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Extending the cDNA Microarray Detection System to Evaluate Genetically Modified Soybean and Traditional Soy Foods

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ABSTRACT

Aggravating controversies of GM (genetically modified) foods on the social, ethical and health aspects have lead to efficient and reliable detection systems to safeguard homogeneity of foods and address legal disputes. Improvising on the existing detection systems, the present investigation demonstrates the advantages of using cDNA microarray as a detection system for GM food. We have extended the study for the detection of genetic modification in commercial GM soybean seeds and in home-made traditional foods derived from these seeds, such as tofu and dried tofu. We looked for common T-DNA regions such as CaMV35S promoter, NOS or 35S terminators, *nptII*, *hph* or *pat* selection marker genes, GUS or GFP marker genes. We also searched for specific traits such as Bt11, T25, CP4EPSPS and four plant internal control genes (invertase, legumin, tubulin, actin gene). Results indicate that our microarray detection system can identify GM soybean seeds as well as processed food made from these seeds, with 100% accuracy. Transformation events identified in the GM soybean seeds were also visible in the processed foods, thereby confirming the accuracy and reproducibility of this procedure to even processed foods. We believe that with more popularity if the cDNA microarray detection system will soon be implemented as a diagnostic kit.

Key words: genetically modified organisms (GMO), cDNA microarray, soybean

INTRODUCTION

Genetic modules identified from various plant sources have shown promise to express faithfully in crop plants. This stimulated interest for its implementation as genetic engineering components to improve plant specific processes in agriculturally important crops, such as soybean, corn, tomato, wheat etc. Soybean (*Glycine max*), a major grain legume, is rich in proteins and thus a nutritious source of food, oil and livestock meal. Engineering strategies have complemented earlier conventional efforts and focused on improving plant specific traits, such as herbicide tolerance and high oleic acid content[(1,2)] in soybeans, thereby promoting multidisplinary studies for producing superior hybrids. Since 1996, USA has emerged as a major GM soybean producing country accounting for 80% of the world's total soybean production[(2)] followed by Brazil, China and Canada.

Although GMO established regulations on the safe release of GM soybean crops and foods, the general public masses has displayed skepticism pertaining to the safety of GM soybean. The controversies are attributable to the fact that GMO regulations are not based on an universal concept, resulting in speculations in developing and underdeveloped countries regarding the homogeneity of non-GMO soybeans imported from developed countries. An Australian pilot survey of corn and soy revealed that GM components were detected in soy milk samples with no labels. In other cases, genetic modification was evident even in unlabelled soy derivatives (http://www.foodstandards.gov.au). Such incidents provided ample proof that either some GM producing nations did not comply with the regulations or non-GM crops were contaminated by neighboring fields.

This has instigated awareness in the scientific community to develop efficient detection systems for monitoring intercontinental gene flow and addressing controversies related to GMO debate, where the public was not complacent with the biosafety regulations of neighboring countries. Establishing such detection systems will not only benefit the country of origin but also preserve consumer's choice in par with non-GMO foods. Developing a reliable detection system is attributable to the presence of commonly used genetic modules in the T-DNA region such as promoter (*CaMV35S*), selections markers (*nptII*, *hph*), reporter (*gus*, *luc*, *gfp*), terminator (*nos*), which function mechanistically upon integration in the plant gene

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pool via transformation. Among all 235 reported methods, 223 were based on the polymerase chain reaction (PCR) and were used to detect certain DNA sequences. Of these, 46 methods could be used to screen the presence of certain genetic elements, e.g. promoter or terminator sequences, while 177 were suitable to detect genetic modifications. On the other hand, 12 of the reported methods were based on the detection of a heterologous protein resulting from genetic modifications and 58 methods could be used to quantity recombinant DNA[3].

In recent years, import of food crops and their derivatives into Taiwan have grown exponentially. The major resource of soybean and maize in Taiwan is from USA In order to safeguard the nation from unwanted GM soybean, it is imperative to develop an efficient and through screening procedure for GM soybeans and even traditional foods in the future. The present investigation is exemplified by demonstrating the feasibility of using microarray as a reliable and efficient method to detect varieties of GM soybean developed by USA-based seed companies. We further demonstrate the reproducibility and feasibility of our protocol by weeding out similar GM soybean components in processed traditional foods.

