Real-time PCR array as a Universal Platform for the GM crop detection and its Application in Identifying Unapproved GM crops in Japan

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Abstract

We developed a novel type of real-time PCR array with TaqMan® chemistry as a platform for the comprehensive detection of genetically modified (GM) crops. Thirty TaqMan® PCRs for the specific detection of GM events, recombinant DNA segments, endogenous reference genes and donor organisms, were designed as the component reactions of real-time PCR array and their analytical performances were evaluated. Furthermore, we developed a Microsoft Excel® spreadsheet application for the easy assumption of unapproved GM crop contamination in analytical samples from the results of the real-time PCR array.

Introduction

Many kinds of genetically modified organisms (GMOs) are already in practical use and, especially, the number of commercially available genetically modified (GM) crops is increasing rapidly (James 2008). In Japan, a total of 76 GM events have been approved for open field cultivation or provision as food, feed or ornamental plants as living modified organisms (LMOs) under the Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of LMOs as of July 8, 2009. Additionally, a total of 98 GM events have been approved for food under the Food Sanitation Act as of April 30, 2009. Under these circumstances, it is desirable to develop GM crop testing methods that are capable of collecting a lot of information regarding GM crops at once and has the potential to be easily updated as the situation demands. In Japan, the Food Sanitation Act, the Act on Safety Assurance and Quality Improvement of Feeds and the Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of LMOs impose a policy of strict restriction of unapproved GM crops. Nevertheless, the incidents of contamination by unapproved GM events have occurred sporadically and have caused considerable concern worldwide. However, no perfect detection system for all unapproved GM crops has yet been developed because there are no biological or chemical characteristics common in such crops.

We have already proposed real-time PCR array with TaqMan® chemistry, i.e., 96-well PCR plate prepared with a different primer-probe in each well, as a universal platform of GM crop detection and have evaluated the specificity and sensitivity of the developed system. Furthermore, we investigated the application of the system in identifying the unapproved GM event (Mano et al. 2009). In the presentation, we review the detection system, adding some new knowledge.

Materials and methods

Test Materials

We used the following as representative GM maize events: Bt11, Event176 (E176), GA21, MON810 (M810), MON863 (M863), NK603, T25, TC1507, MIR604, DAS-59122 (D59122), and MON88017 (M88017). Representative GM soy events were the following: 40-3-2 (Roundup Ready Soybean, RRS), A2704-12 (A2704), and A5547-127(A5547). We used RT73 as a representative GM canola event, and LLRICE62 as a representative GM rice event. The analytical samples and pre-treatment methods were concretely described in the previous report (Mano et al. 2009). Besides them, the conventional seeds of buckwheat, amaranth, millet and

sorghum purchased in Japan were subjected to the grinding with a Multi-beads shocker (Yasui Kikai Co., Osaka, Japan) and the subsequent DNA extraction with a GM quicker 2 DNA extraction kit (Nippon Gene Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol designated for canola sample. The DNA concentration was determined by measuring ultraviolet (UV) absorbance with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA) and calculated as 1 optical density unit at 260 nm equal to 50 ng/μl. All extracted DNAs were diluted by 20 ng/μl.

Primers and TaqMan® Probes

For GM event detection, the representative GM maize and soy events were selected as targets. For recombinant DNA (r-DNA) segment detection, we selected 10 target segments commonly introduced into GM events: the 35S promoter region (P35S) derived from Cauliflower Mosaic Virus (CaMV), the terminator region of the nopaline synthase gene derived from *Rhizobium radiobacter* (TNOS), the 35S promoter region of Figwort Mosaic Virus (PFMV), the intron region of the rice Actin 1 gene (AINT), a region of the neomycin phosphotransferase II gene (NPTII), a region of the phosphinothricin-N-acetyltransferase gene derived from *Streptomyces hygroscopicus* (PAT), a region of the phosphinothricin-N-acetyltransferase gene derived from *Streptomyces Viridochromogenes* (BAR), a region of the glyphosate oxidoreductase gene derived from *Ochromobactrum anthropi* strain LBAA (GOX), a region of 5-enolpyruvylshikimate-3-phosphate synthase gene introduced into NK603, M88017, and RRS (EPSPS1), and a region of the gene introduced into RT73 (EPSPS2). For the endogenous reference gene detection, the following target regions of genes were selected: a region of the starch synthase IIb gene of *Zea mays* (SSIIb), a region of the lectin 1 gene of Glycine max (Le1), a region of the high mobility-group protein I/Y gene of rapeseed (HMG), a region of the sucrose phosphate synthase gene of *Oryza sativa* (SPS), and a region of the 18S rRNA gene common in crop plants (18SrRNA). As the target of donor organism detection, CaMV is selected. The primers and probes are reported previously (Mano et al. 2009). The oligonucleotide DNA for PCR primers and TaqMan® probes was synthesized by FASMAC Co., Ltd. (Kanagawa, Japan) and Applied Biosystems, Inc. (Foster City, CA, USA). The probes were labelled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamin (TAMRA) at the 5' and 3' ends, respectively, except that the CaMV-MGB probe was labelled with FAM at the 5' end and with non-fluorescent quencher linked with minor groove binder at the 3' end.

