A new polymerase chain reaction - capillary gel electrophoresis (size and color) method for simultaneous detection of genetically modified maize events

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RUNNING TITLE: Multiplex PCR-CGE-SC for detection of Transgenic Maize

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ABBREVIATIONS:

6-FAM 6-carboxyfluorescein

AGE	aarose gel electrophoresis
bp	base pairs
CGE	capillary gel electrophoresis
CGE-SC	capillary gel electrophoresis (size and color)
CRM	certified reference material
CTAB	hexadecyltrimethylammonium bromide
ENGL	European Network of GMO Laboratories
EU	European Union
GM	genetically modified
GMO	genetically modified organism
HEX	hexachloro-6-carboxyfluorescein
IRMM	Institute for Reference Materials and Measurements
JRC	Joint Research Centre
LIF	laser-induced fluorescence
LOD	limit of detection
P-35	culiflower Mosaic virus 35S promoter
PCR	polymerase chain reaction
POP-4™	performance optimized polymer
T-Nos	nopaline synthetase NOS terminator
TAMRA	carboxy-tetramethyl-rhodamine
TET	tetrachloro-6-carboxyfluorescein
(w/v)	weight / volume

KEYWORDS:

Multiplex PCR; capillary electrophoresis, genetically modified organism (GMO); maize; fluorescence

Summary

We present a novel multiplex PCR assay for simultaneous detection of multiple events of genetically modified (GM) maize. Initially, five detection primer pairs specific to events Bt11, GA21, MON810, and NK603; and maize species were included. The event specificity was based on the amplification of transgene / plant genome flanking regions i.e. the same targets as for validated real-time PCR assays. These short and similarly sized amplicons were selected to achieve high and similar amplification efficiency for all targets; but at the same time, its unambiguous identification was a technical challenge. We achieved its clear distinction by a novel capillary gel electrophoresis approach that combined the identification by size and color (CGE-SC). In one single step, all five DNA targets were amplified and specifically labeled with three different fluorescent dyes. The assay was specific and its sensitivity was adequate to fulfill legal thresholds established e.g. in the EU (i.e. reliable detection of all targets down to 0.1%). Furthermore, our CGE-SC based strategy in combination with an adequate labeling design had the potential to simultaneously detect higher numbers of targets. As an example, we present the detection of up to 9 targets (also including Bt10 and CBH351 maize events) in a single run. Our multiplex PCR-CGE-SC method can be performed using a conventional sequencer device and enables automation and high-throughput. In addition, it proved to be transferable to a different laboratory.

The number of authorized GMO events is rapidly growing; and at the same time the acreage of GM varieties cultivated and commercialized worldwide is rapidly increasing. In this context, our multiplex PCR coupled with CE can be a suitable assay for screening GM contents in food.

1. Introduction

During the last decade, the development of biotechnology has revolutionized agriculture by the introduction of GM organisms (GMO) with characteristics of interest. They are extensively cultivated reaching 81 million hectares in 2004 in 17 countries [1]; and their derived products have reached the global marketplace. This has led to the establishment of labeling and coexistence regulations in some countries intended to protect the rights of consumers. Policies for labeling GMO foods differ among countries, for example thresholds levels for unintended mixing of GMO in non-GM has been defined as 0.9% in European Union [2], 3% in Korea [3] and 5% in Japan [4]. Adequate detection tools are required to enforce these regulations. The most accepted GM detection methods are based on specific DNA sequence detection by means of polymerase chain reaction (PCR) techniques, able to detect even small amounts of transgene sequences in raw materials and processed foods [5-7]. PCR targets include transgenic elements (e.g. Cauliflower Mosaic virus (CaMV) 35S promoter (P-35), nopaline synthetase NOS terminator (T-Nos), modified *cry* genes), junctions of contiguous transgenic elements (e.g. 35S promoter and *cry*), and junctions between plant genome (including some recombinations) and transgenic sequences. Only the latter can be considered as event-specific targets, and therefore they are recommended for use by GMO control laboratories. A number of these PCR assays have been validated by official bodies / reference laboratories such as the Joint Research Centre (JRC) in the EU (http://biotech.jrc.it).

An interesting feature of PCR technique is the possibility of multiplexing. Multiplex PCR is a variant of PCR in which two or more target sequences are simultaneously amplified in a single reaction. Last challenges in GMO detection methods trend towards multiplex PCR based approaches since they can save considerable time, effort and cost by decreasing the number of reactions required to assess the possible presence of GMO in a food sample; especially in view of the increasing number of new GMO events approved worldwide [8]. It is well known that optimization of PCR conditions is particularly complex in multiplex format [9,10]: especial care has to be taken in the design of compatible primers, determination of limiting primer concentrations and adjustment of other components' concentrations (e.g. *Taq* polymerase, Mg^{2+}) and

cycling conditions. Nevertheless, a number of multiplex PCR assays have been published for GM maize detection, being the PCR products typically distinguished by size in agarose gel electrophoresis (AGE) with ethidium bromide staining [11-16]. The main advantages of AGE for separation and detection of PCR products are its simplicity and cost effectiveness; however its sensitivity and resolution are limited: amplicons should be dissimilar enough to allow their identification by AGE and therefore long and differently sized DNA fragments are usually chosen as targets. Therefore, alternative technologies are being explored.

