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Full Length Research Paper

Development of a multiplex Polymerase Chain Reaction method for specific detection of Genetically Modified Cotton Events MON 531 and MON 15985

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ABSTRACT
The main objective of this study was to develop a reliable detection method for genetically modified cotton events MON 531 and MON 15985 by using multiplex PCR reaction technique. In this study we have used four primer pairs for the detection of individual gene segments present in transgene cassette of two GM cotton events which include *CaMv35S* promoter, *Nos* terminator, *Cry1Ac* and *Npt II* genes and one pair of primers for cotton endogenous reference gene, fiber specific acyl carrier protein (*fsAcp*). In addition, one event specific primers for MON 531 event and one construct specific primer pair specific for MON 15985 event were also analysed in the integration events. Duplex PCR and multiplex PCR methods were standardized. In duplex PCR individual gene segments were co-amplified with *fsAcp* gene and the products were resolved by using high resolution agarose gel based on their amplicon sizes. Multiplex PCR was performed with the genomic DNA extracted from the GM seed and leaf samples of the two events. To test the limit of detection, the DNA mixture prepared from each of two GM cotton events and non GM cotton were used. The sensitivity of our assay was 0.03%. The multiplex PCR method reported in the present study is simple, cost effective and time saving. This method could be an effective tool for detection and evaluation of two specific GM cotton events.

Key words: *Genetically Modified Organisms (GMOs); Multiplex PCR; LOD; GM cotton.*

INTRODUCTION

Cotton (*Gossypium hirsutum*) is a major source of textile fiber, grown in 70 countries in the world [1]. GM cotton, the first transgenic crop in India, was introduced by US based company Monsanto in collaboration with the Maharashtra hybrid seed company (Mahyco) and got approval for commercialization in 2002. GM cotton contains the *cry* gene which gives resistance to lepidopteron insects, including American bollworm (*Helicoverpa armigera*), the spotted bollworm (*Earias vittella*), and the pink bollworm (*Pectinophora gossypiella*) [2]. GM cotton increases the yield by reducing the damage caused by pests [3]. In India 5 million farmers are benefited by planting 7.6 million hectares of Bt cotton in 2008. The number of countries adopting GM crops has increased steadily from 6 in 1999, to 25 in 2008 [4]. Consumers are concerned about the safety of GM foods. Hence there is a need to develop potential GMO detection methods [5]. The novel trait incorporated in GM crops should be diagnosed for environmental safety, human health and ethical aspects concern [6]. A number of countries have established their own GM regulation and labeling systems. The main objective being informing the consumers of the presence of GM contents in food products [7]. In India recombinant DNA safety guidelines would be given by the Review Committee on Genetic Manipulation (RCGM), under Department of Biotechnology (DBT). Large scale trials and commercial release would be taken care of the Genetic Engineering Approval Committee (GEAC), under Department of Environment, forests and wild life (DOE) [8].

At present available GMO detection methods can be divided in to two categories: I) DNA based testing

methods for detection of transgene elements can be performed by either the southern blot or polymerase chain reaction technique. II) Protein based testing methods in which the expressed transgenic protein can be detected by western blot and enzyme linked immunosorbent assay (ELISA) [9]. The polymerase chain reaction (PCR) is the technique widely used for the detection and quantification of GM crops [10]. Multiplex PCR is the efficient method of choice for detection of multiple targets DNA sequences present in transgene construct by employing several primer pairs in the same amplification reaction. Recently many multiplex PCR methods have also been developed to detect GM crops such as soya bean, maize and canola [11]. Multiplex PCR technique has also been developed for simultaneous amplification of selectable markers and reporter genes for screening of different GM crops [12].

