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

Table 2. Results of the analyses of individual kernels in all grains of the non-IP maize samples in 2009.

GM trait	Non-IP maize sample lot											
	1		2		3		4		5		Total	
	Kernel number	Content (%)	Kernel number	Content (%)	Kernel number	Content (%)	Kernel number	Content (%)	Kernel number	Content (%)	Kernel number	Content (%)
Bt11	3	1.0	2	0.8	5	2.4	8	3.6	2	0.9	20	1.7
TC1507	4	1.4	16	6.8	15	7.1	7	3.2	13	5.7	55	4.6
MON810	11	3.8	8	3.4	40	18.9	19	8.6	11	4.8	89	7.5
MON863	8	2.8	4	1.7	2	0.9	1	0.5	8	3.5	23	1.9
MON88017	97	33.6	28	11.8	8	3.8	26	11.8	60	26.2	219	18.4
GA21	1	0.3	0	0	1	0.5	2	0.9	0	0	4	0.3
NK603	18	6.2	21	8.9	22	10.4	24	10.9	18	7.9	103	8.7
DAS59122	11	3.8	8	3.4	1	0.5	2	0.9	7	3.1	29	2.4
MIR604	6	2.1	1	0.4	1	0.5	1	0.5	1	0.4	10	0.8
T25	2	0.7	0	0	1	0.5	1	0.5	1	0.4	5	0.4
Single GM	161	55.7	88	37.1	96	45.3	91	41.2	121	52.8	557	46.9
Bt11×MIR604	0	0	0	0	0	0	4	1.8	3	1.3	7	0.6
Bt11×GA21	2	0.7	0	0	1	0.5	2	0.9	0	0	5	0.4
TC1507×MON88017	2	0.7	8	3.4	9	4.2	2	0.9	4	1.7	25	2.1
TC1507×NK603	4	1.4	4	1.7	6	2.8	2	0.9	0	0	16	1.3
TC1507×DAS59122	21	7.3	17	7.2	0	0	8	3.6	16	7.0	62	5.2
MON810×MON863	1	0.3	4	1.7	0	0	4	1.8	1	0.4	10	0.8
MON810×MON88017	42	14.5	54	22.8	24	11.3	52	23.5	26	11.4	198	16.7
MON810×NK603	7	2.4	8	3.4	26	12.3	16	7.2	3	1.3	60	5.1
MON863×NK603	0	0	0	0	2	0.9	0	0	0	0	2	0.2
MON88017×DAS59122	0	0	0	0	0	0	0	0	2	0.9	2	0.2
NK603×DAS59122	0	0	1	0.4	2	0.9	0	0	2	0.9	5	0.4
Bt11×MIR604×GA21	0	0	0	0	0	0	2	0.9	0	0	2	0.2
TC1507×MON810×NK603	0	0	0	0	0	0	0	0	0	0	0	0
TC1507×MON88017×DAS59122	1	0.3	2	0.8	0	0	1	0.5	0	0	4	0.3
TC1507×NK603×DAS59122	0	0	1	0.4	2	0.9	3	1.4	0	0	6	0.5
MON810×MON863×NK603	2	0.7	4	1.7	1	0.5	2	0.9	3	1.3	12	1.0
Stacked GM	82	28.4	103	43.5	73	34.4	98	44.3	60	26.2	416	35.0
GM	243	84.1	191	80.6	169	79.7	189	85.5	181	79.0	973	81.9
Non-GM	46	15.9	46	19.4	43	20.3	32	14.5	48	21.0	215	18.1
Total	289	100	237	100	212	100	221	100	229	100	1,188	100

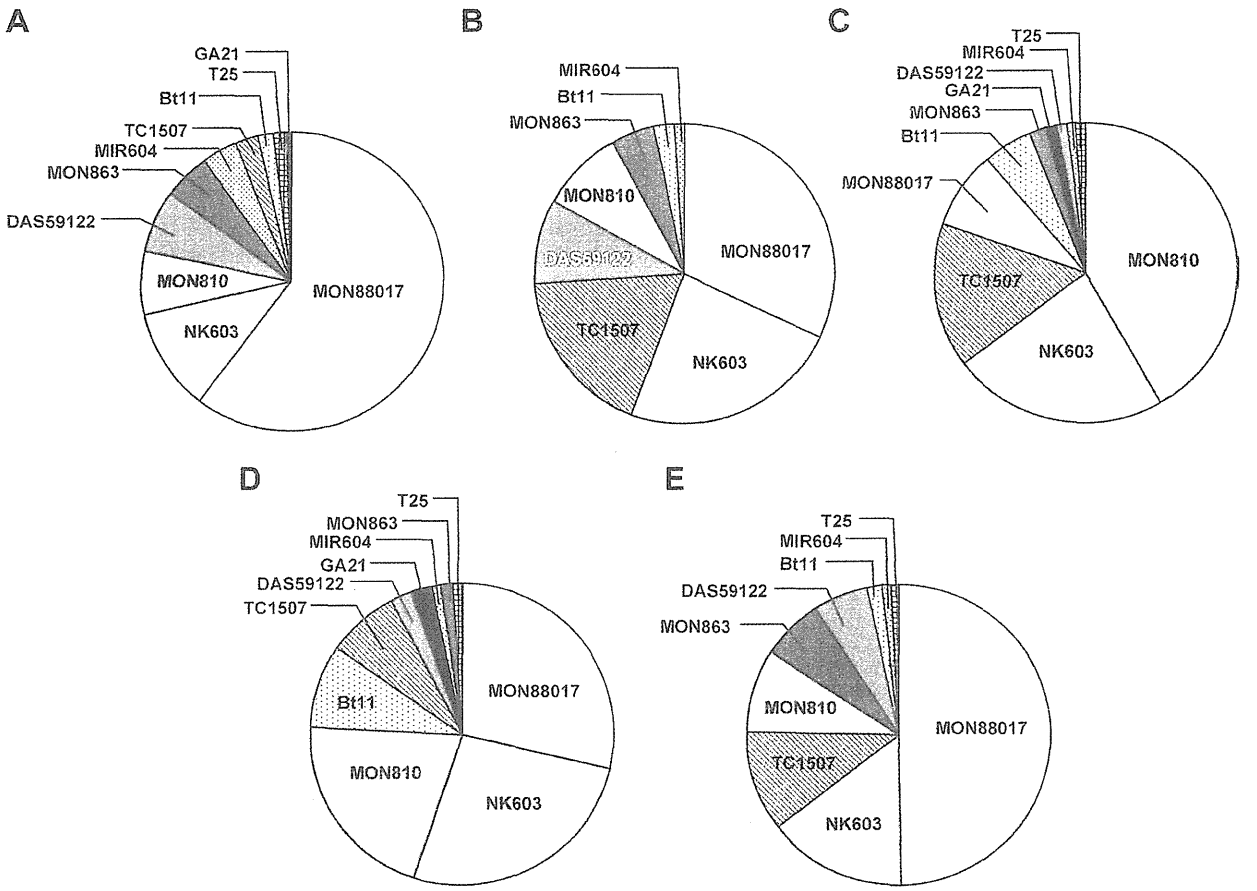

Fig. 1. Pie chart representing the single GM event grain population in five non-IP maize samples in 2009
A–E show the results for sample, No. 1–5, respectively.