As an example, we previously detected simultaneously up to three GM events through a multiplex real-time PCR with the intercalating dye SYBER-Green and subsequent analysis of the melting curves of the amplified products: different amplicons were identified on the basis of their specific melting temperatures [17]. Also, event-specific and species-specific real-time PCR assays with TaqMan® or similar chemistries have been duplexed by labeling each specific probe with a different fluorochrome dye [18]. Real-time PCR based technologies exhibit high throughput and minimal cross-contamination risks. In addition, alternative approaches based on hybridization of PCR products to surface-immobilized DNA or PNA capture probes have been reported to effectively screen complex mixtures of sequences [19-23]. Array-based approaches typically target diagnostically informative sequence motifs flanked by conserved regions.

Capillary gel electrophoresis (CGE) is a promising tool in this field for the analysis of multiplex assays [24]. CGE with laser-induced fluorescence (LIF) has been reported to efficiently resolve PCR products with minimal requirement of sample and reagents [25]; and in addition is highly suitable for automation. Up to five GMO have been simultaneously analyzed by multiplex PCR coupled to CGE-LIF using nucleic acids fluorescent intercalating dyes such as SYBR-Green [26,27]. All amplified fragments could be identified by size: amplicon lenghts varied from 110 to 508 bp with minimal differences among them being 39 bp.

Preferential amplification of one target over another is a known phenomenon in multiplex PCR. Along with other causes, this bias has been attributed to properties of the amplicon size: short amplicons are typically amplified more efficiently than the

longer ones. The simultaneous amplification of similar-sized targets would presumably facilitate similar amplification efficiencies. This would in turn reduce possible interferences of one reaction over another. Moreover, small amplicon sizes are especially recommended for control of GMO in food products since food processing procedures often result in highly fragmented DNA.

Here we present a development of the CGE technology for the simultaneous detection of a mixture of small and similar-sized amplicons. It combines the amplicon identification by size (through CGE) and color using different fluorescent dyes that exhibit distinctly separated emission spectra (CGE-SC approach). Remarkably, amplicon labeling does not require a special reaction but it is performed along the multiplex PCR. We present for the first time a multiplex PCR assay which allows specific identification of four GM events by targeting plant genome / transgenic DNA edge regions; and amplifies short and similarly sized amplicons (which in turn allows high and similar efficiencies) by use CGE-SC. The potential of CGE-SC was prospectively explored by resolving up to 9 amplicons. Our approach offers an alternative tool for routine GM maize identification in raw materials and / or processed food.

2. Materials and Methods

2.1. Plant material

Powdered certified reference material (CRM) of 1507, Bt176, Bt11, MON810, GA21 and NK603 maize and Roundup Ready® (GTS40-3-2) soybean, were from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and commercialized by Fluka (Buchs, Switzerland). Maize NK603 and CBH351 genomic DNA solutions were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Seed powder of maize Bt10 and its wild type isogenic line were provided by the European Network of GMO Laboratories (ENGL). Seeds of other *Zea mays* lines (Aristis, Pegaso, Tietar, Helen, PR33P66) were kindly provided by E.E.A. Mas Badia (Spain). Leaves of *Arabidopsis thaliana* L. ecotype *Columbia*, *Brassica napus* L., *Brassica oleracea* L., *Oryza sativa*, *Solanum tuberosum* L., *Lycopersicon esculentum* L., *Helianthus anuus*, *Hordeum vulgare* L., *Lens esculenta* L., *Triticum aestivum* and *Glycine max* were from plants

cultivated at the IBMB-CSIC greenhouses.

2.2. Extraction of genomic DNA

Genomic DNA was isolated from 100 mg of each sample using a hexadecyltrimethylammonium bromide (CTAB)-based protocol [28]) followed by purification through QIAquick minicolumns (QIAGEN, GmbH, Germany). DNA concentration was quantified by UV absorption at 260 nm using a NanoDrop ND1000 device (NanoDrop Technologies, Delaware, USA). All samples showed a 260/280 nm ratio ranging from 1.9 to 2.1.