In India, many GM crops are under different stages of field trials [DBT webpage: http://www.dbtindia.nic.in]. In this study, we report an efficient multiplex PCR method for reliable detection of two Bt cotton events MON 531 and MON 15985. We have used four primer pairs for the detection of individual gene segments present in tranesgene cassette of two GM cotton events which include *CaMv35S* promoter, *Nos* terminator, *Cry1Ac*, and *Npt II* genes amplified along with cotton endogenous reference gene *fsAcp*. Additionally we used one event- specific primer pairs for MON 531 event, which targets the junction sequence between the transgene cassettes and host genome of the plant. One construct specific primer pair was used for MON 15985, which could amplify junction sequences of *CTP2* and *Cry2Ab2* gene segments of MON 15985event. This multiplex PCR could be an efficient technique for specific detection of two GM cotton events at the same time it could be used to test the artificial mixing of GM seeds of two events. This approach is simple, cost effective and has capability for evaluation of GM seeds.

MATERIALS AND METHODS

Cotton seeds and leaf materials

100% GM seeds of MON 531 and leaf materials of MON 15985 events were kindly provided by Monsanto Company, Hyderabad. The non GM cotton seeds (Mahyco Company) were purchased from local market in Hyderabad. All of these samples were used in both duplex and multiplex PCR assays.

Genomic DNA extraction, purity and concentration of extracts

Seed and leaf samples of both MON 531 and MON 15985 events were grounded by mortar and pestle and fine powders were prepared separately using liquid nitrogen. According to the manufacturer protocol, genomic DNA was extracted from 20 mg of the grounded sample using DNeasy plant mini kit (Qiagen). The concentration of the extracted DNA was measured by a UV spectrophotometer, N1000 (Nanodrop) and confirmed by conventional 0.8% agarose gel. The absorption ratio at 260nm/280nm wavelength was ranging from 1.6 to 1.9 and this quality of extracted DNA was good for PCR.

Preparation of test samples

Three levels of concentrations (50, 100 and 150ng/µl) genomic DNAs were prepared with MON 531 and MON 15985 and were used in singlet, duplex and multiplex PCR assays. DNA extracted from non GM seeds of cotton was used as control. The limit of detection (LOD) was assessed using the serial dilutions of 100ng/µl DNA sample of GM cotton (100%) with 100ng/µl of non-GM DNA(100%) mix was prepared with each of two events with different percentages

i.e. 100%,10%,1%,0.1%,0.05%,0.03% and 0.01%. A volume of 1 µl (100 ng/µl) of serially diluted DNA was used for PCR. The sensitivity tests were performed with the samples of two GM cotton events MON 531 and MON 15985 using event specific and construct specific primers respectively. The DNA extracted from both the events were mixed randomly and used for PCR for detection and differentiation of two events using event-specific and construct-specific primer pairs.

Designing of Oligonucleotide primers

For the amplification of *CaMv35S* promoter, *fsACP* and *cry1Ac* primer pairs were designed by primer-3 software [Primer3 input (version 0.4.0). http://frodo.wi.mit.edu/primer3]. For the amplification of *Nos* terminator, event-specific primers Esp MON 531, *Npt II* and construct-specific primers CSP MON 15985, published primers were used [13]-[14]-[15]-16]. All oligonucleotied primers were synthesized and purified by Oscimum Biosolutions Pvt Ltd, Hyderabad. The dilutions were made for final concentration of 10µM with milli Q water. For multiplex PCR, 4X concentration of primer mix was made by mixing 1.6µM of each primer and finally 1X primer mix of 0.4 µM was used for PCR [12].

Optimization and validation of PCR conditions

Singlet PCR was done in a final volume of 25µl separately for each primer pair using the target genomic DNA for checking the efficiency of the primer pairs with the following reagent concentrations: genomic DNA 50ng, PCR buffer 1X (Accu Taq LA DNA polymerase mix, SIGMA), $Mgcl_2$ 1.5 mM, dNTPs 0.2mM of each (Fermentas), 0.5 U of Taq polymerase (Jump start Accu Taq LA DNA polymerase, SIGMA) and the final concentrations of each primer was 0.4µM.

Duplex PCR was performed in a final volume of 25µl with the following reagent concentrations: genomic DNA 100ng, PCR buffer 1X (Accu Taq LA DNA polymerase mix, SIGMA), $Mgcl_2$ 3.5 mM, dNTPs 0.3mM of each (Fermentas), 0.5 U of Taq polymerase (Jump start Accu Taq LA DNA polymerase, SIGMA) and the final concentrations of each primer was 0.4µM.