MATERIALS AND METHODS

I. *Type of Materials*

Transgenic (Monsanto, GST 40-3-2, Roundup Ready™) and non-transgenic soy bean seeds were provided by American Soybean Association-Taiwan Office. Samples analyzed included soybean seed (transgenic and non-transgenic) and derivatives such as soy milk, dried tofu and deep-fried fermented bean curd.

II. *Preparation of Traditional Soybean Foods*

Soy milk was prepared by soaking 100 g of soybean (transgenic and non-transgenic) in 1 L of water overnight. The seeds were then ground, filtered and boiled for 35 min. Warm soy milk (70-80˚C) was supplemented with 4 g of gypsum (hydrated calcium sulfate, $CaSO_4$-$2H_2O$) suspension and gently stirred until the soy milk curded properly, to obtain dried-tofu later on. The soymilk was fractionated to separate the curded from the liquid phase, and the former condensed under pressure. The bean curd was cut into pieces of suitable size and dried. The bean curd pieces were then soaked in a solution with the starters, *Bacillus pumilus*, *Bacillus subtilis* and *Bacillus cereus*, and fermented for 3 hr. After draining the liquid, the fermented tofu was deep-fried for 2 min to prepare deep fried fermented bean curd[4].

III. *DNA Isolation*

Total genomic DNA was isolated by the standard CTAB protocol[5]. Homogeneous samples (20 g) of each sample (transgenic and non-transgenic) were homogenized in a blender. For isolation of total genomic DNA from soy milk, 300 mL of soy milk was centrifuged in 15000 ×g for 30 min and the genomic DNA was isolated from the precipitate by the standard CTAB protocol. The quality and concentration of DNA was determined spectrophotometrically at 260/280 nm.

IV. *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 by PCR using primers shown in Table 1[5-8]. The PCR cocktail mixture (100 μL) contained plasmid template (300 ng), 10× reaction buffer [200 mM Tris-HCl (pH 8.0), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg/mL nuclease-free bovine serum albumin (BSA)], 5 μL of 100% DMSO, 4 μL of 2.5 mM dNTP, 1 μL of 100 pmole/μL 5'primer, 1 μL of 100 pmole/μL 3'primer and 1 μL of Pfu DNA polymerase (5 units/μL). Amplification was performed in the PCR thermocycler (GeneAmp 2400, Perkin Elmer, California, USA) 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 Viogene kit and cloned into pT7Blue Perfectly Blunt vector, as instructed by of the kit manual.

V. *cDNA Microarray Preparation*

The microarray method was a modification of that described by Seki[9]. 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 μL of PCR products (100-500 ng/μL) were pipetted from the 384-well microtiter plates. Five nL per slide were deposited, onto six slides, spaced 280 μm apart. The printed slides were rehydrated in a beaker with hot distilled water and snap dried at 100˚C for 5-10 sec. The DNA was cross-linked on the slide by using UV cross-linker (150-300 mJ). The slides were placed into a slide rack, which was placed into a glass chamber. The blocking solution, containing 25 mL of 0.2 M sodium borate, pH 8.0, 3.57 g of succinic anhydride (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. Afterwards, the slide racks were transferred into another chamber containing 95% ethanol for 1 min, and centrifuged at 500 rpm for 5 min.

Journal of Food and Drug Analysis, Vol. 12, No. 3, 2004

VI. *Probe Preparation*

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[10] and TIGR[11]. In this modified protocol, 2 μg of genomic DNA