Preparation of Real-Time PCR Array, Reaction Conditions and Data

Analysis

For the preparation of the real-time PCR array, 2 μl of a primer and probe mixture containing 2.5 μM primers and 1μM probe, was added into each well of a 96-well plate and sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems, Inc.). For a negative control test with no primers or probes, sterile distilled water was used in place of the primer and probe mixture. Array plates containing primer and probe mixtures were preserved under -20˚C until just before use. For the assay of sample DNA with the real-time PCR array, the diluted DNA samples described above, TaqMan® Universal PCR Master Mix (Applied Biosystems, Inc.) and sterile distilled water were mixed and added into each well at a volume of 8 μl. Finally, 10 μl of the reaction mixture in each well contained 20 ng of genomic DNA, 5 pmol of 5' primer, 5 pmol of 3' primer, 2 pmol of a probe and 5 μl of TaqMan® Universal PCR Master Mix. The plates containing reaction mixtures were sealed with MicroAmp® Optical Adhesive Film, thermal cycled with the ABI PRISM® 7500 real-time PCR system (Applied Biosystems, Inc.), and then data analysis was carried out using Sequence Detection Software Version 1.4 (Applied Biosystems, Inc.). The thermal cycling conditions were as follows: 2 min at 50˚C, 10 min at 95˚C, 45 cycles of 15 sec at 95˚C and 1 min at 60˚C under 9600 emulation mode. Data analysis was performed using the "Amplification Plot" feature of the analysis software and the detail settings were set at the "Delta Rn vs. Cycle" view with Manual Ct mode (Threshold, 0.256) and Manual baseline mode (start of baseline, 3; end of baseline, 10). Amplification lines crossed with the threshold line were determined as positive.

Evaluation of Analytical Performances

To evaluate the specificity, DNA extraction was performed twice from each ground sample and each DNA sample was analysed 3 times. For A2704, A5547 and LLRICE62, two parallel dilutions of CRM samples were performed and the resultant samples were analysed 3 times each. Specificity was confirmed when the 6 sets of PCR data corresponded to the relevant information.

To evaluate the sensitivity, simulated test samples containing GM crops at several concentrations were prepared. Ground GM crop samples were mixed in ground non-GM crop samples at different mass fractions and the following samples were prepared: 0.1% Bt11, 0.1% E176, 0.1% M863, 0.1% each of 8-event mixtures of GM maize (Bt11, E176, GA21, M810, M863, NK603, T25 and TC1507), 0.25% Bt11, 0.25% E176, 0.25% the 8-event mixture of GM maize, and 0.25% RT73. The ground RRS sample was mixed with the ground non-GM soy sample at a mass fraction of 0.1%. For MIR604, D59122 and M88017, two parallel DNA extractions and dilutions were performed and the samples were mixed with non-GM maize DNA samples (20

ng/μl) at a concentration of 0.25%. For A2704 and A5547, two parallel dilutions of CRMs were performed and the resultant samples were then mixed with non-GM soy DNA samples (20 ng/μl) at a concentration of 0.25%. For LLRICE62, two parallel dilutions of CRMs were performed and the resultant samples were mixed with non-GM rice DNA samples (20 ng/μl) at a concentration of 0.25%. Two DNA solutions for every simulated sample were analysed 5 times each. The sensitivity of CaMV detection was evaluated with the DNA extracts prepared from ground plant leaves infected with the virus. They were then analysed in order to calculate the copy numbers of CaMV by quantitative analysis of the P35S region in accordance with the Japanese standard GMO testing method described in the JAS analytical test handbook (Food and Agricultural Materials Inspection Centre, 2002). Diluted samples containing the CaMV genome at concentrations of 10^4, 10^3, 10^2 and 10 copies were prepared and assayed with real-time PCR array.