Multiplex PCR assay was performed in a final volume of 25µl with the following reagent concentrations: genomic DNA 150ng, PCR buffer 0.8 X (Accu Taq LA DNA polymerase mix, SIGMA), $Mgcl_2$ 3.5 mM, dNTPs 0.4mM each (Fermentas), 1U of Taq polymerase (Jump start Accu Taq LA DNA polymerase, SIGMA) and the final concentrations of each primer was 0.4µM.

The DNA extracted from both the events were mixed randomly and used it for PCR for detection and differentiation of two events. PCR was done in final volume of 25µl and reagent concentrations were same used for duplex PCR. All PCR analyses were performed on DNA Engine, thermal cycler (Bio-Rad laboratories).Thermal cycler conditions were as follows: preincubation at 95° for 5 min, 35 cycles consisting of double stranded DNA denaturation at 95° for 30s, primer annealing at 59° for 50 s, primer extension at 72° for 50s and final elongation at 72° for 5 min.

Agarose gel electrophoresis

PCR products were analyzed using agarose gel electrophoresis. The gel was prepared with 2% agarose (SIGMA, Germany), 2% and 3% agarose high resolution (SIGMA, Germany) for singlet, duplex and multiplex PCR assays respectively, in 1x Tris acetate EDTA (TAE) buffer with 0.5 mg/ml of ethidium bromide (Etbr). The running conditions were constant voltage 120V for 1 hour in 1x TAE buffer.

RESULTS

In this present study, we developed a simple and efficient multiplex PCR method for specific detection of two GM cotton events. Four pairs of primers were used for the amplification of four transgene segments (*CaMv35S* promoter, *Nos* terminator, *Cry1Ac* and *Npt II* genes) present in two GM cotton events. The two cotton events were differentiated by event specific and construct specific primers. This multiplex PCR has been developed for detection of two GM cotton events. The sensitivity of our assay is 0.03%. This could be an efficient technique for evaluation of GM cotton seeds.

Specificity of the primers designed for GM cotton

Three primer pairs (*CaMv35S*, *fsACP* and *Cry1AC*) were designed by primer-3 (version 0.4.0) software and four primer pairs (*Nos, Npt II, ESP 531* and *CSP15985*) were taken from the literature. All oligonucleotide primers were checked with public database (NCBI BLAST search) and confirmed that no match with an unintended DNA sequence was found. The primer pairs used in this study, the corresponding gene bank accession numbers of target genes and PCR amplicon sizes were listed in Table 1**.** All of the primer sets were used for singlet PCR. The efficiencies of all of primer sequences were individually examined using DNA from two GM cotton events. The results showed that each individual primer set for corresponding GM cotton was able to amplify a specific band.

Table 1: Details of primers used for this study

Gene	Gene bank accession No	Primer	Primer sequence 5'-3'	Product size (bp)	Source
CaMv35S promoter	GQ497217.1	*CaMv35S*	F TCCACTGACGTAAGGGATGAC R TCTCCAAATGAAATGAACTTCC	86	Present study
Nos terminator	AB524019.1	Nos	F GAATCCTGTTGCCGGTCTTG R TTATCCTAGTTTGCGCGCTA	180	[13]
fsACP	U48777.1	fsACP	F TTGTGTTGGGACTTGAGGAA R GTTCACACATGATTTCCCCC	210	Present study
Event specific Mon531		ESP 531	F AAGAGAAACCCCAATCATAAAA R GAGAATGCGGTAAAGATACGTC	346	[14]
Cry1AC	AF177675.1	Cry1Ac	F GGGAGGAGATGCGTATTCAA R CTATACCCTGGGCAGAACCA	483	Present study
Npt II	GQ889498.1	Npt II	F GAACAAGATGGATTGCACGC R GAAGAACTCGTCAAGAAGGC	786	[15]
Construct specific Mon 15985		CSP15985	F ATTGAAGAAGAGTGGGATGACGTTA R GACCAGAGTTCAGGACGGAGT T	116	[16]

