

A microarray-based detection system for genetically modified (GM) food ingredients

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Received 16 July 2005; accepted in revised form 23 December 2005

Key words: colorimetric, detection, GMO, GMOchips, microarray

Abstract

A multiplex DNA microarray chip was developed for simultaneous identification of nine genetically modified organisms (GMOs), five plant species and three GMO screening elements, i.e. the 35S promoter, the *nos* terminator and the *nptII* gene. The chips also include several controls, such as that for the possible presence of CaMV. The on-chip detection was performed directly with PCR amplified products. Particular emphasis was placed on the reduction of the number of PCR reactions required and on the number of primers present per amplification tube. The targets were biotin labelled and the arrays were detected using a colorimetric methodology. Specificity was provided by specific capture probes designed for each GMO and for the common screening elements. The sensitivity of the assay was tested by experiments carried out in five different laboratories. The limit of detection was lower than 0.3% GMO for all tests and in general around 0.1% for most GMOs. The chip detection system complies with the requirements of current EU regulations and other countries where thresholds are established for the labelling of GMO.

Abbreviations: CaMV, cauliflower mosaic virus; CTAB, hexadecyltrimethylammonium bromide; DNA, deoxyribonucleic acid; EC, European Commission; EU, European Union; GMO, genetically modified organism; NptII, neomycin phospho transferase originating from *Streptomyces hygroscopicus*; P-35S, promoter 35S originating from cauliflower mosaic virus; Pat, phosphinotricin-N-acetyl transferase; PCR, polymerase chain reaction; RRS, roundup ready® soybean; T35S, terminator 35S originating from cauliflower mosaic virus; Ta, annealing temperature; T-nos, terminator nopaline synthase originating from *Agrobacterium tumefaciens*; UNG, uracil-N-glycosylase

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Introduction

In recent years, the globally cultivated area of transgenic crops has increased dramatically to 81.0 million hectares in 2004 (James, 2004, p. 3). Four major crops are the principal commercial genetically modified plant species: soybean, maize, cotton and rapeseed. The acceptance of GMOs by consumers is still controversial and has pushed the authorities of different countries to implement GMO labelling regulations. In the USA, GMOs are considered to be “substantially equivalent” to conventional foodstuffs and there is no regulatory requirement for GMO labelling. However, the European Union has implemented mandatory rules for labelling foods or food ingredients containing recombinant DNA or modified protein above a ‘de minimis’ threshold of 0.9 % (Regulations EC No. 258/97, 18/2001, 1829/2003 and 1830/2003). Many Asian countries such as Japan, Taiwan and South Korea have established a 5% threshold. South American countries as Brazil adopted a threshold to label all products containing more than 4% of GMO and there are also labelling requirements in other countries from Oceania as Australia and New Zealand.

The development and application of a reliable and specific simultaneous analytical detection method is thus essential in order to guarantee the consumer’s access to information as well as to enforce food labelling by the competent authorities.

PCR-derived amplifications are the methods of choice to detect GMO presence. Several conventional or multiplex PCR, have been reported for qualitative analysis of the GMO content in a sample (Padgett *et al.*, 1995; Zimmermann *et al.*, 1998; Matsuoka *et al.*, 2001). Other PCR-derived technologies such as competitive PCR (García-Canas *et al.*, 2004) or real-time PCR (Terry and Harris, 2001; Rønning *et al.*, 2003; Windels *et al.*, 2003; Hernández *et al.*, 2004) allow the quantification of GMO content in a sample. Multiplex PCR have also been proposed to test for several GMOs (Permingeat, *et al.*, 2002; Germini *et al.*, 2004; Hernández *et al.*, 2005).

Microarrays, also known as DNA chips, allow the analysis of multiple sequence targets in one single assay. Being a highly adaptable tool, it can evolve together with the increasing number of GMOs emerging in the food and feed markets.

The main advantages of DNA microarray technology are miniaturization, high sensitivity and screening throughput. Different DNA microarray approaches have been developed to be used in combination with multiplex PCRs: a multiplex DNA array-based PCR allowing quantification of transgenic maize in food and feed (Rudi *et al.*, 2003); a ligation detection reaction coupled with an universal array technology allows the detection of the Bt176 transgenic maize (Bordoni *et al.*, 2004) or five transgenic events (Bordoni *et al.*, 2005); and recently, a peptide nucleic acid array approach was developed for the detection of five transgenic events and two plant species (Germini *et al.*, 2005). These methods used fluorescent probes, which require costly material and are photosensitive, thus limiting the common use of microarrays for GM detection. We report in this paper the development of a low-density DNA chip using a special technology that makes it a very convenient analytical tool. The aim of this study was to develop a cost effective, highly sensitive, easy to use assay with reagents that avoid the drawback of the fluorescent probes like special conservation precaution. The arrays are glass solid supports containing, on their surface, a series of discrete regions bearing capture nucleotide probes that are complementary to the target nucleotide sequences to be detected (Zammatteo *et al.*, 2000). After target DNA amplification in the presence of biotinylated nucleotides, amplicons are allowed to directly hybridize onto the arrays, and are subsequently detected by a colorimetric system (Alexandre *et al.*, 2001). Such a method complies with the general guidelines and requirements of standardization currently under study by CEN/TC 275/WG 11.

This work has been developed in the framework of a European shared cost project “New technology in food science facing the multiplicity of new released GMO” funded by the European Commission under the Fifth Framework program (GMOchips; contract no. G6RD-CT-2000-00419).

The microarray approach enables the detection and identification of both, authorized GMOs and also the suspected presence of unidentified varieties by the detection of common elements (e.g. 35S promoter from CaMV, nos terminator from *Agrobacterium tumefaciens* or *nptII* gene) in a sample.

The microarray, called from now the GMO-chip, was developed for the identification of nine

GMO events authorized for their commercialization in the European Union (maizes 176, Bt11, GA21, MON810, CBH351 and T25, rapeseeds Topas 19/2 and T45, and RoundupReady soybean). Three small DNA fragments (35S promoter, *nos* terminator and *nptII* gene) present in most GMOs were also added and used for screening purposes. The host plant species, maize, rapeseed, soybean, sugar beet and tomato were also detected using species-specific genes. Special care was taken to use appropriate controls, including the detection of possible CaMV contamination that could lead to false positive results.

Materials and methods

Sample material

Plants. Heterozygous Bt176 and T25 maize seeds were provided by Syngenta Seeds and by Bayer Crop Science (formerly Aventis), respectively, through the French Ministry of Agriculture. StarLink (CBH351) corn and Topas 19/2 rapeseed were provided by Bayer Crop Sciences. GA21 seeds were purchased from American Type Culture Collection (ATCC, USA). Seeds were grown in greenhouses and leaves collected. Certified MON810 (IRMM-413) and Bt11 maizes (IRMM-412) and Roundup Ready soybean (IRMM-410) reference materials (CRM) were purchased from Fluka (Buchs, Switzerland). Presumed OXY235, GT73 and T45 rapeseed lines were distinguished among routine analysis samples. These last samples were identified by PCR testing several parts of the expression cassettes. All GM rapeseeds were checked for the absence of CaMV contamination. Non-GM varieties, i.e. Pactol corn; Drakkar and Goeland rapeseeds were used as negative controls. **Virus.** The cauliflower mosaic virus (CaMV) strain 2 was used and provided by C. Kerlan (INRA, Rennes, France). The freeze dried inoculated cauliflower leaves were re-suspended in a 0.05 M KH₂PO₄ buffer complemented with β-mercaptoethanol (0.4%) to prepare the virus inoculum. Rapeseed (*Brassica napus* L.) plants (six leaves stages) were wounded using sterile carborundum and inoculated using a cotton piece previously immersed in the viral inoculum. The leaves were collected one month

after inoculation and immediately frozen (−20 °C) until DNA extraction.