Table 1. Primers used in PCR amplification

Template	Genes	Primers	Size (bp)	Tin CC)	Ref.
Selection marker					
pCAMBIA 2301	NPTII	sense- 5'TCCGGCCGCTTGGGTGGAGAG anti- 5'CTGGCGCGAGCCCCTGATGCT	470	63.6	This study
pCAMBIA1201	HPH	sense- 5'AGCTGCGCCGATGGT-rTCTACAA anti- 5'ATCGjCCTCGCTCCAGTCAATG	509	60.5	This study
p932A-GUSR	aadA	sense- 5'AAGCGGTGATCGCCGAAGTATCGAC anti- 5'AAAGAGTTCCTCCGCCGCTGGA	455	59.9	This study
pJD4401	PAT	sense- 5'GCGGTCTGCACCATCGTCAA anti- 5'AGTTCCCGTGCTTGAAGCCG	415	63.1	This study
Reporter gene					
pB1221	GUS	sense- 5'CTGCGACGCTCACACCGATACC anti- 5'TCACCGAAGT-rCATGCCAGTCCAG	441	59.5	This study
pMTC54	LUC	sense- 5'GAGAATAACATMGATAGGACCAC anti- 5'GCATAGATTGATACCCCAAG	484	50.8	This study
pCAMBIA1304	GFP	sense- 5'AAGGAGAAGAACTTTTCACT anti- 5'TGATAATGATCAGCGAGTrG	541	51.9	This study
Prornter and Terminator					
pCAMBIA1304	35Sp	sense- 5'CATGGAGTCAAAGATTCAAA anti- 5' ATATAGAGGAAGGGTCTTC	500	47.2	(5)
PJD301	Nost	sense- 5'CGTTCAAACATI-rGGCAATA anti- 5' CCCGATCTAGTAACATAGAT	253	52.5	(5)
pCAMBIA1304	35St	sense- 5'AATTCGGGGG6ATCTGGATT anti- 51 CGATCGACAAGCTCGAGT-rTAT	201	50.4	This study
TetVp16	Ocst	sense- 5'GCTAGCTATATCATCAAT-17AT anti- 5'CCCATCT-rGAAAGAAATATAG	204	44.8	This study
pMTC40	tmIt	sense- 5'TATTAGGTTACGCCAGCCCT anti- 5' TAACACGCACACTTACGATA	240	44	This study
Control Gene					
Rice genomic DNA	Actin	sense- 5'GACTACTACAAGCrGCATCAG anti- 5' CACACCCACTCCAGATGCCT	318	42	This study
Maize genomic DNA	ivr	sense- 5'CCGCTGTATCACAAGGGCTGGTACC anti- 5' GGAGCCCGTGTAGAGCATGACGATC	226	52	This study
Soybean genomic DNA	LE	sense- 5'GCCCTCTACTCCACCCCCATCC anti- 5'GCCCATCTGCAAGCCTrMGTG	118	48	(8)
Tomato genomic DNA	P-tubujin	scnse- 5'CCCGGGCACACTTTGATCCCATTCG anti- 51 CCCCTCTGCATTCTGTCTGGGTACTCTTC	530	50	This study
GM Soybean Maize gene					
Transgenic maize genomic DNA	CBH351	sense- 5'CCTTCGCAAGACCCT TCCTCTATA anti - 5'GTAGCTGTCGGTGTAGTCCTCGT	170	50	(6)
Transgenic soybean genomic DNA	CP4EPSPS	sense- 5'TGATGTGATATCTCCACTGACG anti- 51 TGTATCCCTrGAGCCATGTTGT	172	45	(8)
Transgenic maize genomic DNA	T25	aense- 5'GCCAGT-rAGGCCAGTTACCCA anti- 51 TGAGCGAAACCCTATAAGAACCCT	149	45	(6)
Transgenic maize genomic DNA	Btl I	sense- 5'CCATTTT`TCAGCTAGGAAGT-rC anti- 51 TCGTTGATGTrKGGG'I-rGTTGTCC	110	42	(6)
Transgenic maize genomic DNA	GA21	sense- 5'ACGGTCTGAAGAGTTCAATGTATG anti- 5' TCTCCTTGATGGGCTGCA	270	42	(6)
Transgenic maize genomic DNA	Cry][Ab	sense- 5'ACCATCAACAGCCGCTACAACGACC anti- 5'TGGGGAACAGGCTCACGATGTCCAG	184	50	(7)

Abbreviations

35Sp: promoter from 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: β-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. tmlt: transcription terminator of a tumor morphology large gene from *Agrobacterium tumefaciens*. T25: specific gene of T25 Libery (AgrEvo).

was digested with *Sau3*AI (average size is 500-1000 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 hr 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 μg 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 0.1M DTT, 10mM dNTPs, superscript II RT (Life Technologies, Rockville, USA) were added immediately and mixed thoroughly before incubation in PCR machine for 1 hr at 42˚C. The labeling reaction was cleaned up as described by TIGR standard operating procedure (SOP)[12].

