transgenic sFat-1 pigs. Jiangxi Nongye Xuebao (2012), 24 (5), 127-129.

カテゴリー	魚の種類	導入遺伝子（機能）	開発国	文献
観賞用	ゼブラフィッシュ	青色蛍光タンパク	米国	1
	ゼブラフィッシュ	FP635蛍光タンパク（紫色の蛍光を発する）	米国	2
環境モニタリング	ゼブラフィッシュ	GFP（エストロジェン様物質の検出）	韓国	3
	ゼブラフィッシュ	GFP（PCBの検出）	香港	4
	メダカ	GFP（内分泌撹乱物質の評価）	日本	5

表1　非食用バイオテクノロジー応用魚を作成した研究報告（2012年）

略語：green fluorescent protein (GFP), poly chlorinated biphenyl (PCB)

カテゴリー	生産物	開発国	文献
バイオリアクター	ニワトリ卵白リゾチーム/杉花粉アレルゲンの7つの主要ヒトT細胞エピトープに由来するペプチド	日本	6
	ヒト成長ホルモン	日本	7
	ヒトエリスロポエチン，腫瘍壊死因子受容体，一本鎖Fv /Fc断片	日本	8

表2　非食用バイオテクノロジー応用ニワトリを作成した研究報告（2012年）

略語：fragment crystallizable (Fc)

カテゴリー	導入あるいは改変遺伝子（研究内容）	開発国	文献
臓器移植	GTKO（肝細胞の性質を調べた）	米国	9
	hHO-1(酸化的ストレスから線維芽細胞を守る)	韓国	10
	GTKO, GTKO/CD46（肝をヒヒへ移植）	米国	11
	グルカゴン様ペプチド1（高グルカゴン濃度を達成）	ベルギー	12
	GTKO/TFPI, CD39, ヒルジン, トロンボモジュリン, 内皮細胞プロテインC受容体, CTLA4, A20, FAT-1, 水溶性TNF-α受容体	米国	13
	CTLA-4Igの高親和型（LEA29Y）（島細胞塊をマウスに移植して拒絶反応を抑制）	ドイツ	14
	GTKO/CD46（心をヒヒへ移植）	米国	15
	GTKO/CD46（脂肪の間葉性間質細胞の性質を調べた）	米国	16
	GTKO, GTKO/CD46（肝をヒヒへ移植）	米国	17
	GTKO, GTKO /CD46, GTKO/CD46/CD55, GTKO/CD46/トロンボモジュリン（ブタと霊長類における血液学的、生化学的、凝固のパラメーターを調べた）	米国	18
	GTKO（大動脈内皮細胞の性質を調べた）	米国	19
	ヒトトロンボモジュリン（内皮細胞で発現）	日本	20
	PERV特異的pol2 shRNA（発現を長期間観察）	ドイツ	21
	huTRAIL、huTRAIL/GGTA1KO/CD46（Jurkatリンパ腫細胞へのアポトーシス誘導効果あり）	ドイツ	22
	CD46（ブタウイルスの感染に影響なし）	米国	23
バイオリアクター	ヒトリゾチーム （糞中の微生物叢、土壌への影響を調べた）	中国	24
	血液凝固第9因子（ミルク中で生産）	中国	25
	ヒトI型コラーゲン、ヒトアルブミン	中国	26
	ヒトエリスロポエチン（精巣を調べた）	韓国	27
病原菌耐性付与	SIGLEC1遺伝子、CD163遺伝子不活性化（PRRSV耐性）	米国	28
	O型口蹄疫ウイルスshRNA（線維芽細胞はウイルスを阻害した）	中国	29
	古典的ブタ熱ウイルスの複製を阻害するRNAi	中国	30
	ベータディフェンシン2（抗バクテリア活性）	中国	31
	口蹄疫ウイルス受容体インテグリンb6サブユニット遺伝子ノックアウト（感染率が低下）	中国	32
	PRRSVのORF1b, ORF5, ORF6, ORF7に対するshRNAをコードするDNA（ウイルスに耐性）	中国	33
	IFITM3遺伝子（抗ウイルス活性を持つ可能性あり）	中国	34
その他	線虫Fat-1（脂肪酸組成のn-6/n-3比を下げた）	中国	35
	sFat-1 （導入遺伝子の次世代への伝達を調べた）	中国	36

表3 非食用バイオテクノロジー応用ブタを作成した研究報告（2012年）

略語：a1, 3-galactosyltransferase knockout (GTKO), human heme-oxygenase (hHO), complement regulatory (CD) , tissue factor pathway inhibitor (TFPI), cytotoxic T lymphocyte-associated antigen (CTLA), porcine endogenous retrovirus (PERV), shRNA (small hairpin RNA), human TNF-related apoptosis-inducing ligand (huTRAIL), glycoprotein galactosyltransferase alpha 1, 3 knockout (GGTA1KO), sialic acid binding Ig-like lectin 1(SIGLEC1), RNA interference (RNAi), interferon induced transmembrane protein 3 (IFITM3), fatty acid desaturase (Fat-1), omega-3 fatty acid desaturase (sFat-1)

開発国	非食用GM動物			
	魚	ニワトリ	ブタ	合計
米国	2	0	10	12
中国	0	0	11	11
ドイツ	0	0	3	3
日本	1	3	1	5
韓国	1	0	2	3
ベルギー	0	0	1	1
香港	1	0	0	1
合計	5	3	28	36

表4 非食用モダンバイオテクノロジー応用魚、ニワトリ、ブタについての開発国ごとの分類（2012年）

サンプル		ゲノミックDNA			Ct値(ニワトリ	Ct値
		収量 (μg/g)	A_{260} / A_{280}	A_{260} / A_{230}	cytochrome b遺伝子)	(hEpo遺伝子)
生の鶏肉	ムネ	58	1.85	2.33	16.5±0.2	u.d. (9)
	ササミ	110	1.91	2.36	18.2±0.6	u.d. (9)
	レバー	1000	1.90	2.36	17.2±0.9	u.d. (9)
	モモ	190	1.91	2.35	16.8±0.3	39.1 (1), u.d. (8)
	手羽元	170	1.90	2.41	16.3±0.3	u.d. (9)
	ひき肉	150	1.93	2.36	17.3±0.6	u.d. (9)
加工食品	唐揚げ	290	1.96	2.34	17.0±1.1	u.d. (9)
中の鶏肉	親子丼	430	1.95	2.30	18.2±0.4	39.5 (1), u.d. (8)
	焼き鳥(レバー)	770	1.96	2.31	16.1±0.3	39.4 (1), u.d. (8)
	チキンカツ	100	1.75	2.44	17.5±0.8	u.d. (9)
	照り焼き	69	1.73	2.41	16.8±1.0	39.8 (1), u.d. (8)
	チキンカレー	300	1.84	2.42	17.5±0.2	u.d. (9)

表 5 ニワトリゲノミック DNA の性質と、ニワトリ cytochrome b 遺伝子と hEpo 遺伝子の Ct 値

A260/A280 と A260/A230 はそれぞれ紫外吸収 260 nm と 280 nm の比と 260 nm と 230 nm の比である。リアルタイム PCR はトリプリケートで 3 回測定した。すなわち各サンプルは9つのウエルで測定した。ニワトリ cytochrome b 遺伝子の Ct 値については平均と標準偏差を示した。hEpo 遺伝子の Ct 値における括弧はその結果が得られたウエルの数を意味する。

