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Detection of Genetically Modified Soybean Using Peptide Nucleic Acids (PNAs) and Microarray Technology

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Peptide nucleic acid (PNA) microarrays for the detection of Roundup Ready soybeans in food have been prepared. PNA probes are known to be more efficient and selective in binding DNA sequences than the analogous oligonucleotides and are very suitable to be used for diagnostics in food. PNAs of different lengths were carefully designed and synthesized by solid-phase synthesis on an automatic synthesizer adopting the BOC strategy. PNAs were purified by HPLC and characterized by HPLC/MS. The probes were spotted on a functionalized surface to produce a microarray to be hybridized with PCR products. DNA extracted from reference material was amplified using Cy3- and Cy5-labeled primers, and the fluorescent PCR products obtained were hybridized on the microarray. Two protocols were adopted: the hybridization with dsDNA or with ssDNA obtained by digestion with the enzyme λ exonuclease. The best results were obtained using a 15-mer PNA probe in combination with the ssPCR product derived from enzymatic digestion. The method was applied to the analysis of a sample of certified transgenic soybean flour.

KEYWORDS: GMO; PNA; microarrays; Roundup Ready soybean

INTRODUCTION

In recent years genetically modified organisms (GMOs) have raised a high interest and many political debates about their influence on the environment and their safety as food and feed. Strict regulations have been enforced during the 1990s in the European Union, and even more stringent rules about food and feed labeling and traceability have recently been approved (*1*), whereas other states do not require specific labeling. Therefore, the need for fast, simple, and sensitive methods for the detection and quantification of GMOs in food is increasingly important (*2*).

Two techniques have been mainly developed so far for the detection of GMOs: DNA analysis by Polymerase Chain Reaction (PCR) and protein analysis (*3*). Molecular biology methods routinely used in the food industry, mostly PCR, can sometimes lead to ambiguous interpretations due to the low specificity of the priming sequences or the "carry-over" contaminations that can produce false positives. A recent example of controversial assignment made on the basis of PCR results is the suspect evidence of transgenic contamination of maize in Mexico, which raised a great debate among scientists (*4*) and led to the conclusion that post-PCR controls are often essential to confirm a sequence identity in the case of ambiguous recognition of specific targets (*5*). Nested PCR (*6*), real-time quantitative PCR (*7*), and biosensor technology (*8*) are suitable methods for increasing the specificity of GMO analysis.

In this context, microarray technology can greatly improve the ease and speed of analysis of PCR products. DNA microarrays are analytical systems allowing the simultaneous detection of many nucleic acid sequences (up to thousands) in a sample. Each DNA sequence is represented by an oligonucleotide probe covalently bound to the modified surface of a glass slide (*9*). The probes on the array are hybridized with the fluorescently labeled PCR products. A laser scanning analysis reveals the presence of labeled material containing sequences complementary to those spotted onto the microarray.

A commercial application of this kind of approach is already available on the market (*10*), with oligonucleotides as probes to build a GMO*Chip*.

Recently, peptide nucleic acids (PNAs), analogues of oligonucleotides in which the sugar−phosphate backbone has been replaced by a pseudopeptide chain of *N*-aminoethylglycine monomers, have been reported. These molecules show a higher affinity for DNA than DNA itself and, above all, a higher sequence specificity (*11*−*13*) (**Figure 1**).

PNAs are widely used in molecular biology and biotechnology, as tools in genetic diagnostics, and for the specific regulation of gene expression and are currently investigated as

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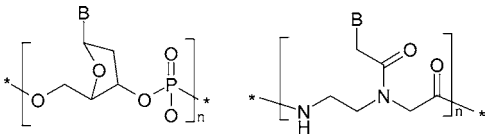

Figure 1. Structures of DNA (left) and PNA (right).

potential antiviral and anticancer drugs (*14*). Unlike oligonucleotide probes, even short PNAs are efficient in hybridizing to target DNA (*15*), being able to perform efficient hybridization of DNA with secondary structures (*16*). Furthermore, the stability of PNA:DNA hybrids is greatly affected by the presence of a single base mismatch (*12*), and they are therefore highly sequence-selective. These properties have been used for detecting specific gene sequences in advanced diagnostic methods (*17*) such as PCR clamping (*18*), real time PCR (*19*), capillary electrophoresis (*20*), MALDI-TOF (*21*), biosensors (*22*−*24*), quartz crystal microbalance (*25*), HPLC (*26*), and microarrays (*27*). A single-molecule detection of DNA from transgenic maize was also performed by means of PNA probes and double-wavelength fluorescence analysis (*28*).

