Development and Validation of an Event-Specific Quantitative PCR Method for Genetically Modified Maize MIR162

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A novel real-time PCR-based analytical method was developed for the event-specific quantification of a genetically modified (GM) maize event, MIR162. We first prepared a standard plasmid for MIR162 quantification. The conversion factor (Cf) required to calculate the genetically modified organism (GMO) amount was empirically determined for two real-time PCR instruments, the Applied Biosystems 7900HT (ABI7900) and the Applied Biosystems 7500 (ABI7500) for which the determined Cf values were 0.697 and 0.635, respectively. To validate the developed method, a blind test was carried out in an interlaboratory study. The trueness and precision were evaluated as the bias and reproducibility of relative standard deviation (RSDs). The determined biases were less than 25% and the RSDs values were less than 20% at all evaluated concentrations. These results suggested that the limit of quantitation of the method was 0.5%, and that the developed method would thus be suitable for practical analyses for the detection and quantification of MIR162.

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Key words: MIR162; event-specific; genetically modified (GM); real-time PCR; maize

Introduction

The utilization of genetically modified (GM) crops has been increasing around the world¹. Many countries including Japan legislate labeling systems of authorized GM crops and/or their derived foods. To enforce the labeling system, it is indispensable to define a practical threshold level and validated quantitative methods for the estimation of unintentional commingling levels of authorized GM organisms (GMO). In Japan, the GMO labeling system is stipulated by the "Act on Standardization and Proper Quality Labeling of Agricultural Forestry Products (JAS Law)"*¹ and the "Food Sanitation Act"*², and the thresholds for approved GM maize and soybean events both have been set at 5%², ³, ⁴. For GM maize, specific quantitative methods for five GM maize events (Bt11, Bt176, GA21, MON810, T25) and a screening quantitative method targeting Cauliflower Mosaic Virus 35S promoter (P35S) and GA21 have been adopted as Japanese standard analytical methods*⁵. MIR162 is an insect-resistant GM maize event and its commercial utilization in Japan was approved in 2010*⁶. In MIR162, a single copy of the modified vip3A gene from Bacillus thuringiensis strain AB88 and a selectable marker gene

*⁶ List of products that have undergone safety assessment and been announced in the Official Gazette (Apr. 10, 2014) of the Department of Food Safety, Ministry of Health, Labour and Welfare of Japan; http://www.mhlw.go.jp/english/topics/food/pdf/sec01-2.pdf
mannose-6-phosphate isomerase (pmi) from Escherichia coli have been introduced without the P35S region (Fig. 1). Thus, a specific detection method for MIR162 is required.

In the present study, we developed a new quantitative method for MIR162 with an event-specific PCR method, and then validated the method in an interlaboratory study.

Materials and Methods

Plant materials

For maize and soybean, MON810, MON863, MON88017, NK603, MON89788, and RBS were kindly provided by Monsanto Company (St. Louis, MO, USA). 3272, Bt11, Event176, GA21, MIR162, and MIR604 were kindly provided by Syngenta Seeds AG (Basel, Switzerland); and TC1507 and DAS59122 were kindly provided by Pioneer Hi-Bred International (Johnston, IA, USA). A2704-12 was kindly provided by its developer and T25 was directly imported from the USA. QC9651 maize obtained from Quality Technology International, Inc. (Elgin, IL, USA) was used as non-GM maize. Seeds of rice (Oryza sativa) variety Kinuhikari, wheat (Triticum aestivum) variety Haruyutaka, and barley (Hordeum vulgare) variety Harrington in Japan were obtained from the market.