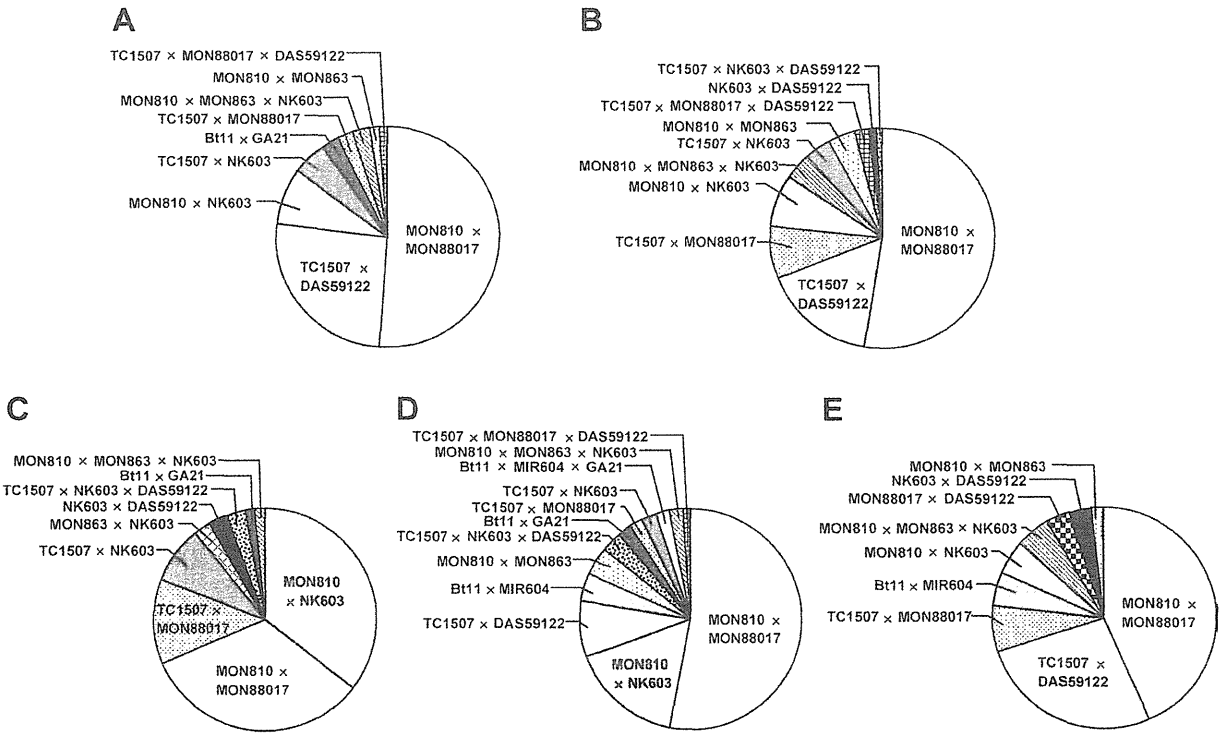

Fig. 2. Pie chart representing the stacked GM event grain population in five non-IP maize samples in 2009
A–E show the results for sample, No. 1–5, respectively.

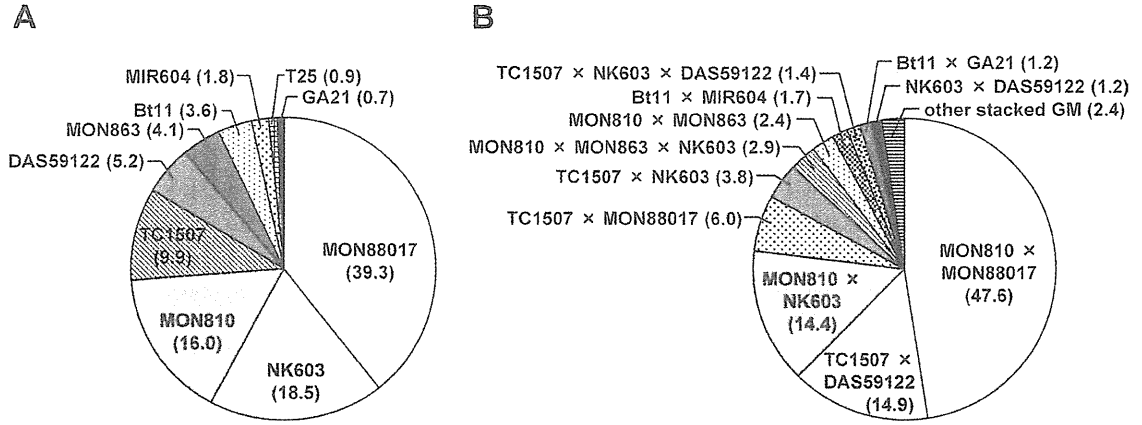

Fig. 3. Pie chart of the average populations of single GM event grains (A) and stacked GM event grains (B) in the non-IP maize kernels analyzed. The values in parentheses show the percentage of each population in the single and stacked GM event grains.

1188 kernels of non-IP samples tested in 2009 are shown in Table 2. The average percentage of GM grains in the samples was 81.9%, and, in the GM grains, the average percentage of the single GM event grains was 46.9% and that of the stacked GM event grains was 35.0%. These results show that the average ratio (35.0%) of the stacked GM event grain population in non-IP maize samples in 2009 was higher than in 2005[5] (12.0%).