Development of the Spreadsheet Application, Unapproved GMO Checker

We used Microsoft Excel® 2007 to construct a spreadsheet application, Unapproved GMO Checker version 2.01. The worksheet is shown in Figure 1. As an input form for an experimental result, the crop name of the analytical sample was designed to be selectable from “Maize,” “Soy” or “Rice” and checkboxes were prepared to input the qualitative results of the real-time PCR array. The “Check” button is linked with a macro that analyses experimental results in the input form. We created three columns, “Validity of the experimental result”, “Comment about approved GMO” and “Comment about unapproved GMO”, for the output of verification results and a checkbox for the output of r-DNA segments in the unapproved GMO. First, the macro confirms consistency of the relationships between the selected crop name and the results of endogenous reference gene detection, and those between the selected crop name and the results of GM event detection. Additionally, it checks whether all the expected r-DNA segments elicited from the results of GM event detection were detected. If all of these conditions are satisfied, the message “Reasonable” appears as the output in the first column, “Validity of the experimental result,” and the macro continues to the next step in the process. If the conditions are not satisfied, the message “Unreasonable” appears as the output in the first column, “Verification was impossible” appears below “Comment about approved GMO” and “Comment about unapproved GMO” and the macro would be finished. Next, contamination of an approved GMO would be examined based on the results of GM event detection. If approved GM crops are detected, the message “Approved GMO was detected” appears under “Comment about approved GMO;” if

not, “Approved GMO was not detected” appears. Finally, unapproved GM contamination would be examined. For this purpose, the expected r-DNA segments deduced based on the results of GM event detection would be compared with the results of r-DNA segment detection. If r-DNA segments other than the expected r-DNA segments are detected, contamination by unapproved GM crops would be suspected, the message “Possible contamination by an unapproved GMO” would appear under “Comment about unapproved GMO,” and the segments expected to be contained in the unapproved GM crop would be shown in the checkbox below “Recombinant segments in the unapproved GMO.”

