

Multiplex Polymerase Chain Reaction-Capillary Gel Electrophoresis: A Promising Tool for GMO Screening—Assay for Simultaneous Detection of Five Genetically Modified Cotton Events and Species

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A multiplex polymerase chain reaction assay coupled to capillary gel electrophoresis for amplicon identification by size and color (multiplex PCR-CGE-SC) was developed for simultaneous detection of cotton species and 5 events of genetically modified (GM) cotton. Validated real-time-PCR reactions targeting Bollgard, Bollgard II, Roundup Ready, 3006-210-23, and 281-24-236 junction sequences, and the cotton reference gene *acp1* were adapted to detect more than half of the European Union-approved individual or stacked GM cotton events in one reaction. The assay was fully specific (<1.7% of false classification rate), with limit of detection values of 0.1% for each event, which were also achieved with simulated mixtures at different relative percentages of targets. The assay was further combined with a second multiplex PCR-CGE-SC assay to allow simultaneous detection of 6 cotton and 5 maize targets (two endogenous genes and 9 GM events) in two multiplex PCRs and a single CGE, making the approach more economic. Besides allowing simultaneous detection of many targets with adequate specificity and sensitivity, the multiplex PCR-CGE-SC approach has high throughput and automation capabilities, while keeping a very simple protocol, e.g., amplification and labeling in one step. Thus, it is an easy and inexpensive tool for initial screening, to be complemented with quantitative assays if necessary.

labeling and traceability regulations set by several countries around the world (1, 2), adequate tools for the identification of GMOs are required. Methods based on specific DNA sequence detection by means of PCR are the most widely used for GMO identification and quantification. They can detect even small amounts of DNA sequences in raw materials and processed foods (3–6). Event-specific detection is ensured by amplifying regions spanning the overlap between the insert and recipient plant genomic DNA or event-specific rearrangements involving the insert or plant DNA. A number of these methods are available and have been validated by official bodies or reference laboratories, e.g., the European Union (EU) Joint Research Laboratory (<http://gmo-crl.jrc.ec.europa.eu/>).

After soybean and maize, cotton is the most widespread GM crop, covering a total of 15 million hectares in 2007, i.e., 43% of the world's cotton. Most GM cotton is grown in the United States and China, but it can also be found in India, South Africa, Australia, Argentina, Mexico, Columbia, and Brazil (7). The production of GM cotton has not yet been approved in the EU but several GM cotton events have been approved for use as food and feed. The two most important GM traits in cotton production are tolerance to herbicides and/or resistance to insect pests (<http://www.agbios.com/main.php>). Examples of events derived from a single transformation are MON1445 [Roundup Ready (RR), Monsanto Co. (St. Louis, MO), a glyphosate herbicide tolerant through an *epsps* gene]; MON531 (Bollgard, developed by Monsanto by insertion of the *cryIAC* gene to be resistant to lepidopteran pests); 281-24-236 and 3006-210-23 (both from DOW AgroSciences LLC, Indianapolis, IN, containing *cryIF* and *cryIAC*, respectively; and the *pat* selectable marker). Event 15985 (Bollgard II, Monsanto) was obtained by a second transformation of MON531 with the *cry2Ab* gene, which gives this cotton resistance to a range of lepidopteran pests, such as the cotton bollworm, tobacco budworm, pink bollworm, and armyworm. In addition, a number of cotton-stacked events are approved, such as

Commercialization of genetically modified organisms (GMOs) is in constant progress. To enforce the GMO

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Table 1. Primers used in the hexaplex reaction

Target	Primer	Sequence (5'-3')	Label	Expected size, bp	Reference
Event Bollgard	MON531F	TCCCATTCGAGTTTCTCACGT	TET	72	http://gmo-crl.jrc.ec.europa.eu/
	MON531R	AACCAATGCCACCCCACTGA	—		
Cotton species	acp1F	ATTGTGATGGGACTTGAGGAAGA	FAM	76	http://gmo-crl.jrc.ec.europa.eu/
	acp1R	CTTGAACAGTTGTGATGGATTGTG	—		
Event Bollgard II	MON15985F	GTTACTAGATCGGGGATATCC	TET	82	http://gmo-crl.jrc.ec.europa.eu/
	MON15985R	AAGGTTGCTAAATGGATGGGA	—		
Event RR	MON1445F	GGAGTAAGACGATTCAGATCAAACAC	FAM	87	http://gmo-crl.jrc.ec.europa.eu/
	MON1445R	ATCGACCTGCAGCCCAAGCT	—		
Event 3006-210-23	3006-f3	AAATATTAACAATGCATTGAGTATGATG	HEX	90	(16)
	3006-r2	ACTCTTTCTTTTTCTCCATATTGACC	—		
Event 281-24-236	281-f1	CTCATTGCTGATCCATGTAGATTC	TET	111	(16)
	281-r2	GGACAATGCTGGGCTTTGTG	—		

