JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Real-Time PCR Array as a Universal Platform for the Detection of Genetically Modified Crops and Its Application in Identifying Unapproved Genetically Modified Crops in Japan

JUNICHI MANO,[†] NATSUKI SHIGEMITSU,[†] SATOSHI FUTO,[‡] HIROSHI AKIYAMA,[§] REIKO TESHIMA,[§] AKIHIRO HINO,[†,⊥] SATOSHI FURUI,[†] AND KAZUMI KITTA*[,†]

National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan; FASMAC Co. Ltd., 5-1-13 Midorigaoka, Atsugi, Kanagawa 243-0041, Japan; and National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

We developed a novel type of real-time polymerase chain reaction (PCR) array with TaqMan chemistry as a platform for the comprehensive and semiquantitative detection of genetically modified (GM) crops. Thirty primer-probe sets for the specific detection of GM lines, recombinant DNA (r-DNA) segments, endogenous reference genes, and donor organisms were synthesized, and a 96-well PCR plate was prepared with a different primer-probe in each well as the real-time PCR array. The specificity and sensitivity of the array were evaluated. A comparative analysis with the data and publicly available information on GM crops approved in Japan allowed us to assume the possibility of unapproved GM crop contamination. Furthermore, we designed a Microsoft Excel spreadsheet application, Unapproved GMO Checker version 2.01, which helps process all the data of real-time PCR arrays for the easy assumption of unapproved GM crop contamination. The spreadsheet is available free of charge at http://cse.naro.affrc.go.jp/jmano/index.html.

KEYWORDS: Genetically modified (GM); real-time PCR array; TaqMan assay

INTRODUCTION

Today many types of genetically modified organisms (GMOs), including microorganisms, animals, and plants, are already in practical use, and the number of commercially available genetically modified (GM) crops is increasing rapidly (*1*). In Japan, a total of 69 lines of GM crops have been approved for open-field cultivation or provision as food, feed, or ornamental plants as living modified organisms (LMOs) under Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Protocol domestic law) that came into effect on January 31, 2008. Additionally, a total of 88 GM crop lines have been approved for food under Food Sanitation Law as of February 12, 2008. Under these circumstances, it is desirable to develop GM crop testing methods that are capable of collecting a lot of information at once. Simultaneous detection methods, such as multiplex polymerase chain reaction (PCR) methods (*2*−*5*), DNA chip analysis (*6*−*14*), and membrane hybridization methods (*15*), have been developed and reported for some GM crops. Although multiplex PCR is one of the most efficient and easiest techniques for multiplex detection, the multiplex reaction is difficult to be applied in practical testing, and false-positive amplifications tends to occur more often than in the simplex reaction (*6, 14, 16*). In addition, 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 (*17, 18*). 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 reevaluate the whole system. This makes it difficult to supply suitable GM testing methods to testing laboratories in a flexible and impromptu manner so that they will be ready to deal with the increasing number of approved GM crops. Given this situation, a universal detection system that permits the simultaneous implementation of many individual validated methods would be an efficient and useful tool for GM analysis. The costs of DNA analysis depend largely on the high price of PCR instruments and reagents. The frequent use of an instrument may pay off. Additionally, the large-scale synthesis of oligonucleotides for primers or probes

* Corresponding author. Tel.: +81-29-838-7369. Fax: +81-29-838-7369. E-mail: kaz@affrc.go.jp.
[†] National Food Research Institute.
[‡] FASMAC Co. Ltd.
[§] National Institute of Health Sciences.
[⊥] Current affiliation: Food Safety Commission Secretariat, 2-13-10 Prudential Tower 6th Floor, Nagata-cho, Chiyoda-ku, Tokyo 100-8989, Japan.

10.1021/jf802551h CCC: $40.75
Published on Web 12/12/2008

may provide superior cost performance to small-scale synthesis. Thus, a universal detection platform with which many analyses can be performed in a single system is also attractive from an economic point of view. Real-time PCR with TaqMan chemistry has been used in various kinds of quantitative detection methods for GM crops (*19, 20*). Furthermore, the validation studies on the detection methods have been reported on the Web site, "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 due to the chemistry with TaqMan probes. Additionally, TaqMan assay development with the Applied Biosystems system does not necessarily require strict optimization of reaction conditions such as thermal cycling or the composition of the reaction buffer (*21*). These characteristics are advantageous for a universal detection platform. The ideal system would be easily updatable and customizable as the situation demands, particularly for the addition of new approved GM lines.

In Japan, Food Sanitation Law and Law Concerning Safety Assurance and Quality Improvement of Feeds require safety assessments of GM crop lines for food and feed, respectively. Cartagena Protocol domestic law requires the assessment of adverse effects on biological diversity. Additionally, these laws impose a policy of strict restriction of unapproved GM crops. Nevertheless, the incidents of contamination by unapproved GM lines 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 specific to such crops.

In this study, we 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 detection and evaluated the specificity and sensitivity of the developed system. We also explored the possibility of adopting this real-time PCR array for the control of unapproved GM crops. Additionally, in order to facilitate the assumption of GM crop contamination, we designed a Microsoft Excel spreadsheet application, Unapproved GMO Checker version 2.01, and made it available on the Internet.

MATERIALS AND METHODS

Cereal Materials and DNA Extracts for Analytical Samples. We used the following as representative GM maize lines: Bt11, Event176 (E176), GA21, MON810 (M810), MON863 (M863), NK603, T25, TC1507, MIR604, DAS-59122 (D59122), and MON88017 (M88017). Representative GM soy lines 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 line and LLRICE62 as a representative GM rice line. F1 generation seeds of Bt11 and E176, and ground F1 generation seeds of GA21, were kindly provided by Syngenta Seeds AG (Basel, Switzerland); F1 generation seeds of M810, M863, and NK603 were kindly provided by Monsanto Company (St. Louis, MO, U.S.A.); and F1 generation seeds of TC1507 were kindly provided by Dow AgroSciences LLC (Indianapolis, IN, U.S.A.). Two certified reference materials (CRMs) in powder form were purchased from the Institute for Reference Materials and Measurements (IRMM; Retieseweg, Belgium): MIR604 (cat. no. ERM-BF-423d; certified value, 98.5 g/kg; uncertainty (coverage factor $k = 2$), −2.6 and +2.9 g/kg) and D59122 (ERM-BF-424d; 98.7 g/kg; −5.8 and +5.9 g/kg). Several CRMs were purchased from the American Oil Chemists' Society (AOCS; Urbana, IL, U.S.A.): nonmodified canola seeds (cat. no. AOCS 0304-A; certified value, below 0.5% of Roundup Ready canola), RT73 seeds (AOCS 0304-B; above 991.9 g/kg of Roundup canola), ground seeds of M88017 (AOCS 0406-D; above 990.5 g/kg), DNA extract of LLRICE62 (AOCS 0306-I; above 999.9 ng/μL of GM DNA), DNA extract of A2704 (AOCS 0707-B; above 999.9 ng/μL of GM DNA), and DNA extract of A5547 (AOCS 0707-C; above 999.9 ng/μL of GM DNA). Plant leaves infected by cauliflower mosaic virus (CaMV) (MAFF Nos. 104018, 104019, and 104021) were obtained as genetic resources from Genebank of the National Institute of Agrobiological Sciences (Tsukuba, Japan). F1 generation seeds of T25 and progeny seeds of RRS were imported directly from the U.S.A. Dry seeds of maize (Quality Technology International, Inc., Elgin, IL, U.S.A.) and dry soybeans harvested in Ohio in 1998 were also imported directly and used as non-GM maize and non-GM soy, respectively. Seeds of the conventional rice variety Kinuhikari (*Oryza sativa*), the conventional wheat variety Haruyutaka (*Triticum aestivum*), and the conventional barley variety Harrington (*Hordeum vulgare*) were obtained in Japan. Non-GM CRMs of cotton (ERM-BF422a; below 0.5 g/kg for GM cotton 281-24-236 and 3006-210-23), sugar beet (ERM-BF419a; 0 g/kg for GM sugar beet H7-1), and potato (ERM-BF421a; 0% for GM potato EH92-527-1) were purchased from IRMM.