The singlet PCR with each primer pair i.e. *CaMv35S* F/R, *Nos* F/R*, fsACP* F/R, *Cry1AC* F/R and *Npt II* F/R were produced a single specific amplicon of expected size with both the events (MON 531 and MON 15985) i.e. 86 bp for *CaMv35S*, 180 bp for *Nos*, 210 bp for *fsACP*, 483 bp for *Cry1AC* and 786 bp for Npt II respectively (Figures 1&2). The primer pairs *ESP 531* F/R, and *CSP 15985* F/R were produced expected product sizes with respective GM events i.e. 346 bp for *ESP 531* in MON 531 event (Figure 1), and 116 bp for *CSP 15985* in MON 15985 event (Figure 2).

Validation of duplex and multiplex PCR methods

In duplex PCR individual transgene segments of two GM cotton events were co-amplified with cotton endogenous reference gene *fsACP* separately. The PCR conditions were cautiously optimized using Jump start Accu Taq LA DNA polymerase. This could avoid interaction between primers and reduce primer dimer formation. As expected, two bands were simultaneously amplified for each gene, one corresponding to the endogenous control (*fsACP*) and the other corresponding to the GM specific amplicons of two GM cotton events (MON 531 and MON 15985) (Figures 3&4). These results suggest that these primers were effective for the specific detection of their respective target events on GM cotton and these would also work well in a multiplex PCR.

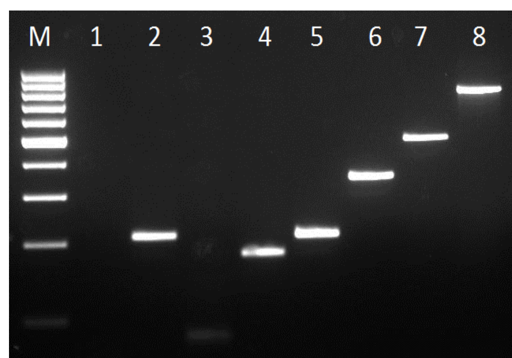

Figure 1. Singlet PCR assay for testing of primer pairs specificity for MON 531 event. Lane M,100 bp ladder; lane 1, negative control; lane 2, non-GM cotton with *fsACP* (210 bp); lane 3 to 8, amplified DNA fragments of 86 bp, 180 bp, 210 bp, 346 bp, 483 bp, and 786 bp correspond to the *CaMv35S* promoter, *Nos* terminator, *fsACP*, *ESP 531*, *Cry1Ac* and *Npt II* genes.

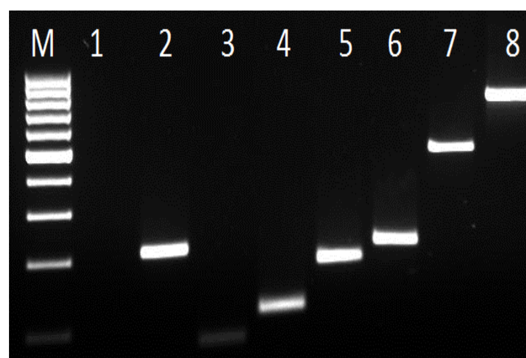

Figure 2. Singlet PCR assay for testing of primer pairs specificity for MON 15985 event. Lane M,100 bp ladder; lane 1, negative control; lane 2, non-GM cotton with *fsACP* (210 bp); lane 3 to 8, amplified DNA fragments of 86 bp, 116 bp, 180 bp, 210 bp, 483 bp, and 786 bp correspond to the *CaMv35S* promoter, *CSP 15985*, *Nos* terminator, *fsACP*, *Cry1Ac* and *Npt II* genes.