2.3. Oligonucleotide primers

We selected a total of seven primer pairs (Table 1) to univocally amplify specific DNA sequences for the six maize events and maize species. They were selected amongst available primer sequences corresponding to validated PCR assays which targeted transgene / plant genome edge regions or rearrangement flanking regions. All primers were *in silico* tested to control their suitability for use in a multiplex system using the *Bimolecular Interactions* tool of the *RNAstructure v 4.11* software [29]. Each forward primer was fluorescently labeled to allow identification of each amplicon by CGE according to the following rule: the most similar sized amplicons were labeled with different dyes. We used 6-carboxyfluorescein (6-FAM), tetrachloro-6-carboxyfluorescein (TET) and hexachloro-6-carboxyfluorescein (HEX). Oligonucleotides were purchased from MWG-Biotech AG (Ebensburg, Germany).

2.4. PCR conditions

Pentaplex PCRs were performed with the TaqMan PCR core reagents (Applied Biosystems-Roche Molecular Systems Inc. Branchburg, NJ) in 50 µl of PCR mixture including 1× buffer II (100 mM Tris-Hcl, pH 8.3, 500 mM KCl), 6 mM $MgCl_2$, 200 µM dNTPs, the adequate primers (10 nM for *GA141F*, *GA212R*, *Adh-F3* and *Adh-R4*; 75 nM for MONF and MONR; and 150 nM for Bt113JFor, Bt113JRev, NK603 primer1 and NK603 primer2), 100 ng tRNA, 1 U of Ampli*Taq* Gold DNA polymerase; and DNA template. Unless otherwise stated, around 10 ng genomic DNA was used per reaction. Reactions were run in a Master Cycler Gradient device (Eppendorf AG, Hamburg, Germany), according to the following program: 10 min at 95ºC; 45 cycles of 15 s at

95ºC and 1 min at 60ºC; and 30 min at 60ºC. All reactions were performed at least in triplicate.

Uniplex PCRs used to optimize pentaplex reactions were performed as multiplex reactions except for the concentrations of primers: the ones reported for each real-time PCR (Table 1) were initially tested and lower concentrations were subsequently assayed.

Uniplex PCRs used to assess the capacity of CGE-SC for simultaneous identification of high numbers of PCR products were performed by slightly modifying available protocols (Table 2): forward primers were 5'-labeled with TET, HEX or FAM; and real-time PCR probes were omitted. Ten ng genomic DNA extracted from either non-GM maize or 0.9 % Bt10, Bt11 or CBH351 were used per reaction.

2.5. AGE conditions

Twenty µl of PCR product was analyzed by 3% (w/v) agarose (Agarose 1000; Invitrogen) gel with 0.5 µg/mL ethidium bromide (Sigma-Aldrich, St. Louis, MO). The electrophoresis was performed at a constant voltage (80 V) for 30 min in 1x TAE buffer. The gel was scanned by the Molecular Image FX system (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.6. CGE conditions

One µl of PCR product was mixed with 0.5 µl carboxy-tetramethyl-rhodamine (TAMRA) labeled molecular weight marker (Genescan-500, Applied Biosystems, Foster City, CA) and 20 µl Hi-Di formamide (Applied Biosystems). The mixture was incubated for 3 min at 95ºC and immediately placed on ice for 3 min. It was subsequently loaded onto a performance optimized polymer (POP-4™, Applied Biosystems) capillary in the ABI PRISM 310 sequencer device (Applied Biosystems) and run according to the following conditions: 5 sec injection; and 24 min at 15000 V, 8 µA electrophoresis current, 60ºC gel temperature. For simultaneous analysis of up to 9 PCR products 1 µl of each reaction was added to the loading mix.

The 310 GeneScan 3.1.2 software was used to determine the size of the fragments. Notice that this is a relative application i.e. the calculated size does not exactly match the real size (a bias of around ± 5 bp is expected, Applied Biosystems). More important than the assigned size is its consistency: for a given device a small S.D. (i.e.

2%, Applied Biosystems) is expected. For that reason, the identity of the amplified fragments was initially assessed by sequencing of the two strands by using the ABI Prism Big Dye Terminator (version 3.1) cycle sequencing kit and the same sequencer device.

3. Results

3.1. Design and optimization of a pentaplex PCR

Our multiplex PCR was designed on the basis of validated real-time PCR assays (i) which targeted transgene / plant genome edge regions (or rearranged regions for GA21) to univocally amplify specific DNA sequences for the GM maize events; and (ii) shown to be suitable for use as maize endogenous control. A total of 13 publicly available primer pairs specifically targeting the events Bt11 (3, [17,30,31]), GA21 (2, [32,33] and http://www.jrc.cec.eu.int), MON810 (2, [34,35]), NK603 (2, [36] and http://www.jrc.cec.eu.int); and maize species (4, [37] and http://www.jrc.cec.eu.int) were initially *in silico* evaluated for bimolecular interactions. The 5 primer pairs presenting the highest calculated ΔG for all possible interactions were selected for experimental optimization of a pentaplex reaction (Table 1). It should be remarked that these primers had been designed for real-time PCR in uniplex format. Therefore, it was important to primarily select the predictably most compatible primer pairs to be combined in a multiplex assay.

