

DETECTION OF GENETICALLY MODIFIED MAIZE BY PCR AND CAPILLARY GEL ELECTROPHORESIS (CGE) USING UNCOATED COLUMNS.

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Abstract

In this work, analysis of genetically modified insect-resistant Bt maize is demonstrated by combining amplification of a DNA fragment by PCR and subsequent detection by Capillary Gel Electrophoresis (CGE). A new CGE method is developed that allows obtaining reproducible separations of DNA fragments using bare fused silica capillaries. The method combines a washing routine of the column with 0.1 M hydrochloride acid followed by a rinsing step with a dissolution containing 1% polyvinyl alcohol. The use of this procedure together with a running buffer containing 2-hydroxyethyl cellulose (HEC) at pH 7.3 gives highly resolved and fast separations of DNA fragments ranging from 80 to 500 bp and great reproducibility. This procedure would allow the simultaneous quantitation of several GMOs in foods in a single run. To our knowledge, this is the first report on the use of PCR combined with CGE to detect transgenic foods.

1. INTRODUCTION

Recently, according to a new European regulation (90/220/CEE), any foodstuff containing more than 1% of genetically modified maize or soya must be labeled as transgenic. To carry out such detection, the usual procedure is to amplify a DNA fragment specific of the genetically modified organism by Polymerase Chain Reaction (PCR) and next to detect it after agarose gel electrophoresis and ethidium bromide staining [1]. The main problem is that both procedures, i.e. PCR and conventional electrophoresis, are essentially semiquantitative. Several procedures have been proposed to overcome the PCR semiquantitative character, e.g., competitive PCR, real time-PCR [2-4]. However, the subsequent use of conventional electrophoresis in competitive quantitative PCR introduces a new source of error [5]. Real time-PCR has proven to be a very powerful tool for quantitative analysis of nucleic acids,

however, it can show limitations for multiplex analysis of several DNA targets due to limitations in the number of different specific probes than can be detected in a single PCR tube (usually not more than two probes are used in the same tube) [6]. Therefore, the use of Capillary Gel Electrophoresis (CGE) can be a good alternative in order to improve the quantitative capabilities of competitive quantitative PCR. This includes the possibility of multiplex analysis of several DNA targets, thanks to the better resolution obtained with CGE related to conventional electrophoresis [7], avoiding the need to use and design specific and expensive fluorescent amplification probes.

CGE has been demonstrated to be a powerful analytical tool for the separation of charged analytes based on their size. Nowadays, polymer solutions have become the alternative of choice to separate SDS-proteins [8,9] and DNA fragments [10] by CGE. These polymer solutions provide well-resolved separations of SDS-proteins as well as single- and double-stranded DNA fragments with the well-known advantages related to the use of electrophoresis in capillary format (i.e., speed of analysis, automation, and quantitative analysis).

However, in order to achieve well-resolved and reproducible separations in reasonable times, CGE separations in polymer solutions have to be performed using capillaries with zero electroosmotic flow (EOF). Capillaries internally coated with a neutral polymer are normally used to eliminate the EOF. However, the price of these coated columns is too high when compared with bare fused silica columns (ca. 20-fold higher), while to home-make such coated tubing is labor intensive [11-13]. Moreover, coated capillaries can degrade with usage affecting both separation reproducibility and resolution [14].

The goal of this work is, therefore, to develop a CGE method able to provide reproducible DNA separations using uncoated capillary columns and to apply it to the detection of transgenic maize in foods by analyzing a specific DNA target previously amplified by PCR.

2. EXPERIMENTAL

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. Tris and EDTA (ethylenediamine tetraacetic acid) from Sigma (St. Louis, MO), 2-hydroxyethyl cellulose (HEC) ($M_{w,av}$ 90000), polyvinyl alcohol (PVA) ($M_{w,av}$ 50000), orthophosphoric from Aldrich (Milwaukee, WI) were used for the CE running. The buffers were stored at 4°C and warmed at room temperature before being used. N-Cetyl-N,N,N-trimethyl-ammonium bromide (CTAB) and sodium hydroxide were from Merck (Darmstadt, Germany). Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA).

The test sample 100-bp ladder was from Biotools (Madrid, Spain) and diluted till a total concentration of ca. 400 µg/ml in PCR reaction buffer (see below) containing Orange G as CGE marker.

