTCGACGATTACACG-3'	Maize genome
GA21esp 5'-1	5'-TGTAATGGGACCTTATCGTTATGC-3'	Maize genome
GA21esp 5'-2	5'-TGGGACCTTATCGTTATGCTATTTG-3'	Maize genome
GA21esp 3'-1	5'-CGATCCTCCTCGCGTTTCC-3'	Rice ACT1 5'-UTR ^a

^a Rice ACT1 5'-untranslated region (UTR) sequence contains the promoter and 1st exon of rice ACTIN1 gene.

Fig. 2. (A) Schematic model of the positions for examined primers

The maize genome and GA21 r-DNA are indicated by a thick line and an open box, respectively. The arrows above the model indicate primers' position. The specificities of primer pairs were examined with GA21 maize.

(B-G) Specificities of examined primer pairs

Each photo shows the electrophoresis image for the amplification with the primer pair described at the top of each photo. Lanes 1–8, amplification of DNAs extracted from the seeds of NK603, Event176, T25, GA21, MON863, MON 810, TC1507 and Bt11, respectively; lane 9, non-GM maize; lane 10, non-GM soy; lane 11, Roundup Ready® soy; lane 12, rice; lane 13, wheat; and lane 14, barley. Lane 15 is the negative control (no template) and lane M shows 100 bp ladder size standards. Each electrophoresis was performed on 3% agarose gel.

on the flanking genome sequence, and used it for amplification with GA21 3'-m primer. Finally, we cloned a 325 bp fragment of the junction sequence that contained 268 bp of native maize genomic DNA and 57 bp of r-DNA of GA21 maize.

Design of the event-specific primers for GA21 maize

The design of the novel event-specific detection primer pair was based on the sequencing result of the clone described above. We designed two forward primers on the maize genomic fragment and a reverse primer on the r-DNA fragment. Then, we evaluated the amplification performance of six pairs of primers, including the primers released from Monsanto Co.*⁵ (Fig. 2A). To harmonize with the methods developed previously, the experimental conditions followed those of the Japanese official PCR method for GM maize detection, including the concentrations of the reaction solution and the thermal cycle program. To evaluate the specificities of these primer pairs at the same time, DNAs extracted from the seeds of each of eight major events of GM maize authorized in Japan and from other cereal crops were used as the templates for PCR. The results indicated that all of tested primer pairs were specific for GA21 maize (Figs. 2B–G). However, some non-specific bands that were different in length were

observed in reactions with GA21 3'-m primer. In addition, the length of this amplification product of this pair is nearly equal into that to the amplification product of the SSIIB03 primer pair (114 bp), which is the taxonomic control reaction of the real-time PCR quantitations of GM maize. As a result, we finally chose an event-specific primer pair, namely GA21 esp5'-2 and GA21 esp3'-1 that amplified a 111 bp of fragment across the artificial junction site (Fig. 1). This primer pair clearly amplified a PCR product using the DNA extracted from GA21 maize as a template (Fig. 2C). In contrast, no product was amplified from other events of GM maize, non-GM maize and other crops, *i.e.*, soy, rice, wheat and barley (Fig. 2C). Therefore, we concluded that the new primer pair was specific to GA21 maize, and demonstrated an equivalent performance with the established Japanese official method under the same experimental conditions.

Quantitation with GA21 maize event-specific primer pair

To apply the GA21 event-specific primer pair to the quantitation method based on real-time PCR system, we designed a TaqMan probe that contained 30 nucleotides of DNA fragment included in the amplification product. To develop the quantitation method following our previous methods¹²⁾, we also constructed a new plasmid molecule for analytical calibration. The calibrant plasmid contained the amplicon of GA21 maize event-specific primer pair and the amplicon of SSIIB03 primer pair in a plasmid vector originated from pUC19

*⁵ Community reference laboratory (CRL) for GM food and feed, E.C. Event-specific method for the quantitation of maize line GA21 using real-time PCR (2005).

Fig. 3. Amplification plots of event-specific real-time PCRs (A and B) and construct-specific real-time PCRs (C and D)

The amplification plots for the plasmid standards are shown in A and C, and those for genomic DNA extracted from GA21 maize are shown in B and D. Horizontal axis indicates the cycle numbers of the thermal cycle reaction and vertical axis indicates the fluorescence intensity level (logarithmic scale).

plasmid, and was diluted to a concentration ranging from 20 to 250,000 copies per reaction, and used for the following experiments. Figure 3 shows typical amplification plots for a series of plasmid calibrants and the genomic DNA extracted from GA21 maize. Comparing amplification plots between the event-specific method and the established method that is construct-specific, no significant difference was found in plots for calibrant plasmids, because a single amplification target on each molecule was used (Figs. 3A and C). On the other hand, in comparison with amplification plots for the genomic DNA extracted from GA21 maize, logarithmic amplification phases of the event-specific method were sharper than that of the established method (Figs. 3B and D). Figure 4 shows the calculated copy numbers of the target in 50 ng of GA21 maize genomic DNA. The result revealed that the single copy of the event-specific target in a set of GA21 maize genome not only reduced the measurement values for the DNA extracted from genuine GM seeds, but also increased the precision of the measurements (Fig. 4). The stability of the measurements also contributed to the higher precision of the conversion factor (Cf). The Cf values were defined based on the measurement values for the DNA extracted from genuine GM seeds and were calculated by dividing the copy numbers of targets by those of the taxon-specific fragment (*SSIIB*)^{(12), (18)}. In theory, the Cf value should be 0.5 for the event-specific method because of the heterogeneity of the single copy for the target and the homogeneity of the single copy for *SSIIB*, but is 2.5 for the construct-specific method because of the heterogeneous five copies for the target and the

homogenous single copy for *SSIIB*. In this study, we experimentally defined Cf for the event-specific method

