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A Microarray Platform for Parallel Detection of Five Transgenic Events in Foods: A Combined Polymerase Chain Reaction−Ligation Detection Reaction−Universal Array Method

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We recently developed a multiplex polymerase chain reaction (PCR) system for the simultaneous detection of four transgenic maize (MON810, Bt176, Bt11, and GA21), one transgenic soybean (Roundup Ready), and two control genes (lectin and zein). Because PCR can lead to ambiguous interpretations due to low specificity, we have developed the ligation detection reaction (LDR) combined with a universal array as a molecular tool to confirm results of PCR analysis. Here, we describe the PCR−LDR−universal array procedure and demonstrate its specificity in revealing the presence of transgenic DNA in experimental samples, raw materials, and commercial foodstuffs.

KEYWORDS: Genetically modified organism (GMO); ligation detection reaction (LDR); microarray; universal array; multiplex; polymerase chain reaction (PCR)

INTRODUCTION

Within the European Community (EC), strict control regarding commercialization of genetically modified organisms (GMOs) is exercised. Just five GMOs, including four transgenic maize (MON810, Bt176, Bt11, and GA21) and one transgenic soybean (Roundup Ready soybeans, Monsanto), are authorized within the EC. Furthermore, the EC requires labeling of foodstuffs containing GMOs and allows up to 0.9% adventitious presence of authorized GMOs in imported food (EC regulations 258/97 and 49/2000) (*1*). Therefore, sensitive and specific methods are required to enforce these regulations. Outside the EC, several other GMOs have been authorized for cultivation and consumption and are globally available for use in food preparation. Therefore, it is of great importance that detection methods be able to identify several transgenes in a single reaction.

Routinely used GMO detection methods generally involve polymerase chain reaction (PCR) techniques to detect specific DNA sequences (*2*), often in a multiplex format in which two or more targets are simultaneously amplified in the same reaction (*3*). For example, we recently described a robust and specific multiplex PCR method to simultaneously detect five transgenic and two wild-type genes in raw materials and foodstuffs (*4*). This seven-target method detects the four transgenic maizes and one transgenic soybean authorized by the EC as well as two endogenous controls (zein gene for maize and lectin gene for soybean). The simultaneous amplification of multiple sequences in a single reaction saves time, reagents, and efforts in GMO detection analysis and decreases the number of reactions required to assess the presence of all possible GMOs (*5*).

Nevertheless, as recently highlighted by the case of controversial PCR evidence for transgenic contamination of maize in Mexico (*6*), post-PCR monitoring is required to confirm sequence identities (*7*). In this context, microarray technology can greatly improve the ease and speed of post-PCR amplicon analysis and identification.

We recently combined the ligation detection reaction (LDR) with a universal array in a molecular approach to detect and quantify the amplified cryIA(b) gene from Bt176 maize (*8*). Universal arrays consist of a set of synthetic oligonucleotides named Zipcodes (*9*) or tags (*10*) of similar thermodynamic characteristics (melting temperature) but different sequences: The sequences are totally unrelated to those under scrutiny. Gerry et al. (*9*) first proposed LDR combined with a demultiplexing universal array as a powerful tool for sequence discrimination. This procedure requires the design of two adjacent probes specific for each target sequence. The discriminating oligonucleotide probe is 5′-fluorescently labeled, while the common probe is 5′-phosphorylated and contains a unique and artificial 3′-sequence (cZipcode) that is complementary to a Zipcode sequence within the universal array. These oligonucleotides hybridize consecutively along the template; thermostable DNA ligase joins their ends, linking the fluorescent label with a specific cZipcode. This reaction can be cycled to increase sensitivity (**Figure 1A**). The resulting products are hybridized onto the universal array where the cZipcode drives the LDR product to the corresponding Zipcode, and ligated fragments are detected by laser scanning (**Figure 1B**). In