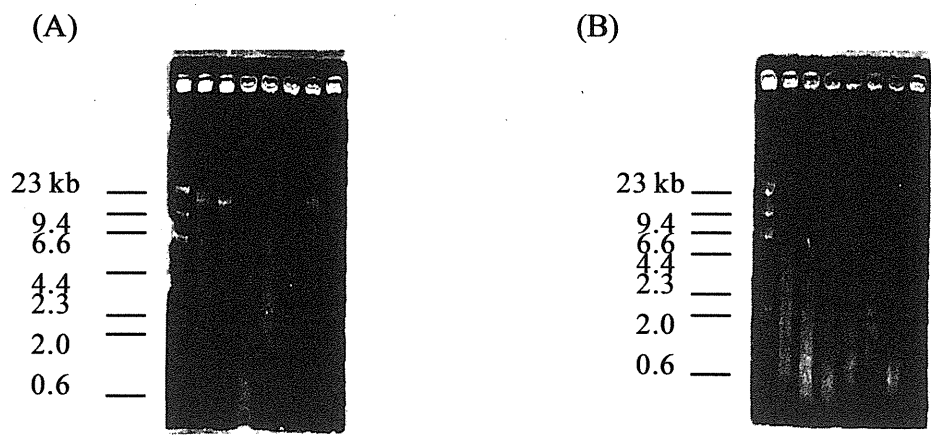

図1 0.8% (w/v)アガロースゲル電気泳動によるゲノミック DNA の分析

(A) 生の鶏肉のサンプルを分析した。左から右にレーンの番号を付ける。レーン1:DNA 分子量マーカー、レーン 2-7:ムネ、ササミ、レバー、モモ、手羽元、ひき肉から抽出したゲノミック DNA。

(B) 加工食品のサンプル中の鶏肉を分析した。レーン1:DNA 分子量マーカー、レーン 2-7:唐揚げ、親子丼、焼き鳥(レバー)、チキンカツ、照り焼き、チキンカレーから抽出したゲノミック DNA。

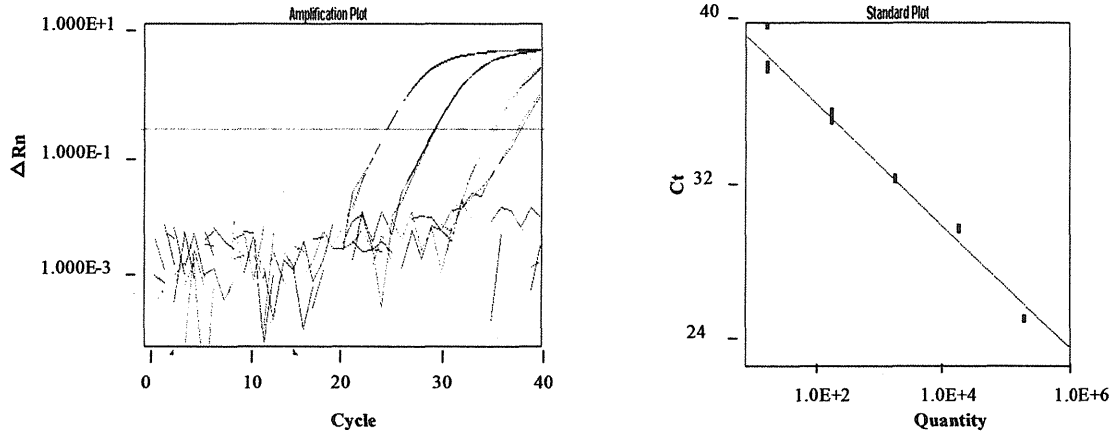

図2 ニワトリゲノミック DNA の非存在下での hEpo 遺伝子のリアルタイム PCR による分析

測定は 5 回行った。スタンダードプロットの式は以下の通りである。y=-3.29x + 42.7 (R^2 0.985), y=-3.21x + 42.4 (R^2 0.971), y=-3.07x + 42.8 (R^2 0.975), y=-3.21x + 42.5 (R^2 0.978), y=-3.21x + 43.3 (R^2 0.970). 最初の測定結果を図に示す。

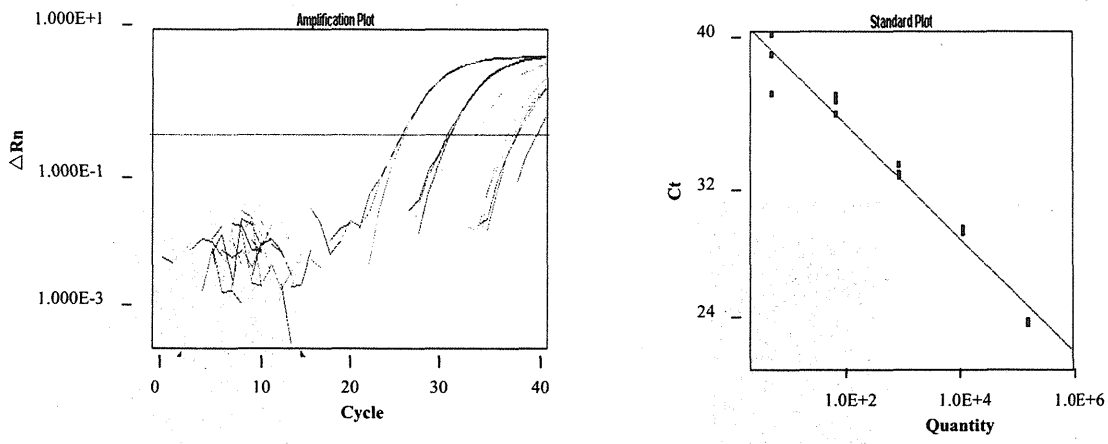

図3 ニワトリゲノミック DNA の存在下での hEpo 遺伝子のリアルタイム PCR による分析

測定は 5 回行った。スタンダードプロットの式は以下の通りである。y=-3.24x + 43.4 (R^2 0.964), y=-3.39x + 43.5 (R^2 0.978), y=-3.15x + 43.1 (R^2 0.965), y=-3.29x + 43.4 (R^2 0.986), y=-3.02x + 42.1 (R^2 0.967) .最初の測定結果を図に示す。

別紙４

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Note

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Comparison of signal enhancement techniques using DNA microarrays for screening GM crops

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Abstract

For the qualification and quantification of genetically modified (GM) crops without PCR, one possible alternative method is the detection of DNA fragments synthesized by random primers by DNA microarrays. Here, we used four signal enhancement techniques adopted in protocols for model target preparation of DNA microarrays and evaluated the detectable copy numbers of the targets. A 100-fold higher detectable copy number of the target was achieved using a fluorescently labeled dendrimer agent with a lower background level than using Cy3-labeled target as the control. This level was estimated to be sufficient for the detection of a single copy gene in GM maize genomic DNA. This model experiment suggests that DNA microarrays will be able to detect introduced genes of GM crops without PCR.