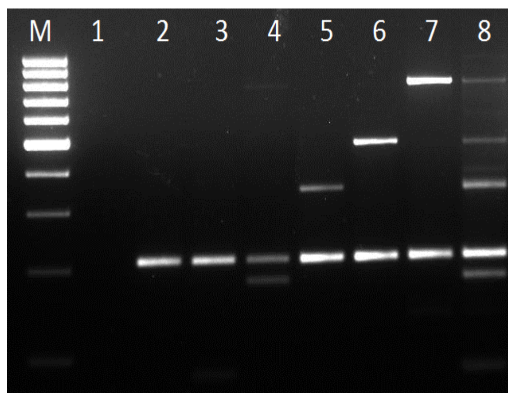

Figure 3. Detection of MON 531 event by duplex and multiplex PCR methods. Lane M, 100 bp ladder; lane 1, negative control; lane 2, non-GM with *fsACP* (210 bp); Lane 3, *CaMv35S* and *fsACP* (86 bp & 210 bp); lane 4, *Nos* and *fsACP* (180 bp and 210 bp); lane 5, *ESP 531* and *fsACP* (346 bp & 210 bp); lane 6, *Cry1AC* and *fsACP* (483 bp & 210 bp); lane 7, *Npt II* and *fsACP* (786 bp & 210 bp). Lane 8, multiplex PCR,amplified DNA fragments of Mon 531 event with 86 bp, 180 bp, 210 bp, 346 bp, 483 bp, and 786 bp correspond to the *CaMv35S*, *Nos*, *fsACP*, *ESP 531*, *Cry1Ac* and *Npt II* genes.

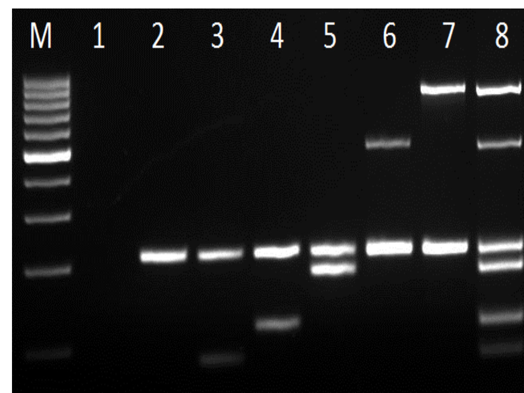

Figure 4. Detection of MON 15985 event by duplex and multiplex PCR methods. Lane M, 100 bp ladder; lane 1, negative control; lane 2, non-GM with *fsACP* (210 bp); Lane 3, *CaMv35S* and *fsACP* (86 bp & 210 bp); lane 4, *CSP 15985* and *fsACP* (116 bp & 210 bp); lane 5, Nos and *fsACP* (180 bp and 210 bp); lane 6, *Cry1AC* and *fsACP* (483 bp & 210 bp); lane 7, *Npt II* and *fsACP* (786 bp & 210 bp). Lane 8, multiplex PCR, amplified DNA fragments of MON 15985 event with 86 bp, 116 bp, 180 bp, 210 bp, 483 bp, and 786 bp correspond to the *CaMv35S*, *CSP 15985*, *Nos*, *fsACP*, *Cry1Ac* and *Npt II* genes.

The multiplex PCR method was used for detection of multiple targets in single reaction and also identifies specific cotton events. To achieve this, we developed a simple and cost effective multiplex PCR for the detection and identification of two insect resistant cotton events (MON 531 and MON 15985). For MON 531 event, primer mix was made with six pairs of primers i.e. *CaMv35S* F/R, *Nos* F/R, *fsACP* F/R, *ESP 531* F/R, *Cry1AC* F/R and *Npt II* F/R), the final concentration of each primer was 0.4µM. The PCR conditions were optimized. The expected amplicon sizes with MON 531 event i.e. 86bp for *CaMv35S*, 180 bp for *Nos*, 210 bp for *fsACP*, 346 bp for *ESP 531*, 483 bp for *Cry1AC*, and 786 bp for *Npt II* (Figure 3).

For MON 15985 event, primer mix was made with six pairs of primers i.e. *CaMv35S* F/R, *CSP* 15985, *Nos* F/R, *fsACP* F/R, *Cry1AC* F/R and *Npt II* F/R, the final concentration of each primer was 0.4 µM. The expected amplicon sizes with MON 15985 event i.e. 86bp for *CaMv35S*, 116 bp for *CSP 15985*, 180 bp for *Nos*, 210 bp for *fsACP*, 483 bp for *Cry1AC*, and 786 bp for *Npt II* (Figure 4).