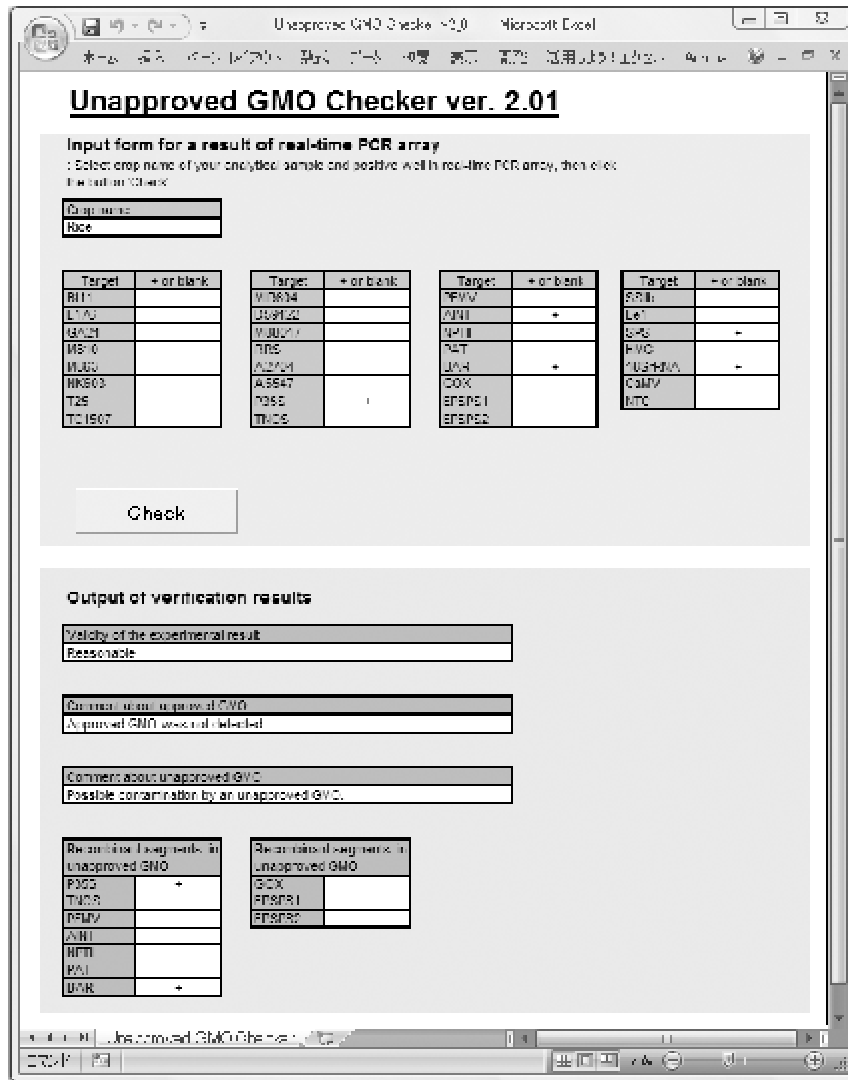

Figure 1. The spreadsheet application, Unapproved GMO Checker version 2.01.

Results and discussion

Design of the Real-Time PCR Array

Simultaneous detection methods for the multiple GMO targets, such as multiplex polymerase chain reaction (PCR) methods, DNA chip analyses and membrane hybridization methods, have been developed and reported. Although multiplex PCR is one of the most efficient and easiest techniques for multiple target-detection, the multiplex reaction is difficult to be applied in practical testing because of several drawbacks. For example, false-positive amplifications tend to occur more than in simplex reaction (Markoulatos 2002). Additionally, the interaction between individual reactions in the multiplex system causes unstable testing results in cases in which there is a big gap between the copy numbers of the target DNAs (Elnifro et al. 2000; Ratcliff et al. 2007). In the development of an analytical method for regulatory use with GMOs, a validation study among participating laboratories is required to evaluate the performance. Validation studies, however, tend to be time- and cost-consuming. The addition of a single individual reaction into a validated multiplex reaction system may require substantial effort to re-evaluate the whole system. This makes it difficult to supply suitable GMO testing methods to testing laboratories in a flexible and impromptu manner. Given this situation, a detection system that permits the simultaneous and efficient implementation of many individual validated methods would be a practical and useful tool for GMO analysis.

Real-time PCR with TaqMan® chemistry has been used in various kinds of quantitative detection methods for GM crops (Kuribara et al. 2002; Holst-Jensen et al. 2003). Furthermore, the validation studies on the detection methods have been reported on the website, "Community Reference Laboratory for GM Food and Feed" (http://gmo-crl.jrc.it/default.htm) under the European Commission. TaqMan® PCR provides higher specificity than conventional PCR or the intercalator-based real-time PCR due to the chemistry with TaqMan® probes. Additionally, TaqMan® assay development with Applied Biosystems-system does not necessarily require strict optimization of reaction conditions such as thermal cycling or the composition of the reaction buffer as described in the manufacturer's protocol of TaqMan® Universal PCR master mix (Applied Biosystems, Inc.). These characteristics are advantageous for a universal detection platform.

Considering the above points, we adopted real-time PCR array analysis based on TaqMan® chemistry i.e., simultaneous implementation of TaqMan® PCRs on a 96-well PCR plate, as a universal detection platform. The individual reactions were designed in order to detect comprehensively GM crops. A total of 30 primer-probe sets were prepared for GM event detection, r-DNA segment detection, endogenous

reference gene detection as a positive control test, and donor organism detection as a negative control test. Primer-probe sets for GM event detection were designed for the detection of representative GM maize and soy events, aiming for the efficient detection of approved GM maize and soy events. They included event-specific and construct-specific detections as previously reported (Holst-Jensen et al. 2003). For r-DNA segment detections, 10 targets were selected as above. Almost all events approved in Japan were expected to contain at least one target of the r-DNA segment detections based on the published information. The set of r-DNA segment detections is expected to be a useful tool for screening GM crops regardless of GM events and would be helpful to strictly distinguish GM and non-GM seeds for the practical coexistence of GM crop farming and conventional farming.