Oligonucleotide primers and probes

For the specific detection of MIR162, a pair of primers (5 ’-GGCGGTGTCTATGTATGTCAAG-3’ and 5’-TGCCTATCGGTGCTTACAG-3’) and a fluorescent dye-labeled probe (5’-TCTAGACATCTAGTACATTTAAAAGTCCCGCA-3’) were used. The sequences of these primers and probe were taken from a report by the Joint Research Centre (JRC, Ispra, Italy). Maize starch synthase IIb (SSIIb) gene was used as a maize-specific endogenous reference DNA for quantitative analysis. For the specific detection of SSIIb, two primers (5’-CAACATCTTGGACATCTGTCC-3’ and 5’-GATCGAGTTGGGTCCCGGA-3’) and a fluorescent dye-labeled probe (5’-AGCAAAATGTCAGGCGGTCAATGCA-3’) were used. The oligonucleotide primers and TaqMan® probes were synthesized by FASMAC Co., Ltd. (Kanagawa, Japan) and Life Technologies (Carlsbad, CA, USA), respectively. The oligonucleotide probes were labeled with 6-carboxyfluorescein (FAM) at the 5’ ends and 6-carboxytetramethylrhodamin (TAMRA) at the 3’ ends.

Preparation of the calibrant plasmid

Target sequence fragments from MIR162 and the endogenous maize SSIIb gene were synthesized as a single oligonucleotide in tandem and inserted into the pUC19 derived vector. The constructed plasmid was purified by equilibrium centrifugation in CsCl gradient. The concentration of the linearized DNA was first measured in terms of ultraviolet (UV) absorbance with a spectrophotometer, DU800 (Beckman Coulter, Brea, CA, USA), and converted to molar concentration. The DNA was then diluted to theoretical concentrations of 20, 125, 1,500, 20,000, and 250,000 copies per 2.5 µL with 5 ng/µL of ColE1 DNA (NIPPON GENE, Tokyo, Japan) solution.

Preparation of test samples and DNA extraction

To evaluate the quantitative method, we used five mixing levels of test materials containing 0%, 0.50%, 1.0%, 5.0%, and 10.0% of MIR162. To prepare the mixed samples, GM and non-GM seeds were separately ground, and then mixed on a weight-basis as described previously. Genomic DNA was extracted from those ground materials using a DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany). The concentrations and qualities of the extracted DNA solutions were evaluated by measuring UV absorbance with a spectrophotometer, ND-100 (Nanodrop Technologies, Wilmington, DE, USA). Maize genomic DNA solutions were adjusted to concentrations of 10 and 20 ng/µL for conventional and real-time PCR analyses, respectively.

Conventional PCR analysis

Conventional PCR using a thermal cycler, GeneAmp PCR system 9700 (Life Technologies), and agarose gel electrophoresis were performed as described previously by Kuribara et al. Twenty-five ng of sample DNA, 200 µM deoxyribonucleotide triphosphate (dNTP), 1.5 mM MgCl₂, 0.625 U of AmpliTaq Gold polymerase (Life Technologies) and 0.5 µM each primer were added to 25 µL of reaction solution. The thermal cycling condition was set as 10 min at 95°C and 40 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C, followed by a final extension at 72°C for 7 min. Five microliters of PCR products were electrophoresed on 3.0% agarose gel supplemented with 0.5 µg/mL of ethidium bromide in Tris-acetate–EDTA (TAE) buffer.

Real-time PCR analysis

TaqMan® real-time PCR assays were carried out using two real-time PCR instruments, the Applied Biosystems 7900HT (ABI7900) and the Applied Biosystems 7500 (ABI7500) (Life Technologies), with 25 µL final volume reactions consisting of 50 ng of sample DNA, 12.5 µL Universal Master Mix (Life Technologies), 0.5 µM primer pairs, and 0.2 µM probe. The thermal cycling condition was set as 2 min at 50°C, 10 min at 95°C and 45 cy-
cles of 30 sec at 95°C, 1 min at 59°C. Each sample was measured in triplicate on the reaction plate.

**Interlaboratory study**

An interlaboratory study was performed with the ABI7900 and ABI7500 independently and consisted of 2 separate stages, a measurement of the conversion factor (Cf) value and a blind test. All measurements were conducted by 10 laboratories for the ABI7900 and 3 laboratories for the ABI7500. Experimental protocols were provided by the National Food Research Institute (NFRI). Quantitative real-time PCR was performed with primers, probes, the Universal Master Mix, and the blind DNA solutions supplied by the NFRI.