Preparation of Test Samples and DNA Extraction. All dry seeds except canola seeds were ground with a P-14 speed rotor mill (Fritsch GmbH, Idar-Oberstein, Germany); canola seeds and plant leaves infected by CaMV were frozen in liquid nitrogen and then ground with an SK mill (Tokken, Inc., Chiba, Japan). The ground materials were stored at −20 °C until DNA extraction. For maize, soy, wheat, barley, rice, cotton, and sugar beet, DNA extraction was performed using a DNeasy Plant Maxi Kit (Qiagen GmbH, Hilden, Germany) as described in our previous report (*19*). For ground samples of canola seeds and potatoes, DNA extraction was performed using a GM quicker 2 (Nippon Gene Co., Ltd., Tokyo, Japan) following the manufacturer's instructions. For ground samples of plant leaves, DNA extraction was performed using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. To evaluate the sensitivity, mixed samples of GM and non-GM ground materials at different mass fractions were prepared and used as described below. The DNA concentrations of solutions were determined by measuring ultraviolet (UV) absorbance with a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, U.S.A.), and quality was evaluated by the absorbance ratios at 260/280 nm and 260/230 nm. The absorbance ratio at 260/230 nm was above 1.7 and at 260/280 nm was between 1.7 and 2.0 for most DNA extracts. DNA concentration was calculated with 1 optical density unit at 260 nm equal to 50 ng/μL. All extracted DNAs were diluted to 20 ng/μL with sterile distilled water.

Primers and TaqMan Probes. The primers and probes used in the present study and the references *19 and 22−29* are listed in **Table 1**. The oligonucleotide DNA for PCR primers and TaqMan probes was synthesized by FASMAC Co. Ltd. (Kanagawa, Japan) and Applied Biosystems (Foster City, CA, U.S.A.). The probes were labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamin (TAMRA) at the 5′ and 3′ ends, respectively, except that the CaMV−MGB probe was labeled with FAM at the 5′ end and with nonfluorescent quencher linked with minor groove binder at the 3′ end. For GM-line-specific detection, 11 GM maize lines (Bt11, E176, GA21, M810, M863, NK603, T25, TC1507, MIR604, D59122, and M88017) and 3 GM soy lines (RRS, A2704, and A5547) were selected as targets. primer-probe set information

Table 1. Primer and Probe Sequences

For GM Line-Specific Detection

crop name	target name	type of detection	evaluation	primer or probe name		sequence (5′-3′)	length (base)	amplicon size (bp)	ref
maize	Bt11	construct-specific	A	Bt11 3-5′	5′ primer	AAAAGACCACAACAAGCCGC	20	127	19
				Bt11 3-3′	3′ primer	CAATGCGTTCTCCACCAAGTACT	23		
				Bt11-2-Taq	probe	CGACCATGGACAACAACCCAAACATCA	27		
maize	E176	construct-specific	A	E176 2-5′	5′ primer	TGTTCACCAGCAGCAACCAG	20	100	19
				E176 2-3′	3′ primer	ACTCCACTTTGTGCAGAACAGATCT	25		
				E176-Taq	probe	CCGACGTGACCGACTACCACATCGA	25		
maize	GA21	construct-specific	A	GA21 3-5′	5′ primer	GAAGCCTCGGCAACGTCA	18	133	19
				GA21 3-3′	3′ primer	ATCCGGTTGGAAAGCGACTT	20		
				GA21-2-Taq	probe	AAGGATCCGGTGCATGGCCG	20		
maize	M810	construct-specific	A	M810 2-5′	5′ primer	GATGCCTTCTCCCTAGTGTTGA	22	113	19
				M810 2-3′	3′ primer	GGATGCACTCGTTGATGTTTG	21		
				M810-Taq	probe	AGATACCAAGCGGCCATGGACAACAA	26		
maize	M863	event-specific	A	M863 1-5′	5′ primer	TGACCCTACTTGTTCGGATGG	21	111	this study
				M863 1-3′	3′ primer	GCATTTGTAGGTGCCACCTTC	21		
				MON863-Taq	probe	CACCCCAAAGTGTACCAAGCTTTCCGA	27		
maize	NK603	event-specific	A	NK603 1-5′	5′ primer	GGCCAGCAAGCCTTGTAGC	19	113	this study
				NK603 1-3′	3′ primer	ATCCCGACTCTCTTCTCAAGCATA	24		
				NK603-Taq	probe	ATGACCTCGAGTAAGCTTGTTAACGCGGC	29		
maize	T25	event-specific	A	PM1	5′ primer	TCAATTGCCCTTTGGTCTTCTGA	23	155	22
				revPM1	3′ primer	TACGACATGATACTCCTTCCAC	22		
				FBP3	probe	TCATTGAGTCGTTCCGCCATTGTCG	25		
maize	TC1507	construct-specific	A	TC1507 1-5′	5′ primer	TGAGTTGATTCCAGTTACTGCCA	23	111	this study
				TC1507 1-3′	3′ primer	ATGTTAGTCGCAACGAAACCG	21		
				TC1507-Taq	probe	ACTCGAGTAAGGATCCGTCGACCTGCAG	28		
maize	MIR604	event-specific	B	MIR604 primer F	5′ primer	GCGCACGCAATTCAACAG	18	76	23
				MIR604 primer R	3′ primer	GGTCATAACGTGACTCCCTTAATTCT	26		
				MIR604 probe	probe	AGGCGGGAAACGACAATCTGATCATG	26		
maize	D59122	event-specific	B	DAS59122-7-rb1f	5′ primer	GGGATAAGCAAGTAAAAGCGCTC	23	86	24
				DAS59122-7-rb1r	3′ primer	CCTTAATTCTCCGCTCATGATCAG	24		
				DAS59122-7-rb1s probe	probe	TTTAAACTGAAGGCGGGAAACGACAA	26		
maize	M88017	event-specific	B	M88017 1-5′	5′ primer	ATCGTGTGACAACGCTAGCA	20	150	this study
				M88017 1-3′	3′ primer	CATATTGACCATCATACTCATTGCT	25		
				M88017-1-Taq	probe	TGCCGGAGTATGACGGTGACGATATATTCA	30		
soy	RRS	construct-specific	A	RRS 01-5′	5′ primer	CCTTTAGGATTTCAGCATCAGTGG	24	121	19
				RRS 01-3′	3′ primer	GACTTGTCGCCGGGAATG	18		
				RRS-Taq	probe	CGCAACCGCCCGCAAATCC	19		
soy	A2704	event-specific	B	KVM175	5′ primer	GCAAAAAAGCGGTTAGCTCCT	21	64	25
				SMO001	3′ primer	ATTCAGGCTGCGCAACTGTT	20		
				TM031	probe	CGGTCCTCCGATCGCCCTTCC	21		
soy	A5547	event-specific	B	A5547 1-5′	5′ primer	CATCGCTATTTGGTGGCATT	20	114	this study
				A5547 1-3′	3′ primer	GAATTATGCAGTGCTGCCATAAC	23		
				A5547-1-Taq	probe	CGCAATGTCATACCGTCATCGTTGTCAG	28		