The reaction conditions were designed by referring to Japanese and EU standard methods. For cost reduction, the volume of the reaction mixtures was set at 10 μl. The total experimental work for one assay including the preparation of the PCR mixtures, thermal cycling and data analysis took only 3 hours. Representative assay results of the present system are shown in Figure 2. The following detections were clearly determined to be positive: 18SrRNA, SSIIb, Bt11, P35S, TNOS and PAT for Bt11 maize; 18SrRNA and SSIIb for non-GM maize; 18SrRNA, SPS, P35S, AINT and BAR for LLRICE62; 18SrRNA, AINT and SPS for non-GM rice. These results are consistent with the publicly available information. The present system successfully distinguished GM and non-GM crops and provided information regarding GM events and r-DNA segments by a simple assay.

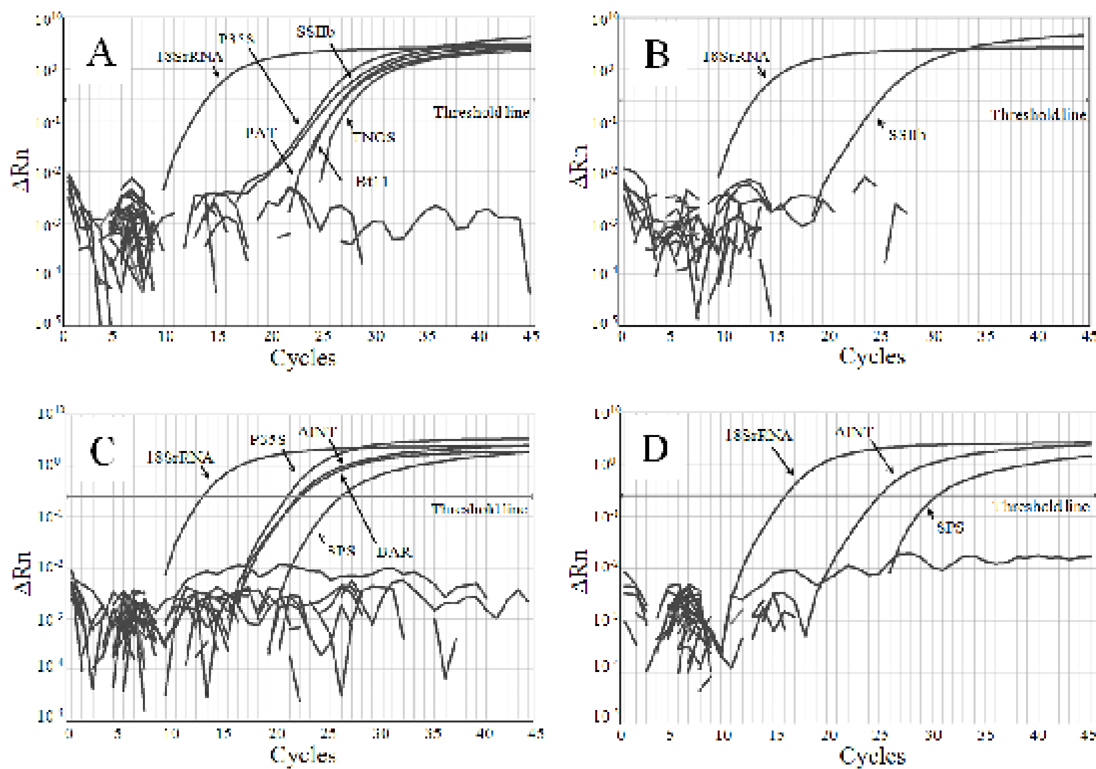

Figure 2. Representative amplification curves in real-time PCR array results. The horizontal axis indicates the cycle numbers of PCR and the vertical axis indicates the ΔRn values, which are the relative values automatically calculated by the analysis software based on signal intensities of FAM dye dependent on the target amplification and ROX passive reference dye. The DNA samples derived from Bt11 (A), non-GM maize (B), LLRICE62 (C) and non-GM rice (D) were assayed for 31 targets as described in the *Materials and Methods* section. The arrows with target names indicate corresponding amplification curves which were determined to be positive.