VII. *Microarray Hybridization and Scanning*

The hybridization methods were also a modification of that described by Seki[9]. The probe samples were placed onto the center of the slide and a cover slip was placed over the entire array surface to avoid bubble formation. Four 5-μL drops of 3 × SSC were placed on separate points on the slide, which were 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 hr. After hybridization, the slides were removed and placed in a slide rack submerged in washing solution 1 (2 × 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 × SSC) carefully to minimize the 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 × SSC), allowed to stand for 2 min, spun at 500 rpm for 5-10 min, and dried.

Microarrays were scanned with a scanning laser microscope (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 Imagene version 5.0 (Gene Spring, Redwood City, CA) software for the microarray data analysis.

RESULTS AND DISCUSSION

Growing popularity of genetically modified food in developing countries have given room for skepticism in developed, developing and underdeveloped nations regarding homogeneity of imported GM and non-GM crops and their derivatives. Skepticism is mainly attributable to the inadvertent toxicity to health and environment and lack of universal GMO regulations. It is imperative that detection systems are developed to bridge the disparity between GMO and non-GMOs and preserve the choice of consumers.

Current GMO detection methods rely mostly on immunoassay to detect proteins and PCR-based techniques to detect DNA. However, immunoassay does not have the advantage of screening complex/mixture samples in one experiment[12]. The low template concentrations or denatured DNA such as processed food can generate significant amounts of non-specific amplification products in PCR-based detection systems. This makes the interpretation of the results more complicated and biased[13]. The official Swiss method (PCR-based) uses detection of cauliflower mosaic virus promoter and the nopaline synthase terminator (NOSt), which are present in most approved GM crops[12]. Most PCR experiments enable detection of one gene/promoter (e.g., CaMV 35S promoter) in each sample at a time and required information of varieties for designing suitable primers. With new molecular tools providing insights into the molecular mechanism of plant specific traits, cDNA microarray has become a desirable tool for the detection of expressed genes during various treatments[9].

In the present investigation, GMO detection method using microarray technology was used to evaluate GM soybean and soy traditional foods. Transgenic soybean constructs Monsanto, GST 40-3-2, Roundup Ready™ and non-transgenic soybean (*Glycine max L.*) provided by the American Soybean Association-Taiwan Office were arrayed for the detection of endogenous genes such as invertase, legumin, actin and β-tubulin, as well as foreign genes such as CaMV 35S promoter, NOSt terminator and CP4EPSPS genes. As evident in Figure 1, the foreign genes were observed in non-transgenic soybean, which served as a negative control. However, in transgenic soybean all the above-mentioned component and endogenous genes of the expression cassette were detectable (Figure 1A right). Non-specific genes were not detected.

A similar experimental setup was used to differentiate homemade soymilk and dried tofu derived from the same non-transgenic and transgenic soybean seeds tested, the former serving as a negative control. As depicted in the results, non-GM soymilk and dried tofu array slide revealed signals of the endogenous genes such as invertase, legumin, actin and β-tubulin. However, no foreign genes of the expression cassette were observed (Figure 2A left and Figure 3A left). On the contrary, transgenic soymilk and dried tofu harboring a reading frame consisting of CaMV 35S promoter, NOSt terminator and CP4EPSPS gene were detectable, in addition to the genes detected in non-transgenic tomato. No non-specific genes were detected on this slide (Figure 2A right and Figure 3A right).

