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Simultaneous Confirmatory Analysis of Different Transgenic Maize (*Zea mays*) Lines Using Multiplex Polymerase Chain Reaction−Restriction Analysis and Capillary Gel Electrophoresis with Laser Induced Fluorescence Detection

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A novel analytical procedure based on the combination of multiplex PCR, restriction analysis, and CGE-LIF to unambiguously and simultaneously confirm the presence of multiple lines of genetically modified corn is proposed. This methodology is based on the amplification of event-specific DNA regions by multiplex PCR using 6-FAM-labeled primers. Subsequently, PCR products are digested by a mixture containing specific restriction endonucleases. Thus, restriction endonucleases selectively recognize DNA target sequences contained in the PCR products and cleave the double-stranded DNA at a given cleavage site. Next, the restriction digest is analyzed by CGE-LIF corroborating the length of the expected restriction fragments, confirming (or not) the existence of GMOs. For accurate size determination of the DNA fragments by CGE-LIF a special standard DNA mixture was produced in this laboratory for calibration. The suitability of this mixture for size determination of labeled DNA fragments is also demonstrated. The usefulness of the proposed methodology is demonstrated through the simultaneous detection and confirmatory analysis of samples containing 0.5% of GA21 and MON863 maize plus an endogenous gene of maize as control.

KEYWORDS: CGE-LIF analysis; multiplex PCR; restriction analysis; GMO detection; MW DNA marker

1. INTRODUCTION

Development and commercialization of genetically modified organisms (GMOs) have rapidly progressed in the past decade. However, food safety, environmental risk, and ethical concerns regarding GMOs continue to grow, and their use is still a controversial issue under debate (*1*). This has led several countries around the world to establish GMO labeling and traceability regulations (1829/2003/CEE and 1830/2003/CEE). Hence, in the past few years, a vast number of Polymerase Chain Reaction (PCR)-based methods have been proposed for the rapid detection of GMOs in foods (*2*−*5*). The major driving force for this incessant development within GMO analysis research is the necessity of high throughput and speed of analysis, as well as increased sensitivity for the detection of the growing number of new GMOs approved worldwide. These goals are reached mainly by automating processes and/or refining existing analytical methods. Regarding this last aspect, a multiplex PCR technique has demonstrated to be useful for reducing the cost and time of analysis with respect to conventional PCR (*6*). This technique involves the simultaneous amplification of more than one target sequence per reaction by mixing multiple primer pairs with different specificities in the same reaction (*7*). The great advantage of multiplex PCR-based methods is that they allow a reduction of the number of reactions that are required to investigate a sample for the presence of several GMO-derived DNA sequences. However, a shortcoming of multiplex PCR, common to most PCR-based methods, accounts for the necessity of sensitively detecting the presence of the target sequence (amplicon) after the amplification. Furthermore, multiplex PCR-based methods are more susceptible to nonspecific product amplification or cross-amplification reactions because several primer pairs are added to the reaction mixture. This constraint, in addition to false-positive signals, may become a major concern when this technique is used as an amplification method for the detection of GMOs in complex matrices.

The majority of multiplex PCR-based methods developed for GMO detection in foods rely on electrophoretic analysis of the PCR product's length (*8*−*20*). The detection of multiplex PCR products has traditionally been performed by agarose gel electrophoresis (AGE) (*8*−*13*). Besides the insufficient resolution and sensitivity of AGE, its semiquantitative feature, the use of carcinogenic substances, and the need to visualize the amplicons make for a non-user-friendly technique difficult to automate. To overcome such limitations, novel approaches based on capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) have been proposed to successfully detect PCR products with high sensitivity, resolution, and automation (*14*−*16*) able to provide

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quantitative results (*17*). In addition, CGE-LIF has demonstrated to be a good alternative for the multiple detection of GMOs in food by using both nucleic acid fluorescent intercalanting dyes (*18*) and derivatized primers with different fluorescent reporters (*19, 20*). However, the aforementioned electrophoretic approaches do not corroborate the specificity of the PCR product as they merely demonstrate whether a band (or peak) of the expected size is obtained or not. The signal could be due to a nonspecific reaction product that happened to be the same size as the expected product. Consequently, methods for confirmatory analysis of the identity of PCR products are required.

In this regard, DNA sequencing is the most reliable and effective method to inspect the identity of a DNA fragment; however, it is expensive, time-consuming, and, therefore, not adequate for a routine analytical setting. Ideally, real-time PCR (RT-PCR) using specific labeled probes should be able to detect and differentiate many different targets. Nevertheless, due to the limited number of fluorophores available and the significant overlap in their emission spectra, the multiplex capabilities of RT-PCR are restricted and often not possible for more than a few targets. On the other hand, owing to its enormous potential for high throughput and multiplexing detection, new analytical trends are focused toward DNA array (*21*−*25*). This technology can improve the specificity in the detection of multiplex PCR products by adding a step of nucleic acid hybridization (*26*). However, the major limitation in the adoption of this approach is the high cost of the technology, which requires a significant investment in equipment and consumables.