DAS-21 23-5/DAS-24236-5 (WideStrike, obtained by DOW AgroSciences LLC by conventional cross-breeding of parental lines 281-24-236 and 3006-210-23). This makes the situation complex from a detection point of view. In this context, analytical methods able to detect several events in a single reaction would be most desirable as an initial screening tool to be then complemented with the existing validated real-time PCR quantitative assays that target particular flanking sequences.

For GMO detection purposes, capillary gel electrophoresis (CGE) in combination with laser-induced fluorescence (LIF) has been reported to efficiently resolve multiplex PCR products with high sensitivity, resolution, and automation (5, 8–11). We previously reported (12) an application of the CGE technology for the simultaneous and unambiguous detection of a mixture of short and similar-sized amplicons, e.g., differing only in 2 base pairs (bp); this resulted in high and similar amplification efficiency for all targets. Identification of the PCR products was achieved by size (through CGE) and color, using different fluorescent dyes (CGE-SC approach). Amplicon labeling did not require a special reaction, but it was performed along with the multiplex PCR. This strategy was recently applied to the detection of 8 GM maize events (13) and further developed to allow restriction-based confirmation of the PCR products (11) or semi-quantification of the targets (14). However, the latter improvements require additional steps to be included in the protocol.

Here we report the development of a multiplex PCR-CGE-SC assay for the simultaneous detection of cotton (*Gossypium hirsutum* L.) and identification of 5 authorized GM cotton events: 281-24-236 and 3006-210-23 from DOW AgroSciences; MON15985 (Bollgard II), MON531 (Bollgard), and MON1445 (Roundup Ready) from Monsanto Co. Its design is based on validated real-time PCR assays that

target either the cotton endogenous reference gene *acp1* or event-specific flanking sequences. In the context of authorized cotton GM events, our multiplex-PCR-CGE-SC is suitable for screening GM cotton contents previous to validated specific real-time PCR quantification, if necessary.

Experimental

Plant Material

Dried cotton (*Gossypium hirsutum* L.) seed powder at <0.5 (0%) and 100 g/kg (uncertainty, 16 g/kg, approximately 10%) mass fraction of genetically modified [281-24-236 3006-210-23 (DAS-21 23-5 DAS-24236-5, WideStrike), DOW AgroSciences, LLC] were produced by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and purchased from Fluka Chemie GmbH (Buchs, Switzerland; references ERM-BF422-A and ERM-BF422-D, respectively). Certified reference materials (CRMs) in the form of powdered seed material of other GM cotton events [MON15985 (Bollgard II), MON531 (Bollgard), and MON1445 (Roundup Ready) from Monsanto] were purchased at American Oil Chemists' Society (AOCS), Champaign, IL. Their references are AOCS 0804-D, 0804-C, and 0804-B, respectively, with certified purities at or above 984.5, 973.9, and 994.0 g/kg, respectively (approximately 100% GMO). DNA extracted from leaf samples of LL cotton 25 (Crop Science, Monheim am Rhein, Germany) was purchased from AOCS (reference, AOCS 0306-E).

Seed or leaf samples of the nontarget species *Arabidopsis thaliana* L. ecotype *Columbia*, *Brassica napus* L., *Brassica rapa* L., *Coffea arabica* L., *Glycine max* L., *Helianthus annuus* L., *Olea europaea* L., *Oryza sativa* L., and *Solanum tuberosum* L. were kindly provided by Centre de Recerca en Agrigenòmica (CRAG). Samples of *Hordeum vulgare* L., *Zea mays* L., *Avena sativa* L., *Secale cereale* L., and *Triticum aestivum* L., and of the common weeds *Fumaria officinalis* L.,