For r-DNA Segment-Specific Detection

crop name	target name	evaluation	primer or probe name		sequence (5′-3′)	length (base)	amplicon size (bp)	ref
consensus	P35S	A	P35S 1-5′	5′ primer	ATTGATGTGATATCTCCACTGACGT	25	101	19
			P35S 1-3′	3′ primer	CCTCTCCAAATGAAATGAACTTCCT	25		
			P35S-Taq	probe	CCCACTATCCTTCGCAAGACCCTTCCT	27		
consensus	TNOS	A	NOS ter 2-5′	5′ primer	GTCTTGCGATGATTATCATATAATTTCTG	29	151	19
			NOS ter 2-3′	3′ primer	CGCTATATTTTGTTTTCTATCGCGT	25		
			NOS-Taq	probe	AGATGGGTTTTTATGATTAGAGTCCCGCAA	30		
consensus	PFMV	A	PFMV 1-5′	5′ primer	ATCAACAAGGTACGAGCCATATC	23	120	this study
			PFMV 1-3′	3′ primer	TGAGGCTTTGGACTGAGAATTC	22		
			PFMV-1-Taq	probe	CCAAGAAGGAACTCCCATCCTCAAAGGTTT	30		
consensus	AINT	B	AINT 2-5′	5′ primer	TCGTCAGGCTTAGATGTGCTAGA	23	112	this study

Table 1. Continued

For r-DNA Segment-Specific Detection (Cont.)

crop name	target name	evaluation	primer or probe name		sequence (5′-3′)	length (base)	amplicon size (bp)	ref
			AINT 2-3′	3′ primer	CTGCATTTGTCACAAATCATGAA	23		
			AINT-2-Taq	probe	TTTGTGGGTAGAATTTGAATCCCTCAGC	28		
consensus	NPTII	A	npt 1-5′	5′ primer	GACAGGTCGGTCTTGACAAAAAG	23	155	this study
			npt 1-3′	3′ primer	GAACAAGATGGATTGCACGC	20		
			NPT-1-Taq	probe	CCCTGCGCTGACAGCCGGA		19	
consensus	PAT	A	pat 1-5′	5′ primer	AAGAGTGGATTGATGATCTAGAGAGGT	27	161	this study
			pat 1-3′	3′ primer	ATGCCTATGTGACACGTAAACAGTACT	27		
			PAT-1-Taq	probe	TGCTTACGCTGGGCCCTGGAAG	22		
consensus	BAR	A	bar 2-5′	5′ primer	ACTGGGCTCCACGCTCTACA	20	186	this study
			bar 2-3′	3′ primer	AAACCCACGTCATGCCAGTTC	21		
			BAR-1-Taq	probe	CATGCTGCGGGCGGCCGGCTTCAAGCACGG	30		
consensus	GOX	B	gox 2-5′	5′ primer	TGCCAGGAAACTTGACTAGCG	21	103	this study
			gox 2-3′	3′ primer	CGAATCAACCAAGGCATGATG	21		
			GOX-1-Taq	probe	TCCAAAGTGGCTTCTTGACCCAATGG	26		
consensus	EPSPS1	A	epsps 1-5′	5′ primer	GCCTCGTGTCGGAAAACCCT	20	118	this study
			epsps 3-3′	3′ primer	TTCGTATCGGAGAGTTCGATCTTC	24		
			EPSPS-1-Taq	probe	TGGACGATGCCACGATGATCGC	22		
consensus	EPSPS2	B	EPSPS2 1-5′	5′ primer	GTCTCGTTTCTGAAAACCCTGT	22	118	this study
			EPSPS2 1-3′	3′ primer	TTAGTGTCGGAGAGTTCGATCTTAG	25		
			EPSPS2-1-Taq	probe	TGATCGCTACTAGCTTCCCAGAGTTCATGG	30		

For Endogenous Reference Gene-Specific Detection

crop name	target name	evaluation	primer or probe name		sequence (5′-3′)	length	amplicon (bp)	ref
maize	SSIIb	A	SSIIb 3—5′	5′ primer	CCAATCCTTTGACATCTGCTCC	22	114	26
			SSIIb 3-3′	3′ primer	GATCAGCTTTGGGTCCGGA		19	
			SSIIb-Taq	probe	AGCAAAGTCAGAGCGCTGCAA		21	
soy	Le1	A	Le1n02-5′	5′ primer	GCCCTCTACTCCACCCCCA	19	118	19
			Le1n02-3′	3′ primer	GCCCATCTGCAAGCCTTTTT		20	
			Le1-Taq	probe	AGCTTCGCCGCTTCCTTCAACTTCAC		26	
rice	SPS	A	SPSF	5′ primer	TTGCGCCTGAACGGATAT	18	81	27
			SPSR	3′ primer	CGGTTGATCTTTTCGGGATG		20	
			SPSP	probe	GACGCACGGACGGCTCGGA		19	
canola	HMG	A	hmg-F	5′ primer	GGTCGTCCTCCTAAGGCGAAAG	22	99	28
			hmg-R	3′ primer	CTTCTTCGGCGGTCGTCCAC		20	
			hmg-P	probe	CGGAGCCACTCGGTGCCGCAACTT		24	
consensus	18SrRNA	A	18SrRNA 2-5′	5′ primer	TGTTGGCCTTCGGGATCGGAGTA	23	111	this study
			18SrRNA 2-3′	3′ primer	GCTTTCGCAGTTGTTCGTCTTTCA	24		
			18SrRNA-2-Taq	probe	TCGGGGGCATTCGTATTTCATAGTCAGA	28		