As we know, there is liability that the genetic material

Journal of Food and Drug Analysis, Vol. 12, No. 3, 2004

and proteins will be denatured during boiling, extracting and deep frying, making detection more difficult. For proof-of-concept purposes, we arrayed a homemade GM and non-GM deep-fried fermented bean cured sample for the above mentioned genes. As depicted in Figure 4A right, in addition to the endogenous genes detected in non-transgenic deep-fried fermented bean curd (Figure 4A left), we could also detect DNA genes such as CaMV 35S promoter, NOSt terminator and CP4EPSPS gene from *Bacillus thuringiensis* in this sample. However, in the present study we obtained unbiased results in the processed soybean food, which was substantiated by comparison with array results obtained from GM soybean seeds. This demonstrates the sensitivity and accuracy of the detection system.

In our study, GMO detection techniques exemplified by the use of a microarray detection system fulfill the previously mentioned limitations encountered in the existing detection systems. Except for the CaMV 35S promoter, NOSt terminator and CP4EPSPS genes, we also included other genes commonly encountered in the present day GMOs. It is hence believed that we can extend our arraying system for the detection of the other GM sources in the functional food or Chinese herbs/medicine. Due to

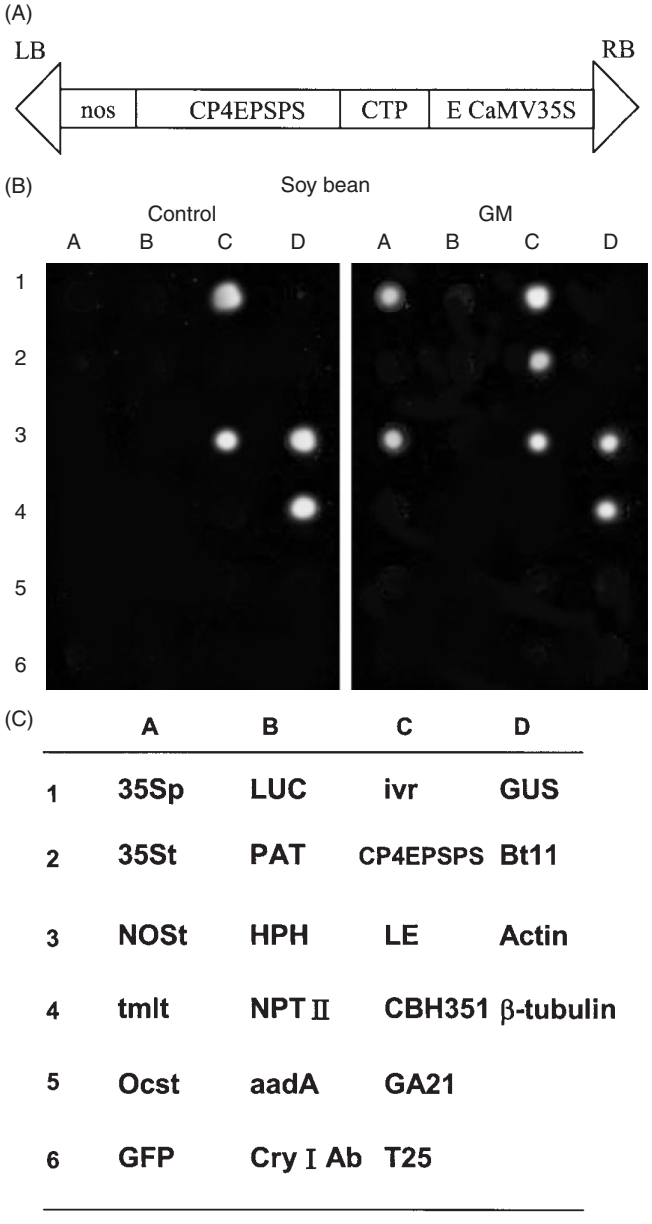


Figure 1. The genetic module of event GST 40-3-2 and arraying of transgenic and non-transgenic soybean seeds (*Glycine max L.*)
Panel A, the genetic construct of GST 40-3-2. The abbreviations are described as follows. E CaMV35S, enhanced CaMV35S promoter, CTP, Chloroplast transit peptide from *Petunia hybrida*, CP4EPSPS and NOSt are as described in Table 1.
Panel B left, non- transgenic soybean was detected for the presence of the endogenous genes: invertase, legumin, actin and β-tubulin gene. Panel B right, transgenic soybean was detected by the presence of additional transgenic genes: 35Sp, NOSt and CP4EPSPS genes. Panel C, the position of specific genes spotted on the cDNA microarray slide.