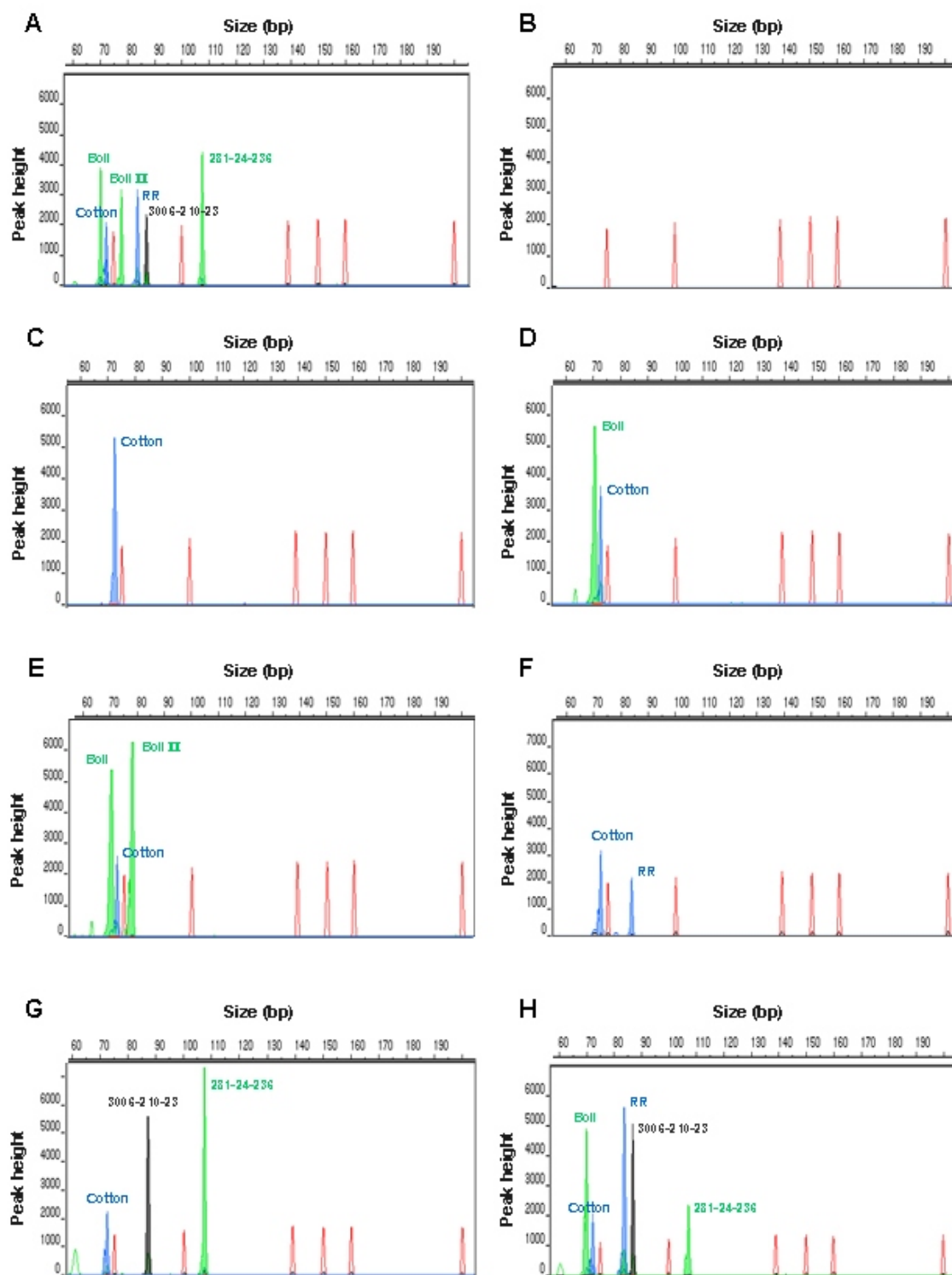


Figure 1. Hexaplex PCR-CGE-SC assay for detection of 5 events of GM cotton (Bollgard, Bollgard II, RR, 3006-210-23, and 281-24-236) and cotton endogenous reference gene (*acp1*). Analysis of 150 ng cotton genomic DNA corresponding to the following simulated mixtures: (A) 0.9% Bollgard, Bollgard II, RR, and WideStrike; (B) no DNA; (C) 0.9% non-GM cotton; (D) 0.9% Bollgard; (E) 0.9% Bollgard II; (F) 0.9% RR; (G) 0.9% WideStrike; and (H) 0.9% Bollgard, RR, and WideStrike. Red, molecular weight markers; black, HEX; green, TET; blue, FAM. Boll, Bollgard; Boll II, Bollgard II; Cotton, *acp1* cotton endogenous reference gene.