For Donor Organism-Specific Detection

crop name	target name	evaluation	primer or probe name		sequence (5′-3′)	length	amplicon (bp)	ref
CaMV	CaMV	A	CaMVF	5′ primer	GGCCATTACGCCAACGAAT	19	89	29
			CaMVR	3′ primer	ATGGGCTGGAGACCCAATTTT		21	
			CaMV-MGB	probe	TTCTCCGAGCTTTGTC		16	

for the specific detection of M863, NK603, and TC1507 were obtained through personal communication with Kodama et al. We designed primer-probe sets for the detection of M88017 and A5547 by referring to the nucleotide sequence information from international patents WO/2005/059103 and WO/2006/108675, respectively. For recombinant DNA (r-DNA) segment-specific detection, we selected 10 target segments commonly introduced into some GM crops approved in Japan: the 35S promoter region derived from CaMV (P35S), 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). With respect to the detection of PFMV and AINT, the primer and probe sets were designed by referring to the nucleotide sequence information from GenBank (Accession Nos. NC003554 and X63830, respectively). For NPTII, PAT, BAR, and GOX detection, the previously reported primers (*30*) were adopted and probes were designed between the primers by referring to the nucleotide sequence information from GenBank (Accession Nos. U00004, DQ156557, X05822, and AR016595, respectively). The primers for EPSPS1 have been previously reported (*30*), and the probe was designed by referring to nucleotide sequences (U.S. Patent 5633435 SEQ ID No. 2 and GenBank Accession No. AB209952). The primers and probe for EPSPS2 were designed by referring to a nucleotide sequence (U.S. Patent 5633435 SEQ ID No. 9). For endogenous reference gene-specific 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). The 18SrRNA gene sequences of some kinds of crops such as *Zea mays* (GenBank Accession No. AF168884), *Hordeum vulgare* (GenBank Accession No. AY552749), *Gossypium hirsutum* (GenBank Accession No. L24145), *Solanum tuberosum* (GenBank Accession No. X67238), *Oryza sativa* (GenBank Accession No. AF069218), *Glycine max* (GenBank Accession No. X02623), *Nicotiana tabacum* (GenBank Accession No. AJ236016), and *Triticum aestivum* (GenBank Accession No. AJ272181) were aligned, and the DNA region that matched completely in these crops was selected for the design of the primer-probe set. For donor organism-specific detection, CaMV was selected as a target.

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 1 μM probe was added into each well of a 96-well plate and sealed with MicroAmp Optical Adhesive Film (Applied Biosystems). 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) 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), and then data analysis was carried out using Sequence Detection Software version 1.4 (Applied Biosystems). The thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 45 cycles of 15 s 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 Specificity. To evaluate the specificity, DNA extraction was performed twice from each ground sample, and each DNA sample was analyzed three times. For A2704, A5547, and LLRICE62, two parallel dilutions of CRM samples were performed and the resultant samples were analyzed three times each. Specificity was confirmed when the six sets of PCR data corresponded to the relevant information. The A- and B-groups of the primer-probe sets listed in **Table 1** were evaluated separately.

Evaluation of Sensitivity. 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-line mixtures of GM maize (Bt11, E176, GA21, M810, M863, NK603, T25, and TC1507), 0.25% Bt11, 0.25% E176, 0.25% the 8-line 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 analyzed five times each. The A- and B-groups of the primer-probe sets listed in **Table 1** were evaluated separately. To evaluate the sensitivity of CaMV detection, DNA extracts were prepared from ground plant leaves infected with the virus (MAFF No. 104019), and they were then analyzed in order to calculate the copy numbers of P35S derived from CaMV by quantitative analysis of the P35S region using the standard method for the detection of GM crops in Japan, as detailed in "The Genetically Modified Food Test and Analysis Manual for Individual Products" by the Food and Agricultural Materials Inspection Center (*31, 32*). 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. Two dilution samples for each concentration were prepared, and each was analyzed five times.

Evaluation of Linearity of Amplification in Semiquantitative Analyses. We evaluated linearity with respect to cycle threshold (Ct) values and the copy number of target DNAs. Detections for Bt11, E176, GA21, M810, P35S, TNOS, and SSIIb were selected as a representative detection system in the

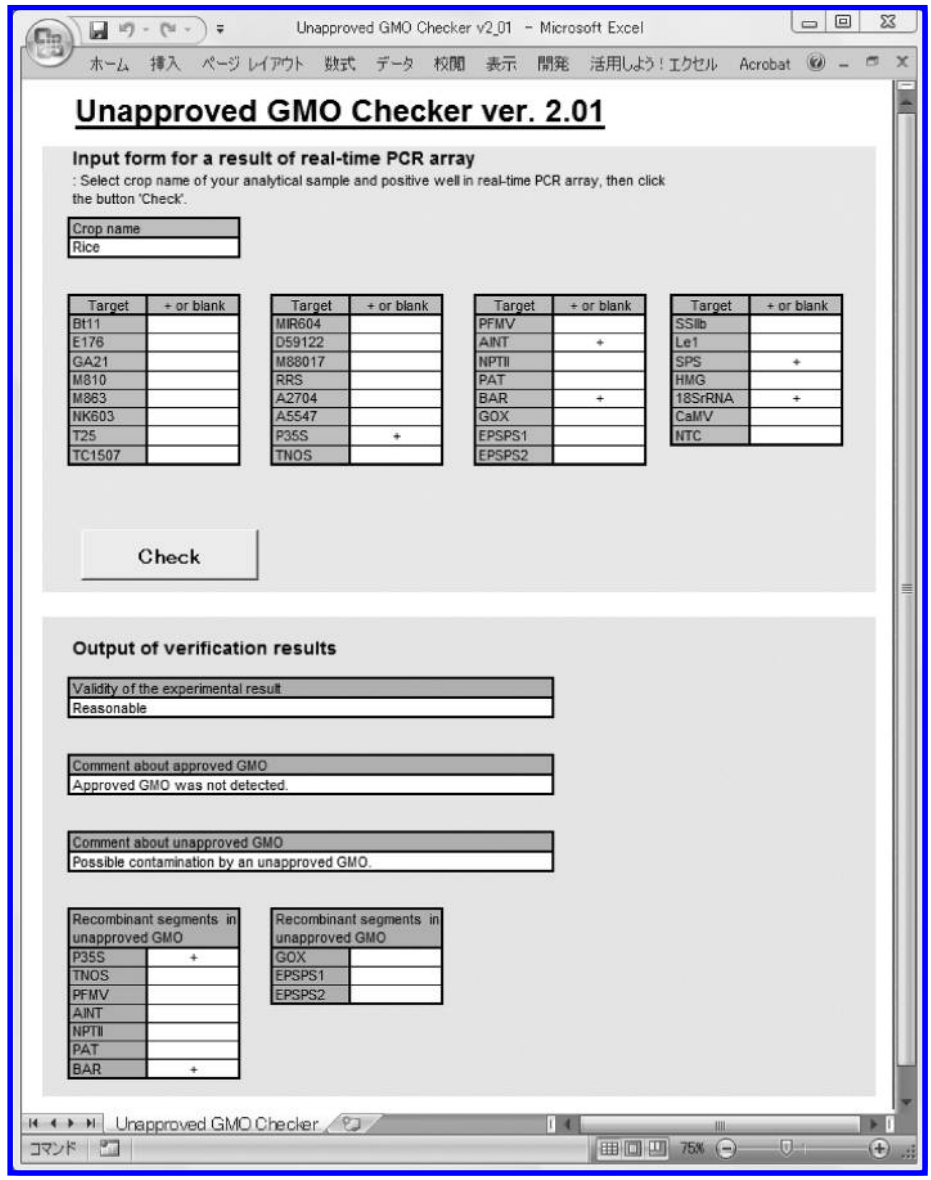

Figure 1. Spreadsheet application, Unapproved GMO Checker version 2.01.

