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Detection and Quantitation of Genetically Modified Maize (Bt-176 Transgenic Maize) by Applying Ligation Detection Reaction and Universal Array Technology

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We have applied the ligation detection reaction (LDR) combined with a universal array approach to the detection and quantitation of the polymerase chain reaction (PCR) amplified cry1A(b) gene from Bt-176 transgenic maize. We demonstrated excellent specificity and high sensitivity. Down to 0.5 fmol (nearly 60 pg) of PCR amplified transgenic material was inequivocally detected with excellent linearity within the 0.1−2.0% range with respect to wild-type maize. We suggest the feasibility of extending the LDR/universal array format to detect in parallel several transgenic sequences that are being developed for food applications.

KEYWORDS: Genetically modified organisms; GMOs; polymerase chain reaction; PCR; ligation detection reaction; LDR; universal array; Bt-176 transgenic maize

INTRODUCTION

Identification of genetically modified organisms (GMOs) in food is becoming an issue of great interest. The introduction in the market of GMO products raises social and ethical concerns, and the European Union has dictated several directives and regulations regarding their liberation and commercialization. This includes the Novel Food Regulation (258/97/CE) for labeling of GMO-containing food-stuff and the Directive 49/2000, which establishes that under 1% of approved GMOs, labeling is no longer necessary as long as their presence in the product is accidental or technically unavoidable (*1*). GMOs could be detected by either polymerase chain reaction (PCR), for direct detection of the transgenic DNA, or immunological methods, for detection of proteins coded by the transgenic sequences. Because of their higher reliability and sensitivity and the higher stability of DNA over proteins, PCR methods are usually preferred (*2*).

PCR has been applied to the detection of FLAVR SAVR tomato (*3*), Roundup Ready soybean (*4*, *5*), Bt-maize (*6*), and gbss-antisense transgene potatoes (*7*). Sequence confirmation of PCR amplicons by proper methods is mandatory to avoid false positives.

With the increasing number of GMOs that are being developed for food application, the ability to detect several transgenes in a single reaction becomes an important feature of any detection method (*8*). Therefore, highly automated and parallel detection systems for the identification of GMO in foods are required. Recently microarray technology has been proposed for several applications in DNA analysis, allowing for the detection of different nucleic acid targets at once.

Rudi et al. (*9*) recently proposed a microarray approach to GMO detection in food by multiplex PCR amplification, GMO specific primer extension, and hybridization of the resulting labeled templates to an oligonucleotide microarray. A different approach based on a universal array could be considered. Universal arrays consist of a set of synthetic oligonucleotides named ZipCodes (*10*) or Tags (*11*) of similar thermodynamic characteristics (melting temperature) but very different in sequence. Their sequences are totally unrelated to those under scrutiny. The ligation detection reaction has been proposed by Gerry et al. (*10*) in combination 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 oligo is 5′ fluorescently labeled, while the 5′ phosphorylated common probe brings a 3′ unique and artificial sequence named cZipCode, which is complementary to a ZipCode sequence included within the universal array. These oligos hybridize consecutively along the template, and a thermostable DNA ligase joins their ends linking the fluorescent label with a specific cZipCode. This reaction can be cycled to gain in sensitivity. The resulting products are hybridized onto the universal array where the