In the present work we report the combination of microarray technology with PNA probes for the detection of genetically modified soybean. Roundup Ready soybean is a transgenic variety of soybean developed to allow the use of glyphosate, the active ingredient of the herbicide Roundup, as a weed control for soybean. This genetically engineered variety contains a glyphosate-resistant form of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) isolated from the *Agrobacterium tumefaciens* strain CP4 (CP4 EPSPS). EPSPS is one of the enzymes of the shikimate pathway, which is involved in the biosynthesis of chorismate, an intermediate for the synthesis of the aromatic amino acids (tryptophan, tyrosine, and phenylalanine) essential for the plant growth. Given the worldwide diffusion of GM soybean [which accounted for >52% of the total GM sowings and nearly one-third of the world total soybean area, in 1999 (*29*)], we focused our attention on this type of transgenic material for testing the effectiveness of PNA microarray technology in food analysis.

Different PNAs were designed, synthesized, and covalently linked to functionalized surfaces to construct a microarray for the identification of the constitutive gene (lectin) and of a gene tract corresponding to the Roundup Ready (RR) GM soybean. The effect of the PNA length on the signal intensity and specificity was evaluated as well as the conditions of detection for either single-stranded (ss) or double-stranded (ds) PCR products. Finally, a model device was prepared and tested with a certified transgenic reference material.

MATERIALS AND METHODS

Reagents. PNA monomers were from Perseptive Biosystems; (4-methylbenzhydryl)amine (MBHA) resin, *O*-(*1H*-7-azabenzotriazolyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU), *N*,*N*-diisopropylethylamine (DIEA), and thioanisole were from Aldrich. *N*-Methylpyrrolidone (NMP), trifluoromethanesulfonic acid (TFMSA), trifluoroacetic acid (TFA), and *m*-cresol were from Fluka. Oligonucleotides (guaranteed oligos grade) used for T_m, phosphorylated, and Cy3- or Cy5-labeled primers were purchased from Sigma Genosys. Blue Taq DNA polymerase was purchased from Euroclone. λ-Exonuclease was purchased from Amersham Biosciences.

Probe Design. The PNA probes were designed to maximize the hybridization efficiency and to minimize any nontarget hybridization. For these reasons the PNA sequence was first checked to evaluate any possibly interfering secondary structure using the on-line available program *Mfold* (version 3.1) by Zuker and Turner (*30*); the sequence specificity was then evaluated using the BLAST homology search system from DDBJ.

Table 1. PNA Sequences and Melting Temperatures of the PNA:DNA Antiparallel Duplexes in Phosphate Buffer (pH 7.0) at a 5 μM Concentration of Each Strand

PNA	abbreviation	sequence	T_m DNA: PNA (°C)
1	lectin 11-mer	H-AAG TCG TCG CT-NH_2	66
2	RR 9-mer	H-AGT CAG CTT-NH_2	50
3	RR 11-mer	H-AGA GTC AGC TT-NH_2	63
4	RR 13-mer	H–CTA GAG TCA GCT T-NH_2	72
5	RR15-mer	H-TGC TAG AGT CAG CTT-NH_2	75

Five PNA probes were designed (**Table 1**): one, PNA 1 (11-mer), was complementary to the lectin gene; the other four, PNA 2 (9-mer), PNA 3 (11-mer), PNA 4 (13-mer), and PNA 5 (15-mer), were complementary to a portion of the Roundup Ready DNA insert.

PNA Synthesis. The PNA oligomers were synthesized using solid-phase synthesis with a 433A peptide synthesizer (Applied Biosystems) with the BOC strategy and HATU/DIEA coupling, as described in a previous work (*26*). Swelling, downloading, and cleavage of the PNAs from the resin were done manually.

The crude products were purified by reversed-phase HPLC using a Phenomenex C18 peptide column (3 μm, 250 mm × 10 mm) with a binary gradient (flow rate = 4 mL/min); eluent A was water /TFA = 100:0.2, eluent B was water/acetonitrile/TFA = 60:40:0.2, and detection was by UV at 260 nm. The products were identified by mass spectrometry (*Micromass ZMD*). PNAs 1 and 3 were synthesized and characterized as previously reported (*25*).

PNA 2: MS (ESI): estimated mass for MH^+, 2451. Found: 2450. Crude yield: 82%.

