Note

Establishment and Evaluation of Event-Specific Quantitative PCR Method for Genetically Modified Soybean MON89788

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A novel real-time PCR-based analytical method was established for the event-specific quantification of a GM soybean event MON89788. The conversion factor (C_f) which is required to calculate the GMO amount was experimentally determined. The quantitative method was evaluated by a single-laboratory analysis and a blind test in a multi-laboratory trial. The limit of quantitation for the method was estimated to be 0.1% or lower. The trueness and precision were evaluated as the bias and reproducibility of the relative standard deviation (RSD_R), and the determined bias and RSD_R values for the method were both less than 20%. These results suggest that the established method would be suitable for practical detection and quantification of MON89788.

Key words: MON89788; event-specific; genetically modified (GM); real-time PCR; soybean

Introduction

Glyphosate is a well-known herbicide which is active against a broad spectrum of plant species, and glyphosate tolerance has been genetically engineered into various crops such as canola, cotton, maize, and soybean¹⁾. A glyphosate-tolerant soybean (GTS), event 40-3-2 [Roundup Ready Soy (RRS)], is one of the most successful genetically modified (GM) crops and has become dominant in several countries where its planting is permitted¹⁾. Indeed, the GM soybean-planted area made up more than half of the total GM-planted area in 2007²⁾. Based on experience of using RRS and the benefits gained from its use, a second-generation GTS, MON89788, has been developed, and is now being commercially cultivated. MON89788 integrates the *cp4 epsps* gene, the chimeric promoter consisting of the enhancer sequence of the Figwort Mosaic Virus (FMV) promoter bound to the *Tsfl* promoter derived from *Arabidopsis thaliana*, and the terminator from pea ribulose 1,5-bisphosphate carboxylase (TRbcS) at a single locus in the soybean genome³⁾. It is reported that MON89788 enables the same flexible, dependable weed

control as the original RRS, but with higher yield potential resulting from advanced gene mapping and insertion technology^{*1}, and the relative crop yield of MON89788 is increased from 7 to 11% over RRS^{*2}. Therefore, MON89788 is expected to become widely used.

There are requirements for labeling GM products in crops or derived products in the European Union (EU), Korea, Japan, Australia, and many countries. The threshold for the unintentional commingling level of GM crops was set at 5% in Japan⁴⁾,^{*3}. To date, real-time PCR-based detection and quantitative methods for several GM maize events and RRS have already been developed and validated by our international inter-laboratory collaborative study⁵⁾,⁶⁾, and adopted as

*1 Monsanto Co., news release
<http://monsanto.mediaroom.com/index.php?s=43&item=687>

*2 Nougyou To Kankyou No. 90, National Institute for Agro-Environmental Sciences
<http://www.niaes.affrc.go.jp/magazine/090/mgzn09008.html>

*3 Notification No. 517 (Mar. 31, 2000); Ministry of Agriculture, Forestry and Fisheries of Japan (2000)

Japanese standard analytical methods^{*4,*5}. However, in Japan there is no standard method for detecting MON89788. An analytical method to detect MON89788 is needed for practical monitoring because MON89788 cannot be detected even by using target sequences from Cauliflower Mosaic Virus 35S promoter (P35S) and the nopaline synthase terminator regions which are commonly used in many GM crops.

In this report, we describe a new quantitative method for detecting MON89788 with an event-specific PCR strategy, and an evaluation of the method.

Materials and Methods

Plant materials

GM soybean and maize seeds, MON89788, RRS, MON 810, MON863, MON88017, and NK603 were kindly provided by the Monsanto Company (St. Louis, MO, USA), A2704-12 by its developer, Bt11, Event176, GA21, and MIR604 by Syngenta Seeds AG (Basel, Switzerland), and TC1507 and DAS59122 by Pioneer Hi-Bred International (Johnston, IA, USA). T25 was directly imported from the USA. Dry soybean seeds which were harvested in the USA in 2004, were purchased from Ryokokushoji Co., Ltd. (Hiroshima, Japan) and used as a non-GM soy sample.