Table 2. Hexaplex PCR-CGE-SC analysis of a simulated mixture of cotton genomic DNA (total, 150 ng) containing 0.9% of each—WideStrike, Bollgard, Bollgard II, and RR^a

Target	Experimental size, bp ^b	SD ^c	RSD ^d
Bollgard	70.39	0.33	0.47
Cotton <i>acp1</i>	72.43	0.18	0.25
Bollgard II	77.97	0.10	0.13
RR	84.02	0.18	0.22
3006-210-23	87.08	0.13	0.15
281-24-236	107.35	0.16	0.15

^a Twenty replicates were performed in 5 independent experiments.

^b The GeneScan software is a relative application, i.e., the calculated size does not exactly match the real size (a bias of around ± 4 bp is expected, Applied Biosystems). More important than the assigned size is its consistency (Applied Biosystems; 12).

^c SD = Standard deviation.

^d RSD = Relative standard deviation.

Papaver roeas L., *Polygonum aviculare* L., and *Raphanus raphanistrum* L. were provided by EEA Mas Badia, Girona, Spain. Plant material from additional species (*Phaseolus vulgaris*, *Lycopersicon esculentum*, and *Lens culinaris*) was purchased at the local market. Powdered CRM of Bt11 and MON810 maize and GTS40-3-2 soybean were from the IRMM (ERM-BF412, ERM-BF413, and ERM-BF410).

Extraction of Genomic DNA

About 5 g seed or leaf material was ground in liquid nitrogen with mortar and pestle. Genomic DNA was subsequently isolated from a 1 g aliquot of each sample using a hexadecyltrimethylammonium bromide (CTAB)-based protocol (15), followed by purification through QIAquick minicolumns (QIAGEN, GmbH, Hilden, Germany). DNA concentration was quantified by UV absorption at 260 nm using a NanoDrop ND1000 device (NanoDrop Technologies, Wilmington, DE). All samples showed a 260/280 nm ratio ranging from 1.9 to 2.1.

All cotton genomic DNA preparations were tested by validated real-time PCR assays (<http://gmo-crl.jrc.ec.europa.eu/>) to confirm the identity and percentage of the GM event. Genomic DNA extracted from non-GM cotton ERM-BF422-A was used to dilute genomic DNA extracted from other GM cotton events to 0.9% GMO and other working concentrations.

Oligonucleotide Primers

We selected a total of 7 primer pairs (Table 1) to specifically amplify DNA sequences for the 6 cotton GM events and cotton species. They correspond to the forward and reverse primers of validated real-time PCR assays, which target transgene/plant genome flanking regions (<http://gmo-crl.jrc.ec.europa.eu/>; 16). All primers were preliminary tested in silico to assess their

suitability for use in a multiplex system using the *Bimolecular Interactions* tool of the *RNAstructure v 4.11* software (17). Calculated ΔG values were, in general, above -6 Kcal/mol for the different possible interactions. Each forward primer was fluorescently labeled to allow identification of each amplicon by CGE. The most similar-sized amplicons were labeled with different dyes. We used 6-carboxyfluorescein (6-FAM), tetrachloro-6-carboxy-fluorescein (TET), and hexachloro-6-carboxyfluorescein (HEX). Oligonucleotides were purchased from MWG-Biotech AG (Ebensburg, Germany).

Hexaplex PCR and CGE Conditions

Hexaplex PCRs were performed in a final volume of 50 μ L, including 1 μ L PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, 200 μ M dNTPs, the adequate primers (125 nM 3006-f3 and 3006-r2, 70 nM Mon15985F and MON15985R, 50 nM Mon1445F and Mon1445R, ACP1F and ACP1R, 40 nM 281-fl and 281-r2, and 30 nM MON531F and MON531R), 1 U of the recombinant *Taq* DNA polymerase (Invitrogen S.A., Merelbeke, Belgium); and DNA template. Unless otherwise stated, a total of 150 ng DNA was used per reaction, corresponding to about 5×10^4 haploid genomes of the tetraploid species *G. hirsutum* (18). Reactions were run in a Master Cycler Gradient device (Eppendorf AG, Hamburg, Germany), according to the following program: 3 min at 95 C; 40 cycles of 15 s at 95 C and 1 min at 60C; and 60 min at 60 C. All reactions were performed at least in triplicate. PCR products were resolved by CGE according to the conditions previously reported in Nadal et al. (12). A 1 μ L amount of PCR product was loaded onto a 47 cm \times 50 μ m 310 capillary (FG, Capillary 310GA USA) containing optimized polymer POP-4TM (Applied Biosystems, Foster City, CA) in the ABI PRISM 310 sequencer device (Applied Biosystems). Samples were injected for 5 s at 15 000 V and run for 24 min at the same voltage (8 μ A electrophoresis current), 60 C gel temperature. After each run, the polymer was discarded and 15 μ L POP-4