DNA purification

Small scale genomic DNA was extracted using a CTAB-based method (Rogers and Bendich, 1985) and purified through silica columns (QIAquick, QIAGEN). Large scale genomic DNA was isolated from leaves of non-GM rapeseed variety Drakkar, GM maize Bt176 variety Pactol and GM maize MON810 according to Dellaporta *et al.* (1983).

Genomic DNA was quantified spectrophotometrically using a GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) and analyzed by 1% (w/v) agarose gel electrophoresis in 1× TAE (0.04 M Tris-HCl pH 8.5, 0.001 M EDTA, and 0.02 M acetic acid) with ethidium bromide staining.

PCR and labeling

PCR reactions were carried out in a GeneAmp™ PCR System 9600 (Perkin-Elmer Cetus Instruments, Emeryville, CA) using TaqMan™ PCR core reagents (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, NJ). Primer Express™ 1.5 software (Applied Biosystems Division of Perkin-Elmer Corp., Foster City, CA) was used to design the oligonucleotides. Unless otherwise stated, labelling PCRs were performed in 50 μL PCR mixture containing: 1× buffer II (100 mM Tris-HCl pH 8.3, 500 mM KCl), MgCl₂, 400 μM each dATP, dCTP and dGTP and 800 μM dUTP, 10 μM each biotinylated dATP and dCTP, corresponding primer pair, 1.25 U of AmpliTaq Gold™ DNA polymerase, 1 U of uracil *N*-glycosylase and 100 ng DNA template. Cycling conditions were: 10 min at 20 °C (UNG incubation), 10 min at 95 °C, 40 cycles of 30 seconds at 95 °C, 30 seconds at the corresponding annealing temperature and 90 seconds at 72 °C with a final extension step of 10 min at 72 °C. Specific conditions for amplification and labelling were: for Bt176, T25, T45 and Topas 19/2 lines, Ta 56 °C, 2 μM *OPP35S1*, 2 μM *OPT352*, 1.8 mM MgCl₂ and 9% DMSO, respectively; for Bt11, StarLink corn and Roundup Ready soybean, Ta 56 °C, 0.8 μM *OPP35S1* (5'-CGTCTTCAAAGCAAGTGGATTG-3'), 0.8 μM

OPTnos2 (5'-ATCTTAAGAACTTTATTGCC AAATGT-3'), 3.75 U of AmpliTaq Gold™ DNA polymerase and 4 mM MgCl₂, respectively; for MON810, Ta 53 °C, 0.9 μM *OPhsp* (5'-CGATG CCTTCTCCCTAGTGTG-3'), 0.9 μM *OPery2c* (5'-CACGAACTCGCTGAGCAGGA-3') and 5.5 mM MgCl₂; GA21 maize, Ta 56 °C, 0.3× buffer II, 1 μM *OPEPS1B* (5'-CCAAGTCGCTT TCCAACCG-3'), 1 μM *OPTnos2*, 3.75 U of AmpliTaq Gold™ DNA polymerase and 4 mM MgCl₂; for *nptII*, Ta 57 °C, 0.2 μM *nptCSIC1Fb* (5'-CTCGACGTTGTCACTGAAG-3'), 0.2 μM *nptCSIC1Ra* (5'-GATGGATACTTTCTCGG-CAG-3') and 2.8 mM MgCl₂; for the PCR duplex system conditions for simultaneously amplification of P35S and T-nos, the mixture consisted of 0.5 μM forward primer (*SF*, 5'-CGTCTTCAAA GCAAGTGGATTG-3' and *nosCSIC2F*, 5'-TTGAATCCTGTTGCCGGTCTT-3') and 0.5 μM reverse primer (*SR*, 5'-TCTTGC GAAGG ATAGTGGGATT-3' and *nosCSIC1R*, 5'-CGCT ATATTTTGTCTATCGCG-3'), together with 2.8 mM MgCl₂ at Ta 56 °C; for CaMV system, Ta 56 °C, 1 μM *CaMV3F* (5'-ACGTAAG TGCCACTAGTATGGCTAATCTTAATCAGA TCC-3'), 1 μM *CaMV3R* (5'-GTTGTTCTATTA GTTGCTCTTT-3') and 2.5 mM MgCl₂; for plant species systems (SS and RA) we used independently the same PCR conditions for each system, 0.5 μM each primer *c-raf/c-rar* (5'-ACAACCAGA TGGTBAACGC and 5'-GCCAGTAYAART TCTCCA, respectively) and PPss3/ PPss4

(5'-GGTTTGGAGARRGGNTGGGG and 5'-TC CAADATGTAVACAACCTG, respectively), and 2.5 mM MgCl₂ at Ta 56 °C. The amplicon sizes were: Bt176 (200 bp), T25 (801 bp), T45 (807 bp), Topas 19/2 (808 bp), Bt11 (1041 bp), Roundup Ready soybean (1849 bp), GA21 (1335 bp), MON810 (233 bp), StarLink (800 bp), *nptII* (105 bp), P-35S (79 bp) T-*nos* (around 170 bp), CaMV (261 bp), sucrose synthase and rubisco activase systems (around 300 bp and 180 bp, respectively).

Low-density microarray design

The array was present on a glass slide with 20 different capture probes covalently attached by an amino group located at the 5' end onto an aldehyde functionalized slide forming spots of 250 μm diameters (Figure 1). All capture probes were spotted in triplicate, as generally recommended (Lee *et al.*, 2000). Capture probes contained 20- to 30-nucleotide sequences complementary to their specific targets and were designed to minimize secondary structures, hetero and self-dimerizations, and to have melting temperature values which are ranging between 77 and 82 °C. They are based on the technology developed by Eppendorf Array Technologies, S.A. (EAT, Namur, Belgium) (Hamels *et al.*, 2001).

The GMOchip contained different types of capture probes according to the detection to be performed. The first class was capture probes

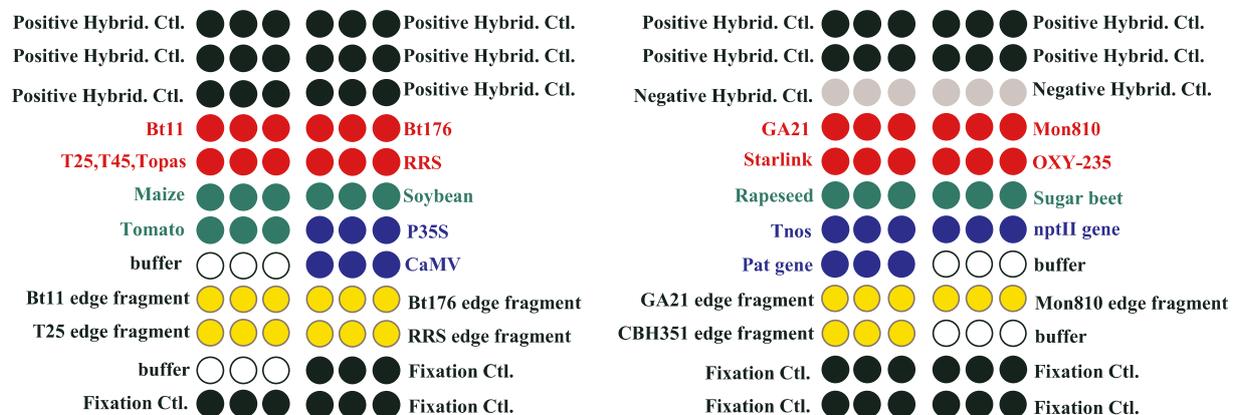


Figure 1. Schematic representation of the GMOchips content. The GMOchip is divided into several Parts according to the specificity of the capture probes: Part 0 for the various controls (●), Part 1 for the specific GMO probes (●), Part 2 for the plant probes (●), Part 3 for the small elements (●) and Part 4 for the edge fragments (●). Order of the capture probes can vary from one image to another. To avoid any misunderstanding below, each capture probe giving a positive signal is indicated by an arrow and its name.