The populations of single GM event grains and stacked GM event grains are indicated in the pie chart of Fig. 3. For single GM events, MON88017 (39.3%) and the NK603 grains (18.5%) were mainly detected, followed by MON810 (16.0%), TC1507 (9.9%) and DAS 59122 grains (5.2%). Most of the detected stacked GM event grains were MON810×MON88017 (47.6%), followed by the TC1507×DAS59122 (14.9%), MON810×NK603 (14.4%), TC1507×MON88017 (6.0%), TC1507×NK603 (3.8%) and MON810×MON863×NK603 (2.9%).

Discussion

To date, many GM maize events have been authorized for import into Japan. The allowed single GM events include resistance to feeding damage by the European corn borer (ECB) (Event 176 and Bt11 from Syngenta Seeds AG (formerly Novartis Seeds), MON810 from Monsanto Company), resistance to corn rootworm (*e.g.*, MON863 from Monsanto Company), tolerance to the herbicide phosphinothricin (PPT) (*e.g.*, T25 from Bayer Crop Science), resistance to the ECB and tolerance to the herbicide PPT (TC1507 from Pioneer Hi-Bred International, Inc., Mycogen Seeds/Dow AgroSciences LLC), tolerance to the herbicide glyphosate (GA21 and NK603, Monsanto Company), resistance to corn rootworm and tolerance to the herbicide glyphosate (MON88017, Monsanto Company), resistance to corn rootworm and tolerance to the herbicide PPT (DAS59122 from Dow AgroSciences LLC/Pioneer Hi-Bred International, Inc.). Furthermore, many stacked event maize varieties (*e.g.*, MON863×NK603, MON810×NK603, MON810×GA21, MON810×T25, MON88017×MON810, TC1507×NK603, MON863×MON810 and MON863×MON810×NK603) have already been authorized in Japan.

In this study, we found that GM maize grains of non-IP maize samples produced in 2009 were present at a high level, in the range of 79.7% to 85.5%. In addition, we found that the ratio of the stacked GM event grains in the non-IP maize samples in 2009 was higher than in 2005[5]. The evidence implies that the cultured area of GM maize in the USA has increased, and in particular, the ratio of the stacked GM maize grains in non-IP maize samples has increased from 2005 to 2009.

The National Agricultural Statistics Service (NASS) reported that the percentage of the cultivation area planted with any GM maize events in 2005, 2008 and 2009 in the USA was 52%, 80% and 85%, respectively, and the percentage for stacked GM events in 2005, 2008 and 2009 was 9%, 40% and 46%, respectively[*9, *10]. Thus, the percentage of the cultivation area planted with GM maize events has increased substantially from 2005 to 2009. In particular, the area planted with GM maize events in Illinois, Indiana and Ohio has increased more than that in other states. In addition, the percentage of the cultivation area planted with stacked GM maize events has significantly increased from 2005 to 2009. We estimated that our multiplex qualitative PCR analyses can cover almost all the GM maize events cultivated in 2009, since it has been reported that other authorized GM maize events such as DBT41 and DDL25 are not cultivated anymore. Thus, it appears that our results are reasonable and probably reflect the average GM grain mixing in 2009.

For the analyses of GM events, we clarified that MON88017 grains (39.3%) and NK603 grains (18.5%) were the major single GM events and that MON88017× MON810 grains were the major stacked GM event in non-IP samples in 2009. We could not follow the

[*9] Acreage, No. 06.30.2009, 2009, National Agricultural Statistics Service, Agricultural Statistics Board, USA. Department of Agriculture.

[*10] http://www.ers.usda.gov/Data/BiotechCrops/ExtentofAdoptionTable1.htm

MON89034 event because a detection method for the MON89034 event had not yet been developed. However, we considered that MON89034 had not yet been widely cultivated in 2009 because the event was authorized worldwide from 2008 to 2010.

This study showed that the major cultured single GM maize event changed from MON810 to MON88017 and the major stacked GM event changed from MON810×NK603 to MON88017×MON810 between 2005[5] and 2009. The MON88017 event maize has both resistance to corn rootworm and tolerance to the herbicide glyphosate, and MON810 has resistance to the ECB. Therefore, MON88017×MON810 event maize has three advantageous features: resistance to corn rootworm, tolerance to the herbicide glyphosate and resistance to the ECB. Therefore, we presumed that the MON88017×MON810 event maize may be more productive than other conventional events and so the area planted with MON88017×MON810 may have been increased. So far, there is little information on the ratio of areas planted with each GM maize event, or which GM maize event is the main planted maize in the USA. NASS reported that the percentage of the cultivation area planted with insect-resistant (Bt) traits in 2005, 2008 and 2009 in the USA was 26%, 17% and 17%, respectively, and the percentage of herbicide-tolerant traits in 2005, 2008 and 2009 was 17%, 23% and 22%, respectively[*9, *10]. The data do not indicate which events for insect-resistant (Bt) traits or herbicide-tolerant traits were planted. However, the data suggests that herbicide-tolerant traits are becoming more popular than insect-resistant (Bt) traits over the period from 2005 to 2009.

In addition, Marra *et al.* reported a survey of the average shares of total corn acres planted by the survey respondents to each GM maize event by agronomic zone in the USA in 2009. The report indicated that MON88017×MON810 (Monsanto's YieldGard VT Triple hybrids) made up the largest share of total planted acres in the survey in 2009[8]. The stacked GM maize event with the second largest share by agronomic zone varied, with MON810×NK603 (TieldGard Con Borer Roundup Ready hybrids) in the western corn belt agronomic zone (13.8%), and TC1507×DAS59122 (Herculex Xtra) in the central corn belt agronomic zone (8.2%) and the east corn belt agronomic zone (13.1%). Among single GM maize events, NK603 made up 15% of planted corn acres in the western corn belt agronomic zone, 12.2% in the central corn belt agronomic zone, and 13.2% in the east corn belt agronomic zone[8]. According to this information, we considered that MON88017×MON810 is the largest share event and TC1507×DAS59122 or MON810×NK603 is the second largest share event among stacked GM maize events, and NK603 is the largest share event as a single GM maize event, in GM maize planted in the USA in 2009. Considering the survey[8] and Fig. 3, we presumed that MON88017 grains or MON810 grains mainly detected as single GM events in the present study could be derived from the cultivation of MON88017×MON810 maize seeds. Consequently, it appears that the results of the present analysis of GM maize events are reasonably consistent with the data on actual planting in the USA in 2009 (Fig. 3).

