

Validation of cDNA microarray as a prototype for throughput detection of GMOs

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ABSTRACT. As a step towards developing an efficient genetically modified organism (GMO) detection system, the present investigation proposes microarray as a prototype for high-throughput detection of pure GMO samples. Common T-DNA regions of the expression cassette such as 35S promoter, 35S terminator, nopaline synthase terminator (NOST), hygromycin, and kanamycin selection marker genes were detected in the transgenic rice, tomato, and potato developed in our laboratory. For proof-of-concept purposes, market-introduced GM potato, GM maize, and cornflakes were screened. The non-GM potato and maize control detected only the presence of endogenous genes while the GM targets detected the presence of transgenic genes such as *CaMV 35S* promoter, 35S terminator, NOST and *nptII*, *pat*, *cp4 epsps*, *cry1 Ab* genes on chips. Moreover, it was observed that the sensitivity of this system for serially diluted GM potato tubers was up to 0.01-0.002% of the mass fraction. Due to its high accuracy and speed, it is believed that the microarray detection system will play an important role in routine, high-throughput detection of pure GMO samples in the future.

Keywords: cDNA microarray; Genetically modified organisms (GMO); GM potato; GM maize.

Abbreviations: **35Sp**, promoter from the cauliflower mosaic virus; **35St**, CaMV35S poly (A) signal; **aadA**, streptomycin-resistance; **Actin**, rice actin gene; **BAR**, gene coding for a phosphinothricin acetyltransferase from *Streptomyces hygroscopicus*; **Bt11**, specific gene of Bt11 (Novartis); **β -tubulin**: tomato β -tubulin gene; **CBH351**, specific gene for CBH351 (StarLink, AgrEvo); **CryIAb**, delta-endotoxin from *Bacillus thuringiensis* subsp. *Kurstaki*; **EPSPS**, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* strain CP4; **GA21**, specific gene of GA21 (Monsanto); **GFP**, green fluorescent protein gene; **GUS**, β -glucuronidase gene; **HPT**, hygromycin phosphotransferase gene; **ivr**, maize invertase gene; **LUC**, luciferase gene; **LE**, soybean legumin protein gene; **NOST**, terminator of nopaline synthase gene from *Agrobacterium tumefaciens*; **NPT II**, neomycin phosphotransferase gene; **Oest**, octopine synthase terminator; **tmlt**, transcription terminator of a tumor morphology large gene from *Agrobacterium tumefaciens*; **T25**, specific gene of T25 Libery (AgrEvo); **E35S**, enhanced CaMV35S promoter; **BP**, bacterial promoter; **bla**, beta lactamase; **pat**, phosphinothricin-N-acetyltransferase from *S. viridochromogenes*; **bla**, beta-lactamase gene; conveys resistance to beta-lactam antibiotics; from Tn3; **Zmhsp70**, maize HSP 70 intron; **Cpsp**, chloroplast transit peptide from *A. thaliana* EPSPS gene; **P-ract1/ract1**, P-ract1/ract1 intron; **Actin 1**, rice actin I promoter; **RuBisco-sp**, ribulose-1,5-bisphosphate carboxylase oxygenase derived chloroplast transit peptide sequence; **AI**, Actin 1 intron sequence; **IVS 2**, IVS2 intron from the maize alcohol dehydrogenase gene; **IVS6**, IVS6 intron from the maize alcohol dehydrogenase gene.

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INTRODUCTION

Recombinant DNA technology has hastened crop improvement strategies through precise genetic manipulation involving plant specific processes. Previously, such improvements were only possible through cumbersome crosses between crops and their wild-relatives. However, the introduction of genetically modified food, certified by legally competent authorities as food or feed since 1994 (Bertheau et al., 2002) has caused unforeseen apprehension among the public, due to a lack of stringent biosafety regulations and reported allergic responses to genetically modified food by humans (CDC, 2001; Soboleva et al., 2003). As a step towards addressing controversies pertaining to GM foods, GMO regulation bodies in different parts of Europe and Brazil are considering whether to demarcate genetic engineering technology from other technological processes (Oda and Soares, 2000). A bill passed in the California senate, AB 2962, requires the labeling of genetically engineered fish in supermarkets (Campaign, 2002). China is undertaking extensive field trials of transgenic crops to implement new rules on GMO, according to the China Daily, 21 March 2002.

GMO technology for developing and introducing genetically modified food, from a social standpoint, has necessitated GMO detection primarily to address concerns related to safety, but also those related to ethics, sociology, and intellectual property rights. It is further intended to keep the consumer's choice on a par with non-GM foods and facilitate the monitoring of the measurable effects of gene contamination in indigenous species, by competent authorities. As a bio-safety measure it is also important for testing the homogeneity of imported seeds, grain, commodities and organic products of conventionally grown crops imported from other countries. Detection methods primarily need to look for genes engineered in crops, such as the promoter sequence, foreign genes coding for a desired trait, and the terminator sequence which functions as a stop signal in the reading frame.

Reported GMO detection methods include immunoassay to detect proteins and PCR analysis to detect DNA. Immunoassays lack the advantage of screening a large number of samples in a shorter time (Brett et al., 1999). The official Swiss method (PCR based) uses detection of cauliflower mosaic virus promoter (35S promoter) and the nopaline synthase terminator (*nos 3'*), which are present in most GM crops approved today (Hardegger et al., 1999). However, this screening method has limited sensitivity and specificity (Brodmann et al., 2002). Most of the PCR experiments enable detection of one gene/promoter (e.g., CaMV 35S) in each sample at a time. However, with more and more GM foods and products reaching the market, a cost effective and high-throughput method of detecting GM products is urgently needed. The microarray method is time-result oriented, enabling detection of more than one gene, including promoter, foreign gene, endogenous control gene, reporter

gene, selection marker gene, and stop sequence.