Table 1. Specific capture probes used in this study.

Probe	Sequence (5'-3')
Bt11	GCGAGGTGAAGAGGATCTTCGCTA
Bt176	GATAGAAGTATATAGTGAAGGTCCT TATATAGAG
GA21	TTTTGGCAGCTTTGTCCGCTTGC
Mon810	ATACCAAGCGGCCATGGACAACAAC
T25, T45, Topas	CTCCGGAGACATGTCGAACTCTAGA
RRS	CGGGGTCTACGATTTGACAGCACC
Starlink	CGATCTGCTTCGGATCCTAGAACG
OXY-235	TACTAGGCAGCCAGGCAGGCCATG

specific for the GMOs and was named “Specific GMOchip” (Part 1). Six capture probes were devoted to the specific recognition of six GMO events: Bt11, Bt176, GA21, MON810, Roundup-Ready Soybean and StarLink. An additional capture probe was designed for the simultaneous detection of T25, T45 and Topas19/2 (Table 1).

The second class of capture probes (“Plant GMOchip”) allowed the specific identification of five plant species, i.e. corn, soybean, rapeseed, tomato and sugar beet (Part 2).

The third class was named “Screening GMOchip” (Part 3). Four capture probes specifically hybridize with DNA elements often occurring in GMOs, i.e. CaMV 35S promoter, *nos* terminator, *nptII* and *pat* genes. The Screening part of the GMOchip includes a CaMV probe aimed at the identification of 35S promoter-positive samples caused by infection of the host plant with this common plant virus.

Finally, additional controls included: (i) positive hybridization capture probes spotted at 10 different concentrations spanning high- and low-labelled spots; (ii) positive fixation controls, biotinylated capture probes spotted at seven different concentrations to control the efficiency of the detection system; and (iii) negative hybridization controls, i.e. non-specific capture probes.

Edge fragments capture probes were introduced but were not used in the study (Part 4).

The final GMOchips were produced by Eppendorf Array Technologies, S.A. (EAT, Namur, Belgium).

DNA array hybridization and colorimetric detection

The assays were carried out as follows: 20 μ L of PCR product were mixed with 5 μ L of positive

hybridization control and subsequently denatured with 10 μ L of 0.175 N NaOH (freshly prepared) for 5 min at RT. The solution was neutralized with 35 μ L of hybridization solution (Genomic Hybrid-Buffer[®] Eppendorf.AG, Hamburg, Germany) and transferred onto the hybridization chamber delimited by the frame. The chamber was closed with a plastic cover slip and the chip was incubated for 1 hour at 60 °C to allow hybridization. After removal of the cover and the frame, the arrays were detected by a colorimetric detection method using the Silverquant[®] reagent following the instructions of the manufacturer (Eppendorf.AG, Hamburg, Germany). The slides were placed in a Silverquant Box[®] containing Washing Buffer[®] and washed three times for 1 min at RT. They were then incubated in pre-blocking buffer for 10 min at RT, after which the procedure was carried out in the dark. The slides were incubated with buffer containing streptavidin conjugated-nanogold particles for 45 min at RT and subsequently washed four times with Washing Buffer[®] and once more with Rinsing Buffer[®]. They were treated with the Silverquant[®] revelation mixture for 5 min, rinsed twice for 30 seconds with distilled water and dried vertically. They were finally scanned with a Silverquant[®] Scanner and the obtained data was analyzed with the Silverquant[®] software (Eppendorf, Hamburg, Germany).

The intensity of each spot was estimated by averaging the value of all pixels present inside the spot boundaries. The values of the averaged background were subtracted from the spot values. The value for a specific probe was the average of the three spot replicates, plus/minus the standard deviation.

The cut-off value for the spots was taken as the mean value of the negative controls plus 2.5 times the standard deviation. The values of the negative controls were very low (having a value between 5000 and 3 on a total of 65,536).

Results

Consensus amplification

The principle of GMOchip analysis based on PCR amplification and the identification on the chips is shown in Figure 2. The method combines the amplification of several target sequences using

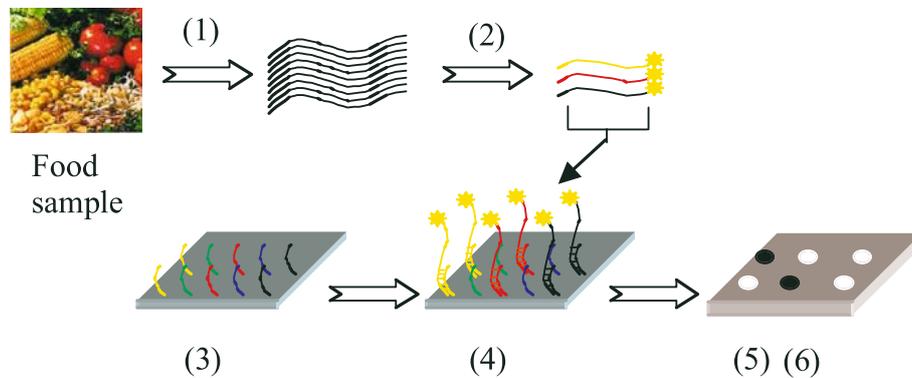


Figure 2. Principle of the GMOchips analysis. (1) DNA is extracted from leaves or powder. (2) A PCR amplification is performed with consensus primers. During this amplification, the DNA is labelled with biotin. (3) Capture probes, complementary of the amplified sequences, are grafted on a chemically treated glass slide. (4) The amplified product is laid down on the biochips. The amplified sequences will hybridize with their complementary capture probes. (5) After the washing steps, the biotinylated sequences, which stay hybridized on the capture probe, are detected by a colorimetric technique (silver salt precipitation). (6) Comparison of the hybridization pattern.

various consensus primer pairs, followed by their discrimination by hybridization on specific capture probes present on the GMOchips.

Specificity of the assay

The hybridization conditions, such as temperature, denaturation mode and incubation time were optimized based on the previous work of Hamels *et al.* (2001).

The specificity of the capture probes located on the “Specific GMOchip” was checked by hybridization of the corresponding PCR products obtained from the nine different GMOs and amplified by the four primer pairs. Figure 3 presents typical microarray pictures of the results. The hybridization and detection controls were correctly detected on the chip. The controls are present on the upper and lower part of the array. In some experiments the positive control signals were low but present. The PCR products for Bt11, RRS and CBH351 were amplified with the same primer pair (*OPP35S1/OPTnos2*) and hybridized on the array (Figure 3a). The PCR product from Bt11 hybridized well to the Bt11 specific capture probe and to the capture probes specific for P-35S and *pat* gene which recognize, respectively, the 35S