It should be noted that there are limitations to the present study in terms of estimation of the ratio of GM maize content and GM maize events in a non-IP maize sample. There are many factors that would influence the estimation, such as the time and place of the sampling for non-IP maize. The present study is only based on analyses of limited numbers of samples obtained in 2009 in Japan. However, to our knowledge, this is the first report of analysis of GM maize content and GM maize events in recent maize samples on a kernel basis, except for our study published in 2008[5]. Although, the samples used in the present study may not be a representative group, they provide important information about the GM maize content and the main GM maize events in non-IP maize samples in 2009.

In conclusion, we successfully determined the GM maize grain content on a kernel basis in non-IP maize samples imported from the USA in 2009 using the individual kernel detection system[2,3]. In addition, we analyzed the GM events in GM maize grains of non-IP maize samples in 2009 using two multiplex qualitative PCR detection methods[4,6] coupled to microchip electrophoresis and partially real-time PCR array analyses[7]. MON88017 and NK603 were the major single GM events and MON88017×MON810 was the major stacked GM event in the non-IP samples. This type of study should provide useful information on GM maize mixing in imported maize samples on a kernel basis and the method permits precise quantification of the GM maize content in GM maize kernels for labeling regulation. It will be necessary to obtain the latest information on the GM maize ratio and GM maize events in non-IP maize grains to investigate the level of GM mixing and the probability of stacked GM maize mixing.

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Extraction Method and Determination of Sudan I Present in Sunset Yellow FCF by Isocratic High-Performance Liquid Chromatography

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ABSTRACT

A method to extract and analyze Sudan I present in Sunset Yellow FCF (SYF) products was developed and validated. The method included the simple extraction of Sudan I from the SYF product using water, acetonitrile, and ethyl acetate and high-performance liquid chromatography (HPLC) analysis with isocratic elution using acetonitrile:water (7:3) with a photodiode array detector at 485 nm. This method was found to remove most of the excess SYF colorant and other impurities before injection to the HPLC instrument, making it easy to maintain precision control in routine laboratory tests for Sudan I in the SYF colorant. The detection limit of Sudan I in SYF products was 0.2 μg/g. A survey conducted to determine Sudan I in 13 commercial SYF samples from Japanese manufacturers from 1970 to 2010 showed that the levels of Sudan I ranged from 0.3 to 1.9 μg/g in products manufactured from 1970 to 1996 and were below the limit of detection in products manufactured after 2005.

Keywords: Sudan I; Sunset Yellow FCF; HPLC

1. Introduction

Sunset Yellow FCF (SYF) is a synthetic yellow azo dye that is allowed worldwide as a food coloring agent and is known by designations such as FD&C Yellow No. 6, Food Yellow No. 5, E110, and Color Index No.15985. SYF is a disulfo monoazo dye prepared by the coupling of diazotized 4-aminobenzenesulfonic acid with the sodium salt of 6-hydroxy-2-naphthalenesulfonic acid. During the manufacture of SYF, 1-(phenylazo)-2-naphthalenol (Sudan I) may be produced by the diazotization and coupling of aniline, an impurity in technical and refined sulfanic acid, with 2-naphthol [1]. Sudan I is an unauthorized colorant and an undesirable substance in food because of its reported possible carcinogenicity and genotoxicity in Japan, the United States, and the European Union (EU) [2]. In 2003, Sudan I was detected in chili products in France. After this report, in the EU and China, Sudan I and other unauthorized Sudan dyes (Sudan II, III, and IV) have been detected in samples of hot chili and other related foods. Consequently, various analytical methods have been established to determine and identify unauthorized Sudan dyes in foods [3-6].

In 2005, the EU adopted a limit of <0.5 μg/g for Sudan I in SYF [7]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) conducted a survey from 17 international manufacturers of the amount of Sudan I in 28 SYF samples. Seventy-five percent of SYF samples contained Sudan I at concentrations ≤ 1 μg/g. Based on these data, JECFA established a maximum limit of 1 μg/g for Sudan I in SYF [8,9]. The US Food and Drug Administration (FDA) developed an analytical method for the detection of Sudan I in SYF, which involves the direct injection of SYF by reversed-phase liquid chromatography [1]. This method was also adopted by JECFA in an SYF monograph for the determination of Sudan I [8]. Although the method is simple and rapid, high concentrations of an SYF solution have to be injected to determine low levels of Sudan I in the SYF. Since injecting high concentrations of the SYF solution can contaminate the line, sample loop, and column in the high-performance liquid chromatography (HPLC) instrument, maintaining precision control in routine SYF laboratory tests is difficult. To reduce the risk of contamination to the analytical equipment from SYF, a method for extracting Sudan I from SYF was developed for cosmetic use. In the method, Sudan I is extracted using chloroform as a solvent and analyzed by reversed-phase HPLC [10]. However, owing to the toxicity of chloroform, it needs to

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be replaced with a less toxic solvent to reduce health hazards and environmental contamination. In this study, we developed a simple analytical method for detecting Sudan I in SYF using a less toxic organic solvent. The method was validated by recovery testing using two different concentrations of Sudan I and was applied to the analysis of commercial SYF samples in Japan.

2. Experimental

2.1. Reagents and Equipment

Sudan I (Color Index No. 12055) from Kanto Chemical Co, Ltd. (special grade, Tokyo, Japan) and Wako Pure Chemical (standard for HPLC, Osaka, Japan) was used for an investigation of purity. The Sudan I from Kanto Chemical was recrystallized from ethanol (5 g/150 mL) to obtain pure Sudan I that was used as a standard for the analysis. Of 13 commercial SYF samples that were obtained from five Japanese companies for surveying the Sudan I content, one of them (No.10) did not contain Sudan I and was used for the recovery test of Sudan I.

The SYF sample for the recovery test was obtained from Daiwa Kasei Corporation (Saitama, Japan). The ammonium acetate and ethyl acetate were special grade reagents (Wako Pure Chemical Industries, Ltd.), the acetonitrile was liquid-chromatography (LC) grade (Merck KGaA, Darmstadt, Germany), and the ultrapure water used for LC was purified with the MilliQ® Gradient system equipped with a Millipak® 0.22 μm filter (Milliopore, Billerica, MA, USA). Parts of the solution for LC were filtered through a polytetrafluoroethylene (PTFE) membrane (Millex®-LH, 13 mm, 0.45 mm; Millipore, Billerica, MA, USA) using a 2.5 mL polypropylene/polyethylene syringe (Terumo syringe; Terumo Corp. Tokyo, Japan). Certified reference material 1,4-bis (trimethylsilyl) benzene-d_4 (1,4-BTMSB-d_4) (Code No. 024-17031, Lot. DCP5375, purity 99.8% ± 0.3%) was obtained from Wako Pure Chemical Industries, Ltd. Chloroform-*d* was purchased from Isotec Ltd. (Miamisburg, OH, USA).