DNA microarrays are analytical systems allowing the simultaneous detection of many nucleic acid sequences (up to thousands) in a sample. This technology is based on the hybridization of complementary probe and target genes / cDNAs. The cDNAs or PCR products are arranged on a solid support (e.g. glass slide) as probes. The probes on the array are then hybridized with the fluorescently labeled PCR products or genes from GM/non-GM samples (called targets). Laser scanning analysis then reveals the presence of labeled material containing sequences complementary to those spotted on the microarray. This type of microarray, was first developed at Stanford University (Schena et al., 1995; Shalon et al., 1996). During years of study, quite a few types of DNA microarrays have been developed into promising tools for the genome-wide analysis of transcripts (Roy et al., 2002). The application of DNA microarray has extended to nutrigenomics, toxicology, and food safety, in areas such as genetically engineered foods and food-borne pathogens (Liu-Stratton et al., 2004).

Although the public has not displayed complacency regarding the introduction of GM technology, large expanses of cultivated land are still being used in transgenic field trials resulting in market introduction of GMOs and their derivatives, either as import or export commodities. Hence, it is felt that apart from the existing detection methods, a more efficient high-throughput detection method is needed, to screen GMOs being introduced to the market. In this study we propose microarray technology as a prototype for high-throughput detection of pure GMOs. The transgenic tomato, potato, and rice in our laboratory were screened for genes such as promoters, candidate genes, endogenous control genes, reporter genes, selection marker genes, and termination signals by a microarray chip. For proof-of-concept we extended practicality to the detection of genetic modifications in market-introduced GM maize and cornflakes. Results clearly portray microarray as a potent and reliable GMO detection system for pure samples, including monocots and dicots.

MATERIALS AND METHODS

Type of materials

Transgenic and non-transgenic rice, potatoes, and tomatoes developed in our laboratory were used. Samples analyzed included seed and leaf materials in rice, fruit and leaf materials of tomato, and tuber and leaf materials of potato. Transgenic maize seeds (Bt11, T25 and NK603) were kindly provided by the National Laboratories of Foods and Drugs, Taiwan. Wild type maize and wheat provided by Dr. Shih (Bureau of Food and Drug Analysis, Department of Health Executive Yuan, Taipei, Taiwan) served as a non-transgenic control. Cornflakes and wheat samples obtained randomly in the market were a product of California, USA.

Raw material dilution

Raw material of transgenic and non-transgenic potato tubers were sliced and mixed by weight in the proportion of 1:20, 1:100, 1:1000, 1:10,000 and 1:50,000 for the sensitivity assay of our system. Twenty gram of mixed samples was homogenized for isolation of total genomic DNA.

DNA isolation

Total genomic DNA was isolated by the CTAB (cetyl trimethyl ammonium bromide) method. DNA from maize seeds was extracted by the method described by Majchrzyk (Majchrzyk, 2002). Homogeneous samples (20 g) of each crop type (transgenic and non-transgenic) were homogenized in a blender. DNA from potato tubers were extracted as described by Wulff et al. (2002). The quality and concentration of DNA was determined spectrophotometrically at 260/280 nm.

Cloning and construction of candidate genes

The vector used for cDNA library construction was pT7Blue perfectly blunt vector (Novagen, Darmstadt, Germany). Inserts of DNA clones were amplified using the PCR primers shown in Table 1 (BgVV, Berlin, Germany, 1998; Hupfer et al., 1998; Lipp et al., 1999; Matsuoka et al., 2001). The PCR cocktail mixtures (100 μ l) contained plasmid (30 ng) or genomic DNA template (300 ng), 10 \times reaction buffer [200 mM Tris-HCl (pH 8.0), 100 mM KCl, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 20 mM MgSO_4 , 1% Triton X-100, 1 mg/ml nuclease-free bovine serum albumin (BSA)], 5 μ l 100% DMSO, 4 μ l 2.5 mM dNTP, 1 μ l 100 pmole/ μ l 5' primer, 1 μ l 100 pmole/ μ l 3' primer, and 1 μ l *Pfu* DNA polymerase (5 units/ μ l). Amplification was performed in the PCR thermocycler (GeneAmp 2400, Perkin Elmer, California, USA) and consisted of 35 cycles (94°C for 3 min; 95°C for 1 min, 72°C, 30 sec, and 72°C for 3 min). Amplification was monitored by fractionating in a 1% agarose gel, stained for visibility with ethidium bromide. The purified PCR product was eluted using a Viogene kit (Viogene, Taipei, Taiwan) and cloned into pT7Blue Perfectly Blunt vector, as instructed by the kit manual.

cDNA microarray preparation

The microarray methods were a modification of those reported by Seki (Seki et al., 2002). The cDNA library products were arrayed from 384-well microtiter plates onto poly-L-lysine-coated micro slide glass (GAPSII, Corning, USA) using the PixSys4500 System gene tip microarray stamping machine (Cartesian Technologies, USA). About 0.5 μ l of PCR products (100-500 ng/ μ l) were pipetted from the 384-well microtiter plates. Five nl per slide were deposited onto six slides, spaced 280 μ m apart. The printed slides were rehydrated in a beaker with hot distilled water and snap dried at 100°C for 5-10 sec. DNA was cross-linked on the slide using a UV cross-linker (150-300 mJ). The slides were placed into a slide rack, which was in turn placed into a glass chamber.

The blocking solution, containing 25 ml of 0.2 M, pH 8.0 sodium borate, (Sigma, Missouri, USA), and 225 ml of 1-methyl-2-pyrrolidone (Sigma, Missouri, USA), was poured into the glass chamber. The slide racks were plunged up and down five times, shaken gently for 15 min, transferred into a chamber with boiling water, and allowed to stand for 2 min. The slide racks were then transferred into another chamber containing 95% ethanol for 1 min, and centrifuged at 500 rpm for 5 min.