PNA 4: MS (ESI): estimated mass for MH^+, 3533. Found: 3534. Crude yield: 66%.

PNA 5: MS (ESI): estimated mass for MH^+, 4090. Found: 4091. Crude yield: 68%.

Melting Temperature Measurements. Stock solutions of PNA and DNA in doubly distilled water were prepared, and their concentrations were measured by absorbance using the following ϵ_{260} (M^{-1} cm^{-1}) for the nucleobases: T 8800, C 7300, A 10400, and G 11700. Each sample containing a PNA:DNA antiparallel couple was first incubated at 90 °C for 5 min and then slowly cooled to room temperature. All hybridization experiments were carried out using samples at a 5 μM concentration of each strand in a 10 mM phosphate buffer, 100 mM NaCl, 0.1 mM EDTA, pH 7. Melting curves were recorded on a Perkin-Elmer λ BIO 20 spectrophotometer, equipped with a PTP 6 Peltier temperature programmer and a cell changer (Perkin-Elmer, Norwalk, CT). The samples were heated (1 °C/min, 5 points/min), and the absorbance at 260 nm was recorded. Melting temperatures were evaluated as the maxima of the first derivative of the melting curves.

Microarray Preparation. Each PNA was dissolved in water at a concentration of 10 pmol/μL and spotted by a piezoelectric spotter (Gesim) onto a commercial slide (CodeLink activated slides, Amersham Biosciences). Each probe was spotted 10 times. Water was spotted after each PNA, to avoid memory effects. Two matrices were spotted on the same slide. Slides were stored, blocked, and rinsed as described by the producer's protocol.

Quality Control. To check the deposition efficiency of the PNAs, the array functionalized with the PNA probes (1−5) was hybridized with the two complementary 11-mer Cy3-labeled oligonucleotides. Solutions of the oligonucleotides (1 pmol/μL) in a 5× SSC buffer containing 0.1% SDS and 0.1 mg/mL salmon sperm DNA were prepared. These solutions (40 μL) were incubated for 2 min at 100 °C and put on the array, which was then covered by a plastic cover-slip (22 × 22 mm). Hybridization was performed for 3 h at room temperature in a moist chamber. After hybridization, the slides were rinsed in a 4× SSC solution and dried by centrifugation.

DNA Extraction. DNA was extracted and purified from soybean powder 5% Roundup Ready (catalog 76913, Fluka, certified reference material IRMM-410S-3) using a Wizard Plus Minipreps DNA purifica-

tion system (catalog A7100, Promega). Quantity and purity of the DNA extracted were evaluated by measurement of the UV absorption at 260/280 nm.

PCR for Double-Stranded Products. Amplification was carried out with the primers SL1 (5′ATG GGC TTG CCT TCT TTC TC 3′) and SL2 (5′CCG ATG TGT GGA TTT GGT G 3′) for lectin and GM07 (5′ATC CCA CTA TCC TTC GCA AGA 3′) and GM08 (5′ TGG GGT TTA AAA TTG GAA 3′) for RR (*31*); both the reverse primers SL2 and GM07 were labeled with Cy3 or Cy5. The 40 PCR cycles were as follows: denaturation at 95 °C for 50 s, annealing at 58 °C for 50 s, and elongation at 72 °C for 1 min. The amplification products were analyzed on a 2% agarose gel in TBE 1× buffer with ethidium bromide staining and then purified and concentrated with GFX columns (Amersham-Pharmacia). Quantification of the PCR fragment was performed with the Bioanalyzer 2100 (Agilent Technologies).

PCR for Single-Stranded Products. The procedure used was the same above-reported for the double-stranded product except that the forward primers (SL1 and GM08) were 5′-phosphorylated. The PCR products obtained were digested with λ-exonuclease at 37 °C for 30 min. After blocking the reaction at 75 °C for 15 min, quantification of the single-stranded PCR fragment was performed with the Bioanalyzer 2100 (Agilent Technologies).

Hybridization of Single- and Double-Stranded PCR Products. For the hybridization experiment we used ∼300 ng of dsPCR product (a similar amount was used for exonuclease digestion generating a ssPCR fragment of corresponding molar concentration). These were dissolved in 65 mL of 0.2× SSC buffer containing 0.1 mg/mL of salmon sperm DNA, denatured at 100 °C for 2 min, spread on the array, which was then covered with a plastic cover-slip (22 × 22 mm) and allowed to stand for hybridization overnight at room temperature in a moist chamber.