Keywords : DNA microarray, genetically modified organism (GMO), signal enhancement

I Introduction

Statistical data on the worldwide area under cultivation with genetically modified (GM) crops showed it was less than five hectares in 1996, but the area has been increasing, and the acreage of GM crops was over 148,000,000 hectares in 2010[1]. The year 1996 was memorable in Japan in that the first three GM crops were introduced with genes for herbicide and harmful insect resistance, traits allowed as safe for food use according to Japanese guidelines for the safety assessment of foods derived from plants containing recombinant DNA. Since then, new GM crops have been commercially developed and more than one hundred GM crops have been authorized through a safety assessment for commercial use in Japan. In particular, in GM maize, two or more individual GM plants having different traits such as herbicide and harmful insect resistance have been hybridized using conventional breeding and many varieties of hybridized GM crops, called stacked GM crops, have been produced. In 2011, 20 GM maize varieties produced by a single-gene introduction event were authorized as safe; 75 hybrids varieties hybridized in combination with these 20 GM maize varieties have completed safety assessment in Japan. The number of such stacked GM crops having two or more transgenes derived from hybridization of single-event GM varieties has increased[2], and the genetic structure of stacked GM crops is getting more complex as a result of multiple rounds of hybridization of single-event GM varieties.

Only GM crops authorized by a food and feed safety assessment can be imported and commercially distributed in most countries. In order to manage the risk regulation of GM crops to accept authorized ones but prohibit non-authorized ones on the market[3], detection methods have been required to identify individual GM crops. At the beginning of GM crop history, when the number of GM crop varieties was limited and single-event GM varieties but not hybridized varieties were commercially used, simple features that could be detected as proof of a GM crop were the introduced nucleic

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acid sequence and translated proteins derived from transgenics using PCR and immunological detection kits. PCR is mainly used to detect GM crops because the nucleic acid sequences often remain in some processed foods[4, 5], and immunological detection is faster, cheaper and more convenient than PCR but the proteins are degraded after food processing[6]. Detection methods based on PCR are highly sensitive, and not only qualitative but also quantitative analysis can be performed by real-time PCR. Because methods based on PCR require one pair of primers per target gene sequence, it is performed basically to detect the nucleotide sequence of a single target transgene in one tube. In order to enable it to detect multiple transgene nucleotide sequences per tube, detection methods combined with multiplex PCR have been developed[7, 8]. However, the number of transgenics that can be analyzed by multiplex PCR is limited; it is difficult to detect the dozens of transgene nucleotide sequences in complex hybrids.

DNA microarrays are used generally as a comprehensive analysis tool. It is possible to detect several tens of thousands of target gene sequences in one detection reaction with DNA microarrays. Because their sensitivity for detection of transgenes in GM crops is inferior to PCR-based methods, preamplification of target gene sequences may be necessary for detection using DNA microarrays[9, 10]. Specific primer sets are used to amplify objective target regions of genomic DNA before hybridization to DNA microarrays[11]. However, the recent increase in the variety and complexity of transgenics in GM crops makes it difficult to prepare primer sets to amplify the specific regions of individual transgenes and requires preparation of a large number of reaction tubes for the numerous primers.

The other defect of PCR for target gene sequences is the differing efficiency of the amplification, depending on the nucleotide sequences[12]. This difference effect on the amplified labeled products corresponds to the different targets applied to the DNA microarray, with the result that the detection signal strength is different but not quantitative for each target on the array. In order to avoid the quantitatively heterogeneous amplification of the labeled targets by PCR, in this study, we investigated a method of signal enhancement for more sensitive detection of target DNAs prepared without PCR amplification, in order to take advantage of the comprehensive and quantitative detection of DNA microarrays. We investigated four signal enhancement techniques on DNA microarrays using model DNAs corresponding to nucleotide sequences harbored in GM crops.

Ⅱ Materials and Methods

1. Plant materials and chemicals

Non-GM maize (dent) was obtained from National Institute of Health Sciences, Division of Food Additives. Cy3-, biotin- and digoxigenin (DIG)-labeled random nonamers (Cy3-9N, biotin-9N and DIG-9N, respectively) were purchased from Operon Biotechnologies, Tokyo, Japan. 9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate, diammonium salt (DDAO phosphate) was purchased from Life Technologies Corporation (Carlsbad CA, US). Lumi-Phos PPD, containing 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane], disodium salt was obtained from Lumigen Inc. (Southfield, MI, US), CDP-Star, containing disodium 2-chloro-5-(methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate, was from Roche Diagnostics (Mannheim, Germany), and 3DNA dendrimer was from Genisphere LLC (Hatfield, PA, US).

2. Preparation of DNA microarrays

All probes were diluted to a final concentration of 5 μM in PrimeSurface spotting solution (Sumitomo Bakelite Co., LTD., Kobe, Japan) and spotted onto PrimeSurface plastic slides using a MicroSys 4100 non-contact type spotter (Cartesian Technologies, Irvine, CA, US). To detect maize endogenous genes, we used *Zea mays alcohol dehydrogenase* (*adh*, 5'-AATCAGGGCTCATTTTCTCGCTCCTCA-3') and *Zea mays starch synthase II beta subunit* (*SSIIb*, 5'-AGCAAA GTCAGAGCGCTGCAATGCA-3') DNA fragment sequences, which were the probe sequences for the detection of GM maize using a quantitative PCR system[13]. For a negative control, two kinds of spots were spotted. One is blank spot for evaluating background level of the DNA microarray substrate. Another is a DNA fragment sequence of *Bacillus subtilis gyrase B* (5'-GACAGATGCCGAT-3') for evaluating nonspecific detection of signal enhancemet reaction on the DNA microarray. A 3'-Cy3–labeled oligo-DNA probe (Operon Biotechnologies, Tokyo, Japan) (5'- GACAGATGCCGAT-Cy3-3') was used for positioning markers spotted on PrimeSurface plastic slides. Their positions on the PrimeSurface slides are shown in Fig. 1.

3. Preparation of chemically labeled target DNA by Klenow fragment and hybridization

The genomic DNA of maize was extracted from kernels using a modified *N,N,N*-cetyltrimethylammmonium bromide method[14]. A 134 base pair DNA fragment of *adh* was amplified by PCR using the primers 5'-CCTCGTTTCCCATCTCTTCCTCC-3' and 5'-CCACTCCGAGACCCTCAGTC-3'. Amprified fragment was analysed by Agilent Bioanalyser 2100 system (Agilent Technologies, Inc., Santa Clara, CA, US), and subsequently its concentration was determined. The amount of the amplified DNA was measured and the total copy number was calculated by the length and concentration of the amplified fragment (data

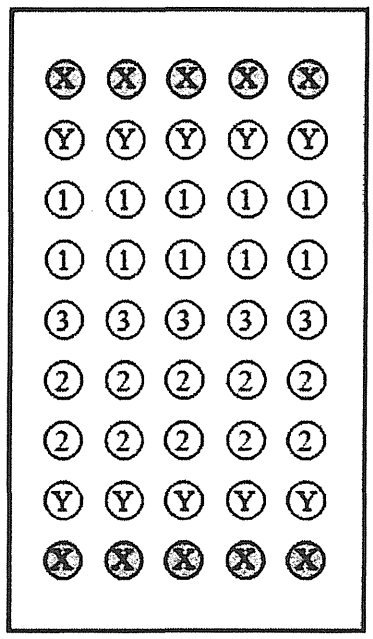

Fig. 1. A schematic presentation of DNA microarray spotted with specific probes
X, marker; Y, blank spot; 1, *adh*; 2, *SSIIb*; 3, *BsGyrB*

not shown). The DNA solution was diluted to 10^4 to 10^9 copies in 14 μL of water containing 4 μM Cy3-9N, biotin-9N or DIG-9N and denatured at 95°C for 5 min, then chilled on ice for 3 min, followed by the addition of 2.5 μL of 10 × Klenow buffer (Random Primer DNA Labeling Kit Ver. 2, TaKaRa Bio Inc., Shiga, Japan), 2.5 μL 0.2 mM dNTP mix, 1 μL Klenow fragment (2 U/μL) and 5 μL water in a total of 25 μL. After incubation at 37°C for 1 h, the reaction was terminated by heating at 65°C for 10 min. The target synthesized by Cy3-9N was for a control without signal enhancement, DIG-9N was for signal amplification by DDAO phosphate, CDP-Star, and Lumi-Phos PPD, and biotin-9N was for signal amplification with a 3DNA dendrimer.