Oligonucleotide primers and probes

For specific detection of MON89788, the primers (MON89788-F; 5'-TCCCCTCTAGCGCTTCAAT-3' and MON89788-R; 5'-TCGAGCAGGACCTGCAGAA-3') and fluorescent dye-labeled probe (MON89788-P; 5'-CTG-AAGGCGGAAACGACAATCTG-3') were used for real-time PCR. The sequences of these primers and probe were taken from the report of the European Commission's Joint Research Center (JRC, IRMM, Retieseweg, Geel, Belgium)^{*6}. The soy lectin1 (*Le1*)⁷ was used as a soybean-specific endogenous reference DNA for qualitative and quantitative analyses. For specific detection of *Le1*, the primers (Le1n02-5'; 5'-GCCCTCTACTCCACCCCA-3' and Le1n02-3'; 5'-GCCCATCTGCAAGCCTTTT-3') and fluorescent dye-labeled probe (Le1-Taq; 5'-AGCTTCGCCGCTTCCTT-CAACTTCAC-3') were used. The maize *starch synthase IIb* (*SSIb*) gene was used as a maize-specific endogenous DNA for qualitative analysis. For specific detection of

SSIb, the primers (*SSIb*3-5'; 5'-CCAATCCTTTGACATCTGCTCC-3' and *SSIb*3-3'; 5'-GATCAGCTTTGGGTCCGGA-3')⁶ 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-carboxytetramethylrhodamine (TAMRA) at the 3' ends.

Preparation of calibrant plasmid

The specific sequence fragments from MON89788, endogenous soybean *Le1* gene⁸, and P35S⁸) were synthesized as a single oligonucleotide in tandem and inserted into the pUC19 vector. The constructed plasmid, designated as pSCS, was purified and diluted with 5 ng/ μ L ColE1 DNA (Nippon Gene, Tokyo, Japan) solution to 20,125,1500,20,000, and 250,000 copies per 2.5 μ L, based on the copy number of the *Le1* segment.

Preparation of test samples

For evaluation of the quantitative method, DNA solutions consisting of different concentrations of extracted MON89788 DNA in non-GM soybean DNA were used. MON89788 and non-GM soybean genomic DNA was extracted from ground seeds using GM Quicker (Nippon Gene) according to the manufacturer's manual. The concentration and quality of the extracted DNA solutions were evaluated by measuring the ultraviolet (UV) absorbance with a spectrophotometer, DU800 (Beckman Coulter, Fullerton, CA, USA) as described previously⁶. Both MON89788 and the non-GM soybean genomic DNA solutions were adjusted to a concentration of 20 ng/ μ L, and then mixed. Samples containing a mixture of the genomic DNA solutions at concentrations of MON89788 of 0, 0.1, 0.5, 5.0, and 10.0% in volume ratio were prepared. The mixed samples of each concentration were divided into 24 sample tubes, and six tubes were then randomly selected for a single laboratory evaluation and multi-laboratory trial, respectively.

Qualitative PCR

Qualitative PCR using a thermal cycler, GeneAmp PCR system 9700 (Life Technologies) and agarose gel electrophoresis were performed as described by Kuribara *et al.*⁸.

Quantitative PCR

TaqMan real-time PCR assays were carried out using an ABI PRISM 7900HT (Life Technologies), in 25 μ L final volume, with 50 ng of sample DNA, 12.5 μ L Universal Master Mix (Life Technologies), 0.5 μ M primer pairs and 0.2 μ M probe, according to the following step-cycle program: 2 min at 50°C, 10 min at 95°C, 45 cycles, 30 sec at 95°C, and 1 min at 59°C. In the reaction plate, each sample was quantified in triplicate.

^{*4} Notification No. 110 (Mar. 27, 2001); Department of Food Safety, Ministry of Health, Labour and Welfare of Japan (2001)

^{*5} Japanese Agricultural Standard (JAS) analytical test handbook: genetically modified food quality, labeling analysis manual for individual products (2002) The Food and Agricultural Materials Inspection Center, Japan.
http://www.famic.go.jp/technical_information/jashandbook/index.html

^{*6} Event-specific method for the quantification of soybean line MON89788 using real-time PCR. http://gmo-crl.jrc.ec.europa.eu/summaries/MON89788_validated_Method.pdf

Multi-laboratory trial

A multi-laboratory trial was performed at 3 laboratories and consisted of 2 separate stages, a stage to measure the C_f value and a blind test. All experimental protocols were provided by the National Food Research Institute (NFRI). Quantitative real-time PCR was performed with primers, probes, Universal Master Mix and DNA solutions supplied by NFRI.