Our approach consisted on using non-labeled reverse primers in combination with fluorescently labeled (with FAM, TET or HEX fluorochromes) forward primers (Table 1), aiming at the amplification and specific labeling of each PCR product in one single step. Detection and identification of PCR products was subsequently performed by CGE based determination of both, size and color (CGE-SC). The labeling design was intended for unequivocal identification of similar-sized amplicons (as commonly are those obtained in real-time PCR assays) by color.

Genomic DNA from non-GM maize and 0.9 % Bt11, GA21, MON810 and NK603 were used for individual amplification of each target in uniplex PCR format. Each reaction was subsequently analyzed by CGE-SC and a single peak of the expected length could

be observed whose identity was confirmed by sequencing. In some of the reactions and especially for GA21, a minor shoulder was observed that could be attributed to the addition of a terminal A residue by Taq-polymerase. As it could be expected, the same results were obtained by AGE run in parallel (data not shown). Notice that clear and sharp CGE-SC peaks were obtained with as few as 1 µl of PCR products; whereas 20 µl were required to identify such small bands on AGE.

Multiplex PCR requires extensive optimization of the concentrations of the different primers for maximal but equivalent amplification efficiency. Uniplex reactions were used to determine the lowest concentration of each primer consistently giving unique peaks i.e. in the absence of secondary peaks above 50 nt. The optimized uniplex reactions were gradually combined using maize genomic DNA containing 0.9 % of each target GMO; and primers concentrations were slightly re-adjusted to achieve 5 unique peaks of the expected length in a pentaplex assay. In the optimized pentaplex conditions (see Materials and Methods section) all 5 peaks were unambiguously distinguished by a combination of size and color (Figure 1), which was particularly relevant for those corresponding to GA21 and Bt11; and MON810 and NK603 which differed in only 2 bp. Control reactions with non-target DNA produced no peaks above 50 nt.

The repeatability of the assay was assessed by performing a total of 12 replicates of the same pentaplex reaction (with the simulated GM mixture containing 0.9% Bt11, GA21, Mon810 and NK603 genomic DNA) in 3 independent experiments. We consistently detected 5 peaks at the expected position (i.e., S.D. below 1.5 %, Table 3).

3.2. Specificity of the pentaplex assay

The specificity of the assay was further assessed by performing pentaplex PCRs (i.e. with all 5 primer pairs) on genomic DNA extracted from either non-GM maize or only one of the target GM maize events (i.e. Bt11, GA21, MON810, or NK603). In all reactions, only the expected peaks were observed: a single peak corresponding to the maize endogenous control in reactions with non-GM maize and 2 peaks corresponding to maize endogenous control and the corresponding transgene flanking region in

reactions containing 0.9 % of one single target GMO (Figure 1). We subsequently tested a number of artificially prepared combinations of the target GM events at various concentrations e.g. 3 % Mon810 and 1 % NK603; 1 % Bt11, 5 % GA21 and 1 % NK603; 0.9 % Bt11, GA21 and Mon810; and 0.9 % GA21 and Mon810. In all experiments, only the expected peaks were observed.

In addition, pentaplex reactions performed using as template DNA from non-target GM maize events such as Bt10, Bt176, CBH351 and TC1507 (1 % and / or 5 %) produced single peaks corresponding to maize endogenous control. Interestingly, DNA from different non-GM maize lines frequently used with commercial purposes produced the same *Adh1* peak, thus further confirming its suitability for use as maize endogenous control. Finally, when DNA extracted from 11 other species such as soybean, sunflower or rice; and from Roundup Ready® soybean was used, no amplification could be detected. Therefore, we concluded that all five primer pairs were specific to their respective target events (or maize species) in pentaplex format.

3.3. Limit of Detection (LOD) of the pentaplex assay

Next, we assessed the relative limit of detection of our assay i.e. the lowest relative percentage of GM materials that can be reliably detected [38]. We prepared maize genomic DNA solutions containing decreasing relative amounts (0.9, 0.5, 0.3, or 0.1 %) of each of the 4 target events; and performed at least 9 replicate assays with each solution in 3 independent experiments. All 5 specific products were consistently detected down to 0.1 % GMO. Absolute LOD (i.e. the lowest number of copies that must be present at the beginning of the PCR to be correctly detected with 95 % probability) were assayed using serial dilutions of an artificial mixture containing 0.9% of each target DNA; and were placed around 30 copies in all cases (data not shown). These values are similar to published PCR methods and fulfill legal requirements [39].

