Validation Study

Interlaboratory Validation of Quantitative Duplex Real-Time PCR Method for Screening Analysis of Genetically Modified Maize

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To reduce the cost and time required to routinely perform the genetically modified organism (GMO) test, we developed a duplex quantitative real-time PCR method for a screening analysis simultaneously targeting an event-specific segment for GA21 and Cauliflower Mosaic Virus 35S promoter (P35S) [Oguchi et al., J. Food Hyg. Soc. Japan, 50, 117–125 (2009)]. To confirm the validity of the method, an interlaboratory collaborative study was conducted. In the collaborative study, conversion factors (Cfs), which are required to calculate the GMO amount (%), were first determined for two real-time PCR instruments, the ABI PRISM 7900HT and the ABI PRISM 7500. A blind test was then conducted. The limit of quantitation for both GA21 and P35S was estimated to be 0.5% or less. The trueness and precision were evaluated as the bias and reproducibility of the relative standard deviation (RSDR). The determined bias and RSDR were each less than 25%. We believe the developed method would be useful for the practical screening analysis of GM maize.

Key words: screening; quantification; genetically modified (GM); duplex real-time PCR; maize (Zea mays)

Introduction

The PCR technique is widely used to detect and quantify GM crops in foods and feeds. The key factor determining the specificity of a PCR-based method is the choice of a target sequence motif in the GM plant genome. The methods can be classified into at least 3 categories depending on the target; event-specific, construct-specific and screening methods. In event-specific methods, a unique sequence located at the junction between the plant genome and recombinant DNA is used as the target. Construct-specific methods target the junction between adjacent elements in an introduced gene cassette, such as a region between a promoter and a structural gene. Screening methods target commonly conserved elements among many GM events such as Cauliflower Mosaic Virus 35S promoter (P35S), nopaline synthase terminator, 5-enolpyruvylshikimate-3-phosphate synthase, phosphinothricin N-acetyltransferase, and so on. Event-specific methods are the most specific, followed by construct-specific and screening methods. Screening methods often overestimate GM amounts, and it is generally agreed that the best estimates can be obtained by event- or construct-specific quantifications. In fact, construct-specific quantitative methods of five GM maizes (Bt11, Bt176, GA21, MON810, T25) and Roundup Ready Soy (RRS) have been adopted as Japanese standard analytical methods.¹,² However, the cost of genetically modified organism (GMO) testing using specific quantifications

¹ Notification No. 110 (Mar. 27, 2001); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2001).

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will increase in parallel with the number of GM events to be examined. In this regard, screening methods are highly cost- and time-effective for routine monitoring. The Ministry of Health, Labour and Welfare (MHLW) of Japan announced a screening method combining the quantification of a P35S region and the construct-specific quantification of GA21 maize, which has been officially used as a quantitative screening method for GM maize. To further pursue more convenient and efficient methodology, we developed a duplex real-time PCR method which simultaneously quantifies the P35S region and an event-specific segment of GA21. The developed duplex screening method will reduce both the cost and time requirement of routine GMO analysis by half compared to the current screening method. These quantitative methods are based on a real-time PCR technique for relative quantification between target and taxon-specific sequences. In many cases, extracted DNAs from processed foods are severely degraded and the degree of the degradation is not always the same among PCR-targeted sequences, so that GM quantification in processed foods by means of the PCR technique is difficult. In fact, the quantification methods adopted as Japanese standard analytical methods, including the current screening method, are applicable to raw materials but not to processed foods.

In this report, we validated the duplex real-time PCR method for the screening analysis of GM maize by means of an interlaboratory study.

**Materials and Methods**

**Plant materials**

The GM maize seeds, MON810 and GA21 were kindly provided by Monsanto (St. Louis, MO, USA), and Syngenta Seeds (Basel, Switzerland), respectively. MRX 3 maize was purchased from Pioneer Hi-Bred International (Johnston, IA, USA) and used as a non-GM maize sample.

**Oligonucleotide primers and probes**

All primers and probes used in this study were identical to those in the previous single laboratory evaluation. The primers and probe for the event-specific detection of GA21 were as follows: GA21esp 5'-1,5',-TGGAACCTTTATCGTTATGCTATTTG-3', GA21esp3 5'-CGATCCTCCTGCGGTTCCTCC-3'; and GA 21 es-TaqHB; 5' -CGGACCACCTCGTGTGAGAAG-3'. The primers and probes for the detection of the P35S region were as follows: P3SS 1-5',-ATTGATGTGAATATCTCAGTGCCT-3'; P3SS 1-3',-CCTCTGCC-

AAATGAAATGAACTCTCCT-3'; and P3SS-TaqFB, 5'-CCACCATATCTCCTGGAAGCCTCTCTTCT-3'. We used the maize starch synthase IIb (SSIIb) gene as a maize-specific endogenous DNA for quantitative analysis, and the primers and probe for SSIIb were as follows: SSIIb 5',5'-CCAATCTTGTGACATCTGCTCC-3'; SSIIb 5',5'-GATCAGCTTTGTGCGGGA-3'; and SSIIb-TaqFB, 5' -AGCAGAGTACAGGGCCTGAAATGCA-3'. The oligonucleotide primers and TaqMan® probes were synthesized by FASMAC (Kanagawa, Japan) and Biosearch Technologies (Novato, CA, USA), respectively. All synthesized probes were labeled with Blackhole Quencher (BHQ) at the 3' ends, and P3SS-TaqFB and SSIIb-TaqFB were labeled with 6-carboxyfluorescein (FAM), while GA21es-TaqHB was labeled with hexachloro-6-carboxyfluorescein (HEX), at the 5' ends.