Molecular techniques combined with CGE-LIF can be proposed as a good alternative for confirmatory analysis because this combination gives rise to a novel and relatively inexpensive methodology. For instance, Heide et al. have demonstrated the potential of the combination of multiplex PCR, SNaPshot technology, and CGE-LIF for the simultaneous detection of many target sequences with high sensitivity and high-throughput capabilities (*27*). In their recent paper, specific probes were labeled with fluorescent ddNTPs using a primer extension technique upon hybridization with complementary amplified sequences, providing fluorescent probes that were further analyzed by CGE-LIF (*27*).

Besides sequencing and hybridization-based procedures, restriction analysis can provide an easy means to reveal the identity of multiplex PCR products. Restriction endonucleases selectively recognize DNA target sequences contained in the PCR products and cleave the double-stranded DNA at a given site. Then, the resulting restriction fragments are detected by electrophoretic analysis providing a pattern that is distinctive of a given amplicon. Accordingly, it can be expected that a methodology that combines multiplex PCR, restriction analysis, and a high-resolution separation technique such as CGE-LIF can provide interesting advantages for the confirmatory analysis of multiplex DNA amplification.

The goal of this work was to investigate the possibilities of CGE-LIF combined with multiplex PCR and restriction analysis for the simultaneous detection and confirmation of two varieties of transgenic maize plus an endogenous gene of maize as control. Also, a DNA standard mixture was produced in-house for accurate size calibration by CGE-LIF. The suitability of this new mixture for size characterization of PCR products and restriction fragment was investigated.

2. MATERIALS AND METHODS

2.1. Chemicals. All chemicals were of analytical reagent grade and used as received. Tris(hydroxymethyl)aminomethane (Tris) and EDTA were obtained from Sigma (St. Louis, MO); 2-hydroxyethylcellulose (HEC, MWav 90000) was from Aldrich (Milwaukee, WI). Separation buffer was stored at 4 °C and warmed at room temperature before use. Water was deionized by using a Milli-Q system (Millipore, Bedford, MA).

Table 1. Primers and Oligonucleotides Used for Simplex and Multiplex PCR and the Production of the 6-FAM-DNA Marker

name	sequence (5′−3′)	accession no. or ref
I. Primers and Oligonucleotides Used for the Production of the 6-FAM-DNA Marker		
pGFor	6-FAM- ACATGATCCCCCATGTTGTGC	X65308
pGRev	6-FAM- GGTTACATCGAACTGGATCTCAAC	
30bpFor	6-FAM-CTT GTA TTA CTT CAC TAC TCT CTA TCT TGG	this work
30bpRev	CCA AGA TAG AGA GTA GTG AAG TAA TAC AAG	
II. Primers Used for Simplex and Multiplex PCR		
taqze1	6-FAM- GCCATTGGGTACCATGAACC	X07535
taqze3	AGGCCAACAGTTGCTGCAG	
GAFor	AGCGCGCAAACTAGGATAAA	AY255709
GARev	6-FAM- TCCCGACTCTCTTCTCAAGC	
MOFor	6-FAM- CACCCCAAAGTGTACCAAGC	*33*
MORev	CTTTTTCCACGATGCTCCTC	

2.2. Samples. Certified reference materials (CRMs) of non-GM and GM maize GA21 and MON863 were purchased from the Institute of Reference Materials and Measurements (IRMM, Geel, Belgium). Samples with known combinations of GMO content were made by mixing DNA isolated from individual reference materials. For accurate size determination of labeled DNA fragments generated in both amplification and restriction reactions by CGE-LIF, an in-house-produced standard mixture was used. A detailed description of the procedure developed for the production of the standard mixture is given next.