The first stage was the experimental determination of the Cf value, which is the ratio of the copy number of recombinant DNA (rDNA) to the taxon-specific sequence in the GM plant genome. To calculate the Cf value for MIR162, we extracted genomic DNA from genuine GM seeds and determined the copy numbers of rDNA and taxon-specific sequences. The measurement was conducted twice at each laboratory. The Cf value for each real-time PCR instrument was separately determined as the mean of the obtained values.

A blind test was carried out as the second stage. Blind samples designed as blind duplicates of the maize genomic DNAs extracted from 5 different concentrations of MIR162 (0‰, 0.50‰, 1.0‰, 5.0‰, and 10.0‰) were provided to the participants. All participants were requested to submit the data of their real-time PCR analyses. All submitted data were analyzed by Cochran’s test and Grubbs’ test to reject outlier laboratories.

**Results and Discussion**

**PCR specificity for MIR162**

For the specific detection of MIR162, an event-specific segment was amplified at the 3’ flanking region between the native maize genomic DNA and rDNA (Fig. 1). First, the specificity of the primer set was confirmed by a conventional PCR method. The expected 92-bp product was detected only from genomic DNA solution of MIR162, but not from non-GM maize, other GM maize events, GM soybean events, rice, wheat, barley, and the no-template control (Fig. 2 A). Next, the specificity of the primer and probe set was confirmed by a real-time PCR analysis. The expected amplification curves were observed using the genomic DNA of MIR162 but not from those of the other samples (Fig. 2 B).

**Determination of the Cf value for MIR162**

The Cf value is required to convert the copy number ratio of rDNA to taxon-specific DNA into weight-based GMO content. To determine the experimental Cf value for MIR162, we measured the copy numbers of SSIIb and MIR162 in the extracted DNA from MIR162 seeds. The Cf value was determined from the results of 10 laboratories for the ABI7900 and the measurement was repeated twice. All submitted data were subjected to Cochran’s test (p<0.025) and Grubb’s test (p<0.025) to eliminate outlier laboratories with extreme variation and extreme average level, respectively, according to the harmonized guidelines of AOAC. One Cochran outlier

(A) Conventional PCR analysis. This agarose gel (3.0%) electrophoretogram of the amplified PCR product corresponds to the 92 bp of MIR162. Arrowhead indicates the expected amplified product. Lanes 1–11, eleven GM maize events, namely, NK603, Event176, T25, GA21, MON810, TC1507, Bt11, MIR604, MON88017, DAS59122, and MON863, respectively; lanes 12 and 13, non-GM maize and non-GM soy, respectively; lanes 14–16, three GM soybean events, namely, RRS, MON89788, and A2704-12, respectively; lanes 17 and 18, GM maize events, MIR162 and 3272, respectively; lanes 19–22, rice, wheat, barley, and no template, respectively. Lane M shows 100 bp ladder size markers.

(B) Real-time PCR analysis. The same set of plant genomic DNAs described in (A) were used as templates. The real-time PCR analysis was performed in one reaction plate, and the resultant amplification plot is shown. This analysis was performed with the 7900HT. Only the MIR162 genomic DNA gave ideal PCR amplification.
was detected. After removing the outlier, further statistical analyses were conducted. The Cf value was finally determined as the mean of values measured by the remaining laboratories. The determined Cf value was 0.697 (Table 1). We also determined Cf value of 0.635 for MIR162 using ABI 7500 as a reference value from the results of 3 laboratories (Table 1).