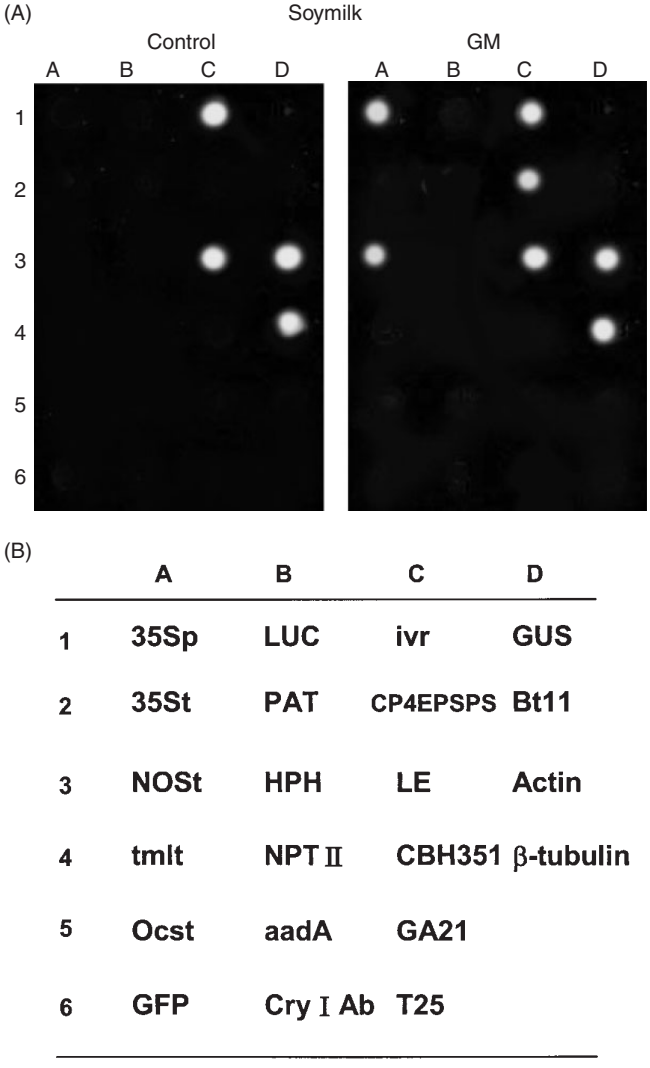
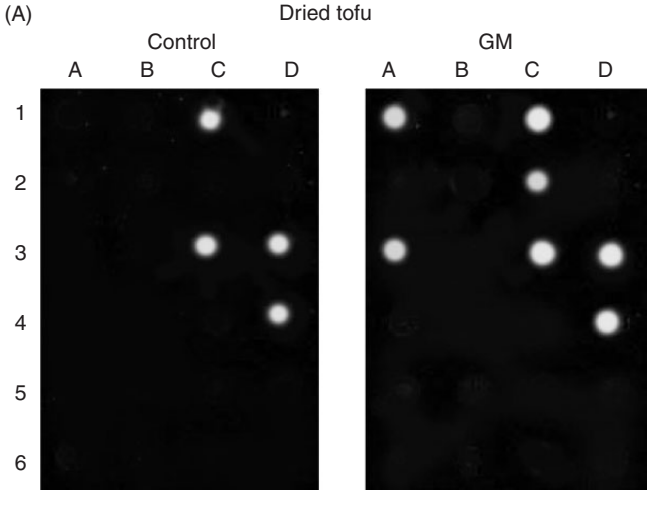


Figure 2. Microarray detection for genetic components in homemade soy milk derived from non-transgenic and transgenic soybean seeds.
Panel A left, endogenous genes were detected in non-GMO soy milk: invertase, legumin, actin and β-tublulin gene. Panel A right, other genetic components detected in GM soy milk: 35Sp, NOSt and CP4EPSPS genes. Panel B, the position of specific genes spotted on the cDNA microarray slide.

its high accuracy, speed and precision, microarray detection method can be effectively implemented for the qualitative detection of GMO in industries and regulatory laboratories to address future GMO disputes, where a large number of samples have to be screened in a timely manner.