2.3. Production of a Low Molecular Range 6-FAM-Labeled dsDNA Molecular Weight Marker for CGE-LIF. To obtain the mixture containing DNA fragments of known size labeled with 6-FAM in a single 5′-end, PCR amplification of a special sequence and its subsequent digestion with restriction endonucleases were applied. First, the amplification of a specific DNA sequence from the commercial vector pGEM-T (Promega, Madison, WI) was performed. To do this, primers *pGFor* and *pGRev* (**Table 1**) were selected to amplify a 344 bp fragment in the sequence of the pGEM-T vector. PCR amplification was performed in 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 2.1 mM $MgCl_2$, 200 μM dCTP, dGTP, dATP, and dTTP, 0.1 μM *pGFor*, 0.1 μM *pGRev*, 1 unit of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA), and 5 ng of pGEM-T vector as DNA template in a final volume of 50 μL. Reaction was run on a Mastercycler Epgradient (Eppendorf, NY) under the following conditions: 10 min at 95 °C (hot-start/denaturation); 40 cycles of 30 s at 95 °C (denaturation), 30 s at 63 °C (annealing), and 30 s at 72 °C (extension). Once the specificity of the amplified product was evaluated by sequencing (sequencing service of Centro de Investigaciones Biológicas, Spanish Council for Scientific Research, Madrid, Spain), two further amplification reactions were performed to obtain the same DNA fragment but having one and both strands labeled with 6-carboxyfluorescein (6-FAM) in the 5′-end. Thus, both amplification reactions were run in parallel, one of them using both primers, *pGFor* and *pGRev*, labeled in the 5′-end with 6-FAM, and the other reaction using *pGFor* labeled in the 5′-end with 6-FAM and *pGRev* without fluorescent reporter. Amplifications were performed under the same conditions mentioned above. After amplification, PCR products were concentrated by ice ethanol and were resuspended in water for further quantification of total genomic DNA by UV spectrometry. In silico restriction analysis was carried out using NEBcutter v2.0 (*28*). To carry out the digestions, two 3 μg aliquots of PCR product containing both strands labeled in the 5′-end were

separately incubated at 37 °C for 2 h with 10 units of each enzyme, *Mnl*I and *Sca*I (New England Biolabs, Ipswich, MA). The reactions were stopped at 85 °C for 10 min. In addition, a DNA fragment of 30 bp labeled with 6-FAM in one of its 5′-ends was prepared by automated synthesis of the two complementary strands (*30bpFor*/*30bpRev*, **Table 1**; Bonsai Technologies). The two strands were combined in a one-to-one ratio to give a duplex DNA fragment. The final DNA mixture for calibration was prepared by combining 8.6 μL of *Sca*I restriction fragments, 8.6 μL of *Mnl*I restriction fragments, 8.6 μL of 344 bp PCR product, and 4.2 μL of 19 ng/μL 30 bp dsDNA fragment, all of them labeled with 6-FAM in a single 5′-end.

2.4. DNA Extraction and Quantification. DNA purification was carried out by the Qiagen DNeasy plant mini kit method (Izasa, Barcelona, Spain) following the instructions of the manufacturer. In this case, DNA from 100 mg of maize powder was recovered in 200 μL of elution buffer. Total dsDNA was quantified in a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA) on the basis of absorption at 260 nm. DNA purity was determined from absorption values at 260 and 280 nm. All samples had an absorption ratio (260/280 nm) ranging from 1.8 to 2.0. Samples containing 0% GM maize DNA, 1% MON863 maize DNA, and 1% GA21 maize DNA or 0.5% MON863 and 0.5% GA21 maize DNA were prepared for amplification. Individual DNA stock solutions at 50 ng/μL were prepared to facilitate the dilution of GMO DNA with DNA from non-GM maize.

2.5. Oligonucleotide Primers. According to unique and specific DNA sequences to GA21 and MON863 events, primer pairs were designed by Primer3 online software (*29*). A total of two primers pairs were selected to amplify short DNA sequences (within the range of 100−200 bp) for GA21 and MON863 maize lines (**Table 1**). In addition, a third primer pair (*taqze1*/*taqze3*) was used as amplification control of a 104 bp DNA fragment in *zein* sequence (*30*). For each primer pair, either forward or reverse primer was labeled with 6-FAM in the 5′-end to allow the detection of each amplicon by CGE-LIF (**Table 1**). The primer pairs were also tested using the FastPCR v.4.0 software (*31*) to control their suitability to be used in a multiplex system. The oligonucleotides were purchased from Bonsai Technologies (Alcobendas, Spain).

2.6. Simplex and Multiplex PCR Conditions. All amplification reactions were performed with a Mastercycler Epgradient. Initially, the efficiency of the primer pairs in amplifying the target sequences was separately tested by performing simplex PCR reactions using each primer pair, *taqze1*/*taqze3*, *GAFor*/*GARev*, and *MOFor*/*MORev*, at the concentrations of 0.05, 0.1, and 0.1 μM, respectively, and several genomic DNA extracts from maize samples with different GMO contents. Reactions were carried out in touchdown, in order to minimize nonspecific amplifications, using a mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.1 mM $MgCl_2$, 200 μM dCTP, dGTP, dATP, and dTTP, 1 unit of AmpliTaq Gold polymerase, 50 ng of genomic DNA, and the primer pair concentration indicated above in a final volume of 50 μL. The touchdown PCR program consisted of an initial denaturation step at 95 °C for 10 min followed by 10 cycles, which involved a denaturation step at 95 °C for 30 s, annealing at 66 °C for 30 s in the initial cycle and at decreasing temperatures by 0.5 °C/cycle until a temperature of 61 °C was reached in the subsequent cycles, and a extension step at 72 °C for 30 s. After the touchdown program, 30 cycles at 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s were performed. Multiplex reactions were carried out under the same conditions described for simplex PCR but including the three primer pairs, *taqze1*/*taqze2*, *GAFor*/*GARev*, and *MOFor*/*MORev*, in the reaction at the same concentrations as those indicated above for simplex PCR. To confirm the absence of contaminant DNA in multiplex PCR reactions, blank (without DNA template) reactions were systematically carried out in all experiments. For multiplex PCR, the primers were premixed to minimize the analysis-to-analysis variability due to pipetting.