**Evaluation of the PCR quantitative method by interlaboratory study**

We performed an interlaboratory evaluation of the developed quantitative method as a blind test performed by 10 laboratories using the ABI7900. All participants received primers, probes and blind samples of DNA solutions consisting of five different concentrations of MIR162, and each measurement was performed twice. The blank sample (0% of MIR162) was used to determine outlier laboratories, but no laboratory was eliminated. The submitted data of 4 individual concentration samples were subjected to Cochran’s test and Grubbs’ test, but no outliers were found. \( R^2 \) value is the coefficient of correlation, which is used to analyze a standard curve obtained by linear regression analysis. We have an acceptance criteria that \( R^2 \) values must be \( >0.990^{*} \). In Europe, minimum performance requirements for analytical methods of GMO testing have been defined by the European Network of GMO Laboratories (ENGL), and \( R^2 \) values should be \( \geq 0.98 \) in the criteria \(*\). We listed the \( R^2 \) values for the standard curves using 20, 125, 1,500, 20,000, and 250,000 copies of standard plasmids from each participant in the two quantitative analyses as blind duplicates (Table 2), and all of the obtained \( R^2 \) values from both SSIIb and MIR162 met the acceptance level. Trueness and precision were determined as previously described\(^{20,35}\). The mean, bias (mean-value, %), repeatability of relative standard deviation (RSDr), and reproducibility of RSD (RSDx) blind samples were measured (Table 3). The determined bias, RSDr, and RSDx for ABI7900 ranged from 12.3% to 21.9%, from 8.4% to 17.0%, and from 9.6% to 17.0%, respectively. RSDr values indicate the variance of the quantification of each concentration, and the variance of the 0.5% sample which was the lowest concentration, was 11.3%. The obtained bias, RSDr, and RSDx here were similar to or within a narrower range than those for previously reported GMO events\(^{20,35}\). Data below 20 copies were extrapolated with a standard curve in our methods because there was no calibrant below 20 copies. For quantitative methods the limit of detection (LOD) is the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time (\( \leq 5\% \) false negative results)*. Twenty copies of standard plasmid were detected by all participants in the two quantitative analyses, indicating that the ratio of the detection was 20/20 and the LOD was 20 copies. All measured copies of 0.50% samples were over 20 copies (Table 3). Therefore, we estimate that the limit of quantitation (LOQ) for MIR162 is 0.50% in this method.

In conclusion, we developed a specific quantification method for GM maize MIR162, using the same primers

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<tr>
<th>Table 1</th>
<th>Experimental conversion factor for MIR162</th>
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<tbody>
<tr>
<td></td>
<td>7900</td>
</tr>
<tr>
<td>Mean</td>
<td>0.697</td>
</tr>
<tr>
<td>SD</td>
<td>0.060</td>
</tr>
<tr>
<td>RSD</td>
<td>8.67</td>
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SD: Standard deviation  
RSD: Relative standard deviation

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<tr>
<th>Table 2</th>
<th>( R^2 ) values of the standard curves drawn by each participant</th>
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</thead>
<tbody>
<tr>
<td>Participant</td>
<td>A</td>
</tr>
<tr>
<td>SSIIb</td>
<td>0.999</td>
</tr>
<tr>
<td>MIR162</td>
<td>0.998</td>
</tr>
<tr>
<td>SSIIb</td>
<td>1.000</td>
</tr>
<tr>
<td>MIR162</td>
<td>0.996</td>
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</tbody>
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<tr>
<th>Table 3</th>
<th>Summary of accuracy and precision statistics for real-time PCR with ABI PRISM 7900HT</th>
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<tr>
<td>% (w/w)</td>
<td>Retained labs</td>
</tr>
<tr>
<td>GMO Amount, %</td>
<td>True value, %</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
</tr>
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<td>10</td>
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\(^{*}\) When RSDr \( \geq \) RSDx, RSDx was considered to have the same value as RSDr.


\(^{**}\) Guidelines on performance criteria and validation of methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods\(^{3}\) CAC/GL 74-2010
and probe sequences for the MIR162 transgene as those described in the method developed by the trait developer*. The experimentally determined Cf value was 0.697 for the ABI7900. The LOQ, trueness, and precision of this method were at the same level or better than those reported in previous studies**. Therefore, we conclude that the developed method would be applicable for the detection and quantification of MIR162 to monitor the validity of the food labeling system in Japan.

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- Japan Grain Inspection Association, Tokyo, Japan
- Life Technologies Japan, Tokyo, Japan
- National Center for Seed and Seedlings, Ibaraki, Japan
- National Food Research Institute, Ibaraki, Japan
- National Institute of Health Sciences, Tokyo, Japan
- Yokohama Plant Protection Station, Kanagawa, Japan

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