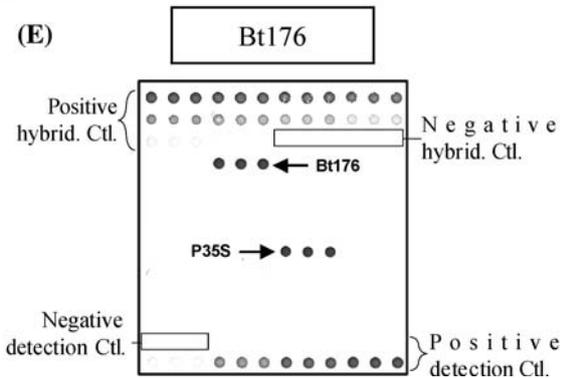
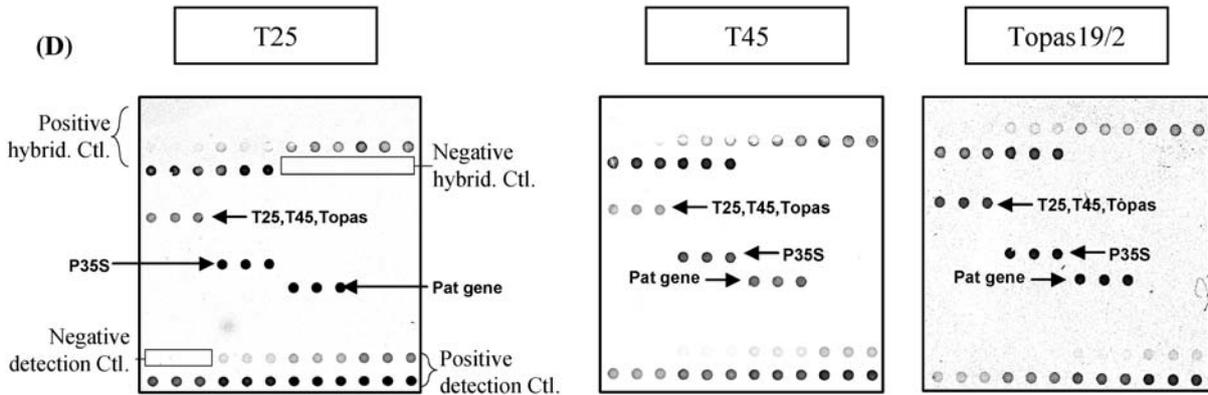
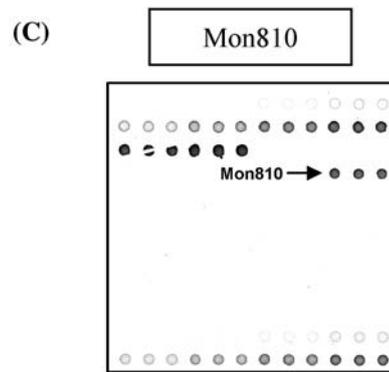
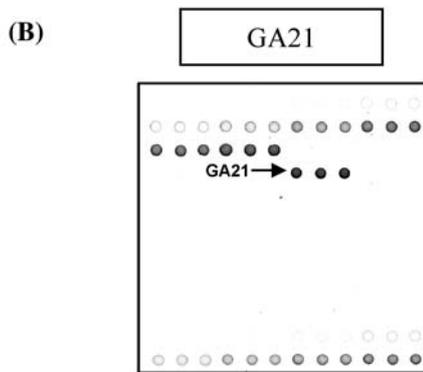
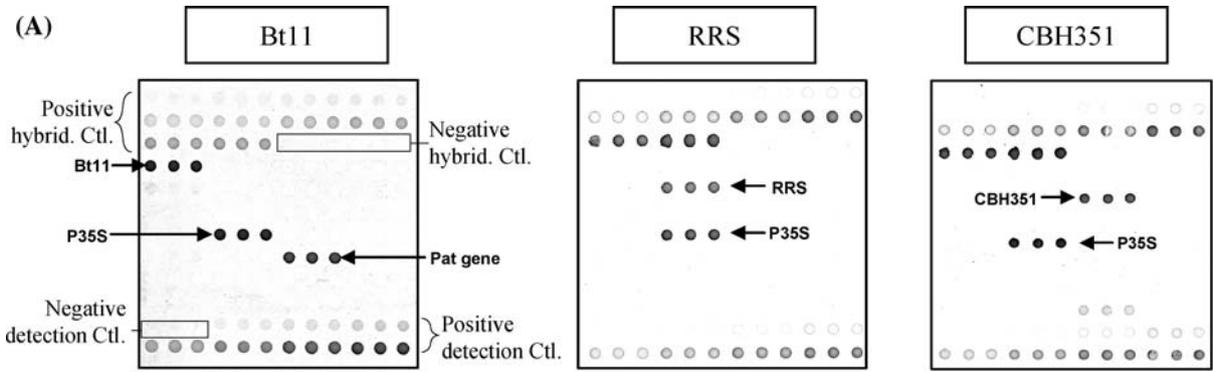
promoter region and the *pat* encoding region present in these amplified sequences. The *PP35S1/OPTnos2* primers amplified a 1000 bp of the Bt11 genome which contains the P-35S, IVS2, the *pat* gene and part of the T-*nos* gene. There was no cross-reaction with the other probes.

The RRS and CBH351 target sequences hybridized to their specific capture probes and to the P-35S capture probe but not to other capture probes. Results are in agreement with the elements included in the amplified sequences. The amplicons from RRS were 1600 bp and included the P-35S, the CP4-EPSPS gene, and part of the T-*nos*. The CBH351 amplicons have a size of 790 bp and also included the P-35S, the *bar* gene and part of the T-*nos*.

The GA21 target sequence was obtained by PCR with the *OPEPS1B/OPTnos2* primer pair and hybridized on the array (Figure 3b). The amplified product hybridized only to the GA21 specific capture probes. The amplified sequence contains the EPSPS gene and part of the T-*nos*.

For MON810, the PCR product was obtained by the use of the *OPhsp/OPcry2c* primer pair. After hybridization on the array, only the MON810 specific capture probe gave a specific signal (Figure 3c). The amplified sequence contains the HSP70 elements and the *cry1A(b)* gene.

Figure 3. Specificity of GMOs detection on the GMOchip. DNA (0.1 µg) extracted from individual GMOs was amplified by PCR and 20 µL of the amplicon solution were hybridized to the GMOchip. Colorimetric detection of biotinylated products was performed after hybridization with PCR products amplified by *OPP35S1/OPTnos2* (A), *OPEPS1B/OPTnos2* (B), *OPhsp/OPcry2c* (C) and *OPP35S1/OPT352* (D and E).



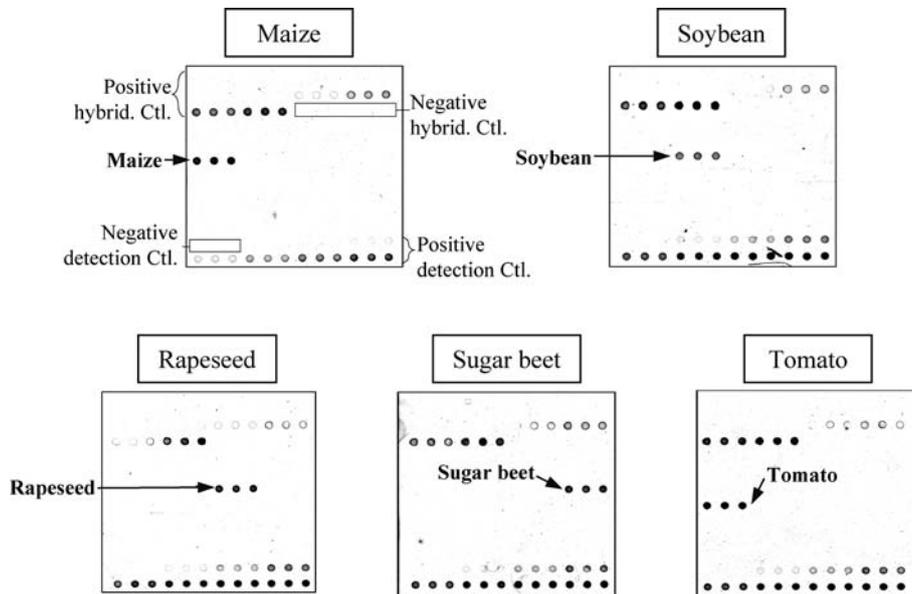


Figure 4. Identification of the plant species on the GMOchip. The DNA of various plant species was extracted (0.1 μ g) and processed for PCR amplification. A 20 μ L of the amplicon solution were hybridized to the GMOchip.

The *OPP35S1/OPT352* primer pair allowed the amplification of four GMOs present on the GMOchip: Bt176, T25, T45 and Topas19/2. The hybridization of the PCR products of T25, T45 and Topas19/2 gave the same hybridization patterns on the array (Figure 3d). This is explained by the extremely high homology between the sequences of the amplified fragments of these three GMOs. Only three base pair differences were found in the three amplified sequences. They also include a 35S promoter, the *pat* gene and the 35S terminator.