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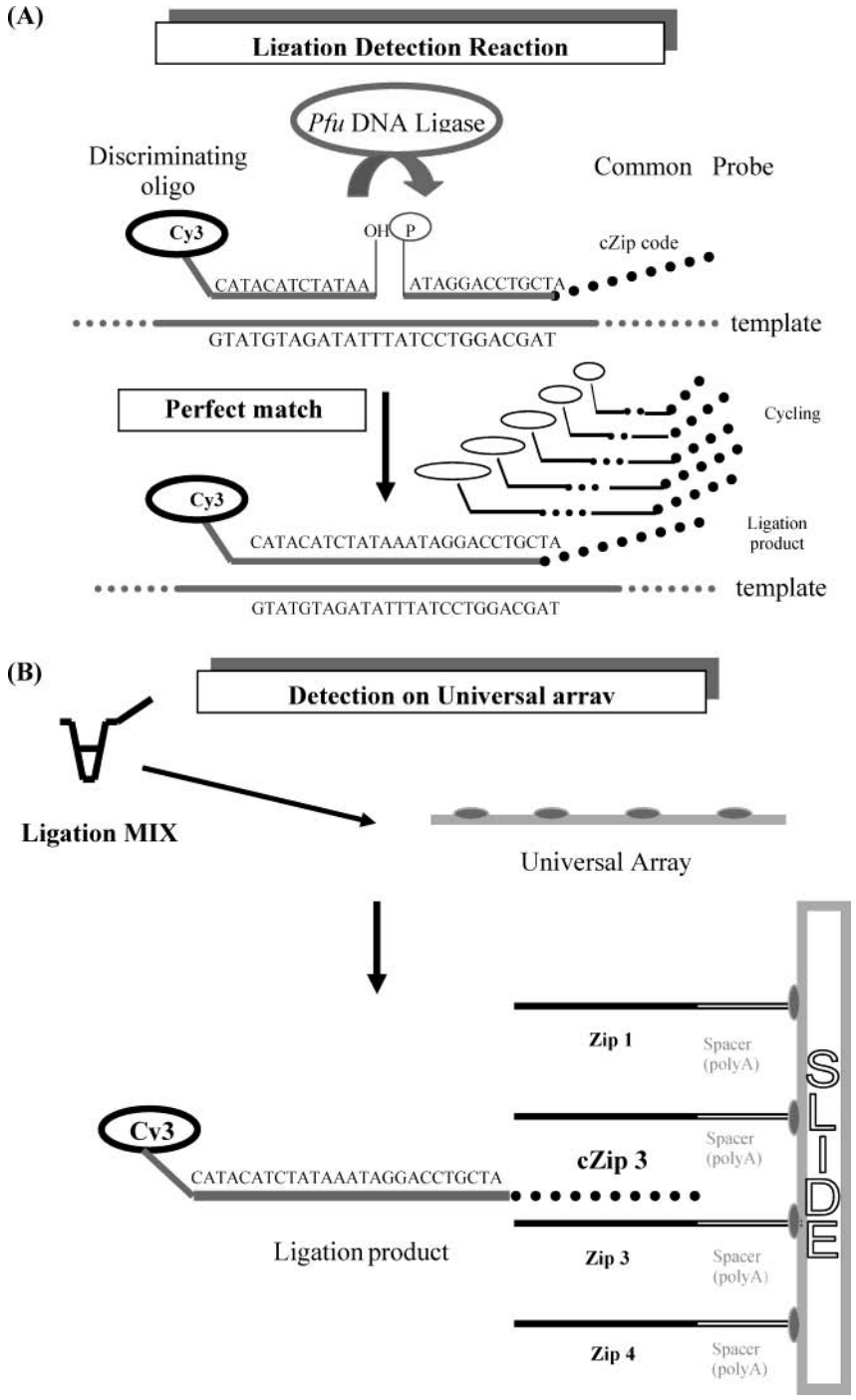

Figure 1. (A) Ligation detection reaction (LDR). (B) Hybridization of LDR products onto a universal array.

cZipCode drives the ligation detection reaction (LDR) product to the corresponding ZipCode and where ligated fragments are detected by laser scanning (**Figure 1**).

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

We have already applied such an approach to bacterial discrimination (*12*), polymorphisms detection in HLA (*13*), and Alzheimer disease (*14*). Here, we extend this approach to the detection of the cryIA(b) gene from insect-resistant Bt-176 transgenic maize, previously amplified by PCR. We have investigated in detail quantitative issues that are of the highest relevance in this field. Excellent results in terms of sensitivity and linearity of response of the procedure are reported, demonstrating the feasibility of the detection of transgenic material in food by means of the LDR/universal array approach and the possibility of multiplexed analysis.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical reagent grade and used without further purification.

Tris[hydroxymethyl]aminomethano (Tris), sodium dodecyl sulfate (SDS), guanidine hydrochloryde, ethylenediaminetetraacetic acid (EDTA), saline sodium citrate (SSC) buffer, salmon sperm DNA, proteinase K, and RNAse A were purchased from Sigma-Aldrich (Italy).