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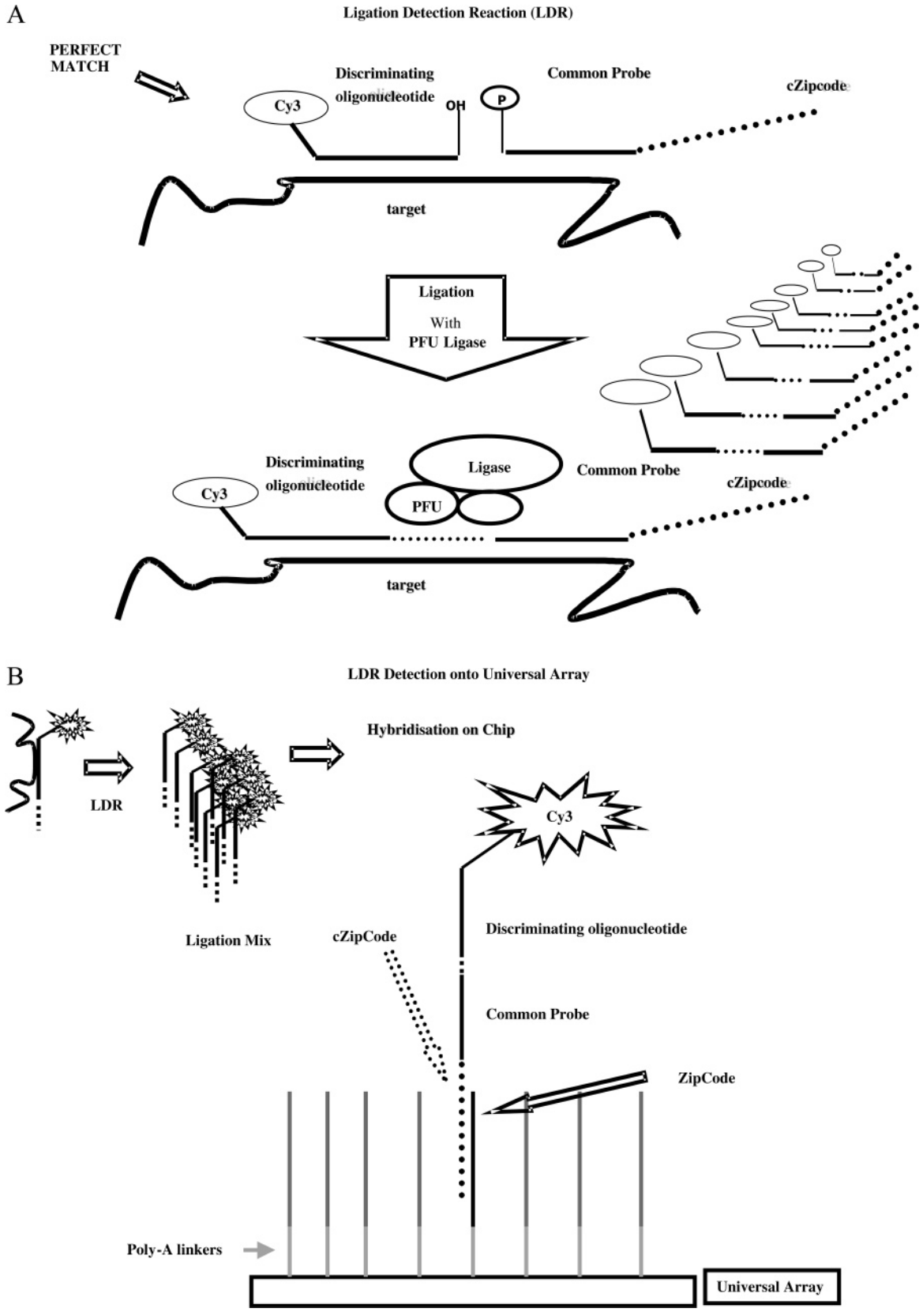

Figure 1. LDR–universal array approach. Scheme of the LDR (**A**) combined with detection on a universal array (**B**).

combination with a universal array, the PCR−LDR approach allows for the unequivocal detection of several amplicons in a single experiment.

We have already applied such an approach in bacterial discrimination (*11*), HLA polymorphism detection (*12, 13*), and Alzheimer's disease genotyping (*14*) and have demonstrated excellent specificity and sensitivity down to the single base substitution. To determine whether the PCR−LDR−universal array approach can be extended to the detection of transgenic material in foods, we studied its feasibility and specificity in analyzing experimental samples and commercially available food products.