2.2. qHNMR Measurement

First, 10 mg of Sudan I and 4 mg of 1,4-BTMSB-d_4 were accurately weighed and then dissolved in 800 μL of chloroform-*d*. Next, approximately 600 μL of the solution was introduced into a nuclear magnetic resonance (NMR) tube with a 5 mm outer diameter (Kanto Chemical Co., Inc.) and subjected to a quantitative proton nuclear magnetic resonance (qHNMR) measurement using a JNM-ECA spectrometer (600 MHz; JEOL, Tokyo). The purity of the Sudan I was calculated by the equation

$$\text{Purity}\ (\%) = \frac{I_s/H_s}{I_{BTMSB}/H_{BTMSB}} \times \frac{M_s/W_s}{M_{BTMSB}/W_{BTMSB}} \times 100 \quad (1)$$

where I_S and I_{BTMSB} are the integrated signal intensity values of Sudan I and 1,4-BTMSB-d_4, respectively; H_S and H_{BTMSB} are the number of proton signals from Sudan I and 1,4-BTMSB-d_4, respectively; M_S and M_{BTMSB} are the molecular weights of Sudan I and 1,4-BTMSB-d_4, respectively; and W_S and W_{BTMSB} are the weights of Sudan I and 1,4-BTMSB-d_4, respectively. The qHNMR measurement was conducted with the following optimized parameters: irradiation frequency, 600 MHz; probe temperature, 25°C; spinning, off; number of scans, eight; spectral width, 20 ppm; auto filter, on (eight times); acquisition time, 4 s; relaxation delay, 60 s; pulse angle, 90°; pulse width, 12.2 μs; free induction decay (FID) data points, 49152; and ^{13}C decoupling, multipulse decoupling with phase and frequency switching (MPF-8). The data were processed with qNMR analysis software, Alice 2 for qNMR "PURITY" (JEOL). The signal integral value calculated using the software was used for quantitative analysis. The chemical shift of all data was referenced to the 1,4-BTMSB-d_4 resonance at δ_H 0.23.

2.3. Sample Solutions and Calibration Curve

After 500 mg of the SYF product was dissolved in 10 mL of water by ultrasonic wave in a 50 mL glass centrifuge tube, 5 mL of acetonitrile was added and mixed well, followed by the addition and mixing of 30 mL of ethyl acetate. The centrifuge tube was centrifuged for 1 min at 3000 rpm, and the ethyl acetate layer was collected in a round flask; another 30 mL of ethyl acetate was added to the residue, and the procedure was repeated. The collected ethyl acetate layer was evaporated at a reduced pressure at 40°C. The residue was dissolved in a 2 mL volumetric flask by sonication and was diluted with acetonitrile: water (7:3) up to the 2 mL mark. A portion of the sample solution was filtered into a vial using a PTFE membrane syringe filter.

2.4. Standard Stock Solution

To obtain a standard stock solution containing 1 mg/mL of Sudan I, recrystallized Sudan I was dried in a desiccator for 24 h under reduced pressure, and 50 mg of Sudan I was weighed and dissolved completely with 45 mL of acetonitrile by ultrasonication in a 50 mL volumetric flask.

2.5. Standard Calibration Curve

The Sudan I standard stock solution was diluted with acetonitrile:water (7:3), and 0.05 - 50 μg/mL standard solutions of Sudan I were prepared. These standard solutions were used to check the linearity of the calibration curve. One microgram of Sudan I in 1 mL of a standard solution was pipetted into 1, 2, 4, and 10 mL of appropriate standard solutions in a series of volumetric flasks and diluted to the 20 mL mark with acetonitrile:water

(7:3). The standard solutions contained 0.05, 0.1, 0.2, and 0.5 μg of Sudan I/mL, and the calibration curve was used for the recovery test and the Sudan I content survey of commercial SYF products.

2.6. Recovery Test and Validation

To evaluate the accuracy of the developed method, recovery tests were performed. A 0.5 mL quantity of the 0.5 and 0.3 μg/mL Sudan I standard solutions was added to 500 mg of a commercial SYF product in the absence of Sudan I. The sample was left at room temperature overnight and then was treated as described above.

Calibration curves were prepared with the Sudan I standard solutions at concentration levels of 0.05 - 50 μg/mL to check the linearity of the calibration curve. Intra-day and inter-day precisions were assessed by analyzing five replicates of a sample during a day and five replicates on three different days, respectively. The limit of quantification of Sudan I was estimated to the limit of confirmatory detection allowed by the PDA detector.

2.7. Sudan I Content Survey of Commercial SYF Products

The amounts of Sudan I in 13 commercial SYF products produced as food additives in Japan from 1970 to 2010 were analyzed by the method described above in *Sample solution.*

2.8. HPLC

The LC system consisted of a Hewlett Packard 1100 series, G1315A photodiode array detector (PDA; monitored at 485 nm for Sudan I), L-column octadecylsilane (ODS; 150 × 4.6 mm id, particle size 5 μm, pore size 12 nm; Chemicals Evaluation and Research Institute, Tokyo, Japan), and a column heater set at 40°C. The eluent was acetonitrile: water (7:3) in the isocratic mode. The injection volume was 20 μL, and the flow rate was 1.0 mL/min. The apparatus was controlled and data were collected and analyzed by use of Agilent Chemstation software.

3. Results and Discussion

3.1. Purity Determination by qHNMR Measurement

To investigate the purity of Sudan I, we analyzed the commercial Sudan I standard by HPLC and the special grade Sudan I by HPLC at 230 nm to see the colorless impurities, previously reported. As shown in **Figure 1(a)** and **(b)**, some unknown interference peaks between 2 and 5 min were detected in addition to a main peak at 12.5 min, which corresponds to Sudan I on the HPLC chromatogram. To remove impurities from the special grade Sudan I, recrystallization was performed in ethanol, and

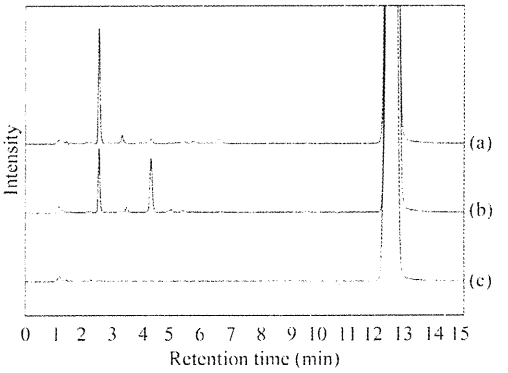

Figure 1. HPLC chromatogram of Sudan I and recrystallized Sudan I at 230 nm. (a) Commercial Sudan I standard for HPLC (Wako Pure Chemical Co.); (b) Special grade Sudan I (Kanto Chemical Co.); (c) Recrystallized Sudan I from Kanto Chemical Co.

recrystallized Sudan I was obtained. As shown in **Figure 1(c)**, a few impurity peaks were detected on the HPLC chromatogram of Sudan I after the recrystallization.