2.7. Restriction Digestion of PCR Products. Restriction digestions were performed by adding 10 units of each enzyme, *Sca*I and *Mnl*I, 3 μg of bovine serum albumin, and 1× NEBuffer#2 to 25 μL of PCR products in a final volume of 33 μL. Reactions were incubated at 37 °C for 2 h and, then, inactivated at 85 °C for 10 min.

2.8. Capillary Gel Electrophoresis−Laser-Induced Fluorescence (CGE-LIF). The analyses were carried out in a PACE-MDQ (Beckman Coulter) equipped with an Ar^+ laser working at 488 nm (excitation wavelength) and 520 nm (emission wavelength). Bare fused-silica capillaries with 75 μm i.d. were purchased from Composite Metal Services (Worcester, U.K.). Injections were made at the cathodic end using a N_2 pressure of 0.5 psi for a given time (1 psi = 6894.76 Pa). The PACE-MDQ instrument was controlled by a PC running the 32 Karat software from Beckman. Before first use, any uncoated capillary was preconditioned by rinsing with 0.1 M HCl for 30 min. The following conditions were used for both PCR product and restriction fragment separations: separation buffer [20 mM Tris, 10 mM phosphoric acid, 2 mM EDTA, and 4.75% HEC (Mw 90000; Aldrich, Spain) at pH 7.3]; temperature of separation, 45 °C; running electric field, −217 V/cm. Between injections, capillaries were rinsed using water for 5 min followed by 0.1 M HCl for 4 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.

3. RESULTS AND DISCUSSION

3.1. Production of the 6-FAM Marker for Size Calibration of DNA. CGE-LIF is used for the separation of analytes having the same charge-to-mass ratio, as, for instance, DNA fragments. This is done by using polymers in solution to create a molecular sieve that allows the separation of analytes by size. Besides, the size of analytes can be estimated if a suitable "size" or molecular weight (MW) marker is used for calibration. DNA markers are common reagents that contain DNA fragments of known size useful for size determination of unknown fragments. However, recent analytical approaches comprising novel combinations of molecular methods with CGE-LIF demand the availability of more refined DNA markers showing the same special features, such as fluorescent moieties, as the interrogated DNA fragments in the sample. Thus, owing to the poor availability of adequate commercial 6-FAM-labeled DNA markers that meet the requirements for the present study, we developed an easy and fast procedure to produce a mixture containing short 6-FAM-labeled DNA fragments for calibration purposes.

For the production of a mixture containing DNA fragments of known size ranging from 30 to 344 bp, all of them labeled with 6-FAM in a single 5′-end, a procedure based on the combination of an amplified PCR product with its restriction digested fragments was developed. First, in silico restriction analysis of pGEM-T vector was carried out to select a suitable sequence for amplification and subsequent digestion. The designed *pGFor*/*pGRev* primer pair for amplification of pGEM-T DNA spanned 344 bases of the vector sequence (GenBank accession no. X65308, 1713−2057 region). In silico restriction analysis of the amplified sequence with *Mnl*I endonuclease provided the fragment pair 56/288 bp, whereas digestion with *Sca*I provided the fragment pair 163/181 bp. To obtain detectable signals of the amplified 344 bp DNA and restricted fragments by CGE-LIF, amplifications were performed using 6-FAM-labeled primers. The CGE-LIF analysis of the amplification reaction obtained using 6-FAM-labeled *pGFor* and *pGRev* primers and pGEM-T vector as DNA template indicated the presence of a single peak (**Figure 1A**) that disappeared when the reaction was subjected to digestion with *Mnl*I (**Figure 1B**) and *Sca*I (**Figure 1C**). Furthermore, electrophoregrams obtained by CGE-LIF of restriction-digested samples demonstrated that depending on the restriction endonuclease used, two different restriction patterns were obtained. As expected, both patterns showed two peaks indicating complete digestion of the 344 bp fragment to yield the fragments pairs corresponding to 56/288 and 163/181 bp DNA for *Mnl*I and