Table 1. Molecular Sequences of the Discriminating and Common LDR Probes for Analysis of Transgenic Constructs[a]

amplicon	discriminating probe (5′→3′) 5′-Cy3	common probe (5′→3′) 5′-phosphate cZipcode sequence	Zipcode number (ref)
MON810	CTCACTCCGCCCTCTGCCTTT	GTTACTGCCACGTTTCTCTGAATGC**CCGTACCCTTCCGCTGGAGATTTAC**	23 (*15*)
Bt176 a	CCTGTACCGTCGACCTTTCAACA	TCGGCATCAACAACCAGCAGC**GGTCCGATTACCGGTCCGATGCTG**	23B (*9*)
Bt176 b	TGTACCGCAAGAGCGGCAC	CGTGGACAGCCTGGACGAGAT**GGTCTACCTACCCGCACGATGGTC**	25B (*9*)
Bt11 a	CTTCTTGGCGGCTTATCTGTCTC	AGGGGCAGACTCCCGTGTTC**AGATTGGGATGCGGTCGCGATACCG**	4 (*15*)
Bt11 b	CGACATGTCTCCGGAGAGGAG	ACCAGTTGAGATTAGGCCAGCTAC**CTTGAGCGATGACGGACGGGAAAAG**	8 (*15*)
GA21 a	AACAAGAAGTTCGAGACGCTGTCG	TACCTGCCGCCGCTGTCTATG**GAGGATCTGTAGCGCCTCTTCGAGC**	5 (*15*)
GA21 b	TCCTCCAGAAGCCTCGGCAAC	GTCAGCAACGGCGGAAGGATC**ACCGCGCAAATGGACAGTGTGGCCA**	32 (*15*)
RRS a	TTGGAATTGGGATTAAGGGTTTGT	ATCCCTTGAGCCATGTTGTTAATT**GCTGAGGTCGATGCTGAGGTCGCA**	1B (*9*)
RRS b	TTGTATCCCTTGAGCCATGTTGTTA	ATTTGTGCCATTCTTGAAAGATCTG**GCTGCGATCGATGGTCAGGTGCTG**	3B (*9*)
lectin a	ATGGGCTTGCCTTCTTTCTCGCA	CCAATTGACACTAAGCCACAAACACA**ATCTTGCGCGGCAGCTCGTCGACCG**	2 (*15*)
lectin b	CGCTGTTGAGTTTGACACTTTCCG	GAACTCTTGGGATCCACCAAATCC**TGTGCTTACCGCACCTCGCAGTCGT**	35 (*15*)
zein a	TATACATTGTCTTTGTTGCCTGC	ATACATCTATAAATAGGACCTGCTAGATCAATC**CGCATACCAGGTCGCATACCGGTC**	15B (*9*)
zein b	TCTTTGTTGCCTGCATACATCTATAA	ATAGGACCTGCTAGATCAATCGCA**GGTCAGGTTACCGCTGCGATCGCA**	21B (*9*)

[a] For each amplicon (except MON810), two pairs of probes were designed. In bold are the cZipcodes that are complementary to the sequences attached onto the universal array (Zipcodes). Zipcode numbers are those in the cited references.

MATERIALS AND METHODS

Oligonucleotide Probes for LDR. Array Designer 1.1. software (Premier Biosoft International, Palo Alto, CA) was used to design two pairs of probes specific to each amplicon of multiplex PCR (**Table 1**). We considered the thermodynamic features and hairpin and self-dimerization values in order to design oligonucleotides with similar thermodynamic features and melting temperature (T_m) values of 65 °C. Each pair consisted of one discriminating probe and one common probe; the latter probe contained a randomly assigned Zipcode sequence at the 3′-terminus. To check the specificity of the selected probes, we verified their lack of homology with other sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Discriminating oligonucleotides carried Cy3 fluorochromes at their 5′-termini, while common probes carried phosphate groups in the same position. Oligonucleotide probes were synthesized by Thermo-Hybaid (Dreieich, Germany) and were high-performance liquid chromatography purified and matrix-assisted laser desorption/ionization mass spectrometryMALDI-MS checked.