Nucleotide labeling

The procedure for labeling genomic DNA by Klenow reaction and Cy3- or Cy5- nucleotides were a slight modification of protocol described by Eisen and Brown (Eisen and Brown, 1999) and TIGR. In this modified protocol, 2 μ g of genomic DNA was digested with *Sau3* AI (average size is 500-1,000 bp for improving labeling efficiency). Purification of the digested DNA (Qiagen PCR purification kit, Qiagen, Valencia, CA) was performed by adding 1/10 volume of 3 M CH_3COONa (pH 5.2) and 2 volumes of ethanol. The DNA was precipitated at -70°C for 0.5 h or overnight at -20°C, followed by centrifugation at 15,000 g at 4°C. The pellet was dissolved in sterile distilled water. A cocktail PCR mixture containing 1 μ g of purified DNA, oligo-dT, and DEPC-water was incubated in a PCR machine at 70°C for 10 min and snap chilled on ice. Superscript buffer 0.1 M DTT, 10 mM dNTPs, superscript II RT (Life Technologies, Rockville, USA) were added immediately and mixed thoroughly before incubation in PCR machine for 1 h at 42°C. The labeling reaction was cleaned up as described by TIGR standard operating procedure (SOP) (Anklam et al., 2002).

Microarray hybridization and scanning

The hybridization method was also a modification of that reported by Seki (Seki et al., 2002). The probe samples were placed onto the center of a slide, and a cover slip was placed over the entire array surface to avoid bubble formation. Four 5 μ l drops of 3 X SSC were placed on separate points on the slide, which was placed in a humid hybridization chamber to prevent dehydration of the probe mixture during hybridization. The slides were placed in a sealed hybridization cassette (Genetix, Boston, USA) and submerged in a water bath maintained at 65°C, for 12-16 h. After hybridization, the slides were removed and placed in a slide rack submerged in washing solution 1 (2 X SSC, 0.03% SDS) with the array face of the slide tilted down so that the cover slip dropped off without damaging the array surface. The racks were then plunged up and down three times in washing solution 1 and transferred to washing solution 2 (1 X SSC) carefully to minimize contamination of the second chamber because SDS can interfere with slide imaging. The slide chamber was rocked gently for 2 min. The slide racks were then transferred to washing solution 3 (0.05 X SSC), allowed to stand for 2 min, spun at 500 rpm for 5-10 min, and dried.

Microarrays were scanned with a scanning laser micro-

Table 1. The primers designed for PCR amplification of DNA clones.

Template	Genes	Primers	Size (bp)	T _m (°C)	Ref
Selection marker					
pCAMBIA 2301	NPTII	sense- 5' TCCGGCCGCTTGGGTGGAGAG anti- 5' CTGGCGCGAGCCCCCTGATGCT	470	63.6	This study
pCAMBIA1201	HPH	sense- 5' AGCTGCGCCGATGGTTTCTACAA anti- 5' ATCGCCTCGCTCCAGTCAATG	509	60.5	This study
p932A-GUSR	aadA	sense- 5' AAGCGGTGATCGCCGAAGTATCGAC anti- 5' AAAGAGTTCCCTCCGCCGCTGGA	455	59.9	This study
pJD4401	PAT	sense- 5' GCGGTCTGCACCATCGTCAA anti- 5' AGTTCCCCTGCTTGAAGCCG	415	63.1	This study
Reporter gene					
pBI221	GUS	sense- 5' CTGCGACGCTCACACCGATAACC anti- 5' TCACCGAAGTTCATGCCAGTCCAG	441	59.5	This study
pMTC54	LUC	sense- 5' GAGAATAACATTTTGATAGGACCAC anti- 5' GCATAGATTGATACCCCAAG	484	50.8	This study
pCAMBIA1304	GFP	sense- 5' AAGGAGAAGAACTTTTCACT anti- 5' TGATAATGATCAGCGAGTTG	541	51.9	This study
Promter and Terminator					
pCAMBIA1304	35Sp	sense- 5' CATGGAGTCAAAGATTCAAAA anti- 5' ATATAGAGGAAGGGTCTTC	500	47.2	Lipp, 1999
PJD301	Nost	sense- 5' CGTTCAAACATTTGGCAATA anti- 5' CCCGATCTAGTAACATAGAT	253	52.5	Lipp, 1999
pCAMBIA1304	35St	sense- 5' AATTCGGGGGGATCTGGATT anti- 5' CGATCGACAAGCTCGAGTTTAT	201	50.4	This study
TetVp16	Ocst	sense- 5' GCTAGCTATATCATCAATTTAT anti- 5' CCCATCTTGAAAGAAATATAG	204	44.8	This study
pMTC40	tmtl	ense- 5' TATTAGGTTACGCCAGCCCT anti- 5' TAACACGCACACTTACGATA	240	44	This study
Control Gene					
Rice genomic DNA	Actin	sense- 5' GACTACTACAAGGGCATCAG anti- 5' CACACCCACTCCAGATGCCT	318	42	*
Maize genomic DNA	ivr	sense- 5' CCGCTGTATCACAAGGGCTGGTACC anti- 5' GGAGCCCGTGTAGAGCATGACGATC	226	52	This study
Soybean genomic DNA	LE	sense- 5' GCCCTCTACTCCACCCCATCC anti- 5' GCCCATCTGCAAGCCTTTTTGTG	118	48	*
Tomato genomic DNA	β-tubulin	sense- 5' CCCGGGCACACTTTGATCCCATTCCG anti- 5' CCCGGGCATTCTGTCTGGGTACTCTTC	530	50	This study
GM Soybean / Maize gene					
Transgenic maize genomic DNA	CBH351	sense- 5' CCTTCGCAAGACCCT TCCTCTATA anti - 5' GTAGCTGTCCGGTGTAGTCCCTCGT	170	50	Matsuoka, 2001
Transgenic soybean genomic DNA	CP4EPSPS	sense- 5' TGATGTGATATCTCCACTGACG anti- 5' TGTATCCCTTGAGCCATGTTGT	172	45	*
Transgenic maize genomic DNA	T25	aense- 5' GCCAGTTAGGCCAGTTACCCA anti- 5' TGAGCGAAACCCTATAAGAACCCT	149	45	Matsuoka, 2001
Transgenic maize genomic DNA	Bt11	sense- 5' CCATTTTTCAGCTAGGAAGTTC anti- 5' TCGTTGATGTTKGGGTTGTTGTCC	110	42	Matsuoka, 2001
Transgenic maize genomic DNA	GA21	sense- 5' ACGGTGGAAGAGTTCAATGTATG anti- 5' TCTCCTTGATGGGCTGCA	270	42	Matsuoka, 2001
Transgenic maize genomic DNA	CryIAb	sense- 5' ACCATCAACAGCCGCTACAACGACC anti- 5' TGGGGAACAGGCTCACGATGTCCAG	184	50	Hupfer, 1998