Maize Samples and DNA Isolation. Certified reference maize powders, containing 0%, 0.1%, 0.5%, 1%, 1.5%, and 2% insect-resistant Bt-176 transgenic maize, were obtained from Fluka Chemie GmbH (Buchs, Switzerland). Genomic DNA was isolated using the Wizard

Table 1. Sequences of the Two Sets of Zein and CryIA Probes[a]

probe	GenBank accession no.	5′ position	discriminating oligo 5′Cy3	5′ position	common probe 5′pho	cZip	zip no.
109 cryl A	141419	1095	cctgtaccgtcgacctttcaaca	1118	tcggcatcaacaaccagcagc	ggtccgattaccggtccgatgctg	23
212 cryl A	141419	1199	tgtaccgcaagagcggcac	1218	cgtggacagcctggacgagat	ggtctacctacccgcacgatggtc	25
1015 zein	M23537	1014	tctacattgtctttgttgcctgc	1037	atacatctataaaataggacctgctagatcaatc	cgcataccaggtcgcacataccggtc	15
1023 zein	M23537	1023	tctttgttgcctgcatacatctataa	1049	ataggacctgctagatcaatcgca	ggtcaggttaccgctgcgatcgca	21

[a] ZipCode numbers refer to those in ref 10.

DNA extraction kit (Promega, Madison, WI) according to the modifications described by Ziimmermann et al. (*15*). Extracted DNA was quantified spectrophotometrically using a GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

Oligonucleotides Primers and Probes. PCR primers and oligonucleotide probes were purchased from Thermo-Hybaid GmbH (Germany). They were HPLC purified and MALDI-MS checked.

Array Designer 1.1 software (Premier Biosoft International, Palo Alto, CA) was used to design the LDR probes specific to zein and cryIA(b) fragments. Sequences and positions of the selected probes are shown in **Table 1**. All oligonucleotides have similar thermodynamic features and melting temperature (T_m) values of 60−65 °C. Discriminating oligos carried a Cy3 fluorochrome at their 5′ termini, while common probes carried a phosphate group in the same position.

PCR Conditions of Zein and cryIA(b) Fragments. The amplification reactions were performed using 100 ng of genomic DNA at a final volume of 50 μL. The reaction mixture consisted of 0.5 μM forward primer (zein gene, CGCCAGAAATCGTTTTTCAT; cryIA(b) gene, CCGCACCCTGAGCAGCAC), 0.5 μM reverse primer (zein gene, TAGGAAGCAAGGACACCACC; cryIA(b) gene, GGTGGCACGTTGTTGTTCTGA), 1× DynaZyme buffer (10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100), 200 μm dNTPs, 1.5 mM $MgCl_2$, and 0.04 U/μL DynaZyme II DNA polymerase (Finnzymes OY, Finland). The sample underwent a thermal cycling procedure consisting of a denaturation step (95 °C for 10 min), 40 cycles of 95 °C for 1 min, 60 °C for 45 s, and 72 °C for 45 s, and a final cycle of 72 °C for 7 min.

The PCR products, 139 bp for zein gene and 189 bp for cryIA(b) gene, were then purified by GFX PCR DNA purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to manufacturer's protocol, eluted in 50 μL of autoclaved water and quantified using agilent bioanalyzer 2100 (DNA 500 kit for 25−500 bp dsDNA sizing and quantification).

Universal Array Preparation. The 5′ amino-modified ZipCodes, carrying an additional poly$(dA)_{10}$ tail at their 5′end, were dissolved in 100 mM phosphate buffer (pH 8.5) at a final concentration of 50 μM and spotted 20 times onto Code-Link slides (Amersham) by a pin contact arrayer (Microgrid II compact BioRobotics, 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 and 0.1 mg/mL salmon sperm DNA at room temperature for 1 h, and then the slide was washed for 15 min in 2× SSC. The fluorescent signal was controlled by laser scanning.

LDR and Hybridization onto Universal Array. 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, 0.01mM ATP, 1 mM DTT, 2 pmol of each discriminating oligo, 2 pmol of each common probe, and 0.5−500 fmol of purified PCR products. The reaction mixture underwent a denaturation step for 2 min at 94 °C, and then 1 μL of 4 U/μL Pfu DNA ligase (Stratagene, La Jolla, California) was added. The LDR was cycled for 30 cycles of 94 °C for 30 s and 60 °C for 4 min in a GeneAmp PCR system 9700 (Applied Biosystems, Foster, CA). The hybridization solution (65 μL) consisting of ligation reaction mix, 5× 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 Silicon isolator, eight wells, Schleicher & Schuell). The hybridization reaction was carried out in the dark at 65 °C for 2 h in a temperature-controlled oven. After removal of the chamber, the array was washed in prewarmed 1× 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, MA) with a green laser using Cy3 dye (λ_{ex} = 543 nm; λ_{em} = 570 nm).