Universal Array Preparation. The 5′-amino-modified Zipcodes, carrying an additional poly(dA)$_{10}$ tail at their 5′-ends, were dissolved in 100 mM phosphate buffer (pH 8.5) at a final concentration of 50 μM and spotted four times onto CodeLink slides (Amersham Biosciences) by a pin contact arrayer (Microgrid II Compact; BioRobotics, Cambridge, England). Printed slides were processed according to the manufacturer's protocols.

Batch quality control was performed by hybridization with 1 μM 5′-Cy3-labeled poly(dT)$_{10}$ in a solution containing 5X SSC, 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg/mL salmon sperm DNA at room temperature for 1 h and then washed for 5 min in 4X SSC. The fluorescent signal was measured by laser scanning.

LDR and Hybridization onto Universal Array. PCR was performed as previously described (*4*). The multiplex PCR products were purified using the GFX PCR DNA purification kit (Amersham Pharmacia Biotech, Piscataway, United States) according to the manufacturer's protocol, eluted in 50 μL of autoclaved water, and quantified using Agilent 2100 Bioanalyzer and the DNA 500 LabChip kit for 25−500 bp dsDNA (Agilent, Palo Alto, CA).

LDR was performed in a final volume of 20 μL containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM $MgCl_2$, 0.1% NP40, 0.01 mM ATP, 1 mM DTT, 2 pmol of each discriminating oligonucleotide, 2 pmol of each common probe, and 10−100 fmol of purified multiplex fragments. The reaction mixture was denatured for 2 min at 94 °C prior to the addition of 4 U (1 μL) Pfu DNA ligase (Stratagene, La Jolla, CA). The LDR was processed for 30 cycles consisting of 30 s at 94 °C and 4 min at 60 °C in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, United States). The hybridization solution (65 μL) consisting of ligation reaction mix, 5X SSC, and 0.1 mg/mL salmon sperm DNA was heated at 94 °C for 2 min and applied onto the universal array using a multisample hybridization chamber (Press-to-Seal Silicone Isolator, eight wells; Schleicher&Schuell Bioscience, Keene, United States). The hybridization reaction was carried out in the dark at 65 °C for 1.5 h in a temperature-controlled oven. After removal of the chamber, the array was washed in prewarmed 1X SSC−0.1% SDS for 15 min at 65 °C.

Fluorescent signals were acquired at 10 μm resolution using a ScanArray 4000 laser scanning system (Packard GSI Lumonics, Billerica, United States) with a green laser for Cy3 dye (λ_{ex} = 543 nm, λ_{em} = 570 nm).

Signal Processing. Images acquired by laser scanning were processed with QuantArray software (Packard GSI Lumonics). The fixed circle option was used to define the grid for spot detection, and the mean signal intensity of each spot was quantified; local background was subtracted. The signal intensity for each Zipcode was expressed as the mean and standard deviation of four spots. Signals of negative controls (different Zipcodes, not assigned to any probe) were assumed to represent a measure of noise related to the overall biochemical procedure in any single array. A signal-to-noise (S/N) ratio of three was defined as the threshold above which signals from specific Zipcodes were included.

Analysis of Experimental and Commercially Available Samples. A ring test trial was performed by four independent European laboratories. Six samples (A−F) were prepared by the laboratory coordinating the trial (HEV, Sion, Switzerland), as previously described (*4*). The samples contained varying amounts of maize and soybean flours. Under blinded conditions, the participating laboratories extracted DNA and amplified the material according to the multiplex PCR protocol (*4*). PCR products were analyzed in our laboratory by capillary electrophoresis (2100 Bioanalyzer) and used as targets in LDR, performed in triplicate for each sample. The presence or absence of a certain transgene was determined on the basis of results from the more sensitive probe pair.

We applied the same procedure to six commercially available food products: soybean drink, maize biscuits, maize nacho chips, organic soybean biscuits, and gluten-free biscuits. No information was available concerning their GMO content.

RESULTS AND DISCUSSION

LDR Probe Design. According to the LDR procedure, for each target sequence, we designed two adjacent oligonucleotides: the Cy3-labeled discriminating oligonucleotide and the common probe with a cZipcode tail at the 3′-end. The probe sequences were chosen in order to set up a universal array with homogeneous characteristics and distinct chemical identities that prevented cross-hybridization. As listed in **Table 1**, we identified two pairs of oligonucleotides for each amplicon of the multiplex PCR, except for MON810; because this is the shortest amplicon (110 bp), we were able to design only one pair of specific probes. cZipcodes (*9*, *15*) were randomly incorporated into the common probes for each sequence in **Table 1**.