The hybridization solution was 220 μL, consisting of 25 μL target solution, 0.1% sodium dodecyl sulfate (SDS), 0.45 M sodium chloride, 45 mM sodium citrate, pH 7.0 (3 × SSC), which was denatured at 95°C for 5 min, then chilled on ice for 3 min following hybridization. Hybridization was performed at 55°C for 4 h at 6 rpm using a G2534A hybridization gasket (Agilent Technologies, Santa Clara, CA, US), which was washed successively with three washing solutions: 3 × SSC, 0.1% SDS at 47°C for 5 min, 3 × SSC at 47°C for 5 min, then 0.2 × SSC at room temperature (RT) for 1 min.

4. Microarray detection

To detect the signals of targets synthesized by Cy3-9N, slides containing microarrays were air-dried after washing and placed in the detection instrument.

To detect targets synthesized by DIG-9N, after washing, the surface of the slides was left wetted with the final washing solution. A 30 μL aliquot of buffer containing 0.1 M maleic acid, 0.15 M sodium chloride, pH 7.5 was added to the slide, a CG00024 coverglass (Matsunami Glass Ind., Ltd., Osaka, Japan) was set on the slide, and it was incubated at RT for 5 min. After floating off the coverglass, 30 μL of anti-DIG antibody conjugated with alkaline phosphatase (AP) (anti-digoxigenin-AP, Fab fragments, Roche Diagnostics, Mannheim, Germany) was added to the slide at a final concentration of 150 mU/mL, which was covered with a new coverglass and incubated at RT for 30 min. After incubation, the coverglass was floated off and the slide was washed with washing buffer containing 0.1 M maleic acid, 0.15 M sodium chloride, 0.3% v/v Tween 20, pH 7.5 twice for 15 min. The washing buffer on the slide was exchanged with 30 μL of detection buffer containing 0.1 M maleic acid, 0.1 M sodium chloride, pH 7.5, the slide was covered with another coverglass and incubated for 5 min in order to equilibrate the DNA microarray on the slide.

For fluorescence detection by DDAO phosphate, after floating off the coverglass and detection buffer, being careful to leave the surface of the slide wet, 30 μL of 1 μg/mL DDAO phosphate was added to the slide, enough to cover the area of the DNA microarray, and the coverglass was immediately placed on the DNA microarray, which was set into the detection instrument as soon as possible.

For chemiluminescence detection by Lumi-Phos PPD or CDP-Star, 30 μL of Lumi-Phos PPD, diluted to 1/10 of the purchased solution with detection buffer, or 30 μL of undiluted CDP-Star was added to the slide, enough to cover the area of the DNA microarray; the cover glass was immediately placed onto the DNA microarray, which was set into the detection instrument as soon as possible.

To detect targets synthesized by biotin-9N using a 3DNA dendrimer, slides were hybridized using the same procedure described in paragraph 3 except that the DNA was synthesized with biotin-9N. After hybridization and washing, 30 μL of anti-biotin/Oyster-550 (900) labelled 3DNA dendrimer signal enhancement reagent, the concentration of 3DNA dendrimer is 2 ng/μL in 0.1 M maleic acid, 0.15 M sodium chloride, 0.3% (v/v) Tween 20, pH 7.5, was added to the slide, which was covered with a coverglass. After incubation at RT for 2 h, the coverglass was floated off and the slide was washed twice with 500 μL 0.1 M maleic acid, 0.15 M sodium chloride, 0.3% (v/v) Tween 20, pH 7.5 for 15 min, and finally 0.2 × SSC at RT for 1 min. After washing, the slide was air-dried and set into the detection instrument.

5. Detection instruments

The arrays were analyzed by an MB Biochip Reader™,

a charge-coupled device-based imaging system prototype (Yokogawa Electric Corporation, Tokyo, Japan)[15].

For the fluorescent detection of Cy3, we incorporated the proper filter set and laser system into the MB Biochip Reader™[15].

For the chemifluorescent detection of DDAO, we used MB Biochip Reader™ that had a filter set for emission at 656 nm and a laser system at 649 nm for fluorescent excitation of DDAO.

For the chemiluminescent detection of Lumi-Phos PPD or CDP-Star, we used an MB Biochip Reader™ without any filter set in order to detect chemiluminescence at all wavelengths.

Detection time was determined by the signal intensity of each spot on the DNA microarray in order to prevent the saturation of detection signal. The images obtained were analyzed using Image Pro and Array Pro Analyser software (Media Cybernetics, Inc., Bethesda, MD, US).

Ⅲ Results and Discussion

In order to investigate a model system to detect GM genes using DNA microarrays without labeling by PCR, we prepared an *adh* DNA fragment (134bp), which was obtained by PCR and labeled with Klenow fragment, as a target. For oligo-DNA probes immobilized on the DNA microarray, DNA fragments corresponding to two maize endogenous genes, *adh* and *SSIIb*, were selected because they are often used as taxon controls for GM gene detection systems[13]; in this study as we used only adh *adh* PCR products, the function of *ssIIb* spots was a kind of negative contorol. *BsGyrB* was selected as a negative control probe, described as II. Materials and Methods, paragraph 2.

In this study we defined a detected spot on the DNA microarray as a spot that presented larger fluorescence than that of *BsGyrB* and *SSIIb*. In addition, the mean value of fluorescence intensity of detected spot plus its value of standard deviation of must be larger value of thats of negative control spots.

First, target DNA fragments conjugated with Cy3 were synthesized by adding a Cy3 fluorescent molecule directly to the 5' end of the primer (Cy3-9N). One copy of the target DNA synthesized using Cy3-9N was conjugated to one molecule of Cy3 fluorescent chemical at the 5' end. This target molecule with Cy3-9N, one fluorescent molecule per target molecule, was considered the standard control for comparison with the other targets described below to amplify the signal intensity. Using the targets synthesized with Cy3-9N, over 1.0×10^8 copies of *adh* fragment in the reactions were detectable (Fig. 2A).