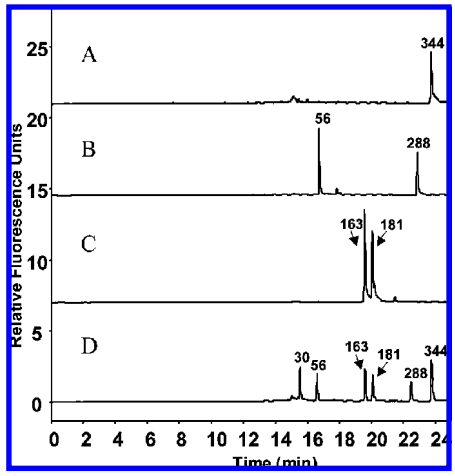

Figure 1. CGE-LIF analysis of (**A**) PCR amplification using the *pGFor*/*pGRev* primer pair labeled with 6-FAM in a 5′-end from pGEM-T as a template and restriction digestions of the PCR product with *Mnl*I (**B**) and *Sca*I (**C**); *6-FAM-DNA marker* (**D**). Samples were injected using N_2 pressure (0.5 psi) for 40 s. For other separation conditions, see section 2.8.

*Sca*I reactions, respectively, all of them labeled with 6-FAM in a single 5′-end (**Figure 1B**,**C**).

In a separate reaction, a synthetic 30 bp dsDNA fragment was prepared by hybridization of two complementary single-stranded oligonucleotides *30bpFor*/*30 bpRev* (**Table 1**). The electrophoretic analyses demonstrated that, under the same separation conditions, the 30 bp synthetic fragment and the *30 bpFor* (labeled with 6-FAM) exhibited different migration times (data not shown). Therefore, according to these data, the absence of labeled ssDNA oligonucleotides in the preparation containing synthetic 30 bp dsDNA fragment was confirmed. Next, a mixture containing the five mentioned DNA fragments, namely, 56, 163, 181, 288, and 344 bp, plus the synthetic 30 bp fragment, all having a single 6-FAM label per molecule, was prepared. To do this, 8.6 μL of *Sca*I restriction fragments, 8.6 μL of *Mnl*I restriction fragments, 8.6 μL of 344 bp PCR product, and 4.2 μL of 19 ng/μL 30 bp dsDNA fragment were combined in a single tube. **Figure 1D** shows the separation of the resulting mixture (called 30−344 bp *6-FAM-DNA marker*) by CGE-LIF. As can be seen, good separation of the six 6-FAM-labeled DNA fragments was obtained.

To investigate the sensitivity of the CGE-LIF for the detection of 6-FAM-labeled dsDNA fragments, a 6-FAM marker sample (containing 2.7 ng/μL of the 30 bp fragment) was injected using N_2 pressure (0.5 psi) for 40 s. Under these conditions, the noise signal was measured and the quantity of the 30 bp fragment required to obtain a signal equal to 3 times the noise signal was determined to be equal to 50.3 pg/μL (i.e., the LOD).

3.2. Application of the 6-FAM Marker for Size Calibration and Determination of DNA Fragments by CGE-LIF. To investigate the suitability of the *6-FAM-DNA marker* for size calibration by CGE-LIF of the DNA fragments investigated in this work, calibration curves based on migration times (t_m) of resolved DNA fragments were constructed. To do this, the *6-FAM-DNA marker* was co-injected with simplex PCR products or their corresponding restriction digests and separated by CGE-LIF.

First, a 104 bp well-characterized amplicon of *zein* gene sequence was produced by simplex PCR amplification using *taqze1*/*taqze3* (*32*) in the presence of genomic maize DNA (**Figure 2B**). After CGE-LIF analysis of the co-injection, the

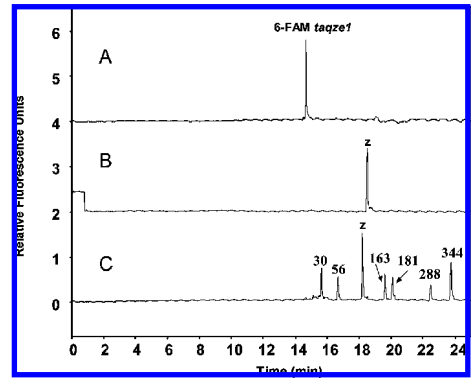

Figure 2. CGE-LIF analysis of simple PCR samples carried out with *taqze1*/*taqze3* and water (**A**) and genomic maize DNA (**B**). Co-injection of B and *6-FAM-DNA* marker (**C**). Samples were injected using N_2 pressure (0.5 psi) for 40 s. All conditions were as in **Figure 1**.

data of migration times corresponding to DNA fragments of *6-FAM-DNA marker* under the separation conditions of **Figure 2C** were used. After least-squares fitting of the plot log(bp) versus $1/t_m$, the equation $\log(\text{bp}) = 4.4503 - 44.652/t_m$ was obtained ($r = 0.988$, $n = 5$). This equation was used to determine the number of base pairs of the *zein* amplicon (*z* peak in **Figure 2C**) as a result of its migration time. The calculated value was 100 bp, which is in good agreement with the theoretical value (i.e., 104 bp).