Molecular Specificity of Selected Probes. To evaluate the specificity of the probes and to investigate probe interference

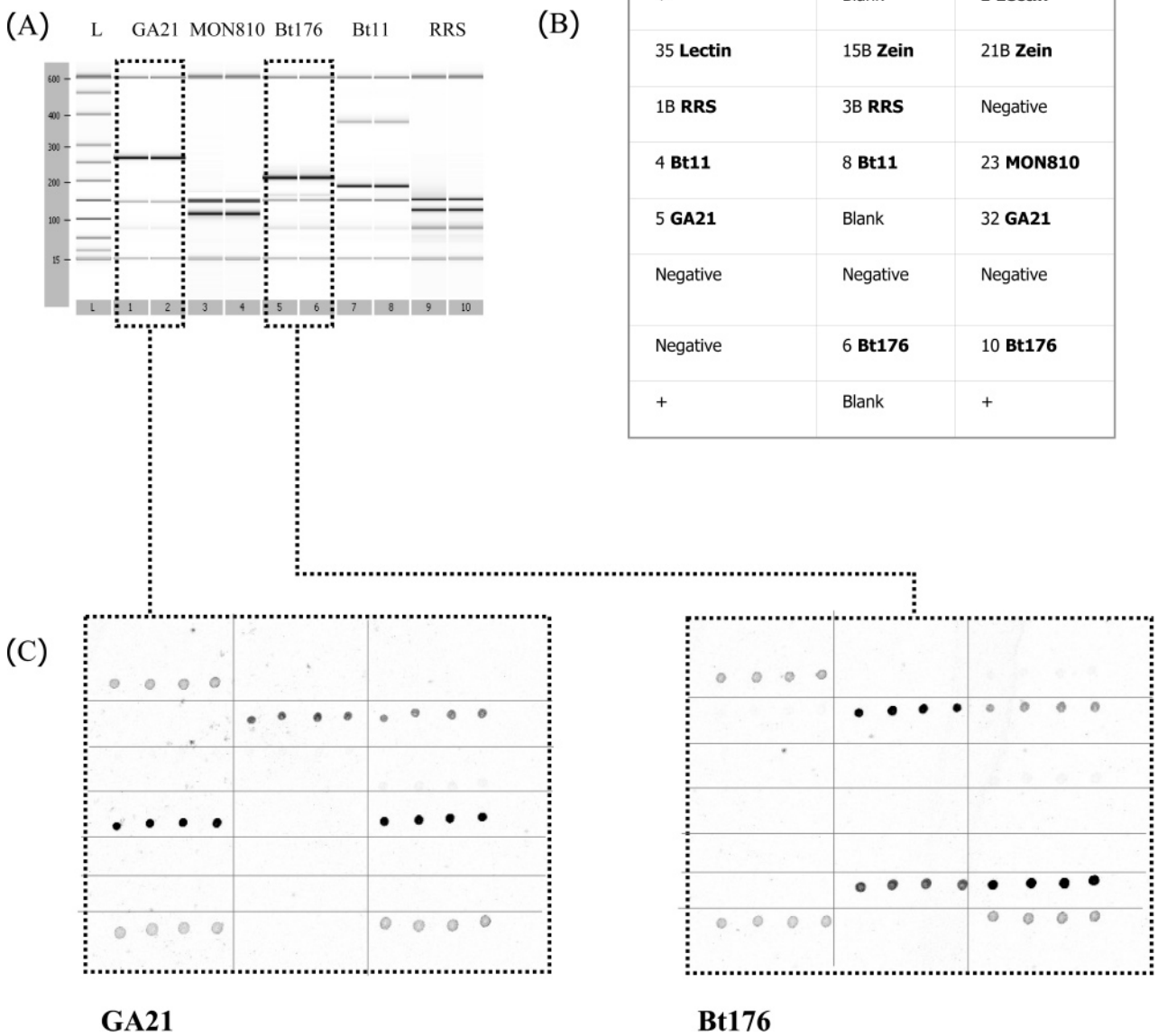

Figure 2. From capillary electrophoresis to array detection. (**A**) Analysis by Agilent 2100 Bioanalyzer of multiplex PCR samples containing a single transgenic DNA and all of the primer pairs. (**B**) Deposition of Zipcodes: Some of these were associated with the amplicons, and others served as negative or hybridization controls. Blank, bidistilled water. (**C**) Two examples of universal array hybridized with ligation products GA21 and Bt176.