3.4. Evaluation of the transferability of our pentaplex assay

The performance of our pentaplex assay was subsequently tested in a laboratory other than the developer (Consorci CSIC-IRTA and UdG, respectively). A series of sample DNA solutions were designed to represent a diversity of possible combinations of maize GM events; and remarkably at the percentages above which labeling is required in the

EU (i.e. 0.9%, [2]). Sample solutions contained (i) 0.9 % Bt11, GA21, Mon810 and NK603 in non-GM maize; (ii) non-GM maize genomic DNA; (iii) 0.9 % GA21 and Mon810; and (iv) 0.9 % NK603 and Bt11. A total of 12 replicates of each sample were subjected to pentaplex PCR and CGE-SC in 3 different days; and this scheme was carried out in the two laboratories. Results were analyzed by determining the numbers of false positive and false negative per pair of primers; and the percentages of false classification were subsequently calculated. No false classification was obtained in any of the laboratories (i.e. rate of false classification below 2 % for each GMO), which proved the capacity of our pentaplex PCR and CGE-SC assay to be transferred to other users.

3.5. Suitability of AGE and CGE-SC for simultaneous identification of multiple GMO amplicons

AGE is widely used for routine methods, because it can be performed simply and cheaply. Specialized agarose polymers have been developed that exhibit high resolution of short DNA fragments. We therefore assessed the performance of AGE with such polymers for simultaneous resolution of the 5 amplicons obtained in our pentaplex assay. As shown in Figure 2, just 3 bands could be visualized and only the one corresponding to maize endogenous control could be identified. Bands corresponding to maize events Bt11 and GA21; and Mon180 and NK603 could not be distinguished. Note that these two pairs of amplicons only differ in 2 bp. The same pentaplex PCR products were subsequently analysed by CGE-SC to confirm the presence of all 5 amplicons. Next, we tested different combinations of our 5 primer pairs in duplex, triplex and tetraplex formats. As it could be expected, tetraplex reactions produced at least one AGE band which could not be identified (data not shown); although triplex and duplex reactions could be selected that allowed clear identification of all PCR products by AGE. It should be remarked that AGE resolution of small fragments requires a highly performant amplification for clear visualization. In addition, small bands corresponding to primer dimmers or unspecific amplification can also mask the results.

3.6. CGE-SC for simultaneous analysis of various multiplex PCR products

We prospectively assessed the capacity of CGE-SC for simultaneously identifying

higher numbers of PCR products. We therefore analyzed in a single CGE-SC run a total of 9 different PCR products obtained through our pentaplex reaction together with other available PCR assays. Such uniplex PCR assays were adapted to be suitable for CGE-SC analysis: each forward primer was labeled with a fluorescent dye; and colors were assigned to allow identification of similar-sized amplicons. The selected uniplex PCR targeted plant genomic – transgene flanking regions of maize GM events Bt10 (3'), CBH351 (3') and Bt11 (5'); and transgenic sequences of event Bt11 (*cryIAb*) (Table 2). All amplicons were clearly identified by CGE-SC (Figure 3).

4. Discussion

The number of authorised GMO events is already high and steadily increasing around the world. In this context, labelling and traceability regulations make multiplexing of qualitative analyses a necessity [39] (http://www.agbios.com). Here we present for the first time a multiplex PCR assay which allows the specific identification of four GM events by targeting GMO flanking sequences together with a species specific control. PCR-based GMO tests can be grouped into at least four categories with different levels of specificity which depend upon their target DNA composition [5]. PCR assays targeting transgenic elements common to the majority of GM plants (e.g. P-35S, T-Nos, resistance to ampicillin *bla* gene, etc.) have wide applications for screening for GM material. Gene-specific assays target the transgene of interest and are more specific than screening methods. Construct-specific assays target junctions between adjacent elements of the transgenic construct, which are often of different origin e.g. between the modified plant gene of interest and the bacterial terminator. These are more specific assays; however the same or a similar construct can be used to produce different GM events (e.g. maize GA21 and NK603 events). The only unique signature of a transformation event is the junction between the inserted sequence and the plant genomic DNA. The integration process may give rise to DNA rearrangements containing transgene, plant genome and / or even plant organelle genomes that can also be considered unique to a certain event. Our pentaplex PCR is to our knowledge the first event-specific multiplex PCR reported to date. Moreover, all five reactions combined in it were designed on the basis of five uniplex real-time PCR assays previously validated by JRC (http://gmo-crl.jrc.it).

The assay was fully specific. GM reactions only recognized their particular targets and did not amplify genomic DNA from other GMO (from all available maize or soybean events) or non-GMO. It is interesting to remark that no false positive or false negative results per pair of primers were obtained (i) in reactions in which single target events were used as sample; and (ii) in reactions where combinations of target events at various concentrations were used, even at high percentages of one target (e.g. 5 %, close to the highest values observed in real coexistence situations, [40]) and only threshold levels of other events (i.e. 0.9 %, limit above which GMO containing food products must be labeled, according to the EU regulations). In addition, the *Adh1* reaction proved to be truly maize species specific since it allowed the detection of all tested maize varieties (including GMO and frequently cultivated conventional varieties) and did not show any false positive result when other species were tested. These are the two main parameters that need to be carefully considered along development of species specific endogenous control reactions [37,41].