For Bt176, we observed a specific hybridization to the Bt176 specific capture probe and to the P-35S consensus capture probe (Figure 3e).

For all of these hybridizations, no cross-hybridizations were observed. These experiments were repeated five times, giving the same results each time.

The part of the “Plant chips” (Part 2) allowed the recognition of five plants species (maize, soybean, rapeseed, tomato and sugar beet) by the use of two target genes: the *rubisco activase* gene (*RA*) and the *sucrose synthase* (*SS*) gene. The specificity of the Plant chips was checked by hybridization with the PCR product from the five species. Each *RA* and *SS* target sequences hybridized to their corresponding specific capture probes as shown in Figure 4. Signal intensity, standard

deviation and the measured cut-off values after hybridization of PCR products onto the GMOchip are given on Table 2. No cross-hybridization

Table 2. Specificity of capture probes for plant species identification.

Capture probe	Signal intensity	Standard deviation
<i>DNA template: maize</i>		
Maize	18,117	1057
Negative control	< 3	–
Cut-off	< 3	
<i>DNA template: soybean</i>		
Soybean	10,572	560
Negative control	218	252
Cut-off	848	
<i>DNA template: rapeseed</i>		
Rapeseed	11,166	276
Negative control	1959	720
Cut-off	3759	
<i>DNA template: sugar beet</i>		
Sugar beet	16,574	650
Negative control	< 3	–
Cut-off	< 3	
<i>DNA template: tomato</i>		
Tomato	30,964	813
Negative-control	< 3	–
Cut-off	< 3	

Other signals were below the cut-off value.

was observed when tested using the following plants: eggplant, pepper, wheat, chenopodium, white beet, rye, barley, potato, papaya, flax, rice, lens, millet, lupin, canola, broccoli, corn, aubergine, carrot, onion and apple.

The part of the “Screening GMOchip” (Part 3) represents a fast screening tool for the presence of GMO in a sample, particularly for processed material due to the small size of the amplified fragment. It is based on the presence or absence of three small conserved fragments (P-35S promoter, T-*nos* terminator and *nptII* gene) commonly used in GMO construction. The most frequently used promoter and terminator in approved GM crops are P-35S and T-*nos* (Hemmer, 1997), isolated from CaMV and from the *nopaline synthase* gene of *A. tumefaciens*, respectively. Thus, we developed a duplex PCR for simultaneous amplification of these two conserved elements, including *nosC-SIC2F/nosCSIC1R* and *SF/SR* primer pairs that yield a 170 bp fragment for T-*nos* and a 79-bp amplicon for P-35S, respectively.

Selectivity of the assay was further tested by the analysis of 100 ng of genomic DNA from the nine target GMOs. Twenty micro liters of PCR were loaded on the biochips. Figure 5 presents examples of microarrays resulting from the hybridization of P-35S and/or T-*nos* sequences from Roundup Ready Soybean and maize GA21. Table 3 shows signal intensity, standard deviation and the measured cut-off values of the micro-arrays presented in Figure 5. Transgenic soybean RRS, transgenic maize lines Bt11, MON810 and CBH351 showed positive signal with both P-35S and T-*nos* capture probes (Table 4). Transgenic maize lines Bt176 and T25 and transgenic rapeseed lines T45 and Topas19/2 showed positive signals only for the

Table 3. Specificity of capture probes for P35S-Tnos screening.

Capture probe	Signal intensity	Standard deviation
<i>DNA template: RRS</i>		
P35S	25,594	1655
Tnos	22,989	1463
Negative control	< 3	–
<i>DNA template: GA21</i>		
P35S	< 3	–
Tnos	21,656	731
Negative control	< 3	–
Cut-off	< 3	

Other signals were below the cut-off value.

Table 4. Specificity of P35S-Tnos screening of the different GMOs.

GMO	Detection of P35S	Detection of Tnos
Bt 11	+	+
Bt 176	+	+
CBH351	+	+
GA21	–	+
MON810	+	+
RRS	+	+
T25	+	–
T45	+	–
Topas19/2	+	–

Detection of P-35S and T-*nos* genetic elements by a PCR duplex system and hybridization on the GMOchips: +, genetic element detected; – genetic element not detected. The experiments were performed as shown in Figure 5.

P-35S element (Table 4). The transgenic maize line GA21 showed a signal only for the T-*nos* element (Table 4). These results are in concordance with the published data about the elements that were

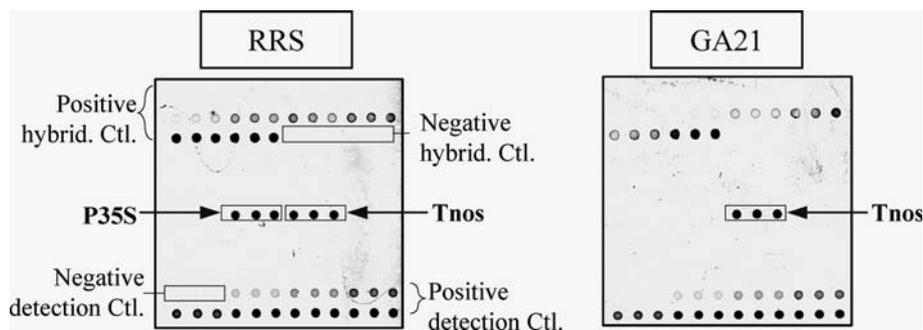


Figure 5. P35S-Tnos screening using the GMOchip. The DNA of individual GMOs was extracted (0.1 μ g) and amplified by PCR and 20 μ L of the amplicon solution were hybridized to the GMOchip.

included in the construct used for transformation of these transgenic lines.

The *nptII* assay was incorporated since it allows the differentiation between two transgenic lines: T45 and Topas19/2. These two GMO were transformed with two identical constructs except for the presence of *nptII* gene in the Topas19/2. The selectivity was similarly tested by analysis of DNA from 15 transgenic lines (not shown). Rapeseed GM lines Topas19/2, MS1, and RF1XRF2 showed positive amplification. These results are in concordance with the published data of the elements contained in the plasmids used for transformation of these lines.

Sensitivity for *nptII* was tested on Topas19/2, MS1, RF1 and RF2. The best sensitivity was obtained on Topas19/2 and was around 5–10 copies. For the three other GMOs, the sensitivity was around 50–100 copies.

A control test was also developed to allow the detection of possible false positive results such as those arising from the presence of P-35S elements from a possible plant infection with CaMV. A CaMV-specific assay already described was used as a contamination control (Fernández *et al.*, 2005). Selectivity of the assay was confirmed by the analysis of 100 ng of genomic DNA extracted from rapeseed leaves infected by CaMV. A 20 μ L of PCR were loaded on the biochips (Figure 6). Two capture probes (CaMV(a) and CaMV(b)) were initially tested. CaMV(b) capture probe giving the most intensive signal (Table 5) was kept on the final design of the microarray.

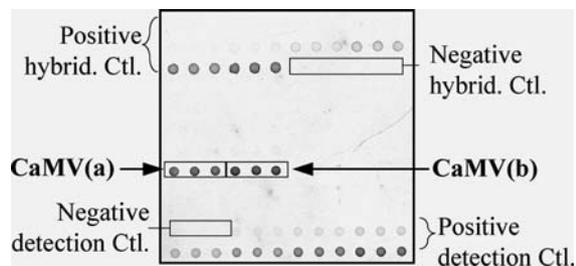


Figure 6. CaMV using the GMOchip. The DNA of CaMV infected-rapeseed leaves was extracted (0.1 μ g) and amplified by PCR and 20 μ L of the amplicon solution were hybridized to the GMOchip. Two capture probes (CaMV(a) and CaMV(b)) were initially tested. CaMV(b) capture probe giving the most intensive signal was kept on the final design of the microarray.

Table 5. Specificity of capture probes for CaMV testing.