Signal Evaluation. Images of the slides were captured by a ScanArray 4000 microarray analysis system (Packard BioScience). Every image was taken at the same conditions: the laser power was set at 80, and the photomultiplayer (PMT) was set at 90 units.

Quantification of the spot intensities was obtained by means of the QuantArray4000 microarray analysis system (Packard BioScience).

RESULTS AND DISCUSSION

The segment amplified by PCR for the lectin gene was a 156 bp internal sequence, wheras the RR construct (169 bp) was located on the junction between the 35S promoter and the CTP4 element; both sequences were previously demonstrated to be specific for these targets (*6*). The PNA probes were chosen to be complementary to internal sequences of these amplicons and were designed to have low self-complementarity. The sequences chosen for the PNAs were unique for the target amplified: no occurrence was found (BLAST software from NCBI) for the entire sequence in any other region of either soy lectin or RR soybean gene.

The length of the PNA complementary to RR was varied (from 9-mer to 15-mer) in order to investigate the effect of the stability of the DNA:PNA hybrid on the microarray responses.

The synthesis of the probes was carried out using solid-phase synthesis and a BOC strategy in an automatic synthesizer. The yields were quite good. Purification by RP-HPLC provided very pure products, as demonstrated by HPLC-MS analysis. The stability of the PNA:DNA and of the DNA:DNA adducts was evaluated by means of UV melting curves; the results are reported in **Table 1**: the melting temperature increases by increasing the length of the PNA probe, as expected.

The PNA synthesized were spotted by means of a piezoelectric spotter onto a commercial activated slide (CodeLink activated slides, Amersham Biosciences). Two matrices were spotted on the same slide in order to compare the results obtained under different conditions. The efficiency of the spotting was checked by hybridization of the PNA probes with the 11-mer Cy3-labeled oligonucleotide complementary to each sequence. The results were good: the spots were homogeneous, and the blank did not show any signal due to contaminations (results not shown), indicating that the spotting process could be performed without appreciable memory effects which could interfere with DNA identification.

Table 2. Interference of Lectin PCR Products on RR PNA Probes in the Microarray, as Percentage of the Specific Lectin/Lectin Signal[a]

	PCR products hybridized on the slides			
PNA	PCR lect ds Cy3	PCR lect ss Cy3	PCR lect ds Cy5	PCR lect ss Cy5
RR 9-mer	5.1	2.6	8.0	3.3
RR 11-mer	2.0	1.4	2.2	0.4
RR 13-mer	1.8	0.0	1.6	0.0
RR 15-mer	0.8	0.0	3.6	0.0

[a] Conditions for hybridization, rinse, and signal evaluation are those reported under Materials and Methods.

Using this model array, we analyzed the DNA content of a soybean flour containing 5% Roundup Ready certified reference material. The DNA extracted was amplified with the specific primers SL1 and SL2 for lectin (present in wild-type soybean and used as control) and GM08 and GM07 for Roundup Ready soybean described above, using either Cy3 or Cy5 as fluorescent labels (dsDNA). Single-stranded PCR products (ssDNA) were obtained using a phosphorylated primer for the unlabeled strand, digesting the resulting duplex DNA with λ-exonuclease.

Hybridization experiments were performed with both dsDNA and ssDNA under different ionic strength, which was decreased from 4× SSC to 0.2× SSC. The results are reported in **Table 2**.

The occurrence of cross-signals in the hybridization experiments was found to be dependent mainly on the type of DNA used, with best results obtained for single-stranded samples, whereas little differences were found for the two fluorophores used (Cy3 and Cy5), due to unspecific interactions (vide infra).

PNAs were showm to bind to complementary dsDNA or ssDNA sequences on the microarray, with high affinity and good specificity. PNA probes are very selective with the ssDNA products (either Cy3- or Cy5-labeled), in particular when they have a certain length (13- or 15-mer). The responses obtained on the microarray using single-stranded lectin and RR amplicons with either Cy3 or Cy5 labeling are reported in **Figure 2**.

As expected, hybridization was found to be selective, although a faint cross-hybridization was observed for the lectin amplicon also with the RR probes (B lectin). The unspecific signals could be due to adsorption of the labeled amplicons through non-Watson−Crick interactions. Because cyanine dyes were found to interact with PNA:PNA duplexes (*32*), it is possible that the same type of interactions occur between the dyes and partially formed PNA:PNA tracts on the array.