To quantitate the fluorescent intensity of spots, we used the QuantArray Quantitative Microarray Analysis software (Packard GSI Lumonics, MA). Quantitation and data analysis were performed as explained in the section "Results and Discussion".

RESULTS AND DISCUSSION

LDR Probe Design. The probe design was performed according to the LDR procedure, requiring the design of two adjacent oligonucleotides (the Cy3-labeled discriminating oligo and the common probe with a cZipcode tail at the 3′ end) specific for each target sequence. In particular, we focused on the thermodynamic features, the hairpin, and self-dimer values of probes in order to set up a universal array with homogeneous characteristics and distinct chemical identities that prevent cross-hybridization. As listed in **Table 1**, we identified two sets of oligos for the reference (zein) gene (1015 and 1023) and two sets for the cryIA(b) gene (109 and 212), the transgenic fragment for Bt176 maize. We randomly assigned cZipcodes to a specific sequence: Zip15 and Zip21 (10) assigned to 1015 and 1023, respectively, to identify the zein probes; Zip23 and Zip25 (10) assigned to 109 and 212, respectively, to identify the cryIA probes.

To check the specificity of the selected probes, we verified the absence of homology with other sequences using BLAST.

Signal Processing. Images acquired by laser scanning were processed as follows. QuantArray quantitation software was used with the fixed circle option to define the grid for spot detection, generating a signal quantitation as a mean intensity for each spot. Local background was then subtracted. Resulting values were averaged (n = 20), and the standard deviation was calculated. Signals coming from a negative control (Zip1 TGCGACCTCAGCATCGACCTCAGC, not assigned to any probe) were assumed to represent a measure of noise (N) related to the overall biochemical procedure in any single array. A signal to noise (S/N) ratio of 3 was defined as the threshold to include/exclude signals from specific ZipCodes.

High Molecular Specificity of Maize Probes. To test the molecular specificity of the selected probes, we used two distinct PCR products (obtained from genomic Bt-176 maize DNA) as substrates in separate ligation reactions: an amplified fragment of 139 bp for zein and a 189 bp amplicon for cryIA(b) genes.

We performed LDR using the entire set of zein and cryIA(b) probes (for a total of 8 oligonucleotides), employing 100 fmol for each specific amplified fragment as target. **Table 2** shows the signal intensity and the standard deviation values obtained after hybridization of LDR products onto a universal array.

Defining a S/N ratio of 3 as a threshold for a positive signal, we found that when 100 fmol of template (either zein or cryIA amplicons) was used, no signal was detectable in the corre-