Capture probe	Signal intensity	Standard deviation
CaMV(a)	33,006	993
CaMV(b)	37,923	460
Negative control	3435	159
Cut-off	3833	

Other signals were below the cut-off value.

Sensitivity of the assay for one individual GMO test

The detection limit of the overall assay was assessed using decreasing concentrations of GM target DNA diluted in non-GM DNA of the corresponding plant species. For sensitivity testing involving only individual GMOs, several concentration levels were tested: 100, 5, 2, 1, 0.3, 0.1, 0.03 and 0%. These concentration levels were chosen for compliance with food labelling thresholds and the expected seed threshold proposed by the Standing Committee on Seeds and Propagating Material of Agriculture, Horticulture and Forestry (SCSP). This seed threshold proposal would allow 0.3–0.7% of incidental presence of GMOs in crops, depending on the variety. This threshold remains to be set. A 100 ng of total DNA were processed for PCR amplification and 20 μ L of the PCR solution were hybridized to the GMOchip. We show in Figure 7, one example of the results and a summary is presented in Table 6. The sensitivity for each GMO was estimated as the lowest concentration giving a signal with its specific capture probe. For example, in the Topas19/2 experiment (Figure 7), a detection limit of 0.03% was obtained for the signals corresponding to this event. The PCR negative controls gave no signal. The sensitivity was also tested for the eight other GMOs. Detection limit for all nine GMOs were found to be lower than 0.3% and could reach 0.03% for five of the target GMOs (Table 6).

Sensitivity of the assay in a GMO mixture containing Bt11, CBH351, RRS and GA21

Since one particular GMO could be present in combination with other GMOs, which could then interfere with the amplification or with the detection steps, we tested the sensitivity of the assay on several GMO mixtures. The four GMOs

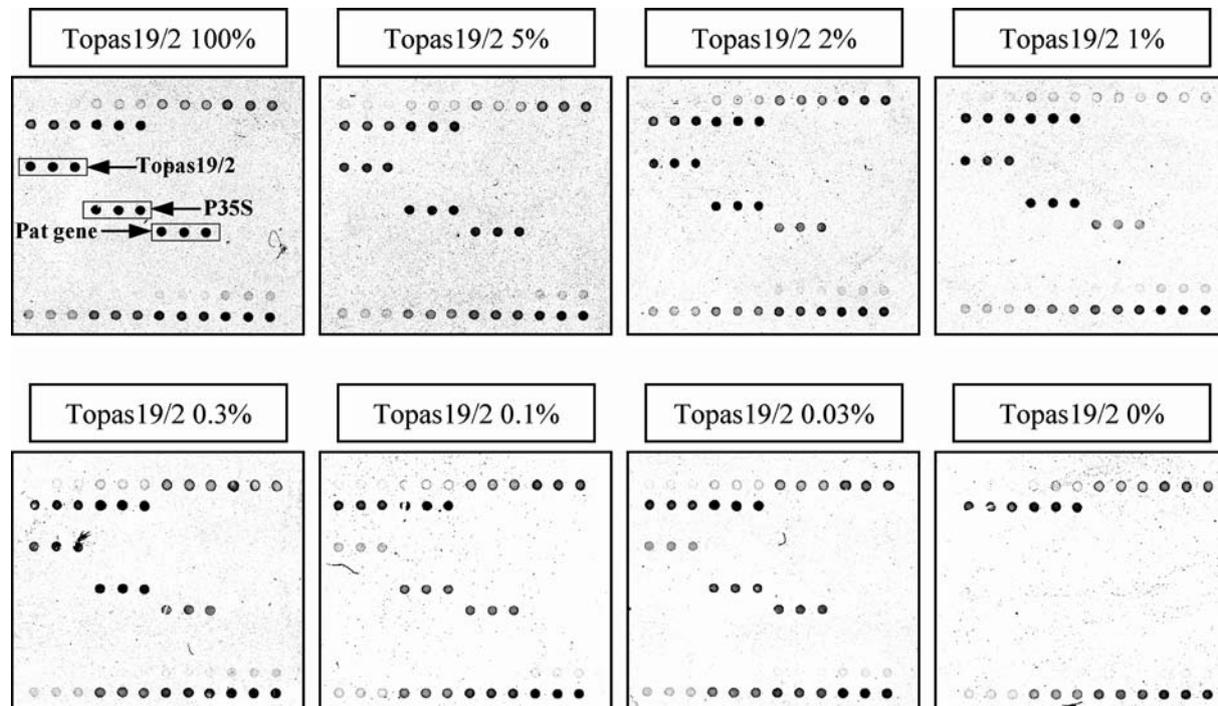


Figure 7. Sensitivity of GMO detection on the GMOchips for individual GMOs. DNA from pure Topas 19/2 Maize was extracted and diluted in non-GM Maize DNA. The DNA mixtures (0.1 μg) were processed for PCR amplification and 20 μL of the solution were hybridized to the GMOchip.

composing these mixtures were grouped together because they contain common sequences (P-35S and/or T-*nos*) so that their amplifications require only three primers (*OPP35S1*, *OPEPS1B* and *OPTnos2*). The composition of these mixtures is shown in Figure 8a. Each of the four GMOs was present in a concentration of 0.3% in the presence of a high amount of the other three. The samples were obtained through mixing the genomic DNA of the different GMOs with non-GM genomic

Table 6. Sensitivity of detection on the GMOchips for different GMOs.

GMO	Sensitivity (%)
Bt11	0.03
Bt176	0.03
CBH351	0.3
GA21	0.03
Mon810	0.03
RRS	0.3
T25	0.1
T45	0.1
Topas19/2	0.03

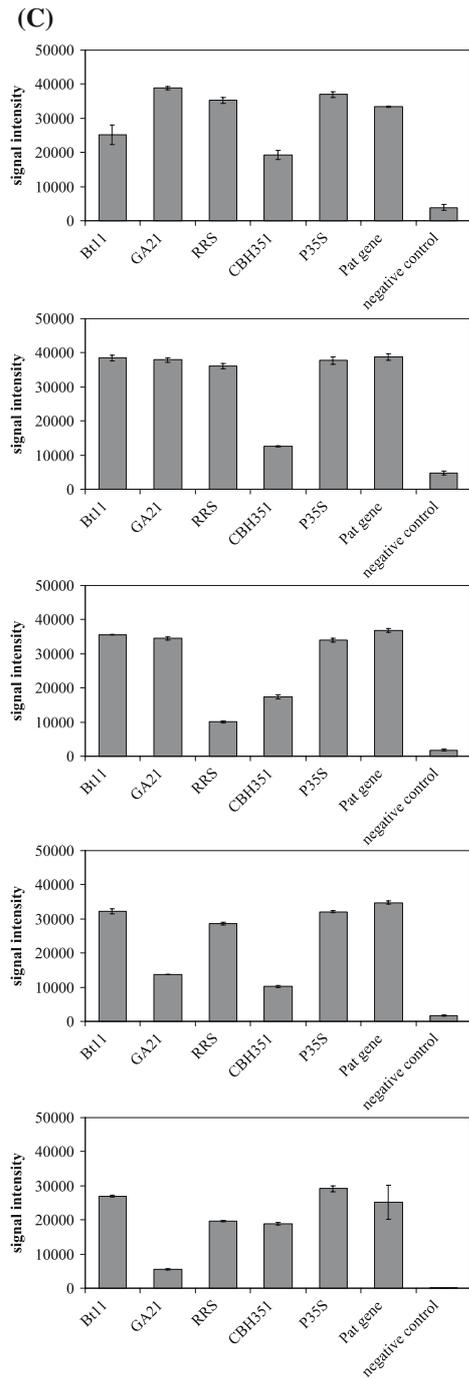
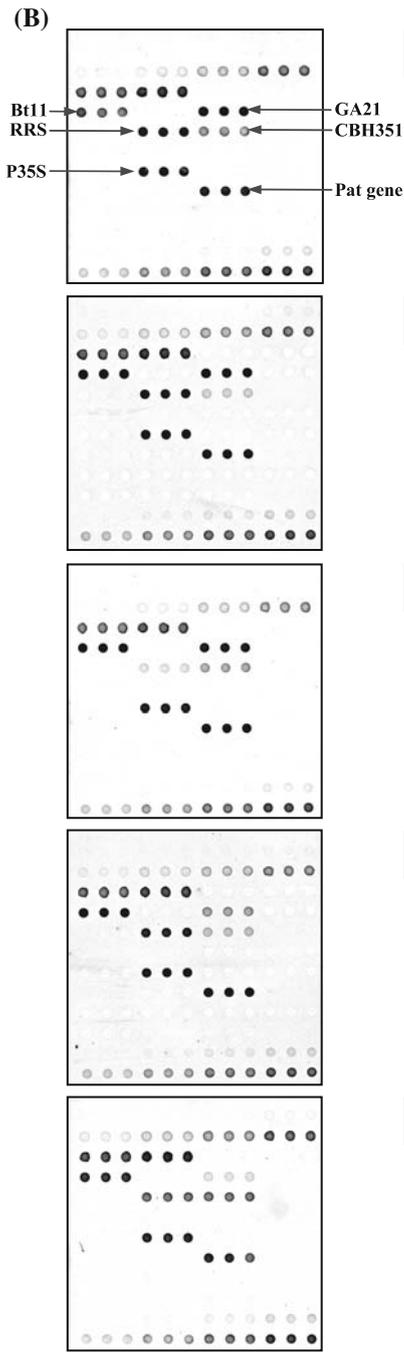
Sensitivity is the lowest concentration giving a positive signal. The experiments were performed as shown in Figure 6.