Certified reference maize powder MZ0 (conventional, i.e., containing 0% transgenic maize) and MZ2 (containing 2% insect-resistant Bt-176 transgenic maize) produced by the Institute of Reference Materials and Measurements were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Oligonucleotides were synthesized at Centro de Investigaciones Biológicas (Spanish Council for Scientific Research, Madrid, Spain). AmpliTaq DNA polymerase, including reaction buffer and MgCl₂ was from Perkin Elmer (Madrid, Spain). Deoxynucleotides were from Amersham Pharmacia Biotech Europe GmbH (Barcelona, Spain).

2.2. DNA extraction

DNA was extracted using a modified CTAB method: 100 mg of MZ0 or MZ2 transgenic maize powder standard were incubated with 300 µl of (2% CTAB, 1.4 M NaCl, 20mM EDTA, 100 mM Tris-HCl pH 8.0, 0.2% β-mercaptoethanol) for 30 minutes at 60°C, then extracted with 300 µl of chloroform:isoamyl alcohol (24:1). The nucleic acids on the aqueous phase were recovered by precipitation with 1 volume of isopropanol, washed with 70% ethanol and dissolved in 50 µl of deionized water. MZ0 DNA was used directly (hereinafter: conventional maize DNA), while MZ2 DNA was diluted 1:1 in distilled water in order to obtain 1% transgenic containing sample (hereinafter: transgenic maize DNA).

2.3. PCR conditions

A test fragment of the modified *cryIA(b)* gene (GenBank accession number I41419) was amplified using primers *cryIA(b)-V3* and *cryIA(b)-V4* (Table 1). Amplification of a fragment of the maize starch synthase gene, used as a control for DNA quality and amplifiability, was performed with primers MSS-S and MSS-A (Table 1).

Reaction mixtures contained 1x AmpliTaq reaction buffer, 25 mM MgCl₂, 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dTTP, 2.5 µM each primer, 10 µl template DNA and 2.5 U of AmpliTaq DNA polymerase. The following thermal parameters were used for each amplification, *cryIA(b)*: first denaturation: 12 min at 95°C, 40 cycles (1 min at 95°C, 30 sec at 58°C, 30 sec at 72°C), terminal elongation 10 min at 72°C; starch synthase: first denaturation: 12 min at 95°C, 40 cycles (1 min at 95°C, 30 sec at 58°C, 30 sec at 72°C), terminal elongation 10 min at 72°C. AmpliTaq DNA polymerase was added after the first denaturation step (manual Hot-start).

2.4. CE conditions

Analyses were carried out in a P/ACE 5500 (Beckman Instruments, Fullerton, CA, USA) CE apparatus, equipped with an UV-Vis detector working at 254 nm. Bare fused-silica capillaries with 75 µm I.D. were purchased from Composite Metal Services (Worcester, England). Injections were made at the cathodic end using N₂ pressure of 0.5 p.s.i. for a given time (1 p.s.i.=6894.76 Pa). The instrument was controlled by a PC running the System GOLD software from Beckman.

Before first use, uncoated capillaries were preconditioned by rinsing with 0.1 M HCl for 30 min. Between injections, capillaries were rinsed using 0.1 M HCl for 4 min, 1% PVA for 2 min. and separation buffer for 4 min. At the end of the day, the capillary was rinsed with deionized water for 5 min and stored overnight with water inside. During the reproducibility study (*vide infra*) the first injection of each day was used as stabilization time for the system.

3. RESULTS AND DISCUSSION

3.1. CGE in uncoated capillaries. Reproducibility study

We intended to develop a new and robust CGE method able to provide good and reproducible DNA separations in bare fused silica capillaries without the disadvantages linked to the use of uncoated columns (i.e., too long washing routines between runs, low reproducibility and usefulness limited at separation pH values lower than 5). To do this, different approaches were combined. Namely, the treatment of the silica wall of the capillary with a strong acid [14] was combined with a treatment with PVA [15] and the use of buffers at nearly neutral pH containing HEC. The method consisted of three consecutive washing steps (i.e., 0.1 M HCl for 4 min, 1% PVA for 2 min and separation buffer for 4 min) that required only 10 minutes of total reconditioning time. Under these conditions, good separations of the fragments up to 500 bp are typically achieved in ca. 20 minutes as shown in Figure 1.

Table 1. Sequence of the primers used in PCR reactions.