In comparison with this detectability as the standard, the signal enhancement was tested using chemifluorescence or chemiluminescence enhancement of DNA microarray detection. The target DNA was synthesized using DIG-9N. After the hybridization and washing, AP-conjugated anti-DIG antibody was reacted with the target DNA synthesized by DIG-9N hybridized to the probe DNA fragments on the DNA microarray. After washing out the excess antibody, detection reagents (DDAO-phosphate for chemifluorescence detection and Lumi-Phos PPD or CDP-Star for chemiluminescence detection) were applied to the microarray. DDAO phosphate could yield detectable level of 1.0×10^8 copies of *adh* fragment (Fig. 2B). The defect of this detection system was that the activated DDAO molecules that fluoresce in the AP reaction are not immobilized on the DNA microarray, so longer incubation of the reaction caused diffusion of DDAO fluorescence in the reaction mixture, resulting in a rise of the background fluorescence during detection. Therefore, the reaction time should be shortened not to improve detection level prolonging the incubation time on the DNA microarray (data not shown) and DDAO-phosphate gave us a lower detectable level than the Cy3 fluorescent control under our experimental conditions (Fig. 2B). In the case of chemiluminescent detection using CDP-Star and Lumi-Phos PPD, which are not diffusible in the reaction mixture and stick to the spots, 1.0×10^6 copies of *adh* target DNA could be detected, whereas chemiluminescent signals were observed for the *SSIIb*, Bs*GyrB* and blank spots as background signals (Fig. 2C, D).

Oyster-550 (900) 3DNA dendrimer conjugated with anti-biotin antibody is capable of making multiple linkages to the biotin molecule of the target DNA in the reaction mixture. After the formation of the linkage, the 3DNA dendrimer molecules are able to yield fluorescence fixed to the spots by air-drying, without diffusion of the molecules. As a result, 1.0×10^5 copies of *adh* could be detected on the microarray and signals were observed clearly, as well as when using the target DNA synthesized by Cy3-9N, without background fluorescence for the *SSIIb*, Bs*GyrB* or blank spots (Fig. 2E).

The copy number of a single copy gene was calculated as 4.0×10^5 copies per μg on the basis of the maze genome size (2.3×10^9 bp per haploid) reported previously[16]. In this study, using 3DNA dendrimer, 1.0×10^5 copies of a gene could be detected with Klenow fragment labeling without PCR amplification. Therefore, in case there are no defferences of labeling efficiency between the use of adh DNA fragment and maize genomic DNA as the templete of target DNA, detection of one copy of the transgene in less than 10 μg of GM maize genomic DNA is thought to be possible using this DNA microarray system.

The levels detectable above background for the signal enhancement techniques using CDP-Star (Fig. 2C), Lumi-

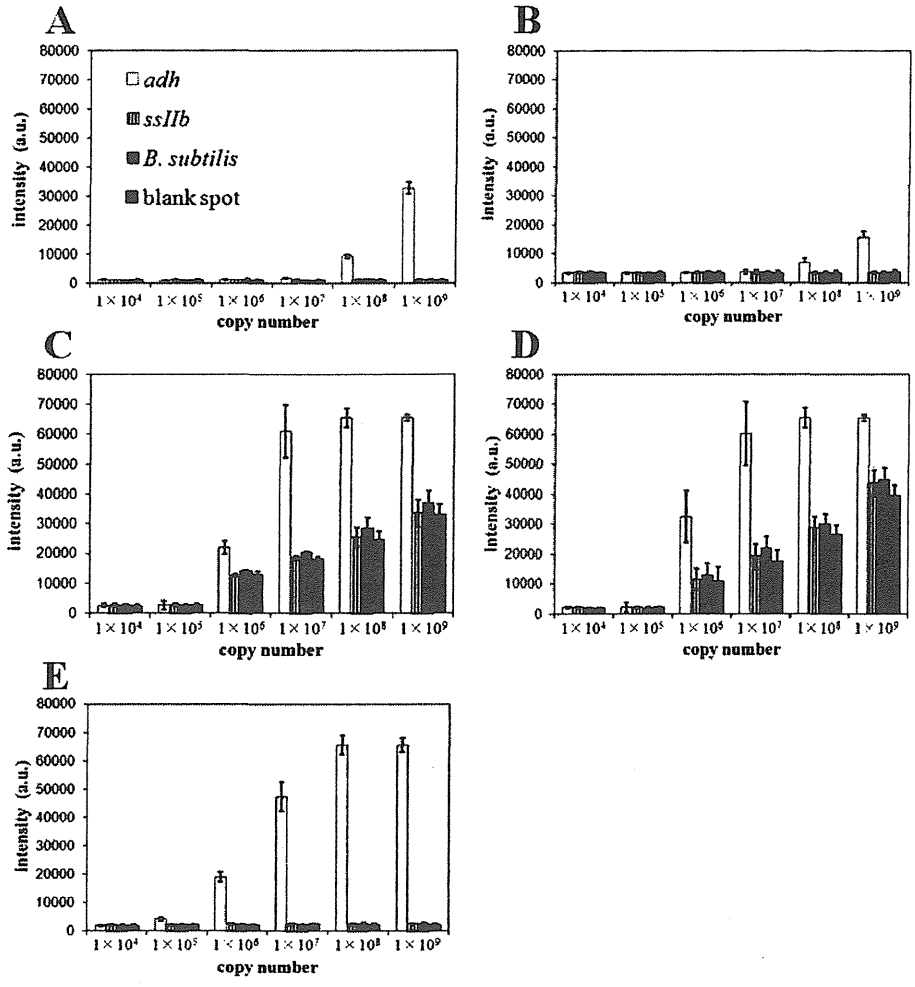

Fig. 2. Fluorescence intensity (A, B, E), and luminescence intensity (C, D) of each spot detected on DNA microarray

As template DNA, 10^4 to 10^9 copies of PCR-amplified DNA fragment of the *adh* gene was used to synthesize three kinds of targets, labeled with Cy3-9N (A), DIG-9N (B–D) and biotin-9N (E) using Klenow fragment and then hybridized to the probes on the DNA microarray. All images were measured by an MB Biochip Reader™ and integrated over 60 sec. A, the fluorescent signal intensity of spots hybridized with Cy3-9N–labeled target DNA was directly measured. B, after reaction with anti-DIG-AP, the chemifluorescence of DDAO-phosphate was detected and measured. C and D, after the reaction of anti-DIG-AP, the chemiluminescence of Lumi-Phos PPD (C) or CDP-Star (D) was detected and measured. E, 3DNA dendrimer conjugated with anti-biotin antibody was reacted with the hybridized microarray and its fluorescence were measured. In B, C, and D, extending the acquisition time over 60 sec caused signal diffusion and led to defective measurements and high background.

□ *adh* ▥ *ssIIb* ▩ BsGyrB ■ blank spot

Phos PPD (Fig. 2D) and 3DNA dendrimer (Fig. 2E) were over 1.0×10^6 copies of the *adh* fragment in the reaction. Although more reaction steps to detect signal were involved in these three methods than in the standard protocol using Cy3-9N, the detectability of these methods was more than 100 times higher than that of the standard protocol (Fig. 3). A lower background level was achieved by the 3DNA dendrimer method compared to the CDP-Star and Lumi-Phos PPD methods. Because fluorescently labeled dendrimers contained in the 3DNA dendrimer agent were immobilized at the spots on the DNA microarray, the free non-immobilized fluorescently labeled dendrimers could be washed out before the reading step and the fluorescent signals could be measured over 60 sec in the MB Biochip Reader™ detector. When using CDP-Star and Lumi-Phos PPD, as these systems are based on chemiluminescent detection, the reaction on the DNA microarray must be detected in real time. Therefore, we attributed the difference in background level to the condition of the materials at detection. Fluorescently labeled dendrimers conjugated with anti-biotin antibody combined with the biotin in the targets hybridized to the oligo-DNA probe on the DNA microarray. On the other hand, AP conjugated with anti-DIG antibody were combined with the DIG in the targets hybridized to the oligo-DNA probe on the DNA microarray, but CDP-Star and Lumi-Phos PPD, as substrates of AP, did not combine with the DIG after activation by AP.