The C_f value is experimentally determined as the ratio of the copy number of recombinant DNA (r-DNA) to the taxon-specific sequence in the GM plant genome. To calculate the C_f value for MON89788, the genomic DNA was extracted from genuine GM seeds, and the copy numbers of r-DNA and taxon-specific sequences were measured. The measurement was done twice at 3 laboratories, and the C_f value was determined as the mean of the 6 values obtained.

A blind test was carried out as described previously⁶⁾. Blind samples which were designed as blind duplicates of the 5 different concentrations of MON89788 DNA, consisting of 0, 0.1, 0.5, 5.0, and 10.0%, were sent to the participants.

All participants were requested to submit their data from the real-time PCR analyses.

Results and Discussion

Specificity of the PCR system for MON89788

For specific detection of MON89788, an event-specific segment was amplified at the junction site between the native soybean genomic DNA and r-DNA (Fig. 1). The specificity of the primer set was confirmed by a qualitative PCR method. No unexpected PCR products were detected using genomic DNA solutions from non-GM soybean, GM soybean RRS and A2704-12, non-GM maize, or 11 lines of GM maize, including glyphosate-tolerant events such as GA21 and NK603 (Fig. 2).

Determination of C_f value for MON89788

To determine the experimental C_f value for MON89788, the copy numbers of *Le1* and MON89788 in the extracted DNA from the MON89788 seeds were measured.

In Japan, one of the features of standard quantitative

methods is utilization of standard plasmid DNA containing both r-DNA and endogenous sequences as reference molecules and calibrators. To harmonize with the Japanese standard method, we developed one novel standard plasmid. The pSCS plasmid includes the specific sequences of MON89788 (Fig. 3), the *Le1* segment, and the P35S segment. The measurements were repeated twice, and the C_f value was determined as the mean from three laboratories. The defined C_f value for MON89788 was 1.21 (Table 1), which was used in the following quantifications.

Single laboratory evaluation of the PCR quantification

We performed single-laboratory and multi-laboratory evaluations of the proposed quantitative method using samples prepared by mixing extracted DNAs of GM and

Fig. 2. Specificity test of the designed primer. Agarose gel (3.0%) electrophoretogram of the amplified PCR products corresponding to the 139 bp of MON89788 (A), 114 bp of *SSIIb* and 118 bp of *Le1* in lanes 1–12 and 13–16 of (B), respectively. Lanes 1–11, GM maize events, NK603, Event176, T25, GA21, MON810, TC1507, Bt11, MIR604, MON88017, DAS59122, and MON863, respectively; 12 and 13, non-GM maize and non-GM soy, respectively; 14–16, GM soybean events, RRS, MON89788, and A2704-12, respectively. M, molecular weight marker.

Fig. 1. Schematic diagrams of the recombinant DNA of MON89788 and RRS. Arrows indicate the target positions in MON89788 (A) and RRS (B). Event-specific target sequence of MON89788 was designed for the 5'-flanking region between the exogenous insert and host soybean DNA.

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cgttactgct gccccacaaa gccctcgaa acttggttct gctccactct tccttttggg 60
cttttttgtt MON89788 - F tcctcgctcta gcgcttcaat cgtggttatc aagctcCAAA CACTGATAGT 120
TTAAACTGAA GCGCGGAAAC GACAATCTGA TCCCCATCAA GCTCTAGCTA GAGCGGCCGC 180
MON89788 - P
GTTATCAAGC TTCTGCAGGT CCTGCTCGAG TGAAGCTAA TTCTCAGTCC AAAGCCTCAA 240
MON89788 - R

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Fig. 3. Sequence between the 5' exogenous insertion and the soybean genome of MON89788. Lowercase letters represent the flanking soybean genomic sequence, and capital letters show the 5' transgenic insert containing the right border and partial FMV promoter sequences. The primers and probe for MON89788 are boxed and underlined, respectively.

non-GM DNA solutions. We decided that using DNA solution-based mixing samples would be easy and suitable for evaluation of the method.