Foot note: 35Sp: promoter from the cauliflower mosaic virus; 35St: CaMV35S poly (A) signal; aadA: streptomycin-resistance; Actin: rice actin gene; PAT: gene coding for a phosphinothricin acetyltransferase from *Streptomyces hygroscopicus*; Bt11: specific gene of Bt11 (Novartis); β-tubulin: tomato β-tubulin gene; CBH351: specific gene for CBH351 (StarLink, AgrEvo); CryIAb: delta-endotoxin from *Bacillus thuringiensis* subsp. *Kurstaki*; CP4EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium tumefaciens* strain CP4; GA21: specific gene of GA21 (Monsanto); GFP: green fluorescent protein gene; GUS: alpha-glucuronidase gene; HPH: hygromycin phosphotransferase gene; ivr: maize invertase gene; LUC: luciferase gene; LE: soybean legumin protein gene; NOST: terminator of nopaline synthase gene from *Agrobacterium tumefaciens*; NPT II: neomycin phosphotransferase gene; Ocst: octopine synthase terminator; tmtl: transcription terminator of a tumor morphology large gene from *Agrobacterium tumefaciens*; T25: specific gene of T25 Libery (AgrEvo).

*: <http://www.methodensammlung-lmbg.de/>

scope (model GenePix4000B; Axon Instruments, Union City, USA). Separate images were acquired for each fluor at a resolution of 10 μm per pixel. In order to normalize the two channels with respect to signal intensity, we adjusted photomultiplier and laser power settings so that the signal ratio of the β -tubulin genes (internal control) was as close to 1.0 as possible. We used Imagen, Version 5.0 (Gene Spring, Redwood City, CA) software for the microarray data analysis.

RESULTS

Set up of array analysis system

The set up of our microarray analysis system was based on the studies of rice, tomato, and potato transgenic plants, which were established by our laboratory. Transgenic and non-transgenic rice (*Oryza sativa*, cultivar TNG67) was arrayed for the detection of endogenous genes such as invertase, LE, Actin, and β -tubulin, in addition to genes such as the CaMV 35S promoter driven mammalian protein gene containing nopaline synthase from *Agrobacterium*, 35S terminator, and a hygromycin phosphotransferase selection marker in transgenic rice, developed in our laboratory. As is clearly evident in Figure 1 (A and B) none of the foreign genes of transgenic rice were observed in the untransformed rice, which served as the negative control (Figure 1B left). However, in transgenic rice all the mentioned above component genes of the expression cassette were detectable (Figure 1B right) in addition to the endogenous genes. Non-specific genes were not detected.

A similar experimental setup was used to discriminate between non-transgenic tomato (*Lycopersicon esculentum*) and transgenic tomato, the former serving as a negative control. Non-GM tomato array slide revealed signals of the endogenous genes such as invertase, legumin, actin, and β -tubulin, but none of the foreign genes of the expression cassette were observed (Figure 2B left). However transgenic tomato harboring a reading frame consisting of *CaMV* 35S promoter driven *Arabidopsis* CBF1 transcription factor, nopaline synthase terminator, 35S terminator, *nptII* selection marker gene, and a *gus* reporter gene were detectable, in addition to the genes detected in non-transgenic tomato. No non-specific genes were detected on this slide (Figure 2B right).

Transgenic potato (*Solanum tuberosum* L.) transformed with a *CaMV* 35S driven phytase gene and containing a nopaline synthase terminator, 35S terminator and a kanamycin selection marker were arrayed along with a non-transgenic potato, which served as a negative control. As expected endogenous genes for invertase, LE, Actin, and β -tubulin were detected in both crop types, but the foreign gene components were observed only in the transgenic potato lines (Figure 3B left). Non-specific genes were not detected on this slide (Figure 3B middle).

For proof-of-concept purposes, we arrayed a market-introduced commercial GM potato sample for the

above mentioned genes. As depicted in Figure 3B right, in addition to the endogenous genes detected in non-transgenic potato, we could also detect T-DNA harboring genes such as 35S promoter, nopaline synthase terminator, *nptII* selection marker, and one foreign gene from *Bacillus thuringiensis*, CryIAb, in this sample. Similarly wild type and market wheat samples were also arrayed for GM detection, but no proof for genetic modification was evident (Figure 4).