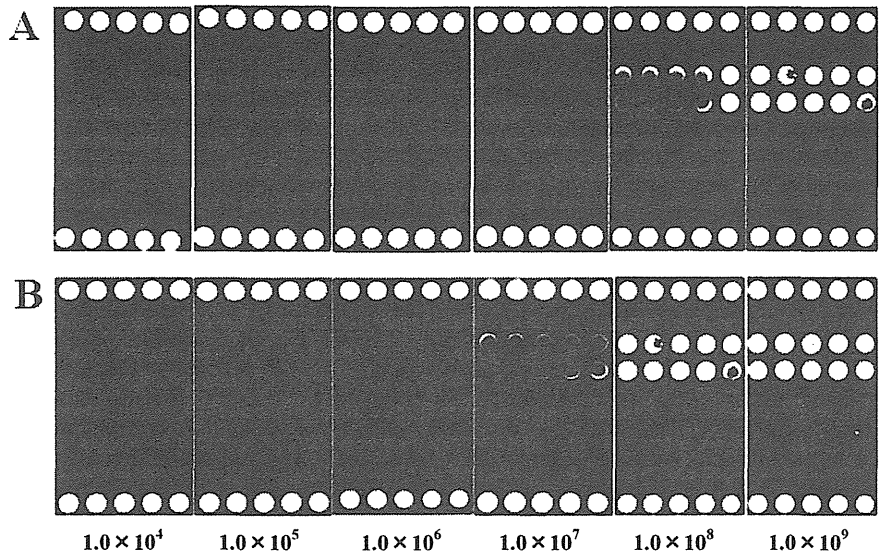

Fig. 3. Fluorescence image of a DNA microarray
A, image of the hybridized target labeled with Cy3-9N primer; B, image labeled with 3DNA dendrimer. All images were measured by an MB Biochip Reader™ detector and integrated for 60 sec.

The great advantage of DNA microarrays lies in the fact that over 10,000 targets having different nucleotide sequences can be qualified and quantified on one slide using a one-tube labeling reaction. The disadvantage of DNA microarrays is lower sensitivity than PCR, and, in order to overcome this disadvantage, recent protocols for target preparation for DNA microarrays require amplifying specific target sequences by PCR labeling[11] or rolling circle amplification[10], which both require preparation of a lot of specific primer pairs to amplify the target region of a lot of individual genes. The chemical signal enhancement system established here has the merit of not requiring specific primers for PCR amplification. Additionally, the different transgenes harbored in GM crops and different combinations of transgenes in stacked GM crops could be identified using a combination of specific probes corresponding to the nucleotide sequence of each individual promoter, coding region and terminator, leading to the possibility that multiple and complex GM hybrids might be distinguishable on one DNA microarray spotted with all probes corresponding to promoter, coding region and terminator sequences.

Ⅳ Acknowledgement

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Original Paper

Quantification and Identification of Genetically Modified Maize Events in Non-Identity Preserved Maize Samples in 2009 Using an Individual Kernel Detection System

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We investigated the GM maize grain content of non-identity preserved (non-IP) maize samples produced in 2009 in the USA using our individual kernel detection system, involving two multiplex qualitative PCR methods coupled to microchip electrophoresis and partially real-time PCR array analysis, to clarify how many GM event maize grains were present in the samples and which GM events frequently appeared in 2009. The average percentage and standard deviation of GM maize grains on a kernel basis in five non-IP sample lots were 81.9%±2.8%, the average percentage of single GM event grains was 46.9%, and the average percentage of stacked GM event grains was 35.0%. MON88017 grains and NK603 grains were the most frequently observed as single GM event grains. The most frequent stacked GM event grains were MON88017×MON810 grains. This study shows that our method can provide information about GM maize events present in imported maize samples on a kernel basis.

Key words: genetically modified maize; event; multiplex qualitative PCR; microchip electrophoresis

Introduction

Genetically modified (GM) crops are currently cultivated widely as sources of food and feed in many countries[1)]. GM crops generally have been assessed and authorized for food use by administrative authorities. In some countries, the labeling of grains, feed and foodstuffs is mandatory if the GM crop content exceeds a certain level of the approved GM varieties. For instance, the European Union, Japan and Korea have set threshold values of 0.9%, 5%, and 3%, respectively, of GM organism material in a non-GM background as the basis for labeling[*1–*7].

In Japan, non-GM crops are segregated as non-GM material and imported from the United States using an identity preserved (IP) handling system that requires documentary certification from US farms to Japanese processing traders. Recently, the production of stacked GM maize grains, in which two or more characteristic

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*1 Regulation (EC) No. 258/97 of the European Parliament and of the Council of Europe, 27 January 1997, *Official J. Eur. Communities* L 043, pp. 1–7.
*2 Commission Regulation (EC) No. 49/2000 of 10 January 2000, *Official J. Eur. Communities* L 6, pp. 13–14.
*3 Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of Europe, 22 September 2003. *Official J. Eur. Communities*. Available from URL: http://europa.eu.int/eurlex/pri/en/oj/dat/2003/l_268/l_26820031018en00010023.pdf
*4 Regulation (EC) No. 1830/2003 of the European Parliament and of the Council of Europe, 22 September 2003, *Official J. Eur. Communities*. Available from URL: http://eurlex.europa.eu/LexUriServ/site/en/oj/2003/l_268/l_26820031018en00240028.pdf
*5 Notification No. 517 of 31 March 2000, Ministry of Agriculture, Forestry and Fisheries of Japan.
*6 Notification No. 79 of 15 March 2000, Department of Food Safety, Ministry of Health, Labour and Welfare of Japan.
*7 Notification No. 2000–31 of 22 April 2000, Ministry of Agriculture and Forestry of Korea.

events have been inserted, has increased in the United States due to enhanced production efficiency[1]. Although the levels of adventitious commingling of GM maize in non-GM maize according to the labeling system refer to GM maize as a weight per weight (w/w) percentage, conventional applicable detection methods, such as quantitative real-time PCR, do not directly measure the w/w percentage of GM maize, but rather provide relative copy numbers between a specific DNA sequence and a taxon-specific DNA sequence, and these values are converted into a w/w percentage using appropriate reference materials. The GM maize content in a maize sample containing stacked GM maize grains, as determined by current quantitative real-time PCR methods, is likely to be overestimated compared to the actual w/w percentage of GM maize in the sample because the relative copy numbers are calculated on a haploid basis. To solve this problem, we have developed an individual kernel detection system that consists of grinding individual maize kernels, DNA extraction from each individual ground maize kernel, multiplex real-time PCR using the extracted DNA samples from individual ground maize kernels for GM detection, and multiplex qualitative PCR using the extracted DNA for GM event detection to analyze the precise commingling level and varieties of GM maize[2)-4)]. The detection system has already been implemented in Japan as an official GM maize detection method[*8].