affecting the final result, we performed LDR using as a target the material amplified by PCR of single DNA samples together with all of the primer pairs. These multiplex PCR products were quantified by capillary electrophoresis to verify the presence of the expected amplicons: GA21 (270 bp), Bt176 (209 bp), Bt11 (189 bp), lectin (157 bp), zein (139 bp), RRS (125 bp), and MON810 (110 bp). We then performed LDR in the presence of the entire set of probes (for a total of 26 oligonucleotides), and we hybridized the ligation products onto a universal array (**Figure 2**). Only the expected spots lit up during laser scanning: those corresponding to the endogenous gene and those to the transgene of the amplified event. Using a S/N ratio of three as threshold, signals were detected only where expected. This is of extreme relevance when trying to detect a minute amount of material (GMOs) in a vast excess of molecular background (wild-type maize and soybean).

The high molecular specificity of the approach was demonstrated by quantifying the signal intensity for each probe hybridized to the universal array (**Figure 3A**−**E**). For each of the five transgenic events, a substantial signal was generated only by the probe pairs specific for the amplicon and for relevant endogenous gene (lectin or zein), while essentially no signal was observed for the other probe pairs. However, the two pairs of probes designed for each amplicon (except for MON810) performed differently (**Figure 3**). For example, zein a probes were more efficient than zein b probes in all experiments performed. This may be due to nucleotide composition or sequence of the probes or to the position of hybridization along the amplicon.

Validation Ring Test. The feasibility of detecting trace amounts of specific transgenes in a heterogeneous sample was tested in a validation ring test involving four independent laboratories. We performed the multiplex PCR technique followed by LDR using all probe pairs (**Figure 4**). The small variation in signal intensity averaged over the results from three arrays indicates the precision of the method. Furthermore, the test confirmed the differing performances of the two probe sets designed for each amplicon. For example, signal intensities from probe pairs zein b (sample A) and Bt176 a (samples D and F) fell below the detection threshold (**Figure 4**). For all samples,