**Preparation of calibrant plasmid**

The standard plasmid pSCM which contains the specific sequence fragments from GA21, P3SS and SSIIb, was prepared according to the previous report and used as the calibrator for the quantification.

**Preparation of test samples**

Non-GM and GM maize mixing samples containing 0.50, 1.0, 5.0, and 10.0% of both GA21 and MON810 were prepared by mixing dried powders in the manner described previously and used as a primary certified reference material. Briefly, washed maize seeds of non-GM, GA21 and MON810 were separately frozen, ground with a high-speed rotor mill (Fritsch, Idar-Oberstein, Germany), freeze-dried in a freeze dryer (FDU-1100; Tokyo Rikakikai, Tokyo, Japan), and then mixed on a weight-to-weight basis.

**Quantitative PCR**

All conditions and PCR instruments were identical to those in the previous report.

**Homogeneity of test samples**

Test samples of each GM mixing level were aliquoted (1 g each) into 200 sample tubes. Ten sample tubes were randomly selected twice from the 200 tubes prepared. DNA was extracted from each sample using the DNeasy Plant Maxi kit (Qiagen, Hilden, Germany) according to the manufacturer's manual, and quantitative PCR was performed using GA21 and MON810 construct-specific methods. The calculated copy numbers were then converted into GMO amounts (%) on a weight basis. The homogeneities of GA21 and MON810 were independently evaluated by one-way analysis of variance (ANOVA) as described previously.

**Interlaboratory study**

The interlaboratory study consisted of 2 separate stages, a measurement of the Cf values and a blind test. Experimental protocols were provided by the Food and Agricultural Materials Inspection Center (FAMIC). The Universal Master Mix, primers, probes, blind samples
and DNeasy Plant Maxi kit were also supplied by FAMIC. The first stage was measurement of the Cf values using the ABI 7900HT (AB 7900) and the ABI 7500 (AB 7500) (Life Technologies, Carlsbad, CA, USA). The Cf value is experimentally determined as the ratio of the copy number of r-DNA to the taxon-specific sequence in the GM plant genome. The Cf values for GA21 and P35S were determined independently from the results of 12 laboratories for the AB 7900, and 5 laboratories for the AB 7500. The measurements were repeated 3 times in each laboratory, and the average values from all the submitted data were defined as the Cf values. After determination of the Cf values, one laboratory withdrew from this study.

The blind test was conducted as the second stage. All measurements were performed by 11 laboratories for the AB 7900. The maize samples were designed as blind duplicates, including 0, 0.5, 1.0, 5.0 and 10.0% of both GA21 and MON810. The blind samples sent to the participants were divided into two sets containing each concentration, and then the measurements were separately performed for each set of blind samples. DNAs were extracted from these blind samples by each participant and then quantitative analyses were carried out. All participants were requested to submit the data from the real-time PCR analyses. All submitted data were analyzed by means of Cochran’s test$^{11}$ and Grubbs’ test$^{12,13}$ as described in the guidelines$^{14}$.  

### Results and Discussion

**Determination of Cf values for GA21 and P35S**

The Cf value for GA21 was determined by measuring the copy numbers of endogenous gene SSIIb and GA21 in the extracted DNA from the GA21 seed. To determine the Cf value for P35S, we used MON810 as a representative of GM maize both because it has been widely used, and because it has only one P35S segment per GM haploid, as the previous single laboratory evaluation described$^{5}$. The Cf values for GA21 and P35S were measured independently with two real-time PCR instruments, the AB 7900 and AB 7500. The Cf values determined are listed in Table 1. The values for P35S with AB 7900 and AB 7500 were very close, and when rounded to the nearest hundredth of a unit both values became 0.36. GA21 contains a single insert
consisting of three copies of its perfect gene cassette and three incomplete copies. In the whole recombinant insertion, five copies of the construct-specific segment of GA21 are supposed to be present (Fig. 1). We obtained the Cf values for the GA21 construct-specific method as between 1.40 and 2.01 in our previous studies. The theoretically expected Cf value for the event-specific method would be one-fifth of these values, and thus would be between 0.28 and 0.40. Both of the Cf values determined for GA21 in this study were within this range.