It is important to investigate the content of GM maize commingled in actual maize samples that contain many GM maize grains, such as non-IP maize samples, in order to determine the main current GM maize and stacked GM maize events and to predict which events are likely to be commingled with IP maize samples. However, there has been little information on the determination of stacked GM maize in non-IP maize samples, because no method is available, except for detection in individual kernels. We previously investigated GM maize content on a kernel basis and determined the varieties of the GM kernels in non-IP maize samples imported from the USA in 2005[5)] using an individual kernel analysis system including a multiplex real-time PCR method[2), 3)], and coupled it to a multiplex qualitative PCR method[4)] followed by analysis using multi-channel capillary gel electrophoresis[5)].

The present study was designed to clarify the GM maize content of non-IP maize samples that contain GM maize produced in 2009, to investigate the content of GM maize grains, and to determine how many stacked GM maize grains are contained therein and which GM maize and stacked GM maize events frequently appeared in 2009 by using the multiplex real-time PCR method[2), 3)], two multiplex qualitative PCR detection methods[4), 6)] both coupled to the microchip electrophoresis and partially real-time PCR array analysis[7)].

[*8] Department of Food Safety, Ministry of Health, Labour and Welfare of Japan. Notice No. 0803, Article 8; Tokyo, 2009.

Experimental

Maize samples

The non-IP maize samples produced in 2009 were purchased from a trading company in Japan. Bt11, GA21 and MIR604 seeds were kindly provided by Syngenta. TC1507 and DAS 59122 seeds were kindly provided by Pioneer Hi-Bred International, Inc. Seeds of MON88017, MON810, MON863, NK603 and stacked maize were kindly provided by Monsanto Co. T25 maize seeds were imported directly from the USA as positive controls of GM maize.

Oligonucleotide primers and probes for multiplex real-time PCR method

Sets of primer pairs and TaqMan® probes for construct-specific and universal GM quantification were described in our previous papers[2), 3)]. The SSIIb-3 system (SSIIb 3–5′ and SSIIb 3–3′ with SSIIb-TaqV) was used for the primers and probe for the detection of the taxon specific gene encoding the maize starch synthase IIb (SSIIb) in the multiplex real-time PCR method, while the p35S-1 system (P35S 1–5′ and P35S 1–3′ with P35S-Taq) and GA21–3 system (GA21 3–5′ and GA21 3–3′ with GA21-Taq) were used in the multiplex real-time PCR method. All sets of primer pairs and Taq-Man® probe p35S-Taq for the detection of the cauliflower mosaic virus (CaMV) 35S promoter sequence (p35S) and GA21-Taq GA21 for the detection of specific sequence were purchased from Fasmac Co., Ltd. (Kanagawa, Japan). SSIIb-TaqV, which is labeled with VIC® at the 5′ and TAMRA™ at the 3′ ends, was synthesized by Life Technologies (Carlsbad, CA, USA) and used as a probe for the detection of SSIIb. The target sequence used by the p35S-1 system to detect the 35S promoter region derived from CaMV is widely found in the recombinant DNA of almost all GM events with the exception of GA21. The GA21–3 system was designed to detect the construct specific sequence of GM maize event GA21[2), 3)].

Grinding and DNA extraction of individual maize kernels

The grinding of individual maize kernels were performed according to previous reports[2), 3)]. DNA extraction and purification were carried out with the GM quicker 96 kit (Nippon Gene Co., Ltd., Tokyo, Japan) using the method described here; genomic DNA extraction from the ground powder of individual kernels was performed according to the kit procedure. GE1 buffer and RNase A solution (100 mg/mL) were mixed to make a working solution at respective volumes of 1.5 mL and 5 μL. A 1.5 mL aliquot of the working solution was added to each sample tube containing the ground maize powder and metal corn. Twenty-four sample tubes were arrayed in the tube holder. The maize powder and working solution were mixed by vigorously shaking the tubes and the metal corn in a multi-bead shocker at 2,000 rpm for 15 s and incubated for 10 min at room temperature. A 180 μL aliquot of GE2-K buffer solution was then added to each

solution. The sample tube was capped to avoid leakage, and vigorously shaken for 15 s in the multi-bead shocker. The tube holder was centrifuged for 10 min at 1,400 $\times g$ using a Metalfuge centrifuge (MBG100; Yasui Kikai Co., Ltd., Osaka, Japan). A 400 μL aliquot of each supernatant was carefully transferred to a 96-well plate. A 250 μL aliquot of GB3 buffer–isopropanol (1 : 1, v/v) was added. A 650 μL aliquot of each sample was then carefully transferred to the 96-well column plate, which was centrifuged for 20 min at 1,400$\times g$. After removal of the filtrate, 650 μL of GW buffer was added to each well. The 96-well column plate was centrifuged for 10 min at 1,400 $\times g$. After removal of the filtrate, the 96-well column plate was recentrifuged for 20 min at 1,400$\times g$. The plate was placed in a collection plate and 50 μL of DW was added to each well. The plate was incubated for 3 min at room temperature, and then centrifuged for 10 min at 1,400$\times g$. For DNA extraction from individual maize kernels, we used a glass-fiber silica-plate base sheet (EPM 2000; GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England) for the 96-well column plate.

Multiplex real-time PCR conditions

To simultaneously detect the genomic DNA from individual GM maize kernels and to confirm the validity of PCR amplification of the extracted genomic DNA, multiplex real-time PCR analyses were performed according to previous papers[2), 3), 5)]. The amplification curves of the target sequence were monitored using a fluorescent dye, which was used to label the designed oligonucleotide probes, using an ABI PRISM® 7900HT sequence detection system (Life Technologies). The reaction volume of 25 μL contained 2.5 μL of the sample genomic DNA (10 ng/μL), 12.5 μL of Universal Master Mix® (Life Technologies), 0.5 μM each primer pair, and 0.2 μM probe (except 0.1 μM for the p35S probe). The PCR step-cycle program was as follows: 2 min at 50℃, and 95℃ for 10 min followed by 45 cycles of 30 s at 95℃ and 1 min 30 s at 59℃.

If an amplification curve indicating GMO detection could be clearly observed after 15 cycles, we considered the sample as positive for GMOs; otherwise, it was considered negative, because we adopted exponential character of the amplification curve after 15 cycles of real-time PCR as the threshold for discrimination of GM and non-GM maize kernels in previous studies[2), 3), 5)]. In this study, the GM Maize Detection Plasmid Set–ColE1/TE– (Nippon Gene Co.) was used as the positive control. This plasmid set contains six concentrations of the reference plasmid pMul5, into which has been inserted the amplification products of p35S, GA21 and SSIIb, diluted with TE buffer (pH 8.0) including 5 ng/μL of the ColE1 plasmid[3), 5)]. The ColE1 plasmid contained none of the amplification GM products, and was used as the negative control. The positive controls were prepared using two concentrations of the plasmid, set at 250,000 and 1,500 copies per plate. In the reaction plate, real-time PCR was performed in duplicate (each two wells) for the negative control, and for one positive control (250,000 copies) and for the other positive control (1,500 copies). The other 90 reaction wells were used for genomic DNA samples extracted from individual maize kernels.