In **Table 3** the intensities obtained for the different DNAs used with all of the probes spotted on the array are reported; in these data, the signal intensity of the background, corresponding to the average of the intensities of the blanks, was subtracted.

The Cy5-labeled amplicons turned out to give rise to higher signals than Cy3, whereas the interferences were equivalent in both cases. Although for the lectin gene the 11-mer PNA probe was able to give rise to good signal-to-noise ratios, with the RR probes an increase in signal intensity was observed by increasing the probe length: whereas the signal obtained for the 9-mer was hardly distinguished from the aspecific hybridization (with lectin amplicon), good results could be obtained with the 15-mer probe targeting the same gene tract.

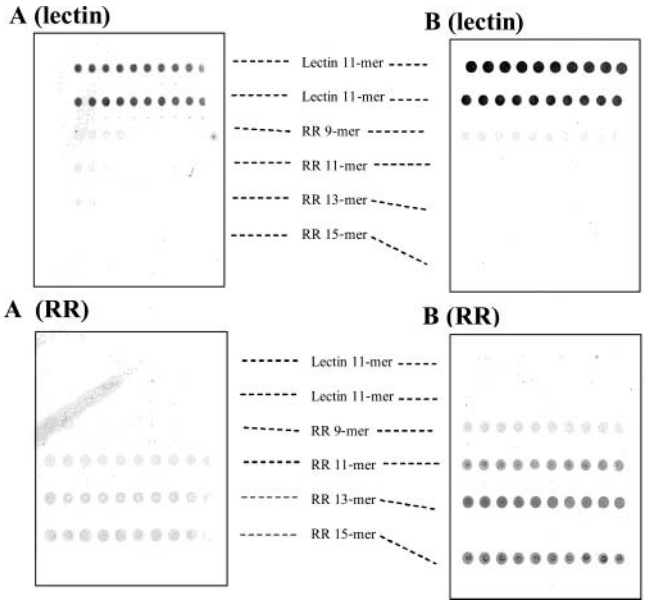

Figure 2. PNA microarray analysis of single-stranded PCR amplicons from the lectin gene (156 bp, top) and of the RR construct (169 bp, bottom), labeled with Cy3 (**A**) or Cy5 (**B**). Conditions for hybridization, rinse, and signal evaluation are those reported under Materials and Methods.

Table 3. Signal Intensity Obtained by Hybridizing Different PCR Products with PNA Probes[a,b]

	Cy3-labeled PCR product				Cy5-labeled PCR product			
	lectin target		RR target		lectin target		RR target	
PNA	ds	ss	ds	ss	ds	ss	ds	ss
lectin 11-mer	12777	17930	826	−260	12223	20463	656	−602
RR 9-mer	657	500	1100	673	981	682	1661	3218
RR 11-mer	265	276	1636	1308	280	85	5137	11632
RR 13-mer	234	−22	2802	2015	201	−11	8601	17394
RR 15-mer	111	−142	3634	2640	442	−87	17983	18832

[a] Values are reported as arbitrary units. Conditions for hybridization, rinse, and signal evaluation are those reported under Materials and Methods. [b] Water was spotted as negative control, and the value was subtracted from the intensities of all probes.

Using this type of approach for analyzing soybean samples containing a definite amount of RR (5%), it was possible to obtain 10 data for each sample at the same time, identifying the amplicon sequence more reliably.

The results obtained by quantifying the signal intensities of the rows on the microarray of **Figure 2** (ssDNA) are reported in **Figure 3A**. High intensities and good signal-to-noise ratios were obtained for all of the probes used. The signal intensity was higher when using Cy5 labeling. In the case of the RR probes of different lengths, the signal intensity was essentially the same when using Cy3 labeling, whereas with Cy5 it increased significantly from 9-mer to 13-mer, and no difference was observed when using 15-mer. Thus, for a ssDNA target, a 13-mer probe could be sufficiently long to generate good signal intensities.