Our multiplex PCR displayed a relative LOD of 0.1 % of each target GMO i.e. below the 0.9 % threshold established by the EU regulations, thus fulfilling European legal requirements. The absolute LOD (with 95% probability) was placed around 30 target copies, similar to the LOD reported for a number of real-time PCR assays (e.g. [37,42]).

Amplicon length is a crucial factor for the detection of DNA in processed foods [43]: DNA degradation during technological treatment can often lead to false negative results due to the presence of highly fragmented DNA. In addition, short sequences are in general amplified with increased efficiency compared to long targets. In a multiplex format, achievement of similar rates of amplification among different targets is crucial for minimal competition and adequate performance of the overall reaction. For this reason, short and similar-sized sequences are ideal targets for multiplex PCR. However, this approach requires the development of adequate techniques for the detection and unambiguous identification of mixed amplified products. We therefore developed a multiplex PCR-CGE-SC technique capable to confer at the same time a very high resolution of short amplicons; and the possibility of confirmation and / or

identification by color. The system incorporates both, target amplification and specific labeling in one single step (i.e. the multiplex PCR); and has been optimized using conventional PCR and sequencer devices, available in many research and routine laboratories. In addition, the method displays high throughput and can be automated.

A number of multiplex PCR assays have been described for GMO analysis coupled to size-based resolution of products. Up to 8 GM events plus 1 endogenous reference control have been amplified by multiplex PCR and resolved by AGE and ethidium bromide staining [16]. CGE proved to be an alternative technique either with UV or with LIF [26,27,44] detection that provided interesting advantages such as better resolution and sensitivity. In addition, it could solve false positives induced by artefacts from multiplex PCR reactions that could not be addressed by AGE. Our multiplex PCR-CGE-SC approach represents a further development of CGE based detection technologies since it incorporates detection by color, which allows identification of amplicons differing in as few as 2 bp.

The usefulness of different techniques to identify PCR products depends upon the characteristics of the DNA fragments to be resolved. Comparison of AGE and CGE-SC in combination with our multiplex PCR assay showed that only the latter could clearly and rapidly discriminate all 5 fragments. However, when compatible triplex reactions were performed (e.g. Mon810, GA21 and *Adh1*) the PCR products could also be resolved by AGE.

Furthermore, our CGE-SC approach was suitable for analysis of higher numbers of PCR products, i.e. at least up to 9. Therefore, it can be considered as one possible tool for resolution of many amplified products as the number of GM events that can be simultaneously amplified in one-tube multiplex PCR continues to grow. Additionally, the simultaneous resolution by CGE-SC of the products of various multiplex PCR (i.e. performed in different tubes) can be considered as an economic, quick and automatable alternative to AGE which can be performed using conventional laboratory equipment.

Finally, we demonstrated the capacity of our pentaplex PCR and CGE-SC assay to be transferred to a different laboratory. Thus, the system is suitable for validation in

multi-laboratory ring trials as recommended by the European Network of GMO Laboratories [45-47] and can be considered as a solid alternative to determine multiple GMO in maize samples in a single run.

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Figure Legends

Figure 1. Pentaplex PCR – CGE-SC assay for detection of four events of GM maize: Bt11, GA21, Mon810 and NK603; and maize endogenous control (*Adh1*). Analysis of maize genomic DNA corresponding to the following simulated GM mixtures: (A) 0.9 % Bt11, GA21, Mon810 and NK603; (B) 0.9 % GA21, Mon810 and NK603; (C) 0.9 % GA21 and NK603; (D) 0.9 % Bt11; (E) 0.9 % GA21; (F) 0.9 % Mon810; (G) 0.9 % NK603; and (H) non-GM maize. In red, molecular weight markers are shown. Black, HEX; green, TET; and blue, FAM.

Figure 2. Multiplex PCR assays coupled to AGE. Lane 2, pentaplex assay for the detection of Bt11, GA21, Mon810, NK603 and maize endogenous control. Lane 3, 4-plex assay for the detection of GA21, Mon810, NK603 and maize endogenous control. Lane 4, triplex assay for the detection of GA21, NK603 and maize endogenous control. Lane 5, NK603 and maize endogenous control duplex PCR. In all reactions, maize genomic DNA simulated mixtures were analyzed that corresponded to 0.9 % of all 4 target events. Lane 1, molecular weight markers.