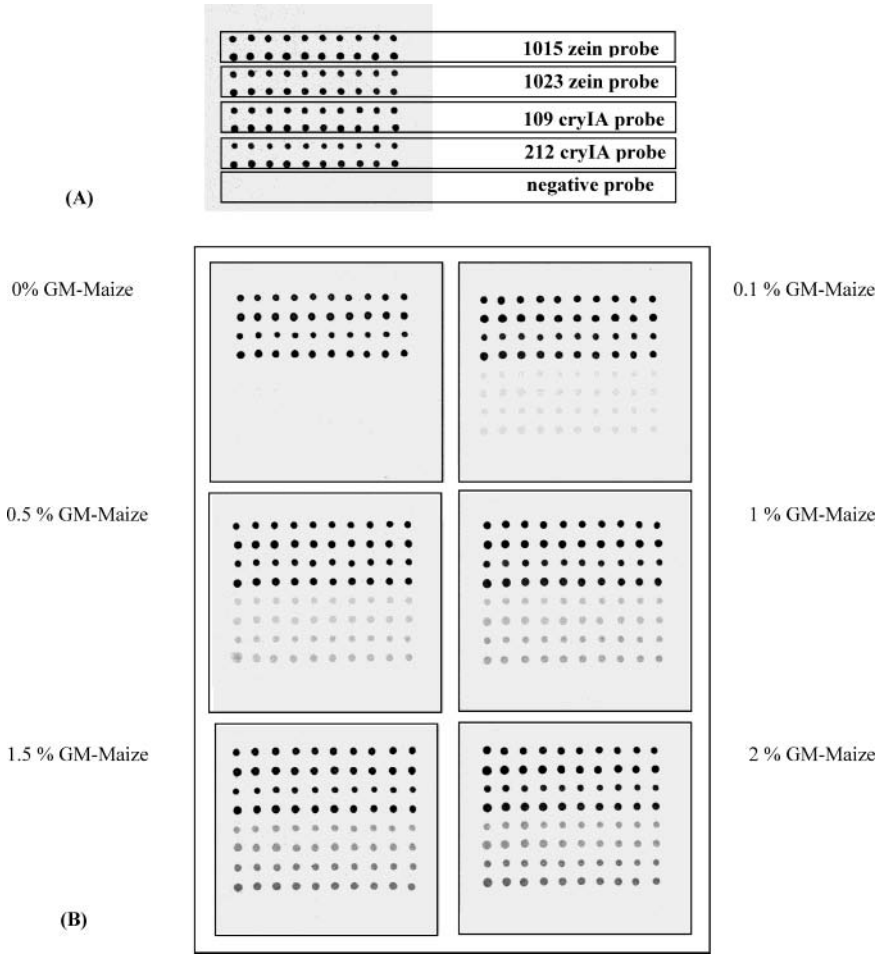

Figure 2. Results of the LDR/universal array approach: (A) ZipCodes deposition scheme; (B) LDR with different GM-maize amplicon percentages hybridized onto the same slide containing six universal arrays.

Table 2. Molecular Specificity of Selected Probes[a]

probe	signal intensity	standard deviation	*S/N*
	(A) LDR Template = Zein Fragment		
1015 zein	38586	1483	185.3
1023 zein	24941	1480	119.8
119 cryIA	390	38	1.9
212 cryIA	231	19	1.1
negative probe	208	44	
	LDR Template = cryIA Fragment		
1015 zein	376	69	2.9
1023 zein	160	13	1.2
119 cryIA	25939	2481	201.3
212 cryIA	42755	2051	331.8
negative probe	129	17	

[a] A gene-specific signal was observed during hybridization of LDR products onto the universal array. Panel A refers to zein fragment as substrate in LDR, and panel B refers to cryIA. *S/N* ratios are calculated using the negative probe as noise. Data are reported in relative fluorescent units (RFU).

sponding negative positions (cryIA or zein, respectively). This is of extreme relevance when trying to detect a minute amount of material (Bt-176 maize) in a vast excess of molecular background (wild-type maize).

High Sensitivity and Linearity of LDR/Universal Array Approach. Since we demonstrated the high molecular specificity of our approach, we intended to test it in a range of Bt-176 maize percentages relevant to regulations of the European Union.

Multiplex PCR of different amplicons is well-known to introduce a bias in the original composition of the target sequences (*10*) and was confirmed within this study by quantitative analysis of multiplexed zein/cryIA PCR amplifications by an Agilent 2100 bioanalyzer. PCR products obtained from DNA extracted from flour at different GM-maize percentages do not maintain the original ratio between the transgene (cryIA) and the reference (zein) gene. This was further confirmed using these amplicons as targets in LDR/universal array approach (with cryIA amplicon being overrepresented with respect to the original concentration) (data not shown). Several approaches have been proposed to overcome this difficulty. Recently Rudi et al. proposed a double round of amplification using specific primers first, followed by a second round with common primers to address the problem with excellent results (*9*). Following these considerations, we addressed the issue of the linear response of LDR, which further amplifies the signal coming from PCR fragments and yield the molecular identity of the fragments under analysis. We prepared unbalanced mixes of zein and cryIA amplicons, which have been separately amplified, accurately quantitated by the 2100 bioanalyzer, and mixed to obtain 0%, 0.1%, 0.5%, 1%, 1.5%, and 2% Bt176-maize PCR standards. Since the detection limit in the LDR is 0.5 fmol of template (data not shown), we performed this reaction using an excess of zein amplicon (500 fmol) and 0.5−10 fmol of cryIA(b) amplicon to reproduce each selected percentage of transgenic material. LDR and hybridization reaction were carried out three times onto three different slides. **Figure 2** shows the ZipCodes deposition scheme (A) and six subarrays (one for each Bt-176 maize percentage) hybridized