The results obtained for the hybridization of the double-stranded amplicons are reported in **Figure 3B**. Also in this case, the best results were obtained using the Cy5 fluorophore. However, the hybridization with PNA becomes less effective, due to the competition between the PNA probe and the long DNA strand for the annealing with the target sequence. Therefore, in this case the best results were obtained by using

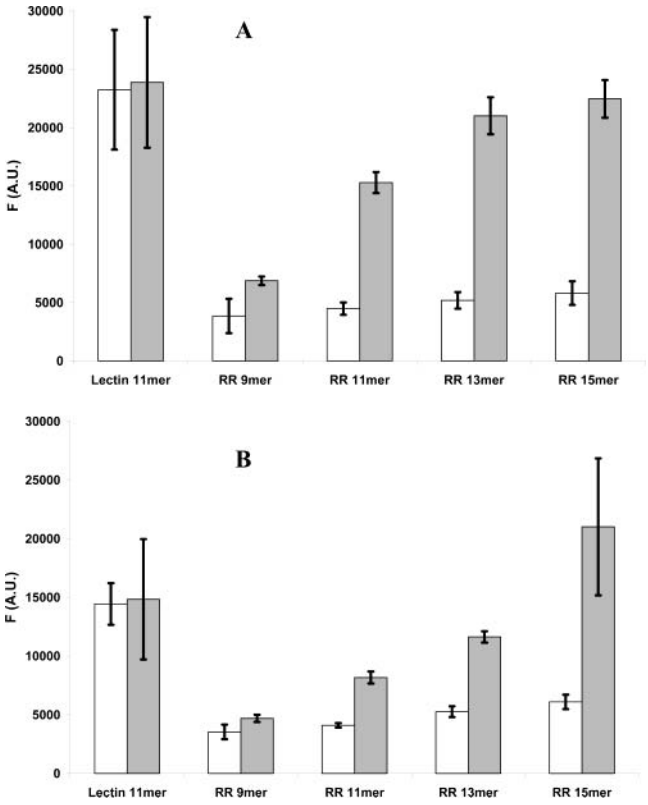

Figure 3. Quantitation of signal intensities obtained by microarray analysis using single-stranded (**A**) or double-stranded (**B**) amplicons labeled with Cy3 (unshaded bars) or Cy5 (shaded bars). Signal intensity for lectin is that observed using the lectin amplicon; all others were obtained using the RR amplicon.

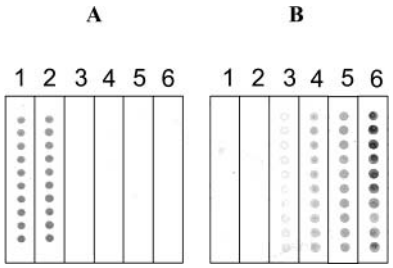

Figure 4. Analysis of double-stranded Cy5-labeled 156 bp lectin (**A**) or 169 bp RR (**B**) PCR products using the model PNA microarray: (lanes 1 and 2) lectin 11-mer probe; (lanes 3–6) RR 9-mer, 11-mer, 13-mer, and 15-mer, respectively. Conditions for hybridization, rinse, and signal evaluation are those reported under Materials and Methods.

the longest probe (15-mer). In any case, with this approach (by direct hybridization of the PCR products on the array) the hybridization protocol becomes easier because it allows the digestion step to be avoided.

A hybridization experiment using the conditions of dsPCR products and Cy5 labeling is shown in **Figure 4**. These conditions can be considered to give the best combination of selectivity, sensitivity, and simplicity in sample preparation. The progressive increase of the signal intensity of the RR amplicon obtained by increasing the length of the PNA probe ensures that the signal obtained is due to specific Watson−Crick base pairing and not to other physical unspecific effects, which should appear on all spots. Thus, it is possible to produce PNA microarrays, which allow the simultaneous detection of DNA sequences in a fast and reliable way.

CONCLUSIONS

In this work we have shown that PNAs and microarrays may be successfully combined to reveal the presence of RR-soybean after PCR amplification. The array format allows one to visualize progressive binding with probes of different lengths, thus preventing the risk of false positives due to unspecific interactions.

The best results were obtained with ssPCR products and with longer PNAs; however, it is possible to analyze the dsDNA in a faster and reliable way using the 15-mer PNA probe.

The advantages of this approach over other technologies are that: (i) PNAs are more efficient and form more stable hybrids than oligonucleotides; (ii) PNAs are highly sequence specific, the PNA:DNA duplex stability being greatly affected even by the presence of a single base mismatch; (iii) microarrays allow the simultaneous molecular analysis of many sequences. In fact, although here we report a microarray designed to detect only GM-soybean, it is possible to design microarrays with PNAs corresponding to other relevant GMOs. Work is in progress to develop a complete chip able to identify, in a single and reliable experiment, most or all of the relevant GMOs eventually present in food via a previous multiplex amplification procedure (*33*).

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