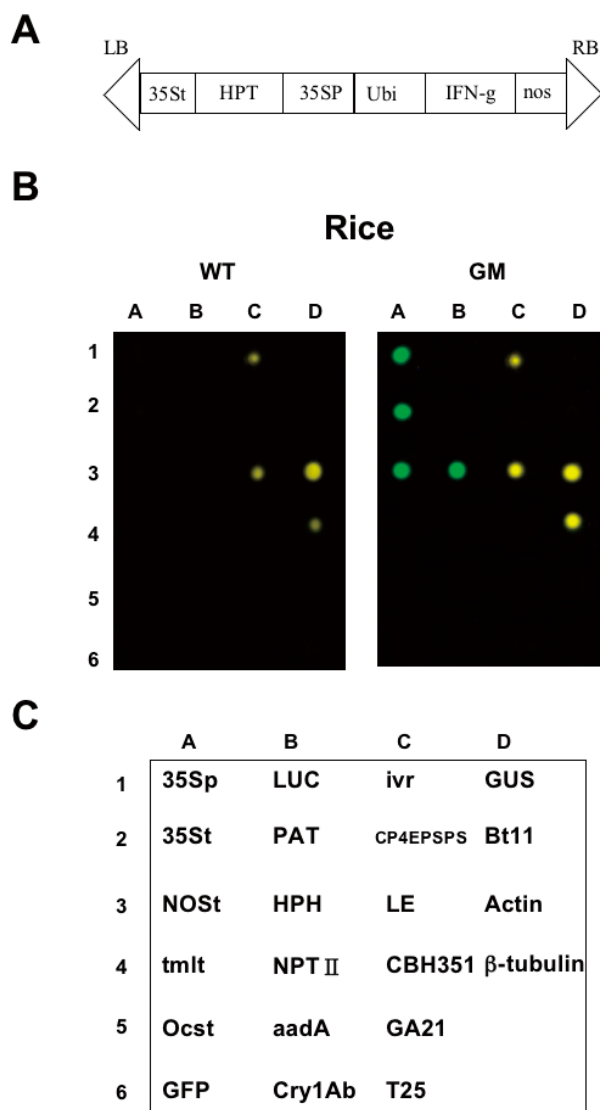


Figure 1. Non-transgenic and transgenic rice detected by microarray slide: Panel A, genetic construct from GM rice. Abbreviations are as follows. Ubi, maize Ubiquitin promoter, IFN-g, human interferon-gamma gene. 35Sp, HPT, nos, and 35St are as described in Table 1; Panel B left, non-transgenic rice (*Oryza sativa*, cultivar TNG67) detected through the presence of *ivr*, LE, Actin, and β -tubulin endogenous genes. Panel B right, transgenic rice (*Oryza sativa*, cultivar TNG67) detected through the presence of additional transgenic genes 35Sp, 35St, Nost, and HPT; Panel C, the position of specific genes spotted on the slide.

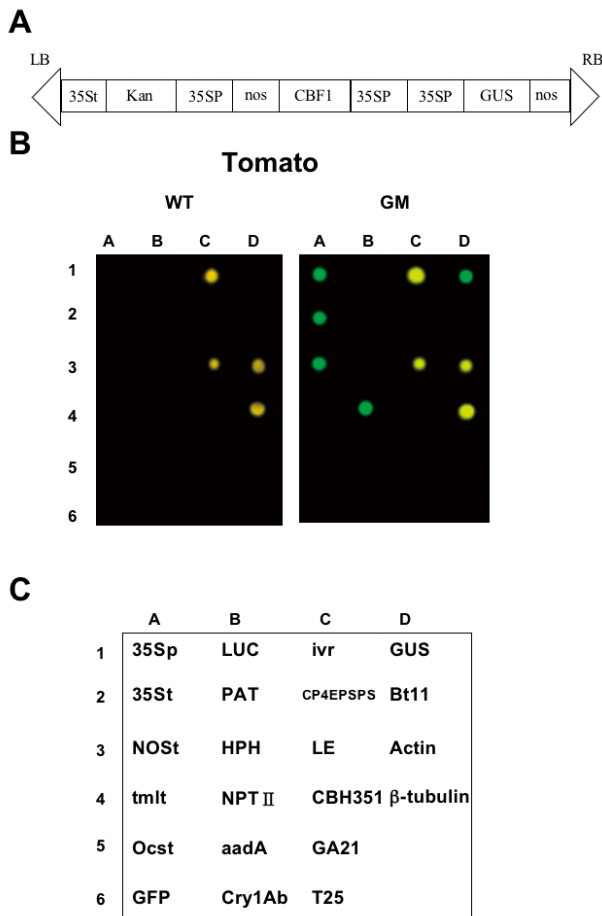


Figure 2. Non-transgenic and transgenic tomato detected by microarray slide: Panel A, The genetic construct of GM tomato. The abbreviation CBF1 indicates the C-repeat/dehydration responsive element binding factor 1 from *Arabidopsis*; Panel B left, non-transgenic tomato (*Lycopersicon esculentum*) was detected through the presence of *ivr*, LE, Actin, and β -tubulin gene. Panel B right, transgenic tomato (*Lycopersicon esculentum*) detected through the presence of additional transgenic genes 35Sp, 35St, Nost and NPTII; Panel C, the position of specific genes spotted on the slide.

Microarray detection of GM and non-GM maize seeds

In the present investigation, we validated cDNA microarray as a detection tool for three varieties of genetically modified maize (T25, Bt11 and NK603). Non-transgenic maize (*Zea mays*) seeds served as a negative control. An array of data depicted in Figure 5 shows that, on the non-GM slide, signals related to endogenous genes for invertase, LE, Actin, and β -tubulin were detected, but not for the mechanistic genes of the T-DNA commonly used in transformation events. However as expected, these genes were detected on our array slide and revealed the presence of CaMV35S promoter gene in the events Bt11 and NK603, and the *bar* gene coding for a phosphinothricin acetyltransferase from *Streptomyces*

hygroscopicus was detected in events T25 and Bt11. The CaMV35S terminator was only observed in event T25 while the terminator of nopaline synthase (*nos*) gene was observed by the events Bt11 and NK603. For the gene of delta-endotoxin, Cry1Ab was detected in event Bt11, and event NK603 was found to harbor the CP4EPSPS gene.

In order to prove the precision of the microarray system in weeding out GMOs from a heterogeneous population, GM maize seeds of the above mentioned transformation events were mixed together in equal proportions. DNA samples arrayed from this germplasm mixture revealed exactly similar patterns as observed individually for each transformation event. A signal for *nptII* was not observed in the array blot, meaning that the event Mon810 was