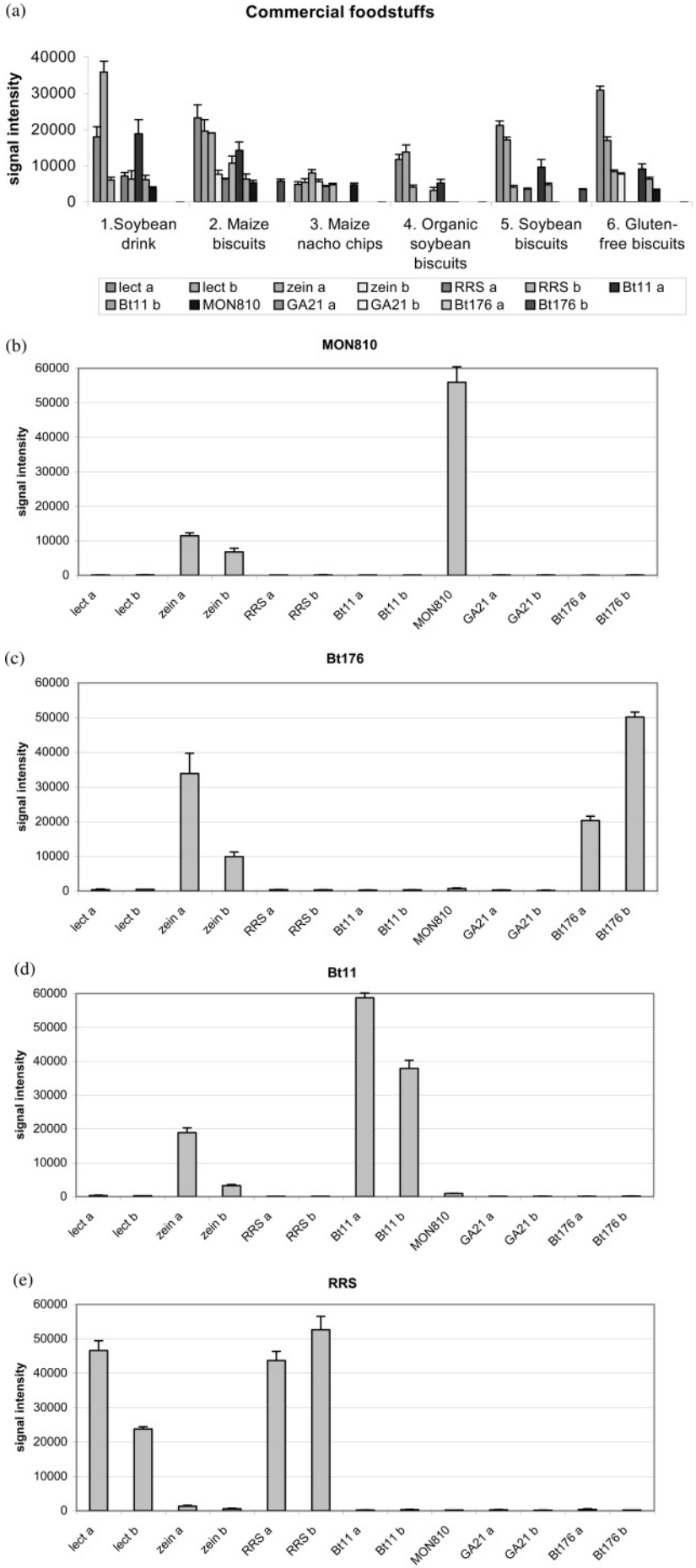

Figure 3. LDR results of each transgenic DNA amplified by the multiplex PCR system.

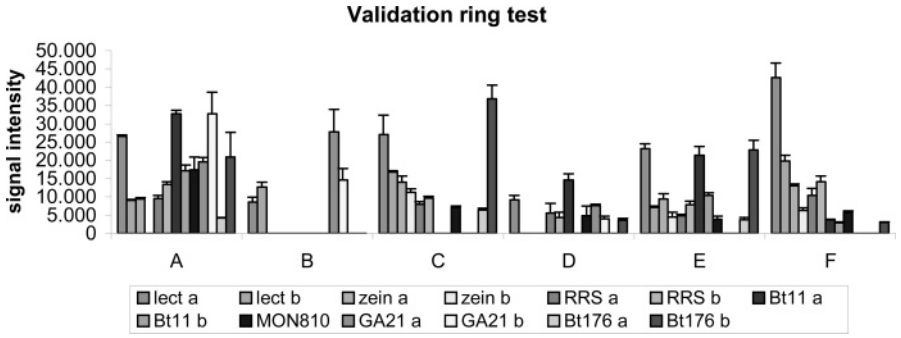

Figure 4. LDR results of six samples blindly analyzed during the ring test. The compositions of the samples were as follows: (**A**) 0.5% Bt176, 0.5% Bt11, 0.5% GA21, 2% MON810, and 2% RRS; (**B**) 5% soybean, 5% maize, 5% GA21, and traces of Bt176; (**C**) 1% Bt176; (**D**) 2% Bt176, 2% Bt11, 2% GA21, 0.5% MON810, and 0.5% RRS; (**E**) corn muffin mix containing soybean oil and traces of transgenic maize; (**F**) flour containing 0.6% RRS and maize. Bars represent the average (and standard deviation) of three experiments (three arrays).