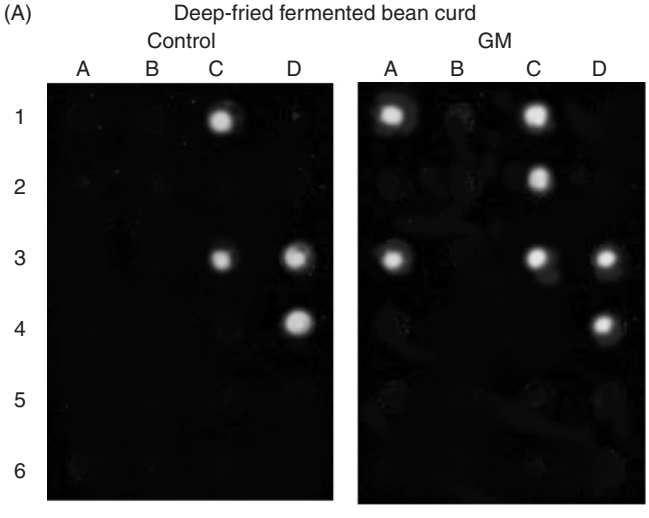
Microarray technology is a powerful method for quantitative analysis and screening of expression genes. Until now, this technique has been used in the laboratory researches for gene expression, new drug development and clinical diagnostics. However, we should not ignore the cost and patent authorization included in each experiments. The current cost of microarray should include the equipments of microarray, the reagents for preparing and detecting of microarray chips and technicians. According to our core microarray facility, the cost of microarrayer and scanner is about NT$ 5.3 million, whereas the cost of reagents for preparing and detecting of microarray chips is about NT$ 1.4 thousand for single chip. Microarray technique developed by Dr. Peck (patent US 6,218,114) is available for NT$ 1 million, but it is authorized for four biotechnical companies. The related patents can be searched in web site: http://www.uspto.gov/patft/ index.html. Any method used in the detection of GMOs would need to be constantly monitored, modified and updated. The field of molecular biology advance at a rapid pace and new methods and technologies also constantly appearing. In the present investigation, we have introduced microarray technology as a powerful tool to detect foreign genes in GMOs, which can be used at commercial level for authenticity certification and also for consumer acceptance of GMOs.



(B)

	A	B	C	D
1	35Sp	LUC	ivr	GUS
2	35St	PAT	CP4EPSPS	Bt11
3	NOSt	HPH	LE	Actin
4	tmlt	NPTⅡ	CBH351	β-tubulin
5	Ocst	aadA	GA21	
6	GFP	CryⅠAb	T25	

Figure 3. Microarray detection of genetic components in homemade dried tofu produced from non-transgenic and transgenic soybean seeds.
Panel A left, endogenous genes were detected in dried tofu derived from non-transgenic soy bean: invertase, legumin, actin and β-tublulin gene. Panel A right, additional transgenic genes were detected the dried tofu derived from transgenic soy bean: 35Sp, NOSt and CP4EPSPS genes. Panel B, the position of specific genes spotted on the cDNA microarray slide.



(B)

	A	B	C	D
1	35Sp	LUC	ivr	GUS
2	35St	PAT	CP4EPSPS	Bt11
3	NOSt	HPH	LE	Actin
4	tmlt	NPTⅡ	CBH351	β-tubulin
5	Ocst	aadA	GA21	
6	GFP	CryⅠAb	T25	

Figure 4. Microarray detection of genetic components in homemade fermented bean curd produced from non-transgenic and transgenic soybean seeds.
Panel A left, endogenous genes were detected in fermented bean curd made from non-transgenic soy bean seed: invertase, legumin, actin and β-tublulin gene. Panel A right, genetic components detected in fermented bean curd made from transgenic soy bean seeds: 35Sp, NOSt and CP4EPSPS genes. Panel B, the position of specific genes spotted on the cDNA microarray slide.

Journal of Food and Drug Analysis, Vol. 12, No. 3, 2004

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