To determine the absolute purity of recrystallized Sudan I, Sudan I was analyzed by qHNMR. As shown in **Figure 2**, the ^{1}H NMR spectrum exhibited signals characteristic of the aromatic protons of Sudan I in δ_H 6.80 - 8.60. Among them, the doublet signals at δ_H 6.87 and 8.57 were assigned to H-3 and H-8, respectively. In qHNMR, it is necessary that the signal for quantification should separate the other intramolecular signals in order to accurately determine the purity of the analyte. Both signals were applied to qHNMR measurement because these were well resolved from the other signals. The absolute purity of Sudan I was calibrated from the ratio of the integrated signal intensity values (signal area values) of each signal to that of 1,4-BTMSB-d_4 at δ_H 0.23. The mean purity value was calculated to be 98.9% ± 0.1% (mean ± SD). These results indicate that the purity of the recrystallized Sudan I is higher than those of the commercial Sudan I standard and special grade Sudan I.

3.2. Extraction of Sudan I from SYF

The self-regulation method of the Japan Cosmetic Industry Association, which uses chloroform to extract Sudan I from SYF followed by reversed-phase HPLC analysis [10], was used. In an effort to replace chloroform with non-toxic solvents, we examined the pretreatment procedure of Sudan I from commercial SYF using methanol and ethyl acetate. The recovery tests were conducted using commercial SYF spiked with the recrystallized Sudan I. Since SYF is soluble in water and Sudan I is only negligibly soluble in water, 0.5 g of commercial SYF spiked with the recrystallized Sudan I (0.5 μg/g of SYF) was dissolved in 10 mL of water, and 10 mL of methanol and 20 mL of ethyl acetate were added. The

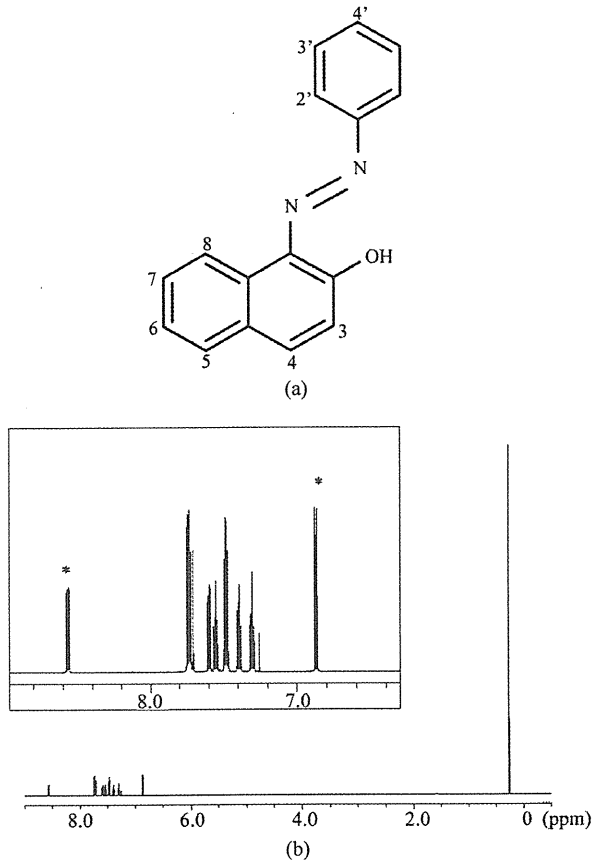

Figure 2. (a) Chemical structure of Sudan I and (b) ^{1}H NMR spectrum of recrystallized Sudan I in chloroform-*d* containing 1,4-BTMSB-d_4. The signals of Sudan I shown on the top are highlighted. Signals marked with asterisks were used for quantification, and the purities were calculated.

resulting solution was mixed using a shaker for 1 min, after which 10 mL of water was added. After centrifuging the solution for 5 min at 3000 rpm, it separated into two layers, and we visually considered the upper layer to contain Sudan I and the lower layer to contain SYF. After taking the upper layer and washing it twice with water, the solution was concentrated and desiccated under a reduced pressure at 40°C, dissolved and made up to a 2 mL volume with acetonitrile:water (7:3), and analyzed with HPLC. The recovery of Sudan I was 89%. To further improve the recovery of Sudan I, 5 mL of acetonitrile was added to the solution instead of 5 mL of methanol, and when extracted with 20 mL of ethyl acetate two times in the same manner, the recovery of Sudan I was 94%. These results suggest that the latter preparation is an improvement over the former preparation in terms of recovery and procedure.

3.3. HPLC Conditions

To determine Sudan I using HPLC, we optimized the HPLC conditions. Tsuji *et al.* [11] reported HPLC conditions to determine raw materials, intermediates, and subsidiary colors in SYF using a gradient system of 20 mmol/L ammonium acetate and acetonitrile:water (7:3).

We utilized the HPLC conditions reported by Tsuji *et al.*, since this gradient system using these solvents will be adopted for the examination of raw materials, intermediates, and subsidiary colors in food colors other than SYF in Japan's Specifications and Standards of Food Additives. Sudan I could not be eluted until 50 min had elapsed because Sudan I appeared to be retained on the reversed-phase column. Upon examination, we found that the use of the acetonitrile:water (7:3) mobile phase led to the efficient separation and elution of Sudan I at 12.5 min under isocratic conditions (**Figure 1**). For the detection of Sudan I, we compared the sensitivity and separation pattern at 230 nm, which is thought to be suitable for the detection of aromatic compounds, and at 485 nm, which is used in the methods reported by Petigara *et al.* [1]. Consequently, the separation pattern of the 485 nm detection had fewer impurity peaks than that at 230 nm, although the sensitivity of the 230 nm detection was higher than that at 485 nm detection (**Figure 3**). Therefore, we chose 485 nm for the detection of Sudan I.