Evaluation of Analytical Performances

To evaluate the specificity, the samples were prepared and 6 assays were performed. None of the qualitative results presented in Table 1 showed any discrepancy with the expected results based on the published information about recombinant DNA from Agbios website (http://www.agbios.com/main.php). In addition to the results shown in Table 1, non-GM wheat, barley, cotton, sugar beet, potato, buckwheat, amaranth, millet and sorghum samples were assayed, and only 18SrRNA was positive as predicted. Throughout the specificity evaluation, no non-specific amplification attributed to the inappropriate design of primers or probes was observed and the detection system was found to be applicable to a broad range of plant species and parts of the plant bodies. With respect to CaMV detection, the DNA samples extracted from 3 types of CaMV-infected plant leaves were assayed and specific amplification was identified.

The results of sensitivity evaluation were indicated in Table 2. False negative results were observed in the assay with several simulated samples containing GM crops at a concentration of 0.1%, specifically, 0.1% Bt11, 0.1% E176 and 0.1% the 8 events of GM maize, but not in the assay with the 0.25% GM samples. The false negative results were considered to have been caused by small copy numbers of target DNAs. We observed high sensitivity of individual reactions for GM event and r-DNA segment detections under low concentrations of target DNA (Table 2, asterisks). Concerning CaMV detection, it was suggested that the minimum concentration of CaMV DNA for a reliable result is 100 copies/μl. We speculate that 0.5% of GM contamination in conventional maize, soy, canola or rice would allow us to obtain accurate results in all wells of the real-time PCR array. The present results demonstrate that the sensitivity of our method is sufficient for the inspection of unintended mixing of approved GM crops under Japanese regulations with a threshold of 5%.

Table 1. Results of specificity evaluation.

Type of detection	Target name	Bt11	E176	GA21	M810	M863	NK603	T25	TC1507	MIR604	D59122	M88017	Non-GM maize	RRS	A2704	A5547	Non-GM soy	LLRICE 62	Non-GM rice	RT73	Non-GM canola
		Sample name																			
GM event detection	Bt11	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E176	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	GA21	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M810	-	-	-	+		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M863	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	NK603	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T25	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	TC1507	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	MIR604	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	D59122	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	M88017	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	RRS	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
	A2704	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	A5547	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
r-DNA segment detection	P35S	+	+	-	+	+	+	+	+	-	+	+	-	+	+	+	-	+	-	-	-
	TNOS	+	-	+	-	+	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-
	PFMV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	AINT	-	-	+	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-
	NPTII	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PAT	+	-	-	-	-	-	+	+	-	+	-	-	-	+	+	-	-	-	-	-
	BAR	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
	GOX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	EPSPS1	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-
	EPSPS2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Endogenous reference gene detection	SSIIb	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
	Le1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
	SPS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
	HMG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	18SrRNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Donor organism detection	CaMV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Negative control	NTC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 2. Results of sensitivity evaluation.

Type of Detection	Target name	Sample name A	B	C	D	E	F	G	H	I	J	K
GM event detection	Bt11	0	0	10	0	10*	0	0	0	0	0	0
	E176	0	0	0	10	10*	0	0	0	0	0	0
	GA21	0	0	0	0	10*	0	0	0	0	0	0
	M810	0	0	0	0	10*	0	0	0	0	0	0
	M863	10*	0	0	0	10	0	0	0	0	0	0
	NK603	0	0	0	0	10*	0	0	0	0	0	0
	T25	0	0	0	0	10*	0	0	0	0	0	0
	TC1507	0	0	0	0	10*	0	0	0	0	0	0
	MIR604	0	0	0	0	0	10*	0	0	0	0	0
	D59122	0	0	0	0	0	0	10*	0	0	0	0
	M88017	0	0	0	0	0	0	0	10*	0	0	0
	RRS	0	10*	0	0	0	0	0	0	0	0	0
	A2704	0	0	0	0	0	0	0	0	10*	0	0
	A5547	0	0	0	0	0	0	0	0	10*	0	0
r-DNA segment detection	P35S	10*	10	10	10	10	0	10	10	10	10	0
	TNOS	10*	10	10	0	10	10	0	10	0	0	0
	PFMV	0	0	0	0	0	0	0	0	0	0	10*
	AINT	10*	0	0	0	10	0	0	10	0	10	0
	NPTII	10*	0	0	0	10	0	0	0	0	0	0
	PAT	0	0	10*	0	10	0	10	0	10	0	0
	BAR	0	0	0	10*	10	0	0	0	0	10	0
	GOX	0	0	0	0	0	0	0	0	0	0	10*
	EPSPS1	0	10*	0	0	10	0	0	10	0	0	0
	EPSPS2	0	0	0	0	0	0	0	0	0	0	10*
Endogenous reference gene detection	SSIIb	10	0	10	10	10	10	10	10	0	0	0
	Le1	0	10	0	0	0	0	0	0	10	0	0
	SPS	0	0	0	0	0	0	0	0	0	10	0
	HMG	0	0	0	0	0	0	0	0	0	0	10
	18SrRNA	10	10	10	10	10	10	10	10	10	10	10
Donor organism detection	CaMV	0	0	0	0	0	0	0	0	0	0	0
Negative control	NTC	0	0	0	0	0	0	0	0	0	0	0