Next, to specifically detect GA21 (Aventis) and MON863 (Monsanto) genetically modified maize, PCR primers were selected to meet the following criteria: (1) allow event specific detection; (2) yield an amplicon containing recognition target sequences for restriction endonucleases that are compatible for simultaneous double digestion of DNA. For detection of GA21 maize, a primer pair (*GAFor*/*GARev*, **Table 1**) was designed to amplify a 144 bp fragment of a sequence that is unique to event GA21 maize because it covers part of a specific multicopy rearrangement occurring during transformation (*19, 32*). The primer pair spans the 3′-end of *T-nos*, a 54 bp sequence of the plasmid vector, and the 5′-end of *r-act* promoter (*32*). In silico restriction analysis of this sequence with *Mnl*I provided two restriction DNA fragments of 104 and 40 bp. For detection of MON863 maize, a primer pair (*MOFor*/*MORev*, **Table 1**) was designed to amplify a 182 bp fragment in the 5′-transgene junction sequence (*33*). In silico restriction analysis of this sequence with *Mnl*I provided three restriction DNA fragments of 23, 27, and 132 bp. The 104 bp sequence of *zein* amplicon showed a recognition target sequence for the enzyme *Sca*I, which is compatible with *Mnl*I digestion conditions, to generate 72 and 32 bp DNA fragments.

To reduce the complexity in the restriction analysis by CGE-LIF of digested multiplex PCR products, and according to the theoretical restriction patterns, the restriction fragments 32, 40, and 132 bp from the respective *zein*, *GA21*, and *MON863* sequences were selected for verification of amplicons identity because these fragments were within the size range covered by the *6-FAM-DNA marker*. To attain this, for each primer pair used in simplex and multiplex PCR, only *taqze1*, *GARev*, and *MOFor* primers (**Table 1**) were labeled with 6-FAM in the 5′-end; in this way the complexity of the resulting CGE-LIF electropherograms is reduced because only one fluorescent fragment could be detected. Panels **A**−**C** of **Figure 3** show the CGE-LIF analysis of simplex PCR amplifications of each primer pair with the corresponding genomic DNA. All primer pairs generated amplicons that could be separated and detected by CGE-LIF (z peak, 104 bp; g peak, 144 bp; m peak, 182 bp).

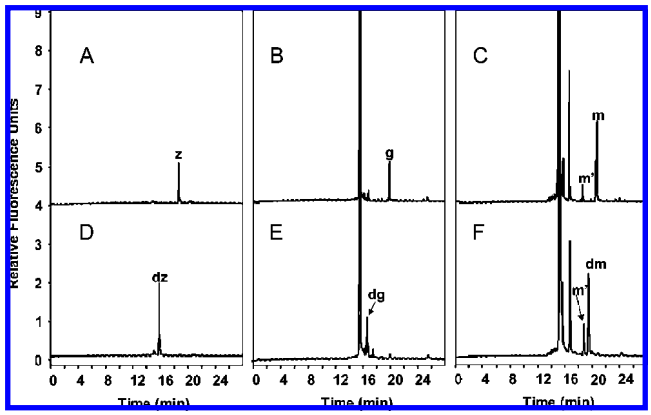

Figure 3. Electrophoretic analysis of a simplex PCR amplification of maize DNA extracts (**A**−**C**) and their corresponding fragments after restriction digestion with *Mnl*I and *Sca*I (**D**−**F**). Zero percent non-GM maize was amplified with *taqze1*/*taqze2* primer pair (**A**); 1% GA21 maize was amplified with *GAFor*/*GARev* primer pair (**B**); 1% MON863 maize was amplified with *MOFor*/*MORev* primer pair (**C**); samples were injected using N_2 pressure (0.5 psi) for 40 s; **D**, **E**, and **F**, restriction digestions of **A**, **B**, and **C**, respectively; samples were injected using N_2 pressure (0.8 psi) for 40 s. Peak identification: z, 104 bp zein maize DNA; g, 144 bp GA21 maize DNA; m, 182 bp MON863 maize DNA amplicons; dz, 32 bp zein maize DNA; dg, 40 bp GA21 maize DNA; dm, 132 bp MON863 maize DNA restriction fragments. All conditions were as in **Figure 1**.