3.4. Method Validation and Recovery Test of Sudan I from SYF

To assess the linearity, the calibration curve for Sudan I

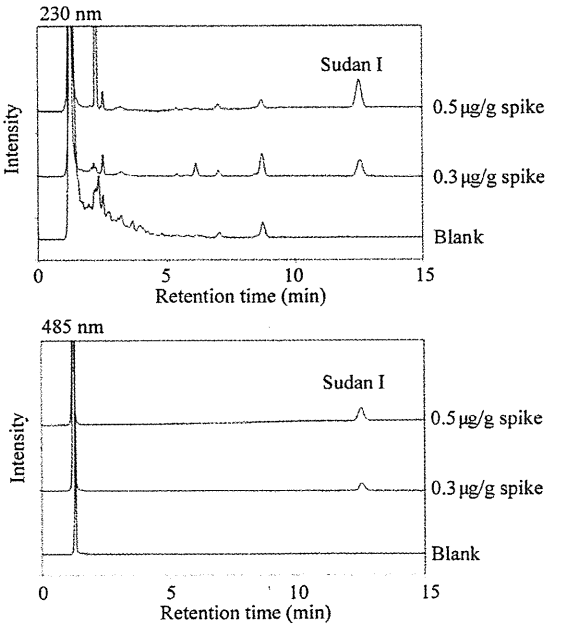

Figure 3. HPLC chromatogram of sample solutions from Sunset Yellow FCF product (blank) and Sunset Yellow FCF product spiked with Sudan I (0.3 and 0.5 µg/g) at 230 nm and 485 nm.

was constructed using concentrations from 0.05 to 50 μg/mL. As shown in **Figure 4**, good linearity was achieved over Sudan I concentrations of 0.05 - 50 μg/mL. The correlation coefficient for Sudan I was $R^2 = 0.9999$.

To assess the accuracy of the developed method, we conducted recovery tests using the SYF product spiked with Sudan I at two levels—0.5 and 0.3 μg—in 1 g of SYF. As shown in **Table 1**, the recovery rates were within the 95.5% - 97.9% range. To assess the precision of the method, intra-day and inter-day precisions were within the 0.4% - 1.8% and 1.1% - 3.8% ranges, respectively, all expressed as relative standard deviations (RSDs) (**Table 1**). The limit of detection of Sudan I was 0.2 μg/g, calculated from the signal-to-noise ratio (S/N > 10) of the peak intensity, and the limit of identification of Sudan I was estimated to be 0.4 μg/g, which was considered to be the limit of confirmatory detection using the PDA detector.

These data clearly demonstrate acceptable linearity, recovery rates, and RSDs, suggesting that the developed method is reliable for the accurate quantitative determination of Sudan I in SYF products.

3.5. Quantitative Survey of Sudan I in Manufactured SYF

A survey determination of Sudan I in commercial SYF products manufactured in Japan from 1970 to 2010 was conducted to confirm the application of the developed method. The results are summarized in **Table 2**. The levels of Sudan I in SYF products ranged from 0.3 to 1.9 μg/g in SYF products manufactured from 1970 to 1996. Using the PDA detector, specific spectra of Sudan I could be confirmed from the peaks of Sudan I in all SYF products except for Sample No. 3 SYF products. Sudan I was below the limit of detection in commercial SYF products manufactured after 2005, which implies that the SYF products manufactured in Japan from 2005 to 2010 contained marginal amounts of Sudan I.

The low level of detection of Sudan I in commercial SYF products manufactured in recent years appears to be attributable to self-regulation by each manufacturer, considering the low limit on Sudan I (<0.5 μg/g) that has been specified by the EU.

4. Conclusion

A rapid, useful analytical method for monitoring Sudan I in SYF products has been developed. The method includes a simple procedure for extracting Sudan I from SYF products and analyzing it by HPLC with isocratic elution using a PDA detector. The method can prevent the contamination of the HPLC instrument and column with excess SYF colorant and other impurities. This method permits the determination of 0.2 μg of Sudan I in 1 g of SYF; thus, it can readily detect the maximum limit of Sudan I in SYF as defined by the EU (0.5 μg/g).

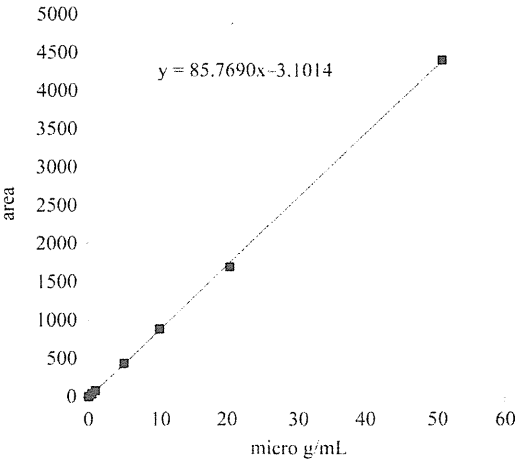

Figure 4. Calibration curve of Sudan I.

Table 1. Intra-day and inter-day precisions in the recovery test of Sudan I in Sunset Yellow FCF.

Spiked level (μg/g)		Intraday (n = 5)			Interday
		Day 1	Day 2	Day 3	
0.5	Recovery (%)	95.5	97.2	96.0	96.2
	RSD[a] (%)	1.2	0.4	1.0	1.1
0.3	Recovery (%)	95.7	97.9	95.7	96.8
	RSD[a] (%)	1.8	1.8	1.8	3.8

[a]Relative standard deviation.

Table 2. Results of the survey determination of Sudan I in commercial Sunset Yellow FCF.

Sample No.	μg/g	Year of manufacture
1	0.4	1970
2	1.6	1972
3	0.3	1972
4	3.1	1976
5	1.1	1985
6	0.8	1985
7	5.0	1989
8	0.7	1990
9	1.9	1996
10	ND[a]	2005
11	ND[a]	2010
12	ND[a]	2010
13	ND[a]	2010

[a]ND = not detected (detection limit < 0.2 μg/g).

5. Acknowledgements

We are grateful to San-Ei Gen F.F.I., Inc. for providing commercial SYF products.