real-time PCR array. Five concentrations (8, 50, 600, 8000, and 100 000 copies per reaction) of control plasmids (*19*) containing the nucleotide sequences of the above-mentioned seven targets were purchased from Nippon Gene.

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 integrates experimental results in the input form and estimates the possibility of unapproved GM crop contamination. 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. The logics in the macro were designed so that the validity of experimental results would be confirmed first. For this purpose, the checker examines the correspondence of all of the relationships between the selected crop name and the results of endogenous reference gene-specific detection, as well as those between the selected crop name and the results of GM line-specific detection. Additionally, it checks whether all the expected r-DNA segments elicited from the results of GM line-specific 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 line-specific 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 line-specific detection would be compared with the results of r-DNA segment-specific 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"

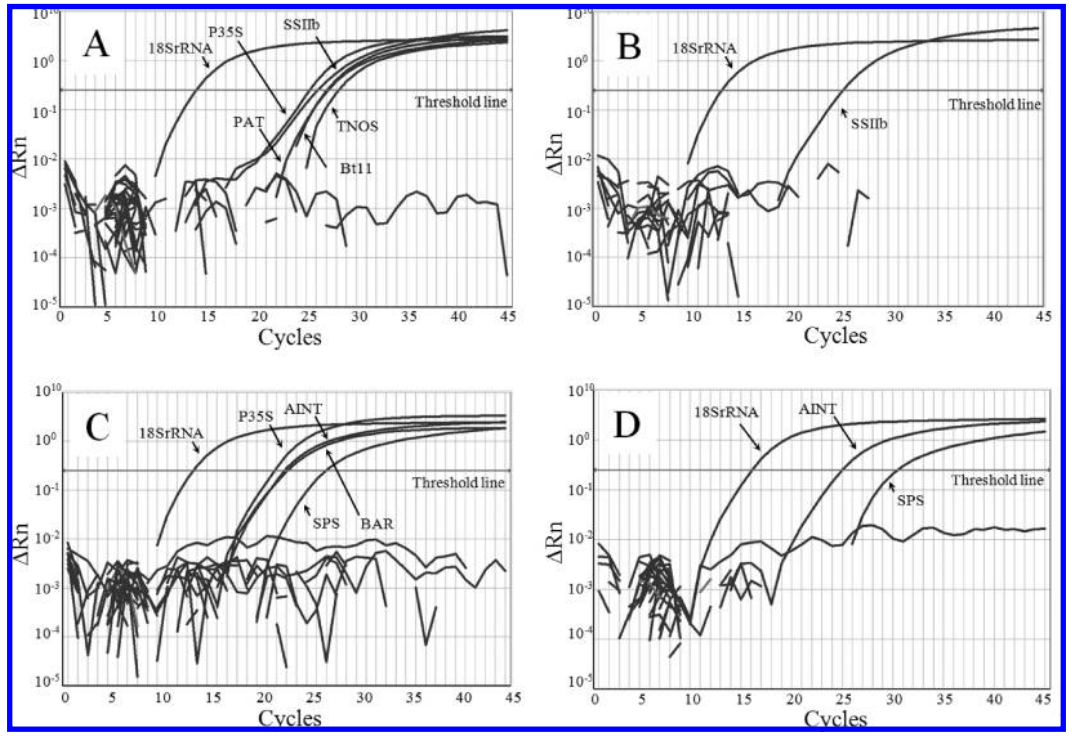

Figure 2. Representative amplification curves in real-time PCR array results. The horizontal axis indicates the cycle numbers of PCR, and the vertical axis indicates the ΔRn values, which are the relative values automatically calculated by the analysis software based on signal intensities of FAM dye 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 that were determined to be positive.

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".

RESULTS AND DISCUSSION

Design of the Real-Time PCR Array. The real-time PCR array was designed for the comprehensive detection of GM crops. In the present investigation, a total of 30 primer-probe sets were prepared for GM line-specific detection including event-specific and construct-specific detections as previously reported by Holst-Jensen et al. (*20*), r-DNA segment-specific detection, endogenous reference gene-specific detection as a positive control test, and donor organism detection as a negative control test. GM line-specific primer-probe sets were designed for the detection of representative GM maize and soy lines, aiming for the efficient detection of approved GM maize and soy lines. Meanwhile, all 69 lines of LMOs approved for open-field cultivation or provision as food, feed, or ornamental plants under Cartagena Protocol domestic law in Japan were expected to contain at least one target of the r-DNA segment detections based on the published information. The r-DNAs common in many GM crop lines were confirmed to be amplified, and r-DNA segment-specific detection was found to be effective for the detection of an extremely broad range of GM crop lines. The present system is expected to be a useful tool for screening GM crops regardless of GM lines 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 standard methods and the validated European methods according to the Web site of "Community Reference Laboratory for GM Food and Feed" (http://gmo-crl.jrc.it/statusofdoss.htm). For cost reduction, the volume of the reaction mixtures was set at 10 μL, which is the smallest volume recommended by the manufacturer of the real-time PCR apparatus. The total experimental work for one assay including the preparation of the PCR mixtures, thermal cycling, and data analysis took only 3 h. Representative assay results of the real-time PCR array with 31 targets containing 30 designed reactions and a negative control reaction with no primer-probe set 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 regarding GM crops. The present real-time PCR array successfully distinguished GM and non-GM crops and provided information regarding the GM lines and the r-DNA segments by a simple assay.

