Development and Interlaboratory Validation of Quantitative Polymerase Chain Reaction Method for Screening Analysis of Genetically Modified Soybeans

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A novel real-time polymerase chain reaction (PCR)-based quantitative screening method was developed for three genetically modified soybeans: RRS, A2704-12, and MON89788. The 35S promoter (P35S) of cauliflower mosaic virus is introduced into RRS and A2704-12 but not MON89788. We then designed a screening method comprised of the combination of the quantification of P35S and the event-specific quantification of MON89788. The conversion factor (Cf) required to convert the amount of a genetically modified organism (GMO) from a copy number ratio to a weight ratio was determined experimentally. The trueness and precision were evaluated as the bias and reproducibility of relative standard deviation (RSDs), respectively. The determined RSDs values for the method were less than 25% for both targets. We consider that the developed method would be suitable for the simple detection and approximate quantification of GMO.

Keywords screening; quantification; genetically modified; real-time polymerase chain reaction; soybean

In the past few decades, a huge variety of genetically modified (GM) crops have been developed and cultivated worldwide.1 Several countries legislate the labeling systems of authorized GM crops, or their derived foods and feeds. To enforce the labeling system, it is indispensable to define a practical threshold level of GM content and to develop validated quantitative methods for the estimation of unintentional commingling of GM organisms (GMO). GM crops are generally classified in terms of event which is identified by what transgene was inserted, and where on the chromosome it was inserted. There are many GM events approved in Japan.2

The quantitative methods of a glyphosate-tolerant soybean (GTS) Roundup ready soy (RRS) and five GM maize events, i.e., Bt11, Bt176, GA21, MON810, and T25, using real-time polymerase chain reaction (PCR) were developed and validated by an interlaboratory collaborative study,3,4,5 and adopted as Japanese standard methods.6

For DNA-based detection of GM crops, screening tests are highly cost- and time-effective methods. The Ministry of Health, Labour and Welfare (MHLW) of Japan announced a combinational quantification of P35S and GA21 maize as a quantitative screening method for GM maize and it has been officially adopted.7 Among approved GM soybean events, RRS has been cultivated since in 2001, and the commercial cultivation of a glufosinate-tolerant soybean, A2704-12 and the second generation of GTS, MON89788, have recently started. To analyze these novel GM soybean events, we have developed individual event-specific quantitative methods.5,6 A practical quantitative screening method for GM soybeans is needed because many food products derived from soybeans are listed for labeling and then frequently analyzed in Japan.3

In this study, we first developed a new quantitative screening method for RRS, A2704-12 and MON89788. The developed method was evaluated with an interlaboratory collaborative study.

MATERIALS AND METHODS

Plant Materials
MON89788 and RRS seeds were kindly provided by Monsanto (St. Louis, MO, U.S.A.), and A2704-12 was kindly provided by the developer. Dry soybean seeds harvested in the U.S.A. in 2004 were purchased from Ryokoku Shoji (Hiroshima, Japan) and used as a non-GM soy sample.

Preparation of Oligonucleotide Primers, Probes and Calibrant Plasmids
The primers and TaqMan probes used in this study are listed in Table 1. We used three standard plasmids, pMulSL2, pLLS and pSCS for the individual quantification of RRS, A2704-12 and MON89788, respectively. pSCS was also used for the P35S quantification. These primers, probes and plasmids were prepared according to the previous reports.3,5,6

Preparation of Test Samples and DNA Extraction
To evaluate the quantitative method, we prepared five test samples that contained equal concentrations of the three GM soybean events: RRS, A2704-12 and MON89788, and each test sample consisted of a different mixing level of test materials, i.e., 0%, 0.50%, 1.0%, 5.0%, and 10.0%. The mixed samples were prepared as described previously.3 Genomic DNA was extracted from those ground materials using a DNasey Plant Maxi kit (Qiagen, Hilden, Germany). The homogeneities of the samples were confirmed using the averages of the one-way analysis of variance according to the previous reports for all mixing levels of each GM soy event.3,5,6 The concentrations and qualities of the extracted DNA solutions were evaluated...
as described previously.  

Quantitative PCR and Interlaboratory Study  TaqMan real-time PCR assays were carried out using the ABI PRISM 7900HT (ABI7900) or the ABI 7500 (ABI7500) (Life Technologies). The interlaboratory study consisted of a measurement of the conversion factor (Cf) values and a blind test. These studies were performed as described previously.  

RESULTS AND DISCUSSION

Determination of the Cf Values for MON89788 and P35S  The Cf value for MON89788 was determined by measuring the copy numbers of endogenous gene Le1 and MON89788. Meanwhile, to determine the Cf value for P35S, we had two choices of GM events that contain the P35S region, that is, RRS and A2704-12. RRS contains the single insertion of the transgene cassette. On the other hand, A2704-12 contains two copies of the whole recombinant segments that include P35S.

We first calculated the Cf values for P35S derived from RRS and A2704-12 in a single laboratory examination with the ABI7900 (Table 2). In the real-time PCR analysis, the obtained amplification plots from RRS and A2704-12 targeting the P35S segment were clearly separated (data not shown). As expected, the evaluated Cf values for P35S from RRS and A2704-12 were close to 1.0 and 2.0, respectively, which are the theoretically estimated copy number ratios of the recombinant per taxon specific region.