PRIMER	SEQUENCE	ACCESSION NUMBER	POSITION
<i>CryIA(b)-V3</i>	5'-CCTGACCAAGAGCACCAACCTGG-3'	I41419	1425-1447
<i>CryIA(b)-V4</i>	5'-GCTCATGGTGGCGCTGAAGTTGC-3'	I41419	1668-1646
<i>MSS-S</i>	5'-TCAACATCCGTGGATTGCATC-3'	AF023159	933-954
<i>MSS-A</i>	5'-TTCAGGGAAATCATCAGTTAATTGC-3'	AF023159	1166-1142

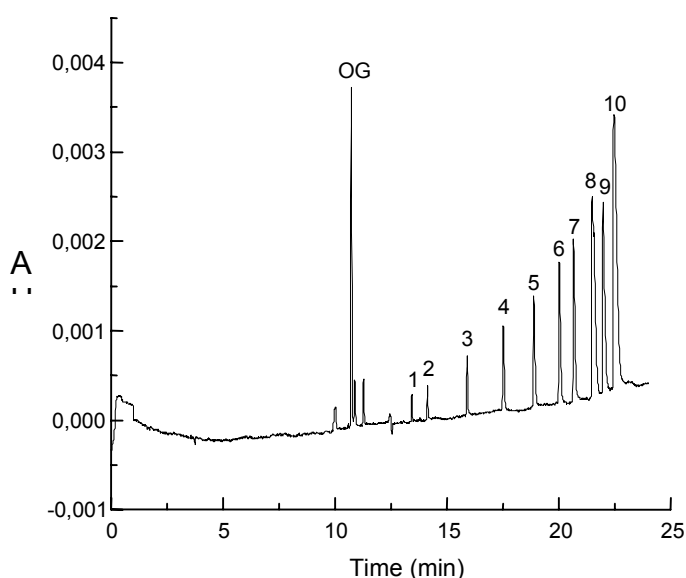


Figure 1. Separation of a 100 bp DNA ladder using an uncoated fused silica capillary with 47 cm of total length, 40 cm of effective length and 75 μm i.d.. Separation voltage: -14 kV. Running buffer: 20 mM Tris, 10 mM orthophosphoric acid, 2 mM EDTA, 4% HEC at pH 7.3. Run electric field: -300 V/cm. Detection at 254 nm. Injection for 50 s using N_2 pressure (0.5 psi) of: 1) 80 bp; 2) 100 bp; 3) 200 bp; 4) 300 bp; 5) 400 bp; 6) 500 bp; 7) 600 bp; 8) 700 bp and 800 bp; 9) 900 bp; 10) 1000 bp.

Reproducibility of this procedure was good, as can be seen in Table 2, where the %RSD values for the same day, four different days and three different capillaries are given for the DNA fragments of 80, 300 and 1000 bp corresponding to the first, an intermediate and the last migrating peak of the DNA test mixture used. As can be seen, high reproducibility was obtained for all cases, with %RSD values up to 0.86 within the same day, 1.61 for four different days (i.e., the worst case) and 1.40 for three different capillaries. Moreover, the efficiency achieved was up to $1.8 \cdot 10^6$ plates/m calculated for the 80 bp fragment in Figure 1. These values demonstrate that the method proposed is reproducible and efficient, and, therefore, can be used with confidence for analyzing

GMOs in foods. To demonstrate that, detection of transgenic maize addition in conventional maize powder was carried out via the amplification of a DNA fragment corresponding to the *cryIA(b)* gene by PCR and subsequent analysis by this CGE method.

3.2. Detection of genetically modified Bt maize.

Figure 2 shows the electrophoregrams obtained for the direct injection of the PCR amplification reactions of the *cryIA(b)* gene fragment from the transgenic (Figure 2B) and conventional (Figure 2C) maize DNA. Figure 2A corresponds to the starch synthase gene fragment amplified from transgenic maize DNA, used to check that the DNA preparation is suitable for PCR amplification and detection. Similar results were obtained for the starch synthase gene amplified from conventional maize DNA (data not shown). Thus, MSS-DNA peak in Figure 2A corresponds to the amplicon obtained with the primer pair *MSS-S/MSS-A* of Table 1, and Bt-DNA peak in Figure 2B corresponds to the amplicon obtained with the primer pair *cryIA(b)-V3/cryIA(b)-V4* both from transgenic maize DNA. A control amplification reaction without template DNA is also shown in Figure 2D.