Table 3. Hexaplex PCR-CGE-SC assay for detection of 5 events of GM cotton (Bollgard, Bollgard II, RR, 3006-210-23, and 281-24-236) and cotton endogenous reference gene (*acp1*)

Target	False positive, % ^a	False negative, %
Bollgard	<1.3	<0.5
Cotton <i>acp1</i>	<0.9	<0.7
Bollgard II	<1.4	<0.5
RR	<1.2	<0.6
3006-210-23	<1.7	<0.5
281-24-236	<1.7	<0.5

^a False classification rates were calculated as percentages of false-positive or false-negative results relative to total expected positive or negative results for each target.

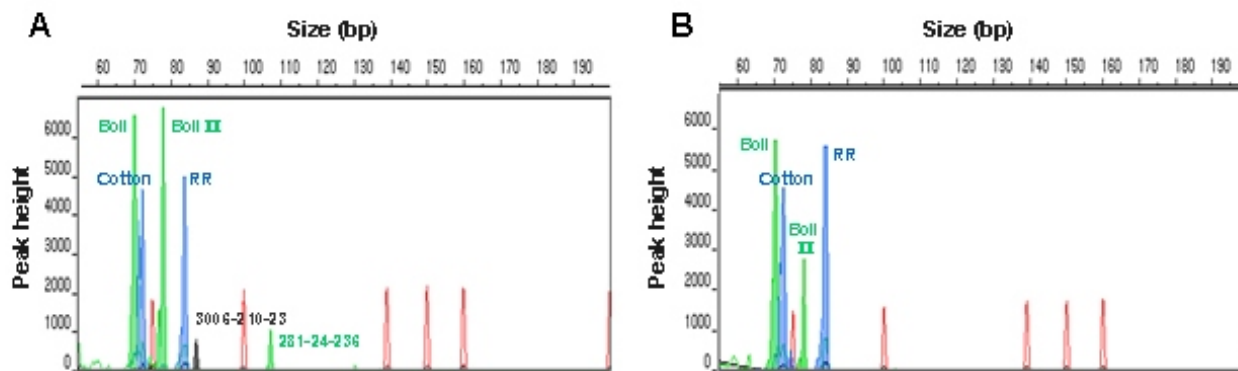


Figure 2. Hexaplex PCR-CGE-SC assay for detection of 5 events of GM cotton (Bollgard, Bollgard II, RR, 3006-210-23, and 281-24-236) and cotton endogenous reference gene (*acp1*). Analysis of 150 ng cotton genomic DNA corresponding to the following mixtures: (A) 1% Bollgard, Bollgard II, RR, and 0.1% WideStrike; (B) 2% Bollgard, 0.1% Bollgard II and RR. Red, molecular weight markers; black, HEX; green, TET; blue, FAM. Boll, Bollgard; Boll II, Bollgard II; Cotton, *acp1* cotton endogenous reference gene.

was injected by pressure (2 min). Excitation and emission spectra of the 4 dyes used are 6-FAM, 494 and 517 nm; HEX, 535 and 553 nm; TET, 522 and 538 nm; carboxy-tetramethyl-rhodamine (TAMRA), 560 and 583 nm.

TAMRA-labeled molecular weight markers (Genescan-500, Applied Biosystems) were used to determine the size of the fragments with the 310 GeneScan 3.1.2 software.

Results and Discussion

Design and Optimization of the Hexaplex PCR-CGE Assay

The aim of this work was to multiplex up to 6 validated real-time PCR assays in a single qualitative reaction. The assays specifically target a cotton endogenous reference gene (fibre-specific acyl carrier protein gene, *acp1*) and the flanking sequences of 5 GM cotton events, 281-24-236, 3006-210-23, MON15985 (Bollgard II), MON531 (Bollgard) and MON1445 (RR). These are the majority of events currently approved for environmental release and food or feed uses. In addition, they are present in most approved stacked events (<http://www.agbios.com/main.php>). As an example, cross-breeding of lines 3006-210-23 (OECD identifier: DAS-21 23-5) and 281-24-236 (OECD identifier: DAS-24236-5) produced the stacked insect-resistant cotton variety commercialized as WideStrike.