Evaluation of Specificity. To evaluate the specificity, the samples were prepared and six assays were performed. None of the qualitative results presented in **Table 2** showed any discrepancy with the expected results based on the published information about recombinant DNA from the Agbios website (http://www.agbios.com/main.php). In addition to the results shown in **Table 2**, non-GM wheat, barley, cotton, sugar beet, and potato samples were assayed, and only 18SrRNA was positive as predicted. Throughout the specificity evaluation, no nonspecific 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 crops including maize, soy, rice, canola, wheat, barley, cotton, sugar beet, and potato. With respect to CaMV detection, the DNA samples extracted from three types of CaMV-infected plant leaves were assayed and specific amplification was identified. In addition

Table 2. Results of Specificity Evaluation

type of detection	target name	sample 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
GM line-specific detection	Bt11	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	E176	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	GA21	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	M810	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	M863	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	NK603	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	T25	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	TC1507	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
	MIR604	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
	D59122	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
	M88017	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
	RRS	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
	A2704	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
	A5547	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
r-DNA segment-specific detection	P35S	+	+	−	+	+	+	+	+	−	+	+	−	+	+	+	−	+	−	−	−
	TNOS	+	−	+	−	+	+	+	+	+	−	+	−	+	−	−	−	−	−	−	−
	PFMV	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
	AINT	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−	+	+	−	−
	NPTII	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	PAT	+	−	−	−	−	−	+	+	−	+	−	−	−	+	+	−	−	−	−	−
	BAR	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
	GOX	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
	EPSPS1	−	−	−	−	−	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−
	EPSPS2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
endogenous reference gene-specific detection	SSIIb	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
	Le1	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
	SPS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−
	HMG	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+
	18SrRNA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
donor organism-specific detection	CaMV	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
negative control	NTC	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Table 3. Results of Sensitivity Evaluation[a]

type of detection	target name	sample name										
		A	B	C	D	E	F	G	H	I	J	K
GM line-specific 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-specific 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-specific 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-specific 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

[a] Sample A, 0.1% M863; B, 0.1% RRS; C, 0.25% Bt11; D, 0.25% E176; E, 0.25% the 8 lines 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.

to the CaMV detection, the P35S, HMG, and 18SrRNA detections were also positive for all infected leaf samples. The detection of P35S was attributed to the genome of infected CaMV, and the detection of HMG and 18SrRNA was caused by plant leaves of *Brassica napus* or a closely related species.

Evaluation of Sensitivity. The preparations of simulated samples and their 10 assays were performed as indicated in **Table 3**. 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 lines 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 line-specific and r-DNA-specific detections under low concentrations of target DNA (**Table 3**, asterisks). Because maize and canola seeds are reproduced through cross-pollination while soy and rice generally self-pollinate, some maize and canola seeds have a commonly heterozygous genome. Therefore, to evaluate the sensitivity for these crops, it is thought to be better to use heterozygous samples. Since the zygosities of the M88017 and RT73 CRMs purchased from AOCS have not been defined, we have to note that the copy number of target DNAs in a 0.25% M88017 or 0.25% RT73 sample corresponds to that of a sample made from absolutely heterozygous seeds with a concentration between 0.25% and 0.5%. Concerning CaMV detection, no false-negative results were obtained in the 10 assays with diluted DNA samples at a concentration of 100 copies/μL of CaMV DNA. Meanwhile, amplification failed 6 times in the 10 assays with the diluted samples at a concentration of 10 copies/μL of CaMV DNA. These results suggest 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 proposed method is sufficient for the inspection of unintended mixing of approved GM crops under Japanese regulations with a threshold of 5%.

Evaluation of Linearity of Amplification in Reactions. We evaluated the linearity of amplification in several representative detections using the control plasmids for the Japanese standard GM analytical method (*32*). The plasmids were tested six times, and the Ct values derived from amplification data were plotted against the log value of the copy number of control plasmids. The linear-regression curves from the means of the Ct values and that of the standard deviation in six assays were obtained (**Figure 3**). We found a relatively large dispersion of Ct values with a small copy number of plasmids. However, all R^2 coefficient values were above 0.99, and linearity of amplification for the seven detections was ascertained. In addition, because the shapes of the amplification curves in the reactions other than these seven reactions indicated in **Figure 3** were also similar and alteration of Ct values dependent on GM crop contents was observed (data not shown), all of the reactions were also expected to demonstrate similar linearity of amplification. Furthermore, TaqMan PCR is generally used for quantitative assays. Therefore, the present system shows potential for semiquantitative use, and further investigation is recommended.

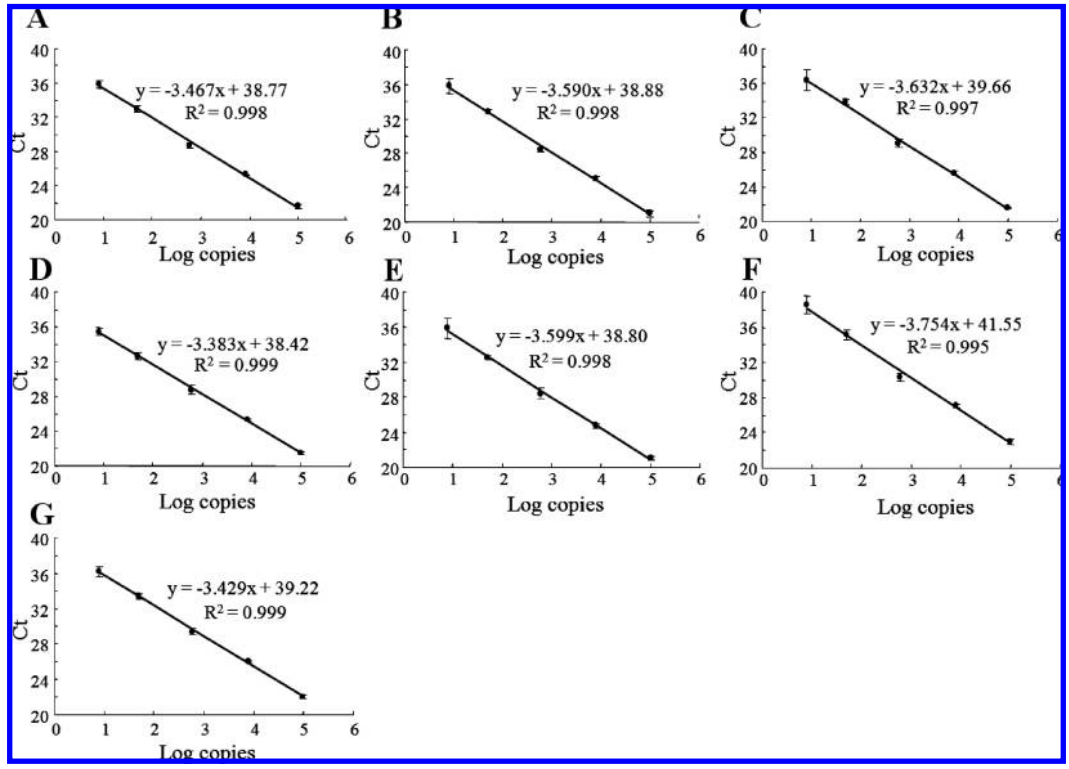

Figure 3. Evaluation of linearity of amplification in real-time PCR array. The calibration plasmids with five different copy numbers were assayed as described in the Materials and Methods section. The mean Ct values with standard deviations, derived from the amplification data of the reactions listed below, are plotted against the log values of the copy number of the control plasmids. The equation and correlation coefficient value for each linear regression curve are also indicated: (A) the Bt11 detection; (B) the E176 detection; (C) the GA21 detection; (D) the M810 detection; (E) the P35S detection; (F) the TNOS detection; and (G) the SSIIb detection.