DNA. A 100 ng of total DNA were used as template in two PCR amplifications using either the *OPP35S1/OPTnos2* or *OPEPS1B/OPTnos2* primer pairs allowing amplification of Bt11, CBH351 and RRS or GA21, respectively. A 10 μL of each PCR solution were loaded on the same array for hybridization. As shown in Figure 8b and c, specific signals for these four GMOs were detected in all five different DNA mixtures, even the GMO present in the low concentrations. The two consensus capture probes designed to recognize the 35S promoter and the *pat* gene were also detected. Indeed, Bt11 PCR fragments hybridized to both P-35S and *pat* gene capture probes. RRS and CBH351 target sequences also hybridized to the consensus P-35S capture probe.

Sensitivity of the assay in a GMO mixture containing MON810, T25, T45 and Topas19/2

The four GMOs composing these mixtures were grouped together because three (T25, T45 and Topas19/2) of them contain two common sequences (P-35S and T-35S) so that their amplifications

(A)	GMO	Bt11	CBH-351	RRS	GA21	DNA mixture label
Concentration level		0.3%	33%	33%	33%	Mix1
		33%	0.3%	33%	33%	Mix2
		33%	33%	0.3%	33%	Mix3
		33%	33%	33%	0.3%	Mix4
		0.3%	0.3%	0.3%	0.3%	Mix5



require only the use of two primer pairs (*OPP35S1/OPT352* for T25, T45 and Topas19/2 and *OPhsp/OPcry2c* for MON810).

The compositions of the different mixtures prepared with different concentrations of the four GMOs are shown in Figure 9a. The samples were obtained through mixing the genomic DNA of the different GMOs with non-GM genomic DNA. A 100 ng of total DNA were processed for two PCR amplifications using either the *OPP35S1/OPT352* or *OPhsp/OPcry2c* primer pairs allowing amplification of T25, T45, Topas19/2 and Bt176 or MON810, respectively. A 10 μ L of each PCR solution were loaded on the same array for hybridization. As shown in Figure 9b and c, specific signals for the four GMOs: T25, T45 and Topas19/2 were well detected in all DNA mixtures, even at the low 0.3% concentrations. The two consensus capture probes designed to recognize the 35S promoter and the *pat* gene were also detected by T25, T45 and Topas19/2 PCR fragments.

All the experiments in this study were repeated at least three times and lead to identical conclusion concerning presence or not of the different GMO. There were some variability in the absolute value of the signal but did not influence the conclusion.

Discussion

Particular emphasis was placed on the reduction of the number of PCR reactions required and on the number of primers present per amplification tube. To reduce the number of PCR, two types of PCR were developed and used in combination: either consensus primers or specific primers for genetic elements present in the GM events. Several primers were designed to hybridize in small conserved elements such as P-35S, T-35S and T-*nos* and to allow amplification of different GMOs simultaneously. Bt11, CBH351 and RRS were amplified with the same primer pairs: *OPP35S1* and *OPTnos2* which were designed to hybridize P-35S and T-*nos* elements, respectively. A primer designed within the sequence of the T-35S (*OPT352*)

allowed, in combination with the primers designed in the P-35S, the amplification of four other GMOs: Bt176, T25, T45 and Topas19/2. For the amplification of GA21 and MON810, two specific primer pairs were used. For the amplifications of the screening elements P-35S and T-*nos*, a duplex PCR system using two specific primer pairs was developed. For the detection of plant species, two degenerated primer pairs designed to amplify both sucrose synthase and rubisco activase genes were used in a single PCR tube. The discrimination between the different PCR products was realized by the use of the micro-array.

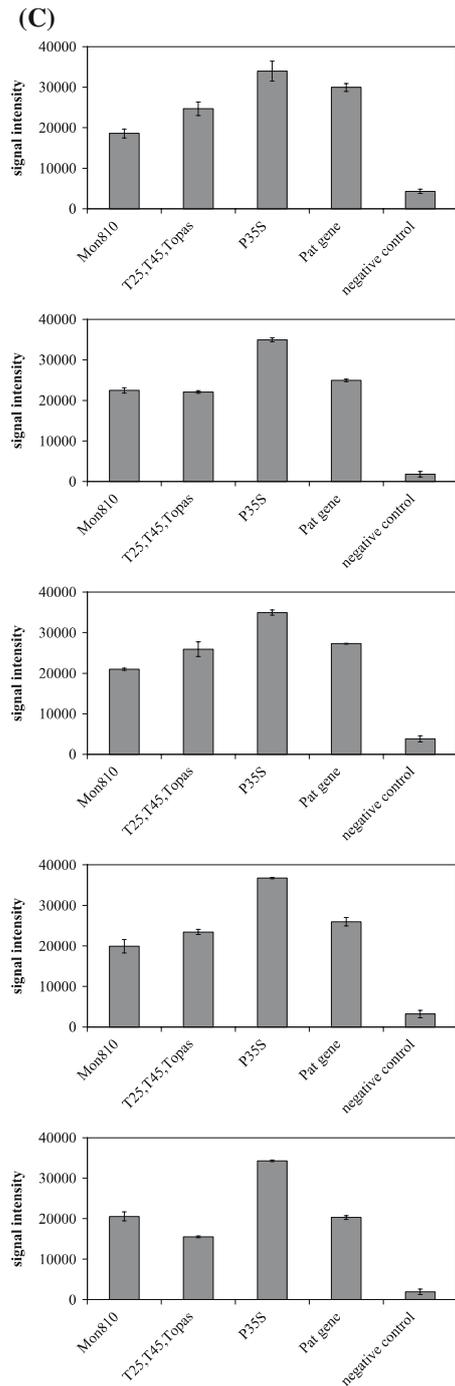
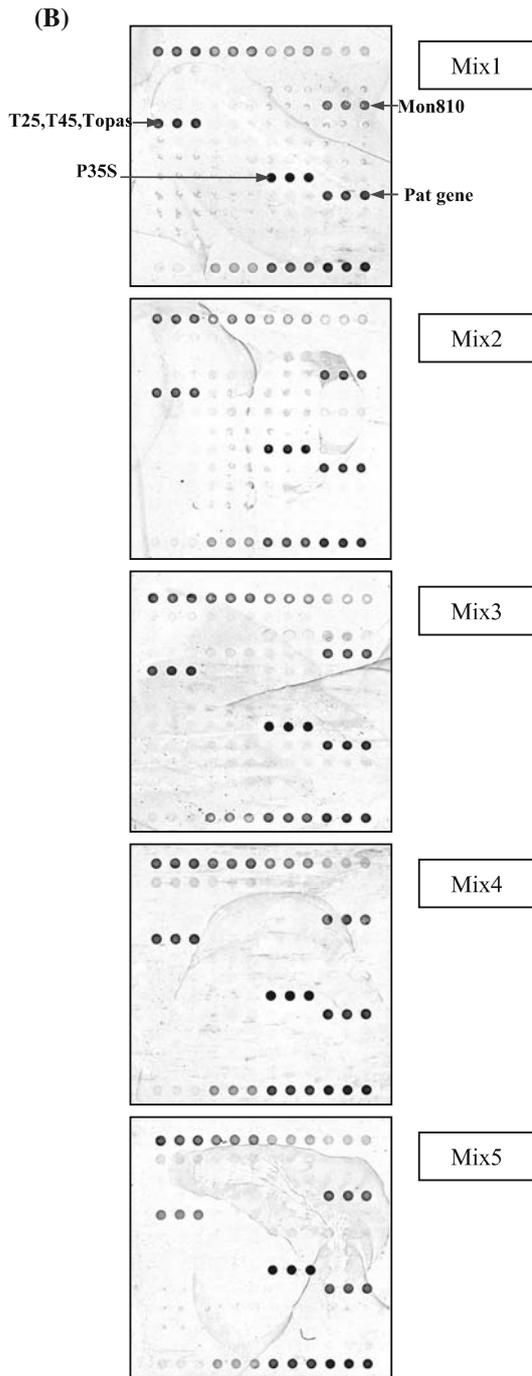
Developing GMO assays is a complex issue for molecular testing since there are several target molecular inserts which could be present in the food, some of them being very different but some being almost identical or homologous. Although several countries such as Japan, South Korea, Taiwan or Brazil have adopted threshold for the labelling of food products containing GMOs, the strictest rules and the lowest threshold of contamination are imposed by the European Union. The 0.9% of contamination that requires the labelling of the food represents only a few thousand copies of inserts to be detected inside a very large plant genome. An already complex situation will be further complicated in the future with the introduction of new GMOs into the market.