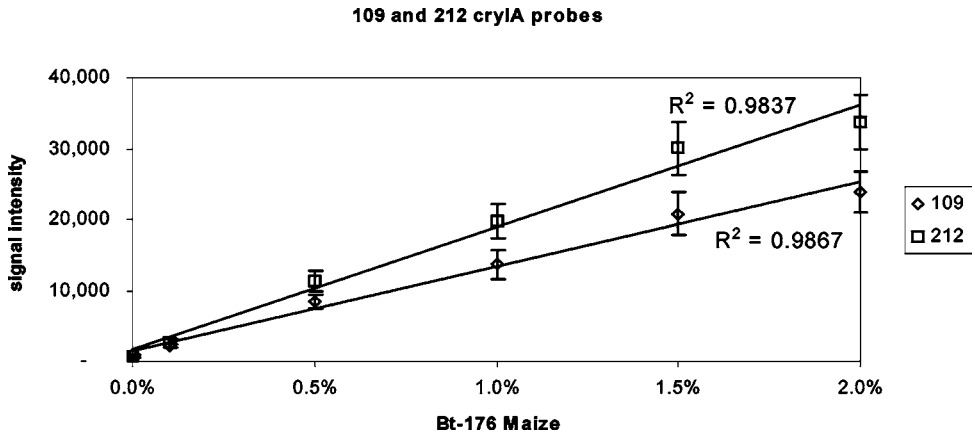

Figure 3. Plot of signal intensity versus GM-maize % for 109 and 212 cryIA probes. Coefficients of correlation (R^2) as well as error bars are reported. Data are plotted using 60 averaged measures (20 spots × 3 replicates in different slides).

onto the same slide (B). We spotted 20 times five ZipCodes corresponding to the zein probes (1015 and 1023), the cryIA probes (109 and 212), and the negative control (Zipcode 1). During microarray scanning, we fixed a photomultiplier gain and a laser power such that signals coming from 0.1% to 2% trangenic materials for the cryIA probe sets were clearly detected (well above the threshold as defined before). This pushed the signal intensity of the zein probes well above the saturation limit. This was done in order to detect also the minimum concentration of the transgenic maize (the 0.5 fmol of 0.1% case). It should be noted that in the analysis of the array for 0% Bt-176, no signals were present for the cryIA probes, thus further demonstrating the very high molecular specificity of the LDR reaction. **Figure 3** shows the LDR results after background subtraction, and an average of 60 spots, obtained from 20 (spots) × 3 (experiments), replicates both sets of cryIA probes (109 and 212). The signal intensity increases linearly with different Bt-176 maize concentrations within the selected range. A very good coefficient of correlation was found for both sets ($R^2 >$ 0.98).

Detection limits of our method were established with the repeated dilution of the transgene amplicon. Down to 0.5 fmol (60 pg) of PCR amplified transgenic material (corresponding to 0.1% Bt176-maize) was detected by LDR/universal array with a very large background of competing PCR amplified zein (500 fmol, 50 ng). This sensitivity enables the detection of very faint samples, undetectable by traditional gel analysis and even by the 2100 bioanalyzer (detection limit is 0.5 ng).

The use of 8 subarrays (which could be extended up to 12) allows for parallel analysis of several samples, increasing throughput and lowering cost. With the increasing number of GMOs that are being developed for food applications, the ability to detect several transgenes in a single, sensitive, and reliable experiment becomes an important additional feature for any detection method (*8*). Our study suggests the feasibility of the LDR and universal array combined approach as a sensitive tool for the analysis of transgenic material in food samples. Furthermore, our experience using such an approach in the HLA region suggests the feasibility of multiplexing up to 27 polymorphisms (Consolandi et al., manuscript in preparation). This suggests that the full potential of microarrays implying a large set of analysis to be performed in parallel could be exploited here in detecting several transgenes at once. Such an attempt is currently under way in our laboratory.

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