Development of the Spreadsheet Application, Unapproved GMO Checker. We developed Unapproved GMO Checker version 2.01 as a spreadsheet application for the assumption of unapproved GM crop contamination (**Figure 1**). In the development of the application, 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 line-specific detections, while r-DNA segment-specific 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-specific detection with those of GM-line specific detection in the real-time PCR array. In the present investigation, unapproved GM crop lines were defined as GM crops that have not been approved for open-field cultivation or provision as food, feed, or ornamental plants under Cartagena Protocol domestic law in Japan. LLRICE62 is an unapproved GM crop line. On the basis of the results obtained by the present 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 regarding GM crops. The application is available and downloadable online (http://cse.naro.affrc.go.jp/jmano/index.html).

Application of the Real-Time PCR Array to the Assumption of Unapproved GM Crops. For the assumption of unapproved GM crop contamination, GM maize, GM soy, and GM rice were selected as targets. 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. Thus, an analytical sample of fewer than 200 seeds may be preferable. Because unapproved GM crops that 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 discern 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 line-specific 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. In addition, since the system is able to add new detection sets, it has great potential for expanding its analytical capacity, making use of feedback information from users about frequently detected approved and unapproved GM lines. A differential quantitative PCR technique was recently reported by Cankar et al. (*33*) as a new approach to unapproved/unknown GMO detection. Their method is based on quantitative assay, and the accuracy of quantitation with real-time PCR is indispensable. Our strategy is based on qualitative results, and higher reproducibility of the assumption of unapproved GM crop contamination is expected. For low concentrations of GM contents, the present qualitative assay strategy is advantageous.

In addition, our system may detect a much broader range of unapproved GM crops by the various r-DNA segment-specific detections. The utilization of semiquantitative analysis in the present method provides great potential for discovery of unapproved GM crops.

In the present investigation, we proposed a universal platform for GM detection. The developed real-time PCR array allows the comprehensive detection of GM crops and the assumption of contamination by unapproved GM crops. This approach is attractive in terms of the specificity of detection, the dynamic range of detection, 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.

ABBREVIATIONS USED

AOCS, American Oil Chemists' Society; A2704, A2704-12; A5547, A5547-127; EPSPS1, a region of 5-enolpyruvylshikimate-3-phosphate synthase gene introduced into NK603, M88017, and RRS; EPSPS2, a region of 5-enolpyruvylshikimate-3-phosphate synthase gene introduced into RT73; GOX, a region of the glyphosate oxidoreductase gene derived from *Ochrobactrum anthropi* strain LBAA; HMG, a region of the high-mobility-group protein I/Y gene of rapeseed; Le1, a region of the lectin 1 gene of *Glycine max*; NPTII, a region of the neomycin phosphotransferase II gene; PAT, a region of the phosphinothricin-*N*-acetyltransferase gene derived from *Streptomyces hygroscopicus*; BAR, a region of the phosphinothricin-*N*-acetyltransferase gene derived from *Streptomyces viridochromogenes*; SSIIb, a region of the starch synthase IIb gene of *Zea mays*; SPS, a region of the sucrose phosphate synthase gene of *Oryza sativa*; 18SrRNA, a region of the 18S rRNA gene common in crop plants; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamin; CaMV, *Cauliflower mosaic virus*; CRM, certified reference material; Ct, cycle threshold; D59122, DAS-59122; E176, Event176; GM, genetically modified; GMO, genetically modified organism; IRMM, Institute for Reference Materials and Measurements; LMO, living modified organism; M810, MON810; M863, MON863; M88017, MON88017; PCR, polymerase chain reaction; r-DNA, recombinant DNA; RRS, Roundup Ready Soybean; AINT, intron region of the rice Actin 1 gene; P35S, 35S promoter region derived from CaMV; PFMV, 35S promoter region of *Figwort mosaic virus*; TNOS, terminator region of the nopaline synthase gene derived from *Rhizobium radiobacter*; UV, ultraviolet.

ACKNOWLEDGMENT

The authors would like to thank Dow AgroSciences LLC, Monsanto Company, and Syngenta Seeds AG for providing GM maize and soy seeds. The authors would like to thank Takashi Kodama (Food and Agricultural Materials inspection center, Saitama, Japan) for kind provision of primer and probe sequence information.

LITERATURE CITED

(1) James, C. Executive summary: Global status of commercialized biotech/GM crops: 2006. *ISAAA Briefs* **2006**, 35.

(2) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A method of detecting recombinant DNAs from four lines of genetically modified maize. *J. Food Hyg. Soc. Jpn*. **2000**, *41* (2), 137–143.

(3) Matsuoka, T.; Kuribara, H.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. *J. Food Hyg. Soc. Jpn*. **2001**, *41* (1), 24–32.

(4) Onishi, M.; Matsuoka, T.; Kodama, T.; Kashiwaba, K.; Futo, S.; Akiyama, H.; Maitani, T.; Furui, S.; Oguchi, T.; Hino, A. Development of a multiplex polymerase chain reaction method for simultaneous detection of eight events of genetically modified maize. *J. Agric. Food Chem*. **2005**, *53*, 9713–9721.

(5) James, D.; Schmidt, A. M.; Wall, E.; Green, M.; Masri, S. Reliable detection and identification of genetically modified maize, soybean, and canola by multiplex PCR analysis. *J. Agric. Food Chem*. **2003**, *51*, 5829–5834.

(6) Rudi, K.; Rud, I.; Holck, A. A novel multiplex quantitative DNA array based PCR (MQDA-PCR) for quantification of transgenic maize in food and feed. *Nucleic Acids Res*. **2003**, *31* (11), e62.

(7) Bordoni, R.; Mezzelani, A.; Consolandi, C.; Frosini, A.; Rizzi, E.; Castiglioni, B.; Salati, C.; Marmiroli, N.; Marchelli, R.; Bernardi, L. R.; Battaglia, C.; Bellis, G. D. Detection and quantitation of genetically modified maize (Bt-176 transgenic maize) by applying ligation detection reaction and universal array technology. *J. Agric. Food Chem*. **2004**, *52*, 1049–1054.

(8) Bordoni, R.; Germini, A.; Mezzelani, A.; Marchelli, R.; Bellis, G. D. A microarray platform for parallel detection of five transgenic events in foods: a combined polymerase chain reaction-ligation detection reaction-universal array method. *J. Agric. Food Chem*. **2005**, *53*, 1049–1054.

(9) Peano, C.; Bordoni, R.; Gulli, M.; Mezzelani, A.; Samson, M. C.; De Bellis, G.; Marmiroli, N. Multiplex polymerase chain reaction and ligation detection reaction/universal array technology for traceability of genetically modified organisms in foods. *Anal. Biochem*. **2005**, *346*, 90–100.