An extra signal (m′ peak) around 18.8 min could be detected (**Figure 3C**). This m′ peak was systematically detected in amplifications carried out with the *MOFor*/*MORev* primer pair of samples containing MON863 maize genomic DNA. However, this unspecific product with a calculated size of 119 bp did not interfer with the detection of the expected amplicons and restriction fragments.

Simplex PCR reactions were simultaneously digested with both *Mnl*I and *Sca*I endonucleases. Digested samples were injected to the CGE-LIF instrument using 0.8 psi for 40 s to compensate a possible decay in the signal for DNA dilution in the digestion step. The results obtained from CGE-LIF analysis of the restriction digests revealed the cleavage of amplicons corresponding to z, g, and m peaks to yield restriction fragments corresponding to dz, dg, and dm peaks, respectively (**Figure 3D**−**F**). In the electrophoregrams obtained from the analysis of simplex PCR amplifications of GA21 and MON863 (**Figure 3B**,**C**), and their subsequent digestions (**Figure 3E**,**F**) as well, major peaks could be observed around 15.0 min that correspond to an excess of 6-FAM-labeled primers, as it was experimentally demonstrated (data not shown). As can be observed, the unspecific amplicon, represented by the m′ peak did not undergo cleavage, indicating the absence of recognition target sequences for endonucleases used (**Figure 3F**), which is a first probe of the usefulness of this approach. The specificity of the primer pairs chosen for each transgenic target was also confirmed by the absence of detectable PCR products on amplified DNA extracts from other transgenic lines including MON810, NK603, and BT11 (data not shown).

To verify whether the sizes of amplicons and restriction fragments correspond to those expected, calibrations with *6-FAM-DNA marker* were performed by CGE-LIF analysis of co-injected samples. To investigate whether the single base 3′ overhang in the 56/288 bp restriction pair (from *Mnl*I digestion of 344 bp amplicon) could affect calibrations, five calibration curves based in five possible combinations for this restriction pair (i.e., 55.5/288.5, 55/288, 55/289, 56/288, and 56/289 bp) were constructed. The five regression curves provided similar equations (data not shown), indicating an insignificant effect of the 3′ overhanged fragments in the *6-FAM-DNA marker*. For the rest of the experiments, we used the 56/288 bp values in the calibration because this pair provided the regression equation with the best linear fit (r value of 0.988 vs 0.986−0.987). **Table 2** shows the equations corresponding to the regression curves used for the calculation of the number of base pairs of amplified and restriction DNA fragments as a result of their migration times. Results indicated a good agreement of the experimentally calculated values with theoretical values. For instance, the estimated size of the *GA21* amplicon represented by g peak deviated from the theoretical value by only 2.7%. Also, the calculated values obtained for zein (z peak) and MON863 (m peak) amplicons and their respective restriction fragments from zein (dz peak) and MON863 (dz peak) showed deviations from the theoretical values of <6.2%. A slightly higher size value of 47 bp was obtained for *GA21* restriction fragment (dg peak, **Figure 3E**) in comparison with theoretical value (40 bp). However, the GA21 amplicon and the corresponding restricted fragment were detected only in amplifications carried out with *GAFor*/*GARev* primer pair when GA21 genomic DNA was present in the reaction. Therefore, we concluded it corresponds to the specific GA21 event sequence.

Table 2. Calibration Curves and Calculated Values of the Number of Pair of Bases of Amplied and Digested DNA Fragments

DNA fragment[a]	equation[b]	calcd size (bp)	theor size (bp)	% deviation[c]
peak z	$\log(bp) = 4.45 - 44.65/t_m$	100	104	3.8
peak g	$\log(bp) = 4.34 - 43.43/t_m$	140	144	2.7
peak m	$\log(bp) = 4.43 - 44.47/t_m$	182	182	0
peak dz	$\log(bp) = 4.39 - 44.42/t_m$	34	32	6.2
peak dg	$\log(bp) = 4.50 - 45.39/t_m$	47	40	17.5
peakdm	$\log(bp) = 4.49 - 45.06/t_m$	128	132	3.0

[a] DNA fragments corresponding to the peaks observed in **Figure 2**. [b] r values were the same for all equations ($r = 0.988$, $n = 5$). [c] % deviation = 100 × (calcd size − theor size)/theor size.