The 6 selected assays produce short and similarly sized amplicons, 72 to 111 bp long (Table 1). These characteristics are expected to make all 6 amplification reactions highly and similarly efficient, even in multiplex format. Resolution and unequivocal identification of the 6 amplification products was achieved by CGE-SC (12). In a single step (multiplex PCR) all 6 targets were amplified and labeled with FAM, TET, or HEX fluorochromes through labeled forward primers

(Table 1). This was expected to make amplicons differing in as few as 4 bp length (e.g., *acp1* and Bollgard) to be correctly identified by color.

Optimization of the hexaplex reaction (primers concentrations, annealing temperatures, and number of cycles) was performed according to Nadal et al. (12). Initially, genomic DNA from non-GM cotton and 0.9% of either WideStrike, Bollgard, Bollgard II, or RR were used in uniplex reactions containing decreasing concentrations of the corresponding primers. Genomic DNA amounts were used that corresponded to approximately 100 copies of each target flanking region and 46 000 copies of cotton endogenous control. The lowest concentration of each primer consistently giving unique peaks of the expected length was used as a basis for subsequent optimization of the multiplex reaction. In the optimal conditions, described above, amplification of a simulated mixture of cotton genomic DNA containing 0.9% of each, WideStrike, Bollgard, Bollgard II, and RR, produced 6 peaks of the expected sizes and colors (Figure 1A). The assay was highly repeatable, as demonstrated by the consistent results obtained in 20 replicate hexaplex reactions performed in 5 independent experiments (Table 2).

The *acp1* target (and often other targets, as well) appeared as a double peak consistently 1 nt (1.07–0.11) apart. This could be attributed to the addition of a terminal A residue by *Taq*-polymerase. Control reactions performed in the absence of DNA produced no peaks above 65 nt (Figure 1B).

Specificity of the Assay

We initially assessed the specificity of the assay by running (in 3 replicates) hexaplex PCR-CGE-SCs using as template genomic DNA either extracted from non-GM cotton or from only one of the following varieties (at 0.9% GMO): Bollgard,

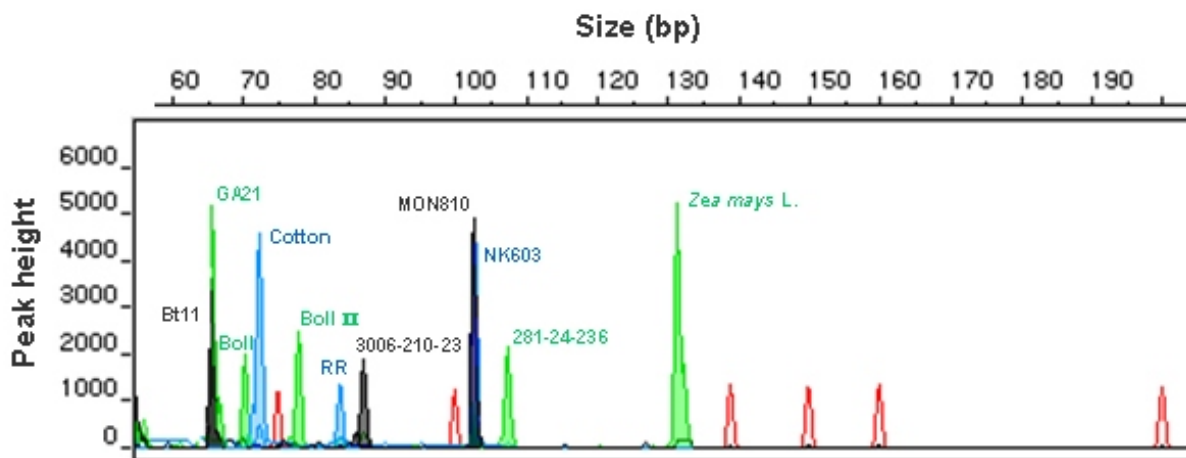


Figure 3. CGE-SC analysis of 11 different amplicons obtained in 2 multiplex PCRs: (1) the hexaplex PCR for detection of 5 events of GM cotton (Bollgard, Bollgard II, RR, 3006-210-23, and 281-24-236) and cotton endogenous reference gene (*acp1*); and (2) a pentaplex PCR for detection of 4 events of GM maize (Bt11, GA21, MON810, and NK603) and maize endogenous reference gene (*adh1*; 12). Analysis of a simulated mixture of cotton and maize genomic DNA containing 0.9% of each target event. Red, molecular weight markers; black, HEX; green, TET; blue, FAM. Boll, Bollgard; Boll II, Bollgard II; Cotton, *acp1* cotton endogenous reference gene.