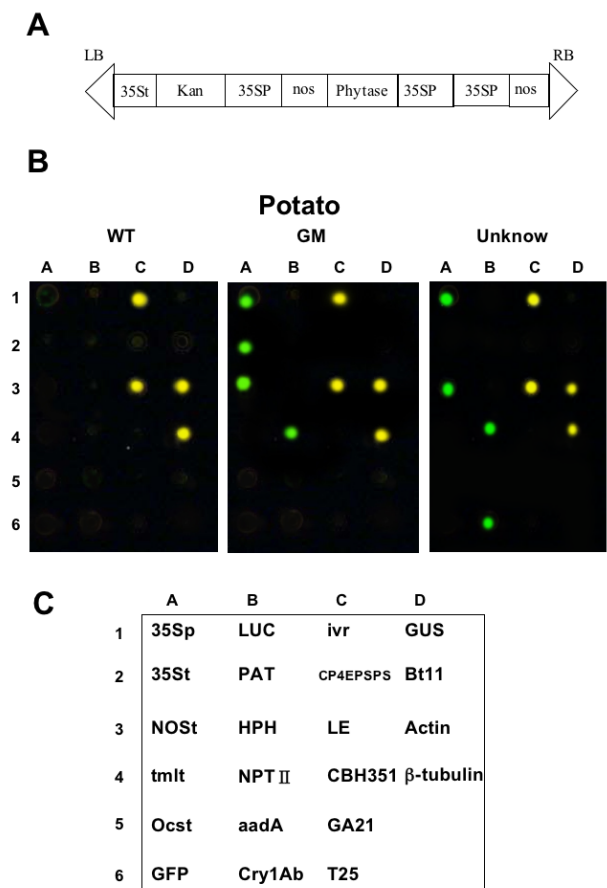


Figure 3. Non-transgenic and transgenic potato detected by microarray slide: Panel A, genetic construct of GM potato. The abbreviation phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is from *Selenomonas ruminantium* (Yanke et al., 1998); Panel B left, non-transgenic potato (*Solanum tuberosum* L.) was detected through the presence of *ivr*, LE, Actin, and β -tubulin gene; Panel B middle, transgenic potato (*Solanum tuberosum* L.) was detected through the presence of additional transgenic genes 35Sp, 35St, Nost and NPTII. Panel B right, unknown potato sample detected through the presence of 35S promoter, nopaline synthase terminator, kanamycin selection marker, and one foreign gene from *Bacillus thuringiensis*, Cry IAb; Panel C, the position of specific genes spotted on the slide.

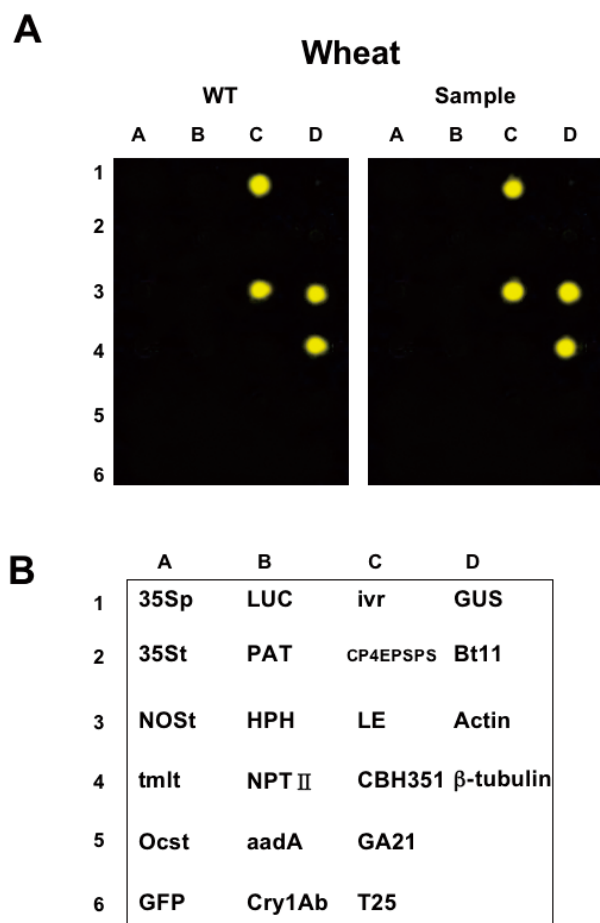


Figure 4. Non-transgenic wheat and marketing wheat sample detected by microarray slide: Panel A left, non-transgenic wheat (*Triticum aestivum* L.) detected through the presence of *ivr*, *LE*, *Actin* and β -tubulin gene. Panel A right, marketing wheat sample detected through the same presence of *ivr*, *LE*, *Actin*, and β -tubulin gene; Panel B, the position of specific genes spotted on the slide.

missed during sampling. Further, the fact that no false genes were observed in the array blot proved beyond doubt that the microarray is a reliable and accurate system for detecting GMOs.

Microarray detection of GM and non-GM cornflakes

Assessing the genetic modification in 3 GM maize exemplifies cDNA microarray as an efficient detection system for GMOs. However for proof-of-concept purposes, a safety assessment measurement was conducted in two types of branded commercial cornflakes introduced to the Taiwan market and imported from USA. As expected, we observed that all the endogenous genes used were detected in the two brands of cornflakes. In addition, gene components corresponding to various transformation events were detected in these flakes as depicted in Figure 6. In the first cornflake brand, sample 1 (blot1), CaMV35S