In the single-laboratory evaluation, the GMO amount (%) of each sample was analyzed in six repetitions, and statistical analyses were carried out. The mean, bias (mean value, %), and relative standard deviation (RSD) at each mixing level were calculated (Table 2). The largest value of determined bias from the true value was 13.3, and the highest value of the RSD was 14.4% for the 0.1% mixing sample.

Blind test

We next evaluated the proposed quantitative method with a blind test involving 3 laboratories. All participants received primers, probes, and the test samples consisting of 5 different concentrations of MON89788, and the measurement was performed twice. The true-

ness and precision were determined as previously described^{5), 6)}. The bias, repeatability RSD (RSD_r), and reproducibility RSD (RSD_R) of the blind samples were measured (Table 3). The determined bias, RSD_r , and RSD_R ranged from -11.7 to 5.00% , from 2.70 to 19.4% , and from 5.92 to 19.4% , respectively. In Europe, the acceptance criteria for full validation of an analytical method are defined by the European Network of GMO Laboratories (ENGL)^{*7}. All of the obtained bias values were below the limit of the trueness acceptable level in

Table 2. Single-laboratory evaluation

%	n	Means	Bias	RSD^a , %	Detection limit
		GMO amount, %	True value, %		Below 20 copies ^b
0.00	6	0.00	0.00	nd	6/6
0.10	6	0.113	13.3	14.4	0/6
0.50	6	0.532	6.34	4.96	0/6
5.0	6	5.00	-0.06	9.03	0/6
10.0	6	9.37	-6.35	8.40	0/6

^a RSD: Relative standard deviation

^b Below 20 copies are expressed as the ratio of the number of retained data below 20 copies/the total number of retained data.

Table 1. Experimental conversion factor for MON89788

7900HT		
Mean	SD	RSD
1.21	0.0866	7.14

SD; Standard deviation

RSD; Relative standard deviation

Table 3. Trueness and precision

% (v/v)	Retained labs	Trueness		Precision		Detection limit
		Means	Bias	RSD _r ^a , %	RSD _R ^b , %	Below 20 copies ^c
		GMO amount, %	True value, %			
0.00	3	0.00	0.00	nd	nd	6/6
0.10	3	0.105	5.00	19.4	19.4	0/6
0.50	3	0.442	−11.7	16.8	16.8	0/6
5.0	3	4.85	−3.00	2.70	5.92	0/6
10.0	3	9.18	−8.20	5.19	6.32	0/6

^a RSD_r : Repeatability relative standard deviation

^b RSD_R : Reproducibility relative standard deviation

^c Below 20 copies are expressed as the ratio of the number of retained data below 20 copies/the total number of retained data.

When $RSD_r > RSD_R$, RSD_R was considered to have the same value of RSD_r ⁹⁾.

*⁷ Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing, European Network of GMO Laboratories (ENGL)

http://gmo-crl.jrc.ec.europa.eu/doc/Min_Perf_Requirements_Analytical_methods.pdf

ENGL (The bias value should be within $\pm 25\%$). The obtained RSD_r and RSD_R here were similar to, or within a narrower range than, those in previously reported GMO events^{5), 6)}. ISO 24276*⁸ specifies that the RSD_R is 25% or less at the limit of quantitation (LOQ) in GMO analysis, and all the obtained RSD_R values were within the criterion, even for 0.10% samples. The data below 20 copies were extrapolated based on the standard curve in our method because there was no calibrant below 20 copies. In Table 3, all the measured copy numbers of 0.10% samples were over 20 copies. Therefore, it was estimated that the LOQ for MON89788 is 0.10% in this method.

In conclusion, the newly developed method is applicable for the detection and quantification of MON89788 to monitor the validity of the labeling. Several GM soybean events such as RRS, MON89788, and gluphosinate-tolerant soybeans have been approved in Japan. The number of approved GM soybean events is expected to steadily increase. Specific analytical methods for novel GM events or a screening method for GM soybean events will be required.

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*⁸ International Standard 24276, Foodstuffs—Nucleic acid based of analysis for the detection of genetically modified organisms and derived products—General requirements and definitions