Sample A: 0.1% M863; B: 0.1% RRS; C: 0.25% Bt11; D: 0.25% E176;
E: 0.25% the 8 events of GM maize; F: 0.25% MIR604; G: 0.25% D59122;
H: 0.25% M88017; I: 0.25% A2704 and A5547; J: 0.25% LLRICE62; K: 0.25% RT73.
The value of each element indicates the number of positive detections in a total of 10 assays.
Asterisks indicate data used for the evaluation of the sensitivity of individual detections.

Application of the Real-Time PCR Array to the Assumption of Unapproved GM Crop Contamination

The unapproved GM crops were conceptually defined as (Unapproved GM crops) = (All GM crops) – (Approved GM crops). Approved GM crops could be selectively detected using GM event detections, while r-DNA segment detections detected various kinds of GM crops ranging from approved to unapproved. Therefore, the assumption of unapproved GM crop contamination is achieved by comparing the results of r-DNA segment detection with those of GM event detection in the real-time PCR array. For the easy assumption of unapproved GM crop-contamination, we developed the spread sheet application, Unapproved GMO Checker version 2.01 (Figure 1). In the version of application, the unapproved GM events were defined as GM crops which have not been approved for open-field cultivation or provision as food, feed or ornamental plants under the Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of LMOs in Japan. Furthermore, GM maize, GM soy and GM rice were selected as targets. LLRICE62 corresponds to the definition of unapproved GM events. Based on the results obtained by the real-time PCR array (Figure 2C), the contamination of unapproved GM crop(s) was assumed, as shown in Figure 1. This result was obtained by the detection of P35S and BAR as r-DNA segments. The result demonstrated no discrepancy between the obtained data and publicly available information. The application is available and downloadable online (http://cse.naro.affrc.go.jp/jmano/index.html).

Our assumption could be accomplished only when the appropriate results in all wells of the real-time PCR array were perfectly obtained. The results of the present sensitivity evaluation indicated that a contamination level of 0.5% would be sufficient to obtain reliable data without false negative results, so that an analytical sample of fewer than 200 seeds may be preferable. Because unapproved GM crops which have become major concerns such as CBH351 maize, Bt10 maize, LLRICE601, and Bt-rice containing r-DNA segments were selected as target DNA in our investigation, our analytical system may have the potential to discover the novel types of unapproved GM crops as well as the already known unapproved GM crops. However, the present method does not necessarily promise the absolute detection of unapproved GM crops because crops constructed of completely unknown r-DNA segments or r-DNA segments with modified nucleotide sequences cannot be detected. Also, GM event detection does not completely cover all the approved GM crops at present. Furthermore, if approved and unapproved GM crops were mixed in a sample and both crops shared all r-DNA segments, the unapproved GM crop would be masked by the approved GM crop. If unapproved GM crop contamination is

suspected, further analysis, such as sequencing of the r-DNA flanking regions, may be required. Despite its many restrictions, the proposed system would serve as an excellent tool to detect unapproved GM contamination.

Conclusions

The developed real-time PCR array allows the comprehensive detection of GM crops and the assumption of contamination by unapproved GM crops. Additionally, the platform was proved to be applicable to the various kinds of plant species. This approach is attractive in terms of specificity of detection, dynamic range, time efficiency, easy manipulation, updatability and customizability. Another important factor for the dissemination of this new technology is that the proposed method requires no extra investment for equipment in many GMO testing laboratories. Further updating of this system by editing detection targets depending on the purpose of a given investigation would provide appropriate testing methods for both regulatory and commercial use. Finally, we remark that the systemization regarding update and validation of the detection system is ongoing.

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