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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

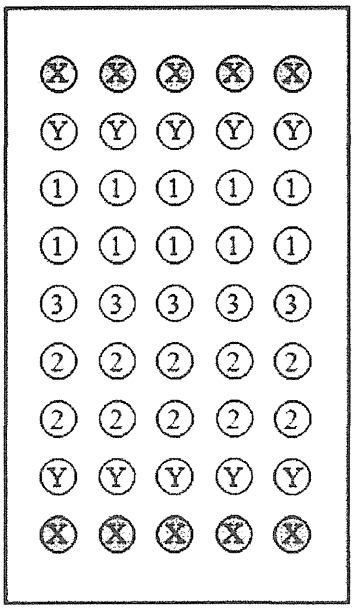

Fig. 1. A schematic presentation of DNA microarray spotted with specific probes
X, marker; Y, blank spot; 1, *adh*; 2, *SSIIb*; 3, *BsGyrB*

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-

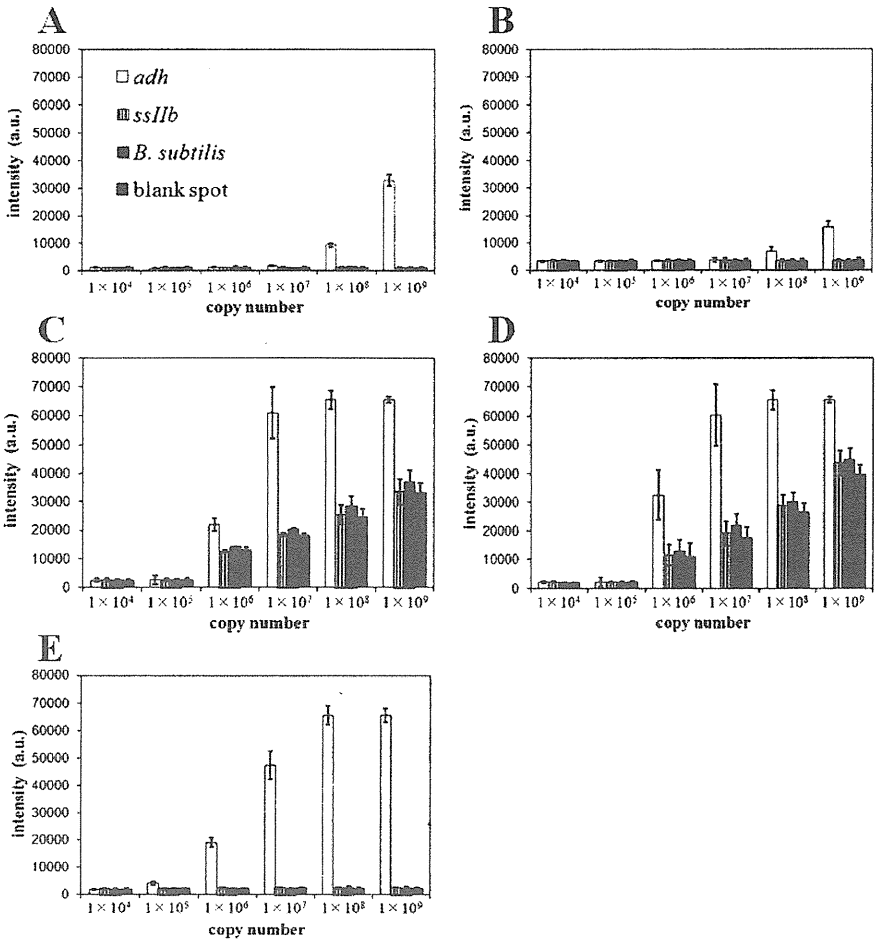

Fig. 2. Fluorescence intensity (A, B, E), and luminescence intensity (C, D) of each spot detected on DNA microarray

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* ▥ *sslIb* ▩ 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.

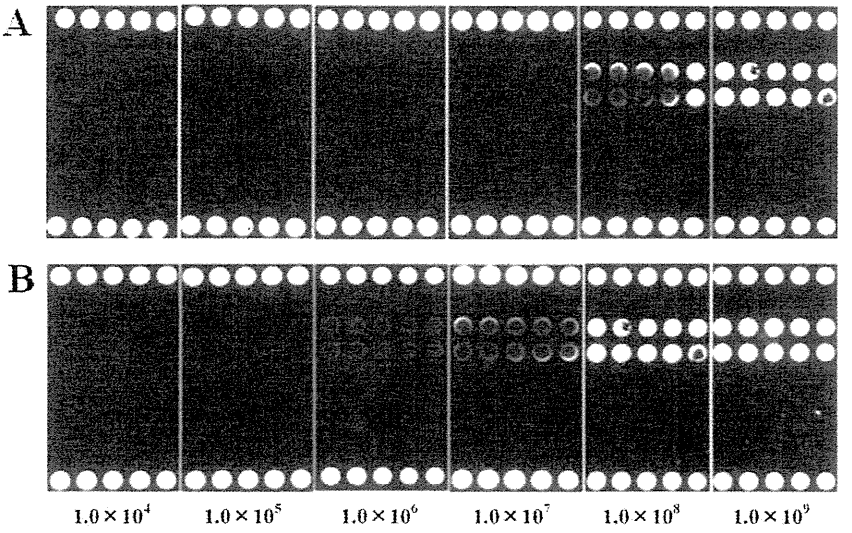

Fig. 3. Fluorescence image of a DNA microarray
A, image of the hybridized target labeled with Cy3-9N primer; B, image labeled with 3DNA dendrimer. All images were measured by an MB Biochip Reader™ detector and integrated for 60 sec.

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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DNAマイクロアレイによる遺伝子組換え作物のシグナル増強検出法の比較

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キーワード：DNAマイクロアレイ、遺伝子組換え作物、シグナル増強

概　要

PCR法に替わる遺伝子組換え作物を検知するための方法として、DNAマイクロアレイを用いてランダムプライマーにより合成された遺伝子断片を検出する方法が考えられる。そこで我々は、目的とするモデル遺伝子断片を検出するための方法として、4つのDNAマイクロアレイのシグナル増強検出法の性能比較検討を行った。蛍光標識DNAデンドリマーを用いた場合、シグナル増強を行わないコントロールと比較して約100倍の検出感度の増大が確認された。バックグラウンドレベルもコントロールと比較して同等であった。蛍光標識DNAデンドリマーを用いた場合、遺伝子組換え作物のゲノムDNA 1分子に1コピー組み込まれていると想定した組換え遺伝子をPCRのような核酸増幅法を用いることなくDNAマイクロアレイにより検出可能であると考えられる。

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