Bollgard II, RR, and WideStrike. In all CGEs only the expected peaks were observed (Figure 1C–G). The cotton endogenous reference gene (*acp1*) peak appeared in all CGEs. Note that Bollgard II was derived by transformation of the DP50B parent variety, which contained event MON531 (Bollgard). Thus, genomic DNA from 0.9% Bollgard II allowed amplification of 2 different flanking sequences corresponding to event MON531 and the new insert incorporated into 15985. For samples giving positive results for these 2 peaks, quantification of the 2 targets by real-time PCR would be recommended to assess the possible mixture of Bollgard II and Bollgard. Samples obtained from the 281-24-236 x 3006-210-23 stacked event WideStrike produced 2 peaks besides the *acp1* peak. The HEX-labeled peak was placed at 87.08 ± 0.13 nt and the TET-labeled peak ran at 107.35 ± 0.16 nt, i.e., the colors and sizes corresponding to events 3006-210-23 and 281-24-236, respectively. No artifacts of these colors and sizes were observed in any multiplex reaction, suggesting that the peaks corresponded to the 3006-210-23 and 281-24-236 amplicons. However, the identity of all 6 peaks was confirmed by sequencing.

We subsequently prepared all possible combinations of 3 DNAs from the following cotton events (0.9% each): Bollgard, Bollgard II, RR, and WideStrike. This resulted in 4 different combinations that were tested by hexaplex PCR-CGE-SC. The whole experiment was performed in triplicate. All reactions exclusively produced the expected peaks (see Figure 1H). All targets present in the samples were unambiguously identified in all cases, and no false-positive results were observed.

The specificity was finally tested against 150 ng DNA extracted from the GM event LL cotton 25 (5%); 2 insect-resistant GM maize events (0.9% Mon810 and Bt11), and RR GM soybean (0.9% GM). A total of 21 plant species frequently found in food products and common weeds were tested, as well. None of the assays produced any peak above 65 nt, except for the *acp1* peak, which appeared in LL cotton 25 samples. We thus concluded that our hexaplex PCR-CGE-SC assay was highly specific, allowing unequivocal identification of all 6 targets. Note that the *acp1* real-time PCR assay was validated for use as cotton endogenous reference gene in combination with Bollgard, Bollgard II, and RR event-specific real-time PCR assays (<http://gmo-crl.jrc.ec.europa.eu/>). In our multiplex PCR-CGE-SC assay we also show its suitability to detect other GM cotton varieties, such as WideStrike and LL cotton 25, and not to detect different species.

Importantly, no false-positive or false-negative results were obtained for any target. These include reactions with no DNA, with nontarget DNA, with one or a few target DNAs, and with target DNAs at very different concentrations. As shown in Table 3, the false classification were <1.7% for all targets.

Limit of Detection (LOD)

To obtain a crude estimate of the LOD of our hexaplex PCR-CGE-SC assay, we initially used simulated mixtures of cotton genomic DNA containing decreasing percentages of Bollgard II, RR, and WideStrike: 0.9, 0.4, 0.2, and 0.1, respectively. In a 150 ng reaction, this represents about 105,

50, 25, and 12 copies of each target flanking sequence (note that the Bollgard sequence is provided by Bollgard II DNA). In triplicate experiments, all 6 specific products were consistently detected down to 0.1% GMO or about 12 target molecules per reaction. All peaks displayed signal-to-noise (S/N) ratios placed above 1500-fold. The obtained LOD values are similar to those obtained in a pentaplex PCR-CGE-SC previously developed for the identification of maize species and various approved maize GM events (12, 13) and to published PCR methods, and they fulfill legal requirements (19).