Limit of detection

A sensitivity assay was carried out to determine the limit of detection (LOD) of two cotton events MON 531 and MON 15985 using event-specific (Esp 531) and construct-specific (CSP 15985) primers respectively. Seven levels of DNA mixture was prepared with each of two GM cotton events and non-GM cotton using serial dilution of 100ng/µl DNA sample of GM cotton (100%) with 100ng/µl of non-GM DNA(100%) with different percentages i.e. 100%,10%,1%,0.1%,0.05%,0.03% and 0.01%. The GMO copy number of cotton was calculated by dividing the copy number of GM by the copy number of the cotton reference gene (*fsACP*) and multiplied by 100 to get percentage value (GM%= GM copy/fsACP copy x 100). The copy number in the sample is determined by dividing the sample DNA weight (in picograms) by the published average 1C value for cotton genome (2.33 picograms) [17]-[18]-[19].The dilutions of GM DNA and corresponding percentage values and copy numbers were listed in Table 2**.** Singlet PCR was performed for the two events. Both event specific (*ESP 531*) and construct specific (*CSP 15985*) primers were used and two expected amplicons (346 bp and 116 bp) could be detected from MON 531 and MON 15985 events respectively. In singlet PCR, 100ng of DNA template of 1µl volume was used from each dilution for two cotton events, whereas bands with 0.03% were weakly visible. This suggests that the GM cotton detection limit of our assay is sensitive to 0.03% (Figures 5 & 6**).** This method is sufficiently sensitive to evaluate GM cotton samples.

Table 2: Calculation of genetically modified organisms (GMO) genome copies in GM cotton

S.No	GM DNA	Non GM DNA	GM%	GMO genome copies
1	100	0	100	42918
2	10	90	10	4291
3	1	99	1	429
4	0.1	99.9	0.1	42
5	0.05	99.95	0.05	21
6	0.03	99.97	0.03	12
7	0.01	99.99	0.01	4
8	0	100	0	0

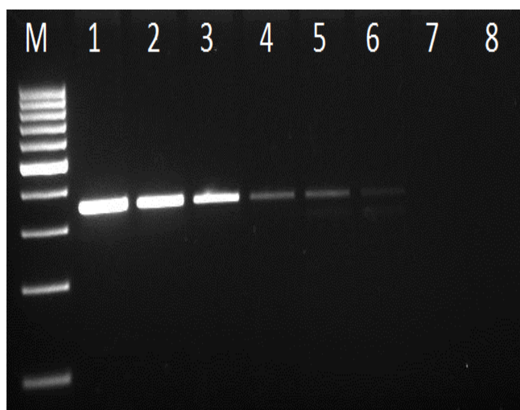

Figure 5. Sensitivity assay of MON 531 event. Lane M, 100 bp ladder; Lanes 1 to 8: serially diluted DNA of MON 531 event containing 100%, 10%, 1%, 0.1%, 0.05%, 0.03%, 0.01% and 0% respectively. Amplified with ESP MON 531 primers amplicon size is 346 bp.

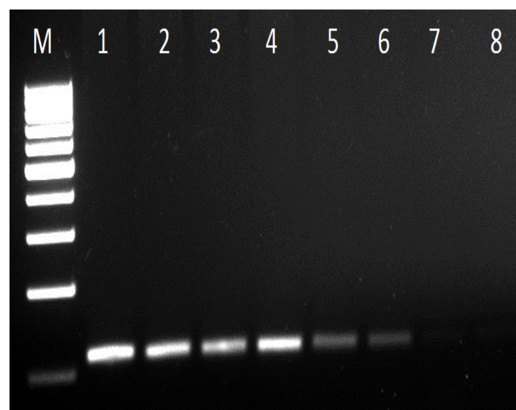

Figure 6. Sensitivity assay of MON 15985 event Lane M, 100 bp ladder; Lane 1- 8: serially diluted DNA of MON 15985 event containing 100%, 10%, 1%, 0.1%, 0.05%, 0.03%, 0.01% and 0% respectively. Amplified with CSP MON 15985 primers amplicon size is 116 bp.