Two multiple qualitative PCR methods

To identify which GM event grains are contained in genomic DNA extracted from individual kernels, two multiple qualitative PCR detections were performed according to our previously reported methods[4)]. The first method was performed for the detection of MON810, NK603, T25, GA21, TC1507, Event176, Bt11, and MON863 (construct specific)[4)]. The reaction mixture for PCR was prepared in a 96-well plate. The reaction volume of 25 μL contained 25 ng genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 1.25 units AmpliTaq Gold® DNA polymerase (Life Technologies), and 15 primers at the following concentrations: 0.2 μmol/L for M810 1–5′, NK603 1–5′ M863 1–5′, M863 1–3′, Bt11 1–5′, and CryIA 1–3′; 0.1 μmol/L for T25 2–5′, T25 2–3′, GA21 1–5′, GA21 1–3′, TC1507 1–5′ and TC1507 1–3′; 0.05 μmol/L for Event 176 1–5′; and 0.045 μmol/L for SSIIb 1–5′ and SSIIb 1–3′. The reactions were buffered with PCR buffer II (Life Technologies) and amplified in a Silver 96-well GeneAmp PCR System 9700 (Life Technologies) thermal cycler in max mode, according to the following PCR step-cycle program: pre-incubation at 95℃ for 10 min, 10 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 65℃ for 1 min, and extension at 72℃ for 1 min; 27 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 60℃ for 1 min, and extension at 72℃ for 1 min; followed by a final extension at 72℃ for 7 min.

The second method was performed for the detection of DAS-59122-7, MIR604, MON863 (event-specific) and MON88017[6)]. The reaction mixture for PCR was prepared in a 96-well plate. The reaction volume of 25 μL contained 25 ng of genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, and 0.625 units AmpliTaq Gold® DNA polymerase (Life Technologies) and nine sets of primers at the following concentrations: 0.50 μmol/L for MON88017-mF and MON88017-mR; 0.40 μmol/L for MON863-mF and MON863-mR, 0.30 μmol/L for DAS59122-7-rb1R; 0.25 μmol/L for DAS59122-7-rb1F; 0.15 μmol/L for MIR604-mF; 0.06 μmol/L for SSIIb 3–5′ and SSIIb 3–3′. The PCR conditions were the same as for the first method described above. Both methods were followed by microchip electrophoresis analysis.

Microchip electrophoresis analysis

The PCR products of multiple samples were analyzed using an MCE-202 MultiNA™ microchip electrophoresis system (Shimadzu, Kyoto, Japan). This system uses microchip technology for automated electrophoretic separation at high sample throughput using a 96-well PCR plate and high-sensitivity fluorescence detection. The analysis was run essentially according to the manufacturer's instruction manual using a DNA-1000 reagent kit (Shimadzu), which consists of Separation Buffer,

DNA Marker Solution (100–1,000 bp), and DNA-100 Ladder solution. The Separation Buffer was used to dilute SYBR® Gold (Life Technologies) 100-fold to prepare the DNA-1000 Separation Buffer solution for analysis. The DNA-1000 Ladder solution was prepared using $\phi\times$ 174 DNA/*Hae*III Markers (Promega, WI, USA), which was diluted 100-fold in TE buffer (10 mM Tris–HCl buffer containing 50 mM KCl, 1.5 mM $MgCl_2$). The PCR products and the diluted DNA-100 Ladder solution in a MicroAmp Optical 96-well reaction plate (Life Technologies) were placed into the instrument alongside the reagents. The samples and reagents were mixed automatically on-chip and run using MultiNA Control and MultiNA Viewer software (Shimadzu).

Preparation of real-time PCR array, reaction conditions and data analysis

To clarify GM events in genomic DNAs from some GM grains that gave ambiguous results in analyses using the two multiplex qualitative PCR detection methods, a real-time PCR array was employed according to our previously reported method with some modifications[7]. The following detection targets were selected for one analysis: Bt11, E176, GA21, M810, M863, NK603, T25, TC1507, DAS59122, M88017, MIR604 and SSIIb. To prepare the real-time PCR array, 2 μL of a primer and probe mixture for each detection target, containing 2.5 μM primers and 1 μM probe, was added into each well of a 96-well plate, which was sealed with MicroAmp® Optical Adhesive Film (Life Technologies). Array plates containing primer and probe mixtures stored at −20℃ until just before use. For assaying sample DNA with the real-time PCR array, the diluted DNA samples described above, TaqMan® Universal PCR Master Mix (Life Technologies) and sterile distilled water were mixed and added to 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 5′ primer, 5 pmol 3′ primer, 2 pmol probe and 5 μL TaqMan® Universal PCR Master Mix. The plates containing the reaction mixtures were sealed with MicroAmp Optical Adhesive Film, and thermally cycled with the ABI 7500 real-time PCR system (Life Technologies) or ABI PRISM® 7900HT Sequence Detection System (Life Technologies). The data were analyzed using Sequence Detection Software Version 1.4 for the 7500 system and Version 2.3 for the 7900HT system. The thermal cycling conditions were as follows: 2 min at 50℃, 10 min at 95℃, 45 cycles of 15 s at 95℃ and 1 min at 60℃ under 9600 emulation mode. Data were analyzed using the "Amplification Plot" feature of the analysis software with detail settings 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 that crossed the threshold were determined to be positive.

Table 1. GM maize grain contents on a kernel basis in five non-IP maize samples in 2009

Non-IP maize sample lot	Kernel number			GM content (%)
	Non-GM	GM	Total	
1	46	243	289	84.1
2	46	191	237	80.6
3	43	169	212	79.7
4	32	189	221	85.5
5	48	181	229	79.0
Total	215	973	1,188	81.9

Results

Determination of GM maize content in non-IP maize samples using multiplex real-time PCR

We randomly sampled 212 to 289 kernels from each of the five non-IP maize samples produced in 2009 and performed single kernel analyses using the multiplex real-time PCR method. The multiplex real-time PCR method allowed us to individually discriminate GM maize from non-GM maize and simultaneously evaluate the quality of the extracted genomic DNA for PCR in one run. As shown in Table 1, the GM maize content on a kernel basis in the five non-IP maize samples was 84.1%, 80.6%, 79.7%, 85.5% and 79.0%, respectively, and their average value and standard deviation were 81.9%±2.8%. This result indicates that the average ratio of GM maize in non-IP maize samples in 2009 was higher than in 2005[5]. In addition, although the GM maize ratios in non-IP maize samples in 2005 varied greatly (28.3–77.2%), the standard deviation (2.8%) of the GM maize ratio in non-IP maize samples in 2009 was small.

GM event analysis using multiplex qualitative PCR

Next, the genomic DNA extracted from positive kernels of five non-IP maize grain samples was individually analyzed using two multiplex qualitative PCR detection methods[4,6] both coupled to microchip electrophoresis and a partially real-time PCR array method[7], to clarify whether these GM events are present as single or stacked events and which event is present in the genomic DNA from each kernel.

The percentage of the single GM event population and that of the stacked GM event population in each non-IP maize sample are shown in Table 2. The values for the single and stacked GM events are also indicated in the pie charts in Figs. 1 and 2, respectively. For the single GM event grains, MON88017 grains and NK603 grains were mainly detected in four samples (No. 1, No. 2, No. 4, No. 5), although MON810 grains were mainly detected in sample No. 3. For the stacked GM event grains, mainly MON88017×MON810 grains and TC1507×DAS59122 grains were detected in four samples (No. 1, No. 2, No. 4, No. 5), although MON810×NK603 grains were mainly detected in sample No. 3.

The total populations of the non-GM grains, single GM event grains and stacked GM event grains in the