Fig. 4. Comparison of calculated copy numbers between the event-specific method and the construct-specific method

The calculated copy numbers of GA21 specific target sequence and the taxonomic control sequence, *SSIIB*, are indicated with solid bars and open bars, respectively. Ten lots of samples were measured in triplicate for each method. The difference of the copy numbers of *SSIIB* is not significant ($p=0.15$). The error bars indicate the standard deviation of the measurements.

Table 2. Homogeneity of simulated mixtures

Method ^a	Mixing level, %	Measured mean, % ^b	95% Confidence limit ^b		<i>F</i> -value	<i>p</i> -value
			Lower, %	Upper, %		
Event-specific	0.5	0.49	0.43	0.56	0.42	0.89
	1.0	0.94	0.88	1.01	1.97	0.15
	5.0	4.29	4.04	4.55	1.91	0.16
	10.0	10.47	9.92	11.03	0.11	1.00
Construct-specific	0.5	0.52	0.50	0.55	1.90	0.17
	1.0	1.01	0.95	1.07	0.43	0.89
	5.0	4.63	4.36	4.93	0.19	0.99
	10.0	9.34	8.91	9.78	1.54	0.25

^a Measurements of ten lots with two replications were transformed using the logit transformation and were analyzed by one-way ANOVA.

^b Mean and 95% confidence limits were calculated based on logistic values and transformed using the inverse logit transformation.

Table 3. Trueness and precision statistics for quantitation method

Method ^a	Mixing level, %	Trueness		Precision	
		Measured mean, %	Bias, relative%	SD ^b	RSD ^c
Event-specific	0.5	0.50	−0.57	0.053	11
	1.0	0.95	−5.3	0.091	9.7
	5.0	4.30	−14	0.34	8.0
	10.0	10.50	5.0	0.28	2.6
Construct-specific	0.5	0.52	4.8	0.034	6.5
	1.0	1.01	1.4	0.057	5.6
	5.0	4.65	−6.9	0.17	3.6
	10.0	9.36	−6.4	0.55	5.9

^a Statistical analyses were conducted for ten lots with two replications.

^b SD; Standard deviation

^c RSD; Relative standard deviation

as 0.45, and that for the construct-specific method as 2.09. The similarity of experimental and theoretical Cf values may imply that the amplification efficiency of the event-specific target for GA21 maize is closely similar to that of *SSIIb*.

Single-laboratory evaluation

A single-laboratory evaluation for the event-specific quantitation method of GA21 maize was planned, referring to the harmonized guidelines of IUPAC, ISO, and AOAC¹⁹⁾. For this purpose, we prepared four sorts of simulated maize mixtures with different commingled levels of GA21 maize as analytical samples. Each level of mixtures was divided into aliquots of about 1 g (about one or two hundred independent packets), and ten packets were chosen at random from each commingled level of samples for this experiment. All of the samples were subjected to quantitation with the event-specific method and the established construct-specific method. The measurement was done twice for each sample. To conduct these measurements, we first evaluated the homogeneity of each simulated mixture by the one-way ANOVA test. Table 2 shows the results of one-way ANOVA tests of the logit-transformed means for each duplicate quantitation. These results showed that the between-lot variations were not different from the within-lot variations in each sort of

simulated mixture (Table 2). Thus, we concluded that the simulated mixtures used in the experiment were sufficiently homogenous within each sort. Then, using these measurements, we performed the preliminary method validation for the event-specific method, and compared the results with those for the existing construct-specific method. Table 3 shows the statistical analysis of the results for the event-specific method and the construct-specific method, respectively. The relative standard deviation (RSD) scores for the event-specific method and the construct-specific method ranged from 2.6% (10% mixture) to 11% (0.5% mixture) and from 3.6% (5% mixture) to 6.5% (0.5% mixture), respectively. The RSD scores of these two methods were found below 20% (Table 3). The results of single-laboratory evaluation tests suggested that the precision of the event-specific method was sufficient for the quantitation of GA21 maize, although interlaboratory collaborative studies should be conducted to confirm this.

In conclusion, the event-specific quantitation method for GA21 maize developed in this study demonstrated better performance than the established construct-specific method that is a Japanese official method. This newly developed method has the advantage that the detection target is present as a single copy per set of GM genome. Based on this study, we are trying to develop a simple and rapid detection method for GM crop

screening.

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