PCR for the detection of artificial event mixing

PCR was standardized using event specific primers of MON 531 event (*ESP 531*) and construct specific primers of MON 15985 event (*CSP 15985*).These primers were co-amplified with endogenous primers *fsACP* separately for two events. For the detection of individual events, the DNA extracted from both events was mixed randomly and PCR was done in final volume of 25 µl. The reagent concentrations in PCR mix were same used for duplex PCR. Here three pairs of primers were used i.e. endogenous (*fsACP*), event specific (*ESP 531*) and construct specific (*CSP 15985*). The expected amplicons (116 bp for CSP MON 15985, 210 bp for *fsACP* and 346 bp for ESP MON 531) were analyzed on agarose gel by electrophoresis (Figure 7).

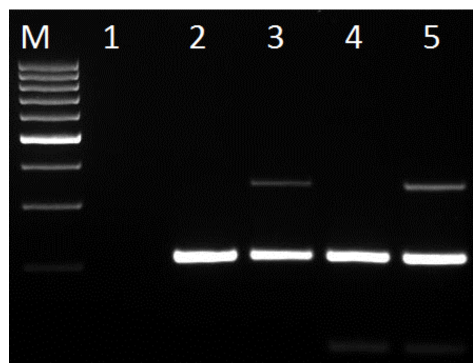

Figure 7. Agarose gel electrophoresis of PCR amplified products of two events for detection of artificial mixing of two cotton events. Lane M, 100-bp ladder; lane 1, negative control; lane 2, non-GM with fsACP (210 bp); lane 3, MON 531 with *fsACP* & *ESP 531*(210bp & 346 bp); lane 4, MON 15985 with *fsACP* & *CSP15985* (210 bp & 116 bp); lane 5, mix of two events with CSP 15985, *fsACP* & *ESP531* (116 bp, 210 bp & 346 bp).

DISCUSSION AND CONCLUSION

ELISA is the method of choice to screen GM foods in raw materials. But processed foods couldn't be detected by this method because ELISA can detect only expressed proteins. However ELISA has lower detection power than PCR methods. It is less sensitive for processed foods [20]. There were many factors affecting optimization and validation of ELISA assay for detection of GM foods [21]. There are several methods recently been developed for detection of GM foods. But Polymerase Chain Reaction remains the technique of choice. These methods are versatile, sensitive, specific and precise. It can detect targets in raw materials as well as processed foods [22]-[23]. The main advantage of PCR techniques is feasibility of multiplexing to save money and time. In multiplex PCR, two or more target sequences are simultaneously amplified in a single reaction. There were many reports of multiple detection techniques through the use of multiplex PCR [24]-[25]-[26]-[27].

The multiplex PCR is the best method of choice to detect multiple targets in a single reaction with less consumption of reagents as compared to singlet PCR. In the present established multiplex PCR assay, same set of primers (*CaMv35S* F/R, *Nos* F/R, *fsACP* F/R and *Npt II* F/R) were used for the detection and identification of two GM cotton events. We also used event specific and construct specific primers in multiplex PCR, where event specific primer pair for MON 531 event targets the junction sequence between the transgen cassettes and host genome of the plant and construct specific primer pair for MON 15985 amplifies the junction sequence of *CTP2* and *Cry2Ab2* gene segments of MON 15985event.These primers are specific for respective cotton events and can differentiate one event from other cotton event. The present multiplex PCR is an efficient technique for specific detection of two cotton events and could also be used to test the artificial mixing of GM cotton events.

In summary, two GM cotton events were tested successfully for the presence of four transegenes (*CaMv35S, Nos, fsACP* and *Npt II*) by multiplex PCR using same sets of primers. The GM cotton detection limit of our assay is sensitive to 0.03%. This technique can be used to meet the GM regulatory obligations. This is a reliable, simple, cost effective and more authentic method which would help for certification of GM cotton seeds in global seed trade.

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