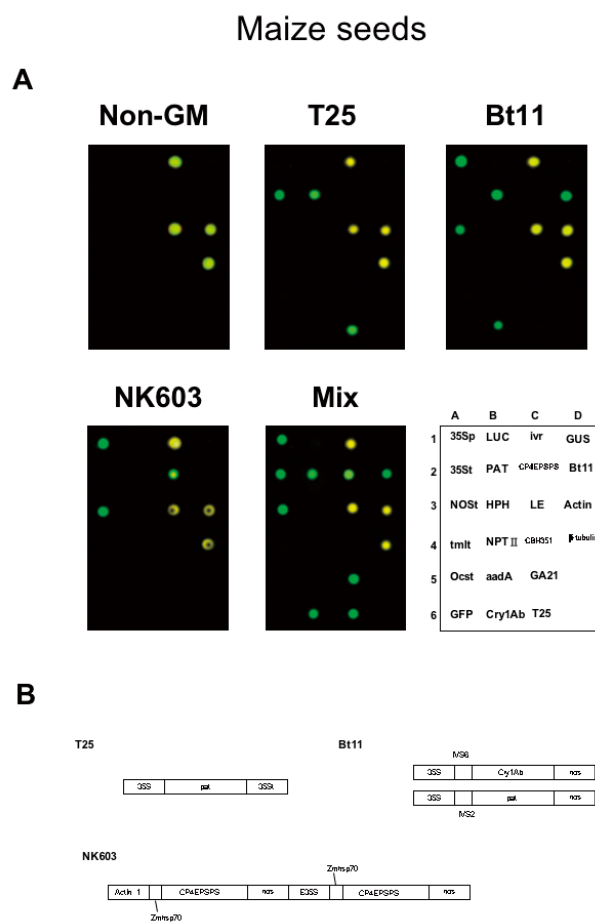


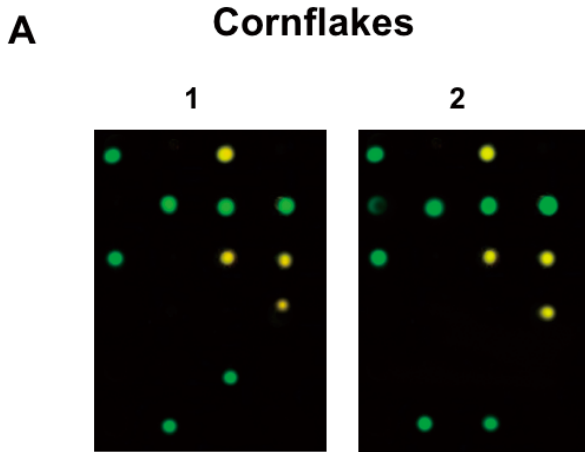
Figure 5. Array data depicting the maize components detected in the non-transgenic and transgenic maize seeds: Blot non-GM: non-transgenic maize, Blot T25, Bt11, NK603: the genes detected in the transgenic maize. Panel A right-corner, details of specific genes spotted on the slide; Panel B, the construction of T25, Bt11, NK603.

promoter, *nos* terminator, *bar* gene, CP4EPSPS, Cry1Ab, Bt11, and GA21 gene were detected. This implies that maize samples of Bt11 and GA21, probably containing event NK603, might also have been slipped in as derivatives. Similarly, in cornflake sample 2, the array revealed the presence of CaMV 35S promoter, *nos* terminator, CaMV 35S terminator, *bar* gene, CP4EPSPS gene, Cry1Ab, Bt11, and T25 gene, implying that the transformation events Bt11, T25, and NK603 were used. Until now, totally 119 samples have been tested in this system including GM and non-GM crops (Table 2). Only one soybean sample showed a false result. That means the mean accuracy percentage is 99%.

Sensitivity assay

To examine the limitation of our detection system, the DNA samples were prepared after mixing transgenic and non-transgenic potato tubers in respective proportion. The

compositions of the samples in Figure 7 were as follows: 5% GM potato tuber; 1% GM potato tuber; 0.1% GM potato tuber; 0.01% GM potato tuber and 0.002% GM potato tuber. DNA samples arrayed from these mixtures correctly identified the foreign genes and endogenous genes from 5% to 0.01% dilution samples. However, two blurred spots (spots of 35S promoter and *nptII* gene) were observed in a further dilution to 0.002% of GM potato tuber, implying that the limitation of this detection system ranged from 0.01 to 0.002% of GM crops in raw material.



B

	A	B	C	D
1	35Sp	LUC	ivr	GUS
2	35St	PAT	CP4EPSPS	Bt11
3	NOS _t	HPH	LE	Actin
4	tmlt	NPT II	CBH351	β-tubulin
5	Ocst	aadA	GA21	
6	GFP	Cry 1Ab	T25	

Figure 6. GM cornflake samples arrayed for the detection of maize components: Panel A, in two cornflake samples the presence of specific endogenous and genetic modifications were detected; Panel B, details of specific genes spotted on the slide.

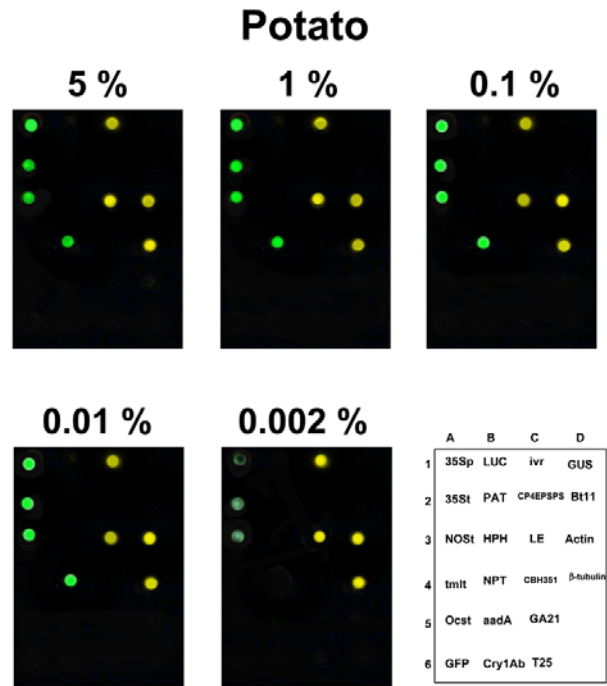


Figure 7. Array sensitivity assay. Raw material of GM potato tubers was mixed in the proportion of 5%, 1%, 0.1%, 0.01% and 0.002%. Endogenous internal control genes *ivr*, *LE*, *Actin*, and β -tubulin genes and transgenes 35Sp, 35St, *Nost*, and *NPTII* were detected clearly in the slides 5%, 1%, 0.1% and 0.01%. Presence of transgenes 35Sp, and *NPTII* was blurred in the slide 0.002%.

DISCUSSION

Today, GM food is a topic of debate in the scientific community and among the public and is a media focus. Legal authentication by competitive authorities regarding use of GM as food/feed has raised concerns among the public due to the lack of strong evidence for the safety of such foods on a long term basis. Though GMO regulations over the release of such food products into the market have been tightened, lack of universality has given rise to skepticism among the public regarding the stringency observed in different countries. Hence the public has demanded a selection of GM-foods that is on a par with non-GM foods. This has necessitated development of an efficient and reliable GMO detection system that will enable high-throughput screening to check intercontinental

Table 2. The GM and non-GM samples detected by this system.