(10) Germini, A.; Rossi, S.; Zanetti, A.; Corradini, R.; Fogher, C.; Marchelli, R. Development of a peptide nucleic acid array platform for the detection of genetically modified organisms in food. *J. Agric. Food Chem*. **2005**, *53* (10), 3958–3962.

(11) Xu, X.; Li, Y.; Zhao, H.; Wen, S. Y.; Wang, S. Q.; Huang, J.; Huang, K.; Luo, Y. B. Rapid and reliable detection and identification of GM events using multiplex PCR coupled with oligonucleotide microarray. *J. Agric. Food Chem*. **2005**, *53*, 3789–3794.

(12) Xu, J.; Miao, H.; Wu, H.; Huang, W.; Tang, R.; Qui, M.; Wen, J.; Zhu, S.; Li, Y. Screening genetically modified organisms using multiplex-PCR coupled with oligonucleotide microarray. *Biosens. Bioelectron*. **2006**, *22*, 71–77.

(13) Leimanis, S.; Hernandez, M.; Fernandez, S.; Boyer, F.; Burns, M.; Bruderer, S.; Glouden, T.; Harris, N.; Kaeppeli, O.; Philipp, P.; Pla, M.; Puigdomenech, P.; Vaitilingom, M.; Bertheau, Y.; Remacle, J. A microarray-based detection system for genetically modified (GM) food ingredients. *Plant Mol. Biol*. **2006**, *61*, 123–139.

(14) Schmidt, A. M.; Sahota, R.; Pope, D. S.; Lawrence, T. S.; Belton, M. P.; Rott, M. E. Detection of genetically modified canola using multiplex PCR coupled with oligonucleotide microarray hybridization. *J. Agric. Food Chem*. **2008**, *56*, 6791–6800.

(15) Su, W.; Song, S.; Long, M.; Liu, G. Multiplex polymerase chain reaction/membrane hybridization assay for detection of genetically modified organisms. *J. Biotechnol*. **2003**, *105*, 227–233.

(16) Markoulatos, P.; Siafakas, N.; Moncany, M. Multiplex polemerase chain reaction: a practical approach. *J. Clin. Lab. Anal*. **2002**, *16* (1), 47–51.

(17) Ratcliff, R. M.; Chang, G.; Kok, T.; Sloots, T. P. Molecular diagnosis of medical viruses. *Curr. Issues Mol. Biol*. **2007**, *9*, 87–102.

(18) Elnifro, E. M.; Ashshi, A. M.; Cooper, R. J.; Klapper, P. E. Multiplex PCR: Optimization and application in diagnostic virology. *Clin. Microbiol. Rev*. **2000**, *13* (4), 559–570.

(19) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically

modified maize and soybean. *J. AOAC Int*. **2002**, *85* (5), 1077–1089.
(20) Holst-Jensen, A.; Ronning, S. B.; Lovseth, A.; Berdal, K. G. PCR technology for screening and quantification of genetically modified organisms (GMOs). *Anal. Bioanal. Chem*. **2003**, *375*, 985–993.
(21) TaqMan Universal PCR master mix protocol. Applied Biosystems, Inc.: Foster City, CA.
(22) Collonnier, C.; Schattner, A.; Berthier, G.; Boyer, F.; Coué-Philippe, G.; Diolez, A.; Duplan, M. N.; Fernandez, S.; Kebdani, N.; Kobilinsky, A.; Romaniuk, M.; de Beuckeleer, M.; de Loose, M.; Windels, P.; Bertheau, Y. Characterization and event specific-detection by quantitative real-time PCR of T25 maize insert. *J. AOAC Int*. **2005**, *88* (2), 536–546.
(23) Community Reference Laboratory for GM Food & Feed. Event-specific method for the quantification of maize line MIR604 using real-time PCR (protocol). 2007, http://gmo-crl.jrc.it/summaries/MIR604_validated_Method.pdf.
(24) Community Reference Laboratory for GM Food & Feed. Event-specific method for the quantification of maize 59122 using real-time PCR (protocol). 2005, http://gmo-crl.jrc.it/summaries/59122-Protocol%20Validation.pdf.
(25) Community Reference Laboratory for GM Food & Feed. Event-specific method for the quantification of soybean line A2704-12 using real-time PCR (protocol). 2007, http://gmo-crl.jrc.it/summaries/A2704-12_soybean_validated_Method.pdf.
(26) Yoshimura, T.; Kuribara, H.; Matsuoka, T.; Kodama, T.; Iida, M.; Watanabe, T.; Akiyama, H.; Maitani, T.; Furui, S.; Hino, A. Applicability of the quantification of genetically modified organisms to foods processed from maize and soy. *J. Agric. Food Chem*. **2005**, *53* (6), 2052–2059.
(27) Ding, J.; Jia, J.; Yang, L.; Wen, H.; Zhang, C.; Liu, W.; Zhang, D. Validation of a rice specific gene, sucrose phosphate synthase, used as the endogenous reference gene for qualitative and real-time quantitative PCR detection of transgenes. *J. Agric. Food Chem*. **2004**, *52* (11), 3372–3377.
(28) Weng, H.; Yang, L.; Liu, Z.; Ding, J.; Pan, A.; Zhang, D. Novel reference gene, *high-mobility-group protein I/Y*, used in qualitative and real-time quantitative polymerase chain reaction detection of transgenic rapeseed cultivars. *J. AOAC Int*. **2005**, *88* (2), 577–584.
(29) Cankar, K.; Ravnikar, M.; Zel, J.; Gruden, K.; Toplak, N. Real-time polymerase chain reaction detection of cauliflower mosaic virus to complement the 35S screening assay for genetically modified organisms. *J. AOAC Int*. **2005**, *88* (3), 814–822.
(30) Matsuoka, T.; Kuribara, H.; Takubo, K.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Issiki, K.; Toyoda, M.; Hino, A. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J. Agric. Food Chem*. **2002**, *50*, 2100–2109.
(31) Moriuchi, R.; Monma, K.; Sagi, N.; Uno, N.; Kamata, K. Applicability of quantitative PCR to soy processed foods containing Roundup Ready soy. *Food Control* **2007**, *18*, 191–195.
(32) *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, 2002;available at http://www.famic.go.jp/technical_information/jashandbook/index.html.
(33) Cankar, K.; Chauvensy-Ancel, V.; Fortabat, M. N.; Gruden, K.; Kobilinsky, A.; Zel, J.; Bertheau, Y. Detection of nonauthorized genetically modified organisms using differential quantitative polymerase chain reaction: Application to 35S in maize. *Anal. Biochem*. **2008**, *376*, 189–199.

Received for review August 19, 2008. Revised manuscript received October 15, 2008. Accepted October 28, 2008. This research was supported in part by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan for the Japan Research Project "Assurance of Safe Use of Genetically Modified Organisms" and by a grant from the Ministry of Health, Labour and Welfare of Japan.

JF802551H