In a complementary approach, a number of artificial DNA mixtures were prepared to represent a diversity of possible combinations of cotton GM events at different concentrations. These samples contained 0.1% of one GM event in the presence of high percentages of other events: (1) 0.1% Bollgard + 0.1% Bollgard II + 2% RR; (2) 0.1% Bollgard + 2% Bollgard II + 0.1% RR; (3) 2% Bollgard + 0.1% Bollgard II + 0.1% RR; (4) 2% Bollgard + 2% Bollgard II + 0.1% RR; (5) 1% Bollgard + 1% Bollgard II + 1% RR + 0.1% WideStrike. They were all analyzed in triplicate experiments and consistently produced the expected results (Figure 3), thus confirming that the previously obtained LOD was achieved as well in the presence of high amounts of other targets.

We observed an unspecific blue peak (i.e., FAM labeled) at position 74.56 nt that was consistently associated with high concentrations of RR cotton and its specific primers. Although it was placed close to the endogenous reference gene (FAM labeled, 72.43 nt) they could be unambiguously distinguished: in a total of 15 replicates performed in 5 independent experiments, mean positions of the cotton and unspecific peaks differed in 2.13 nt, with standard deviation values of 0.18 and 0.21, respectively; therefore, they displayed nonoverlapping 95% confidence intervals.

Reproducibility of the Hexaplex PCR-CGE-SC Assay

We finally assessed the capacity of the developed assay to produce the same results with different operators and test apparatus. We prepared a set of 3 genomic DNA solutions (with non-GM cotton, 0.1% RR cotton, and a mixture containing 0.9% of each event—Bollgard, Bollgard II, RR, and WideStrike)—that were then analyzed by 2 different operators and using 2 different PCR devices (Master Cycler Gradient device, Eppendorf AG, and Applied Biosystems 9600). Each operator analyzed a total of 9 replicates of each sample (on 3 different days) in each PCR device. All 108 reactions produced the expected peaks and no unspecific peaks were observed. This demonstrates that the assay is reproducible, with high success rate (>99.07% per target) in independent experiments conducted by different persons and PCR apparatus.

CGE-SC for Simultaneous Resolution of Various Multiplex PCR Products

We previously showed that the CGE-SC-based strategy, in combination with adequate labeling design, has the potential

to simultaneously detect higher numbers of targets. Our cotton hexaplex PCR-CGE-SC assay was designed to be compatible with the previously published maize pentaplex PCR-CGE-SC assay. Figure 3 shows the simultaneous resolution of 5 maize and 6 cotton targets (amplified in 2 multiplex PCR) in a single CGE-SC run. In 2007, GM maize and cotton covered a global cultivation area of 35.2 and 15 millions of hectares, respectively; this represented >40% of the acreage of GM crops. Multiplex PCR-CGE-SC only requires a conventional PCR and a conventional sequencer device. In this context, it can be considered a simple, quick, economical, and automatable tool for GMO screening that can be complemented with specific quantitative analyses if required.

Conclusions

We developed a multiplex PCR-CGE-SC assay for simultaneous detection of 5 events of GM cotton (Bollgard, Bollgard II, RR, 3006-210-23, and 281-24-236) and the cotton endogenous reference gene *acp1*. It is the first multiplex assay that allows specific identification of the most common transgenic cotton events; it targets the junction between the inserted sequence and the host plant genomic DNA. The assay is fully specific, with accurate detection ratios >98.3% per target. It has LOD values similar to those obtained for validated real-time PCR assays and thus complies with the EU legal requirements for GMO analytical methods. Its sensitivity is kept at different relative percentages of targets.

Adaptation of validated real-time PCR assays to uniplex PCR-CGE-SC was simple, and although multiplexing needs the primers to be compatible and a fine adjustment of the primer concentrations, we have shown it is feasible for 5 maize (12) and 6 cotton targets. This further confirmed the suitability of the multiplex PCR-CGE-SC approach to simultaneously detect numerous targets. It offers many advantages, such as simultaneous detection of many target sequences, high sensitivity and throughput, and automation capabilities, while keeping a very simple protocol, e.g., specific amplification and labeling in one step. Our assay was designed to allow detection of more than half of the EU-approved individual or stacked GM cotton events. It can be used as an easy and inexpensive tool for initial screening to be further complemented with the existing validated real-time PCR quantitative assays that target particular flanking sequences. Furthermore, the products of various multiplex PCRs can be resolved in a single CGE, which makes the approach more economical. Simultaneous detection of 5 maize and 6 cotton targets (2 endogenous reference controls and 9 GM events) is particularly interesting for GMO analysis.

Acknowledgments

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