Figure 3. CGE-SC analysis of nine different amplicons corresponding to the following events of GM maize: Bt11 (3'), GA21, Bt11 (internal), Bt11 (5'), CBH351, Mon810, NK603 and Bt10; and maize endogenous control. Analysis of maize genomic DNA corresponding to a simulated GM mixture containing 0.9 % Bt10, Bt11, CBH351, GA21, Mon810 and NK603. In red, molecular weight markers are shown. Black, HEX; green, TET; and blue, FAM.

Tables

Table 1. Primers used in the pentaplex reaction

Target	Primers	Sequence (5'-3')	Target gene	Amplicon (bp)	Reference
Event Bt11	Bt113JFor	HEX-GCGGAACCCCTATTTGTTTA	3' junction	70	[30]
	Bt113JRev	TCCAAGAATCCCTCCATGAG			
Event GA21	*GA141F*	TET-GGATCCCCCAGCTTGCAT	rearrangement	72	[32]
	GA212R	TTTGGACTATCCCGACTCTCTTCT			
Event Mon810	MONF	HEX-CAAGTGTGCCCACCACAGC	3' junction	106	[34]
	MONR	GCAAGCAAATTCGGAAATGAA			
Event NK603	NK603 primer1	FAM-ATGAATGACCTCGAGTAAGCTTCTTAA	3' junction	108	www.jrc.cec.eu.int
	NK603 primer 2	AAGAGATAACAGGATCCACTCAAACACT			
Maize species	*Adh-F3*	TET-CGTCGTTTCCCATCTCTTCCTCC	*adh1*	136	[37]
	Adh-R4	CCACTCCGAGACCCTCAGTC			

Table 2. Primers used to assess the capacity of CGE-SC for simultaneous identification of high numbers of PCR products

Target	Primers	5' Label	Target gene	Amplicon (bp)	Reference
Event Bt11	*Bt11f*	HEX	*cryIA(b)*	75	[17]
	Bt11r				
Event Bt11	Bt11-1	HEX	5' junction	82	[31]
	Bt115JRev				[30]
Event CBH351	Nos1NEST	FAM	3' junction	100	[48]
	SL2				
Event Bt10	JSF3	FAM	Not described	130	www.jrc.cec.eu.int
	JSR3				

Table 3. Pentaplex PCR – CGE-SC analysis of maize genomic DNA extracted from a simulated GM mixture containing 0.9 % Bt11, GA21, Mon810 and NK603. Mean and S.D. of 12 replicates performed in 3 independent experiments are shown.

Target	Experimental size	5' Label
Event Bt11	65.31 ± 0.74	HEX
Event GA21	66.67 ± 0.62	TET
Event Mon810	102.67 ± 0.41	HEX
Event NK603	103.40 ± 0.44	FAM
Maize species	132.17 ± 0.67	TET

Figures

Figure 1.