3.3. Confirmatory Analysis of Genetically Modified Maize by Multiplex PCR/Restriction Analysis and CGE-LIF. A multiplex PCR system was developed for the simultaneous detection of an endogenous gene of maize and two recombinant DNA constructs of genetically modified crops: GA21 and MON863 lines of GM maize. Multiplex PCR amplifications were tested on maize genomic DNA extracts containing different GMO contents (**Figure 4A**−**D**). Panels **A**−**D** of **Figure 4** reveal the presence of z peak, which corresponds to the expected 104 bp zein amplicon used as control, assuring the amplifiability of each maize genomic DNA extract obtained. **Figure 4D** shows that in the electrophoregram of the sample containing 0.5% DNA from each transgenic variety, GA21 and MON863 maize, g and m peaks, which correspond to the expected 144 bp GA21 and 182 pb MON863 amplicons, could be detected, indicating that the proposed multiplex PCR-CGE-LIF procedure appeared to be suitable for the simultaneous detection of both transgenic maize lines. In addition, peaks g (**Figure 4B**) and m (**Figure 4C**) were detected in samples containing 1% GA21 and 1% MON863, respectively, whereas no peaks other than z peak (**Figure 4A**) could be observed in the analysis of non-GM maize in the region where the amplicons used for transgenic DNA detection should come out (from 19 to 21 min).

To confirm the identity of amplified DNA sequences, multiplex PCR products were simultaneously digested using *Mnl*I and *Sca*I endonucleases. *Zein* amplicon was confirmed in all amplifications carried out from maize genomic DNA by the detection of the dz peak in the electrophoregrams (**Figure**

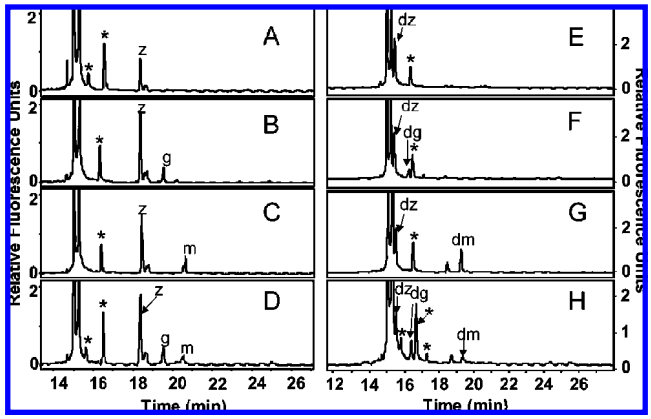

Figure 4. Electrophoretic analysis of a series of multiplex PCR amplification of maize DNA samples (**A**−**D**) and their corresponding restriction digestion with *MnlI* and *ScaI* (**E**−**H**: (**A**, **E**) 0% non-GM maize; (**B**, **F**) 1% GA21 maize; (**C**, **G**) 1% MON863 maize; (**D**, **H**) 0.5% GA21 and 0.5% MON863 maize; (**A**−**D**) samples were injected using N_2 pressure (0.5 psi) for 40 s; (**E**−**H**) samples were injected using N_2 pressure (0.8 psi) for 40 s. Peak identification is as in **Figure 3**. For other separation conditions, see section 2.8.

4E−**H**). After restriction digestion, g peak disappeared, coinciding with the detection of dg peak in the sample containing 1% GA21 maize (**Figure 4F**). Similarly, restriction digestion of the multiplex amplification of a DNA extract containing 1% MON863 resulted in cleavage of amplicon related to m peak to yield a shorter restriction fragment represented by dm peak in **Figure 4G**. As can be seen in **Figure 4H**, disappearance of g and m peaks in the separation of amplification of a DNA extract containing 0.5% DNA from each transgenic variety was accompanied by the appearance of dg and dm peaks, allowing unequivocal and simultaneous differentiation of GA21 and MON863 maize from conventional maize at these very low percentages.

In conclusion, the proposed multiplex PCR−restriction analysis procedure combined with CGE-LIF allows sensitive and confirmatory analysis of the presence of different transgenic maize lines simultaneously at levels below the minimum threshold currently marked by European Regulations (i.e., 0.9% GMO). Unlike other procedures for amplicon sequence identification, the proposed procedure based on multiplex PCR and restriction analysis does not demand tedious optimization steps and can be easily extended to the simultaneous confirmatory analysis of many GMOs. Unlike RT-PCR technique, the proposed multiplex PCR-CGE-LIF methodology does not provide quantitative information; however, it can allow for simultaneous detection and confirmation of a much higher number of DNA targets once adequate amplification and separation conditions have been established.

Moreover, a method is proposed for the rapid production of a *6-FAM-DNA marker* for calibration of PCR products and restriction fragments of a given size. These results were consistent with those obtained by in silico restriction simulation, allowing for confirmation of the identity of amplicons based on amplicon size and the presence of specific restriction sites.

ABBREVIATIONS USED

6-FAM, 6-carboxyfluorescein; AGE, agarose gel electrophoresis; CGE-LIF, capillary gel electrophoresis with laser-induced detection; GMO, genetically modified organism; HEC, 2-hydroxyethylcellulose; PCR, Polymerase Chain Reaction.

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