In the absence of template DNA, no peak could be observed for the PCR control reaction (Figure 2D) in the region where the amplicon used for transgenic DNA detection should come out (about 16 min), indicating that no interferences have to be expected from the PCR reaction mixture. Amplification using the primers of Table 1 corresponding to the maize starch synthase gene (Figure 2A) gave similar results for both types of maize, thus confirming the suitability of both DNA preparations for PCR amplification and detection. By using the *cryIA(b)* primer pair a single peak could be observed for the transgenic maize (Figure 2B) that could not be detected for the conventional one (Figure 2C). This method is, therefore, able to specifically detect 1% of transgenic maize in conventional maize fulfilling the requirements imposed by the European regulation (90/220/CEE). Moreover, this is done via the direct injection of the PCR products in the CGE system without further purification steps.

Using this CGE method, the agreement between the experimental and theoretical size of the *cryIA(b)* amplicon obtained under our PCR conditions can be also

checked. To do this, the data of migration times (t_m) corresponding to DNA fragments of 80, 100, 200, 300, **Table 2.** Reproducibility of migration times of the DNA fragments of 80, 300 and 1000 bp using uncoated capillaries for the same day, four different days and three different capillaries. All the conditions as in Figure 1.

	80 bp		300 bp		1000 bp	
	t_{av} (min)	%RSD	t_{av} (min)	%RSD	t_{av} (min)	%RSD
Same day (n=10)*	13.49	0.66	17.60	0.82	22.57	0.20
Four days (n=40)*	13.43	0.99	17.51	1.29	22.40	1.61
Three capillaries (n=15)	13.58	0.99	17.68	1.30	22.55	1.40

* same capillary.

400 and 500 bp under the separation conditions of Figure 1 were employed. After least square fitting of the plot $\log(\text{bp})$ versus $1/t_m$, the equation: $\log(\text{bp}) = 4.33 - 31.87/t_m$ was obtained ($r=0.998$, $n=6$). This equation was used to determine the number of base pairs of the *cryIA(b)* amplicon based on its t_m . The calculated value was 240 bp, which is in good agreement with the theoretical value (i.e. 244 bp).

Although the sensitivity of the PCR-CGE procedure is enough to detect 1% of transgenic maize in food samples (Figure 2B), it would be convenient to have higher

sensitivity since the peak obtained is too close to the detection limit. A new procedure for detecting DNA fragments from PCR using CGE together with laser induced fluorescence (LIF) as detection is now being developed in our laboratory. Based on the well-known better sensitivity provided by LIF compared to UV detection [16], it is expected to carry out more easily quantitative analysis of several DNA fragments (corresponding to different GMOs) below the mentioned threshold of 1%.

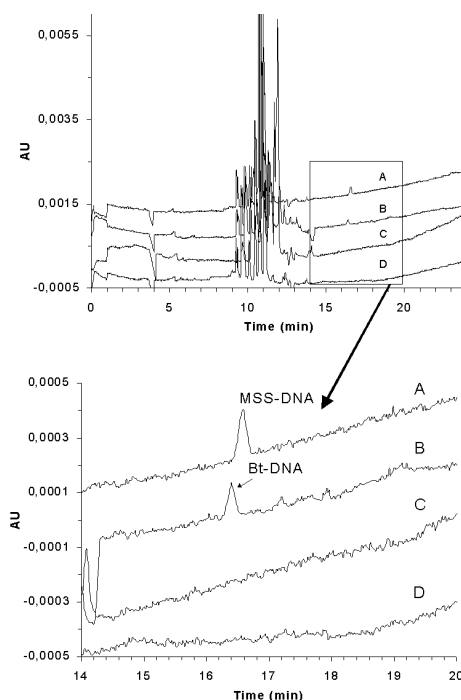


Figure 2. Electrophoregrams obtained for the PCR amplification reactions using: A) transgenic maize DNA and the primer pair *MSS-S/MSS-A* (see Table 1); B) transgenic maize DNA and the primer pair *cryIA(b)-V3/cryIA(b)-V4*; C) conventional maize DNA and the primer pair *cryIA(b)-V3/cryIA(b)-V4*; and D) control amplification reaction without template DNA (i.e., blank) and the primer pair *cryIA(b)-V3/cryIA(b)-V4*. Samples injected for 75 s using N_2 pressure (0.5 psi). Other conditions as in Figure 1.

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