The advantage of the Chip technology is that it combines the miniaturization of the assay with multiple detections. There is almost no limit to the number of capture probes, which can be introduced onto an array. In this work, we first introduced on the array capture probes specific for each GMO and then increased gradually the number of capture probes for plant species determination, for screening of conserved fragments, for the CaMV and for the different types of controls.

We have seen that the technology developed allowed detection and identification of the required targets, being specific either for the GMO inserts themselves or the plant species. The specificity was found to be good with no cross-reactivity observed in the experiments.

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 Figure 8. Sensitivity of GMO detection on the GMOchip in a GMO mixture. DNA from four GMOs (Bt11, CBH351, RRS and GA21) was extracted and different mixtures of the DNA were prepared. The DNA mixtures (0.1 μ g) were amplified in two PCR using either the *OPP35S1/OPTnos2* or *OPEPS1B/OPTnos2* primer pairs. A 10 μ L of each PCR solution was introduced into the GMOchip frame for hybridization. (A) Composition of the mixtures; (B) hybridization results on the GMOchip; (C) corresponding signal intensities. Other signals were below the cut-off value.

(A)	GMO	Mon810	Maize	Topas 19/2	T45	T25	DNA mixture label
		0.3%	25%	25%	25%	25%	Mix1
		25%	25%	0.3%	25%	25%	Mix2
Concentration level		25%	25%	25%	0.3%	25%	Mix3
		25%	25%	25%	25%	0.3%	Mix4
		0.3%	0.3%	0.3%	0.3%	0.3%	Mix5



We obtained a limit of sensitivity below 0.3% contamination and most commonly below 0.1% for the specific probe detection. This level of sensitivity is similar to those obtained in other studies using other detection techniques. The detection limits of protein immunoassay techniques such as Immunoassay strip test, western blot or enzyme-linked immunosorbant assay (ELISA) can predict the presence of modified proteins in the range of 0.3–1% GMOs (Ahmed, 2002). For examples, ELISA could detect Roundup Ready soybeans at 0.3% (Lipp *et al.*, 2000) and strip tests give limits of sensitivity between 0.1% and 0.5% (Stave, 2002) but these techniques give a response only for one event at the same time. A multiplex PCR method allows to detect 0.5% of each of the five lines of GM maize (Matsuoka *et al.*, 2001). In a quantitative-competitive (QC)-PCR methodology, the competition between the internal standard DNA and target DNA generally leads to loss of detection sensitivity (Ahmed, 2002). Nevertheless, detection limits at 0.1% were reported by Rudi *et al.* (2003) for seven different GM maize events and by Garcia-Canas *et al.* (2004) for the Bt176 event using a double competitive PCR approach. Different Real-Time PCR systems allowed to detect down to 100 genome molecules (Hernández *et al.*, 2004).

Levels of sensitivity observed in this study are also similar with those obtained from other microarray approaches. Bordoni *et al.* (2004) reported a sensitivity of 0.1% for the Bt176 transgenic maize and can identify the presence of 0.5% transgenic events within complex mixtures of RRS, Bt11, MON810, GA21 and Bt176 (Bordoni *et al.*, 2005). Recently, a peptide nucleic acid (PNA) array platform was reported for the detection of GMO in food: in this paper (Germini *et al.*, 2005), PNA array showed a high selectivity for several 5% GM events and two specific plant genes (lectin for soybean and zein for maize).

Moreover, sensitivity levels obtained in this study are in compliance with the European food and feed labelling threshold level of 0.9%. The forthcoming regulation concerning the labelling

threshold for seeds (0.3–0.7%), according to the current discussion between the EU and member states might be compatible with the detection limits obtained through the use of these DNA chips. It must be noted, however, that the specific detections were obtained using long amplicons, limiting such results to non-processed food matrices. For processed foods, the screening part of the chip would be more appropriate since amplification is performed on smaller fragments.

The results presented show that the chips detection due to the specificity of the capture probes and its compatibility for multiple probes fit particularly well with the consensus PCR amplification. It results in a specific and sensitive microarray technique for the actual accepted EU GMO lines using a limited number of PCR assays.

Microarrays are presented here as a detection method for the presence of possible multiple amplicons present in the PCR. The method is time and cost effective mainly because 17 different amplicons are identified and quantified in one single step and another eight edge fragments could also be detected. We have shown here that the amplification solutions of four PCR tubes are combined for the simultaneous detection on the arrays. The present method was tested with the same results presented here in five laboratories being part of the EU project and which are authors of this paper.

The present data were presented for the known GMO and one of the challenges of the chips technology would be to investigate how far it can be useful for the detection of the GMO.

Acknowledgement

This work was supported by contract no. G6RD-CT-2000-00419 project funded by the European Community under the “Competitive and Sustainable Growth” Programme (1998–2002). Special thanks to M. Bas and M. García-Aragónés for excellent technical assistance. The help of S. Hamels is acknowledged for the array work.

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Figure 9. Sensitivity of GMO detection on the GMOchip using GMO mixtures. DNA from 4 GMOs (MON810, T25, T45 and Topas19/2) was extracted and different mixtures of the DNA were prepared. The DNA mixtures (0.1 µg) were amplified in two PCR using either the primers *OPhp/OPery2C* or *OPP35S1/OPT352*. A 10 µL of each PCR solution was introduced into the GMOchip frame for hybridization. (A) Composition of the mixtures; (B) hybridization results on the GMOchip; (C) corresponding signal intensities. Other signals were below the cut-off value.

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