It is true that screening quantitative methods can often yield an overestimation of the GMO amount. In the practical monitoring, however, it is most important to exclude the risk of underestimation by which the commingling GMO amounts surpassing the defined labeling threshold could be estimated unduly low. Therefore, we chose RRS to determine the Cf value for P35S. The Cf values for MON89788 and P35S were determined using ABI7900 and ABI7500 independently, from the results of 11 laboratories for ABI7900 and 4 laboratories for ABI7500. The measurement was repeated twice, and the Cf value was determined as the mean of values measured by these laboratories (Table 3).

Interlaboratory Evaluation of the PCR Quantification  We performed an interlaboratory evaluation of the developed screening quantitative method as a blind test performed by 11 laboratories using ABI7900. The blank sample, with 0% RRS, A2704-12, and MON89788 was used to determine outlier laboratories, but no laboratory was eliminated. All the submitted data were subjected to Cochran's test (p<0.025) and Grubb's test (p<0.025) to remove outlier laboratories according to the harmonized guidelines of AOAC as previously described. Three Cochran outliers were detected in 0.5% of the MON89788 and 2.0% and 20.0% of the P35S quantifications. After removing these outliers, we conducted further statistical analyses. The trueness and precision were determined as previously described. The mean, bias, repeatability of relative standard deviation (RSDr), and reproducibility of RSD (RSDs) of blind samples were measured (Table 4). The determined RSDs were similar to or within a narrower range than those in previously reported GMO events. The determined biases in MON89788 quantification were also similar to those in the previously established methods. As envisaged in advance, the bias values obtained from P35S quantification were significantly high. These high bias values must be attributed to the difference of P35S copy numbers between RRS and A2704-12. The P35S contents derived from A2704-12 must have been overestimated by nearly double.

We developed a screening method for GM soybeans which quantitatively detected P35S and MON89788. Screening methods are designed on the assumption that the qualitative or quantitative tests analyze samples that may contain more

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Table 1. Primers and TaqMan Probes for Real-Time PCR Systems

| Target | Name       | Sequence                                             | Length | Reference |
|--------|------------|------------------------------------------------------|--------|-----------|
| MON89788 | MON89788-F | 5’-TCC CGC TCT AGC GCT TCA AT-3’                     | 139bp  | 5)        |
| MON89788 | MON89788-R | 5’-TCG AGG AGG ACC TG CAG A-3’                       |        |           |
| MON89788 | MON89788-P | 5’-FAM-CTG AAG GGC GGA AAC GAC ATG CTG-TAMRA3’       |        |           |
| P35S    | 1–5’      | 5’-ATT GAT GTG ATA TCT CCA CTG ACG T-3’              | 101bp  | 8)        |
| P35S    | 1–3’      | 5’-CCT TCC CA AAT AAA TGA ACT TCC T-3’               |        |           |
| P35S-Taq |           | 5’-FAM-CCT ACT CTT CGC AAG ACC TGT CCT-TAMRA3’       |        |           |
| Le1 (soybean endogenous) | Le1n02–5’ | 5’-GCC CTC TAC TCC ACC CCC A-3’                      |       |           |
|         | Le1n02–3’ | 5’-GCC CAT CTG CA A GCC TTT TT-3’                    |        |           |
| RRS     | RRS 01–5’ | 5’-CCT TTA GGA TTT CAG CAT CAG TGG-3’                |        |           |
| RRS     | RRS 01–3’ | 5’-GAC TTG TCG CGG GGA ATG-3’                        |        |           |
|         | RRS-Taq   | 5’-FAM-AGC TTC GCC GCT TCC TTC AAG TTC AC-TAMRA3’    |        |           |
| A2704-12 | KVM175    | 5’-GCA AAA AGG CGG TTA GCT CCT-3’                    | 64bp   | 6)        |
|         | SMO001    | 5’-ATT CAG GCT CGG CAA CTT TT-3’                     |        |           |
|         | TM031     | 5’-FAM-CGG TCC TCC CCG CTC TCG CCT TAMRA3’           |        |           |

Table 2. Single Laboratory Estimation of Cf Values for P35S

|        | RRS            | A2704-12 |
|--------|----------------|----------|
| Mean   | 0.83           | 1.80     |
| RSD    | 7.22           | 5.16     |

RSD: Relative standard deviation.
than one GM event using PCR-target sequences shared within targeted events. Nevertheless, there are few reports that assess screening methods using test samples containing plural GM events for common target segments. In this study, we quantified and evaluated test samples that contained both RRS and events for common target segments. In this study, we quantified and evaluated test samples that contained both RRS and A2704-12 and MON89788 would be performed –

In the practical monitoring, the total amount of the three types of GM soybean content can be estimated by the developed quantitative screening method whether the commutersing level is less than the labeling threshold or not. If the GM content exceeds the threshold level, individual quantitative analysis for RRS, A2704-12 and MON89788 would be performed for final assessment. We concluded that the developed method would be reliable and practical for the primary stage of the monitoring.

On the other hand, the commercial utilization of other approved GM soybean events such as insect resistant and high oleic acid soybeans would be started at anytime. In the future, it will be necessary to add new detection and quantification methods for novel approved GM soybean events depending on the situation of their commercial cultivation and distribution and, furthermore, other screening methods using commonly introduced promoters or terminators into them may be required, while the time- and cost-effectiveness of P3SS quantification will remain.

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