Examples	No.	GMO	Non-GMO	FALSE
Tomato	18	2	16	0
Rice	16	3	13	0
Maize	25	5	20	0
Soybean	20	6	14	1
Potato	26	2	24	0
Wheat	14	0	14	0

gene flow and solve legal disputes arising from GMO controversies. The present investigation is an attempt to develop an efficient and reliable detection method for GMOs exemplified by the use of cDNA microarray.

In the present investigation, GMO detection techniques, exemplified by the use of a microarray detection system, overcome limitations encountered in the existing detection systems. The microarray detection system we designed has advantages like accuracy, precision, reliability, and reproducibility and is well suited to the high-throughput detection of pure GMO samples. For proof-of-concept purposes, the present study demonstrates the feasibility of detecting mechanistic T-DNA genes in the transgenic rice, tomato, and potato developed in our laboratory. In addition we extended our arraying system for the detection of a market-derived commercial transgenic potato and wheat, with no changes observed in the quality of the detection system. Due to its high accuracy, speed, and precision qualities, microarray detection can be effectively implemented for the qualitative detection of GMOs in industries and regulatory laboratories to address disputes pertaining to GMOs in future, where a large number of samples have to be screened in a time-result manner. The advantages of microarray detection include screening for a large number of different GMOs within a single experiment (Aarts et al., 2002). The feasibility of a microarray detection system to weed out genetic components in processed food and food products has been proven in our lab with soybean and processed soybean foods.

This microarray system also has been used for the detection and identification of three GM maize lines (T25, Bt11 and NK603), the transformation details of which were known. These products were developed by USA-based companies and have been marketed all around the world. According to the results in Figure 4, it can be assumed that microarray will meet internationally prescribed standards for GMO detection.

Past strategies for introducing GMOs depended on consumer requirements, but in the near future GMO development will become more complex. Crossing of approved GM varieties are considered to be novel GM varieties. Due to the easier amplification of specific sequences in the PCR product by universal primers (T7 and U19), the production of our GMO microarray is accomplished.

The strength of any GMO detection assay lies in its accuracy, reliability, and ease of use. Earlier DNA and protein based assays have reportedly been unable to achieve 100% accuracy in detecting the samples. When compared with the traditional food-borne pathogen detection methods, the microarray method displayed a significantly higher sensitivity. Recently, the report of using spotted DNA microarrays labeled with oligonucleotide probes that were complementary to four virulence loci (intimin, Shiga-like toxins I and II, and hemolysin A), *E. coli* O157:H7, a food born pathogen was detected from a less than one-cell

equivalent of genomic DNA (1fg) (Call et al., 2001).

According to a 2002 proclamation of the Taiwan Department of Health, all food products prepared from raw material containing GM soybean and maize greater than 5% are now subject to mandatory labeling prior to public sale (<http://www.doh.gov.tw/cht/index.aspx>). The European Union also introduced legislation stipulating the mandatory labeling of food products with GMO content exceeding 1% (Anonymous, 1998). In this study, we also tested the quantitative sensitivity of our microarray system. A serial dilution of GM potato tuber raw material was used as a model for this assay (Figure 7). The detection limits were in the range of 0.01-0.002% of the mass fraction of GMOs. This result is sufficient for the application of this system to the detection of genetically modified material.

In our results, microarray detection assay was able to achieve almost 100% accuracy, exemplified by its ability to weed out GM maize components in three non-labeled branded cornflake samples. Low template concentrations can generate significant amounts of non-specific amplification products in PCR. This makes interpretation of the results more complicated and biased (Minunni et al., 2000). Microarray analysis involves more than one target in each reaction, enabling a crosscheck of the results in the same reaction with other targets.

Due to the microarray methods high accuracy, speed, and precision, it can be implemented for the qualitative and quantitative detection of GMO in industry and in regulatory laboratories. Though we have not tried to adapt the microarray system to a quantitative detection analysis of GMO, we feel this methodology can be highly useful if used in combination with other effective methods, such as quantitative PCR.

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以 cDNA 微矩陣的方法建立大量篩檢基因改造食品 (GMOs) 的模式

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為了發展出一套有效且快速的基因改良食品檢驗系統，本實驗搜集了一般常見於基因改良作物的外來 DNA 基因片段如花椰菜鑲嵌病毒 *CaMV 35S* 啟動子，*NOS*t 或 *35S* 終結子、*nptII*、*hph* 或 *pat* 篩選基因、*gus* 或 *gfp* 標示基因、*cp4 epsps*、*cry1 Ab* 等已知的轉殖基因和 4 個植物的內標準基因 (如 *invertase*, *legumin*, β -*tubulin*, *actin* 基因) 等，打點於微型晶片上建立出一個大量篩檢基因改良食品的 cDNA 微矩陣系統。研究中先以本實驗室自行完成的轉殖水稻、蕃茄及馬鈴薯做初步的檢測和系統敏感度測試。結果顯示，外來基因 *CaMV 35S* 啟動子、*NOS*t、*35S* 終結子、*nptII* 和 *hph* 等基因都能成功地檢測出來，而相對應的野生種則只檢測出 4 個植物的內標準基因。為了更進一步了解此系統對市售樣品的檢測效率，實驗中也測定 GM 馬鈴薯及 GM 玉米及玉米脆片食品和市售小麥種子，結果同樣可以明確的偵測到外來 DNA 基因的存在。而在以馬鈴薯所做的敏感度測試中也顯示此系統至少可以偵測到原料中只含有 0.01-0.002% 的 GM 馬鈴薯塊莖，因此證明這個 cDNA 微矩陣系統可以發展成一個快速且便於大量篩檢的檢測系統。

關鍵詞：cDNA 微矩陣法；基因轉殖物種 (GMO)；基因轉殖馬鈴薯；基因轉殖玉米。