Interlaboratory validation of the duplex real-time PCR method

After determination of the Cf values, the homogeneities of the blind samples were confirmed by one-way ANOVA. Ten tubes of each mixing sample were randomly selected twice. The DNA was then extracted from these samples, and quantitative PCR analyses of GA21 and MON810 were performed using each construct-specific method. The measured copy numbers were converted into the GMO amount, and one-way ANOVA was then conducted on the data. The F- and p-values were calculated (Table 2). Even at the smallest value, 0.18, obtained by the MON810 specific quantification of the 0.50% sample, the p-values were larger than 0.05, indicating that the contents of both GA21 and MON810 of all the test samples were sufficiently homogeneous and met the requirements for the following interlaboratory study.

The developed duplex real-time PCR quantitative method was evaluated in a blind test performed by 11 laboratories using the AB 7900. The measurements of GA21 and P35S were carried out independently. A blank sample, with 0% GM content, was used to estimate invalid laboratories, and no laboratory was eliminated. All the submitted data except 0% were then handled according to the harmonized guidelines of AOAC to remove outlier laboratories with extreme variation using Cochran’s test and with an extreme average level using Grubbs’ test as previously described. One Cochran outlier was detected in the 1.0% GA21 sample and one Grubbs outlier was detected in the 10.0% P35S sample. After removing these outliers, further statistical analyses were conducted. The trueness and precision were determined as the bias (mean-value, %) and reproducibility relative standard deviation (RSDR) for GA21 and P35S in individual samples (Table 3). The bias and RSDR of GA21 were less than 15% and 20% in all samples, respectively. Both the bias and RSDR of P35S were slightly higher than those of GA21, but were less than 20% and 25%, respectively. These obtained bias and RSDR levels were similar.

Table 2. Homogeneity of the simulated mixtures

<table>
<thead>
<tr>
<th>% (w/w)</th>
<th>Measured mean, %</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA21 0.50</td>
<td>0.44</td>
<td>1.01</td>
<td>0.49</td>
</tr>
<tr>
<td>MON810 0.50</td>
<td>0.48</td>
<td>1.82</td>
<td>0.18</td>
</tr>
<tr>
<td>GA21 1.0</td>
<td>0.74</td>
<td>0.48</td>
<td>1.00</td>
</tr>
<tr>
<td>MON810 1.0</td>
<td>0.95</td>
<td>0.11</td>
<td>0.86</td>
</tr>
<tr>
<td>GA21 5.0</td>
<td>3.71</td>
<td>0.47</td>
<td>0.87</td>
</tr>
<tr>
<td>MON810 5.0</td>
<td>5.26</td>
<td>0.83</td>
<td>0.60</td>
</tr>
<tr>
<td>GA21 10.0</td>
<td>7.67</td>
<td>1.36</td>
<td>0.32</td>
</tr>
<tr>
<td>MON810 10.0</td>
<td>9.77</td>
<td>1.03</td>
<td>0.48</td>
</tr>
</tbody>
</table>

a) Critical value of F is 3.02 (α = 0.05)

Table 3. Summary of accuracy and precision statistics for the duplex real-time PCR method

<table>
<thead>
<tr>
<th>Trueness</th>
<th>Precision</th>
<th>Detection limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Means</td>
<td>Bias</td>
<td>RSDR%</td>
</tr>
<tr>
<td>% (w/w)</td>
<td>Retained labs</td>
<td>GMO amount, %</td>
</tr>
<tr>
<td>GA21</td>
<td>0.50</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>11</td>
</tr>
<tr>
<td>P35S</td>
<td>0.50</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>10</td>
</tr>
</tbody>
</table>

a) RSDR: Reproducibility relative standard deviation
b) Below 20 copies refers to the ratio of the number of retained data below 20 copies/the total number of retained data

Interlaboratory validation of the duplex real-time PCR method

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lar to or even less than those of previously reported GMO events\textsuperscript{9,10}. In terms of the limit of quantitation (LOQ), all the measured copy numbers of the 0.5% samples were over 20 copies and there was no calibrant below 20 copies in this method. Therefore, we estimated that the LOQ for the GA21 event-specific and P 35S in the duplex PCR method was 0.50% or less.

The previously developed duplex real-time PCR method was validated in this interlaboratory study using AB 7900 instruments. The levels of obtained LOQ, trueness and precision were almost the same as those of other established methods\textsuperscript{9,10} and the single laboratory evaluation\textsuperscript{5}. We thus consider that the duplex real-time PCR a good candidate for routine screening for GM maize commingled in agricultural crops.

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- FASMAC, Kanagawa, Japan
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- Japan Food Research Laboratories, Tokyo, Japan
- Japan Frozen Foods Inspection Corporation, Kanagawa, Japan
- Japan Grain Inspection Association, Tokyo, Japan
- Japan Grassland Agriculture and Forage Seed Association, Tochigi, Japan
- National Food Research Institute, Ibaraki, Japan
- National Institute of Health Sciences, Tokyo, Japan
- National Livestock Breeding Center, Nagano, Japan
- Japan Institute of Nutrition of Japan, Tokyo, Japan
- Food and Agricultural Materials Inspection Center, FASMAC, Kanagawa, Japan
- Applied Biosystems Japan, Tokyo, Japan

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Reference


14) Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis. In Official Methods of Analysis of AOAC Int. 17th ed., volume II, Gaithersburg, MD, USA.