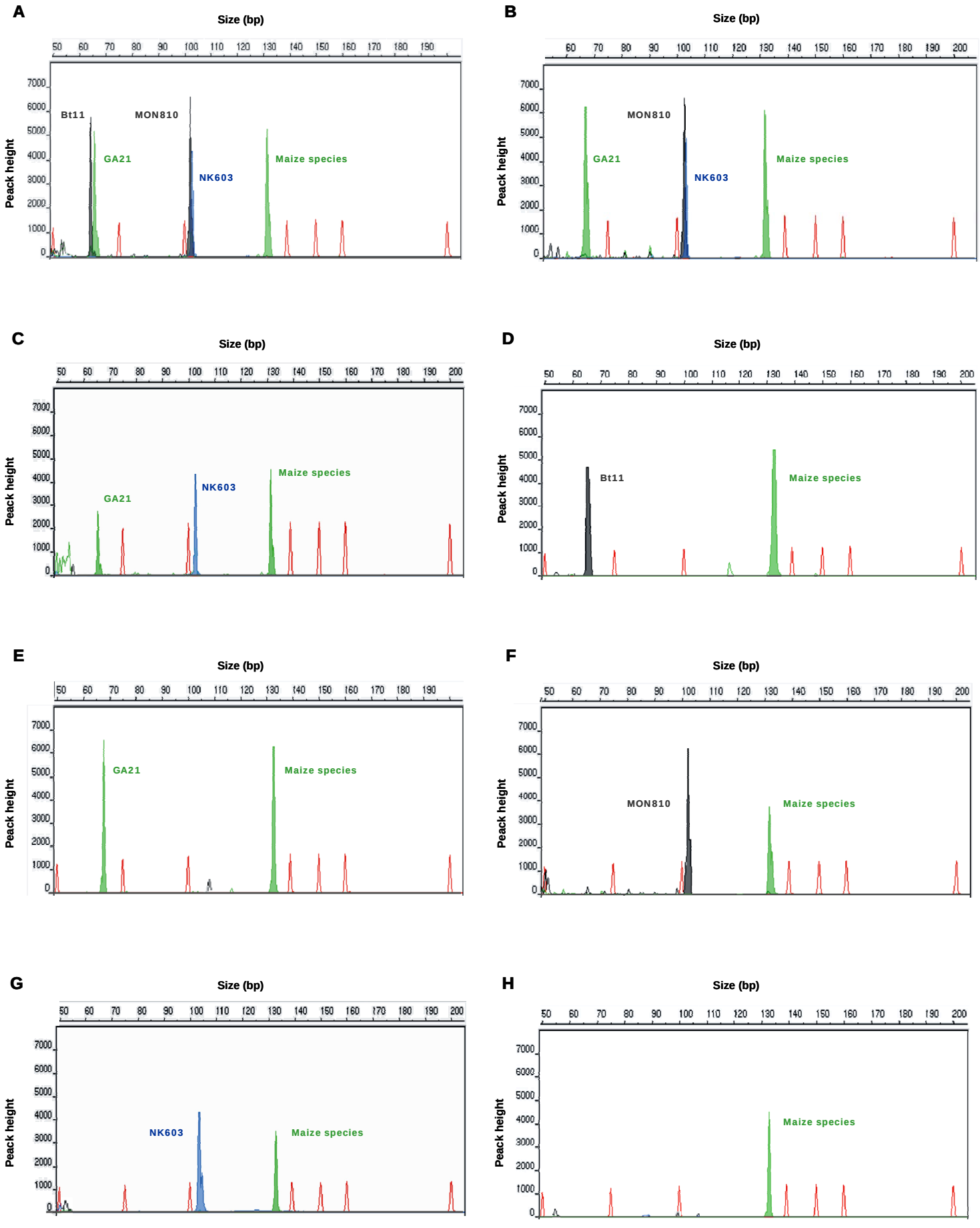


Figure 2.

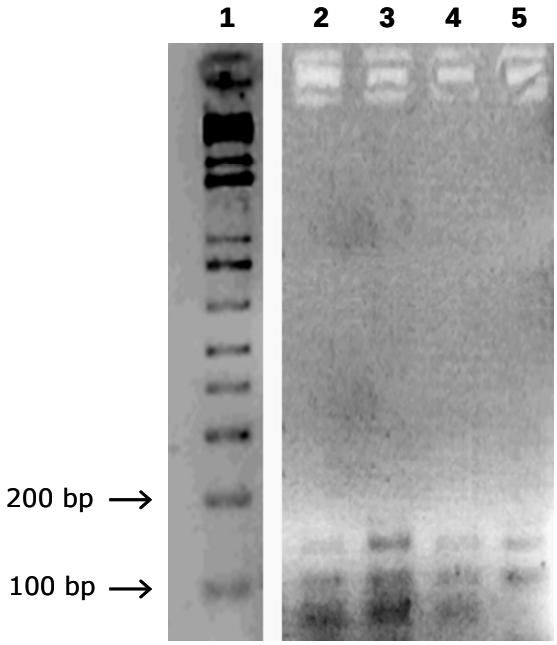
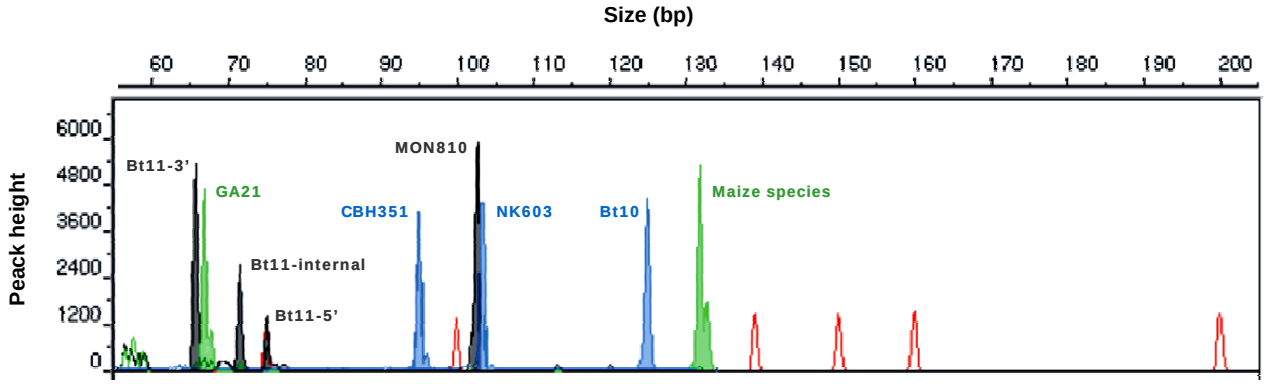


Figure 3.


Size (bp)
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
Peack height
6000
4800
3600
2400
1200
0
Bt11-3'
GA21
Bt11-internal
Bt11-5'
CBH351
MON810
NK603
Bt10
Maize species