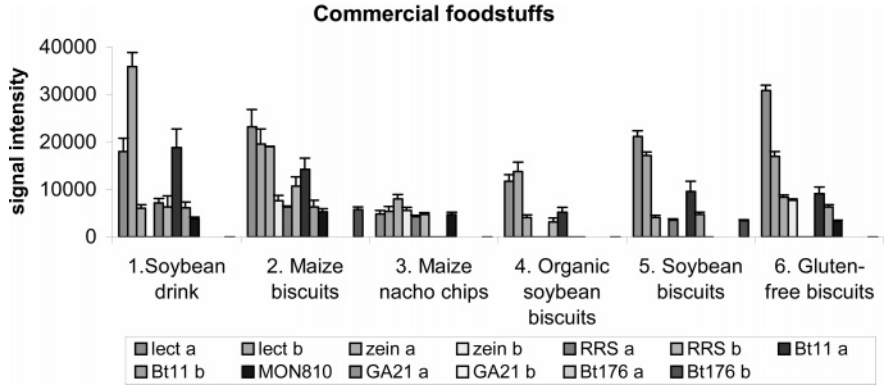

Figure 5. LDR results of six commercial foodstuffs containing maize or soybean. Bars represent the average (and standard deviation) of three experiments (three arrays).

LDR reflected the results obtained by capillary electrophoresis when considering the best probe pair for each amplicon (data not shown). No signal was obtained for the absent traits. Furthermore, there were some unexpected positive results, probably due to contamination, as already observed (*4*). For example, sample F (flour containing maize and 0.6% RRS) was positive for lectin, zein, and RRS probes as expected but also for Bt11, MON810 and Bt176 probes, whose amplicons were identified either by LDR or capillary electrophoresis. Although we have not determined a limit of detection of this multiplex PCR−LDR−universal array approach, during the ring test, we reliably identified the presence of 0.5% transgenic events within complex mixtures.

Application to Commercial Foodstuffs. The PCR−LDR−universal array approach was tested on real samples to evaluate the matrix effect. Six commercially available foodstuffs containing maize or soybean, previously analyzed with multiplex PCR, were submitted to LDR in the presence of the entire set of oligonucleotide probes. We performed LDR in triplicate for each commercial sample. After hybridization onto the universal array, the signal intensity reflected the composition of the multiplex amplified material as highlighted by capillary electrophoresis: No signal was detected for GA21 probes, but the LDR−universal array approach found an unexpected (in comparison to capillary electrophoresis) MON810 signal in samples 1, 2, 3, and 6 (**Figure 5**). This may be due to the concentration of the MON810 amplicon, which was probably below the 2100 Bioanalyzer's limit of detection (0.5 ng/μL). Furthermore, we detected the presence of other transgenic material, such as RRS (samples 1−5), Bt11 (samples 1, 2, and 4−6), and Bt176 (samples 2 and 5). Soybean and maize (through their lectin and zein genes) were correctly identified in accordance with the composition of each commercial sample; however, they were also detected in other samples when they were not listed as an ingredient, probably due to a contamination of the ingredient "vegetable proteins". These findings demonstrate that the LDR−universal array method, when combined with multiplex PCR, is a sensitive tool permitting the detection of faint transgenic events, even those not detected with capillary electrophoresis on the Bioanalyzer.

In conclusion, the LDR−universal array approach proposed in this paper is a valid method for the simultaneous detection of five different GMOs (Roundup Ready soybean and MON810, Bt176, Bt11, and GA21 maize) and two endogenous genes (lectin and zein), after their multiplexed amplification. We compared two pairs of probes for each amplicon (except MON810) to determine which was more efficient. The results obtained in different experiments demonstrate the high molecular specificity of the method and the possibility of applying this approach to complex samples.

With the increasing number of GMOs that are being developed and validated for food applications, there is great interest in the use of multiplex PCR for the simultaneous detection of numerous transgenic events. This approach has recently been applied to the detection of five genetically modified maize lines (*16*) and four transgenic canola lines (*3*). Garcia-Cañas et al. (*17*) reported a highly sensitive method based on PCR, capillary gel electrophoresis, and laser-induced fluorescence to determine in a single run different percentages of five transgenic maizes below the minimum threshold set by the EC regulations. We demonstrated that the LDR−universal array platform is an appropriate tool for parallel analyses of several samples, thereby increasing throughput. The inherent scalability of the universal array—we are currently using a 49-Zipcode array

(*13*)—is well-suited to the needs for testing food containing an increasing number of potential transgenic events. Our next goal is to test the sensitivity of our method and to develop an LDR—universal array platform that fulfills the requirements of the EC regulation.

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