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RESEARCH ARTICLE

PCR BASED DETECTION OF TRANSGENES Cry1Ac AND Cry2Ab IN MON-15985(BG-II) Bt-COTTON IN GUJARAT SAMPLES

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ABSTRACT

Polymerase chain reaction (PCR) is the most widely used and accepted analytical method for GM detection. Duplex & Multiplex PCR, a derivative of conventional PCR, is reliable, efficient and cost-effective qualitative assay, as fewer reactions are required to test the transgenic nature of a crop by simultaneously detecting the target sequences of the inserted gene construct, i.e. specific transgene, marker genes, promoter and terminator gene sequences in a single PCR assay. The present study reports on the development of qualitative and quantitative PCR assays for detection of commercialized Bt - cotton events, which are being widely cultivated in the North, Central and South zones, i.e. MON531 and MON15985 which are under different stages of field trials in India. Several analytical methods such as methods based on the polymerase chain reaction (PCR) for detecting the transgenic DNA have been developed. DNA has higher stability than proteins, it may also be extracted from processed foods therefore it is preferred analyse for screening of both raw ingredients and processed products. PCR has found real application in GMO detection as an acceptable method for regulatory purposes. Therefore, in the present work attempt has been made to develop and standardize different detection of transgenes Cry1Ac, Cry2Ab, npt-II, 35S Promoter & NOS terminator for Bt - cotton using PCR based strategies and applied to the chosen specific contain any GM genes. Lastly, this effort will be of relevance for timely detection of transgenes in GM foods and products (GMOs) that are still not approved to be imported in India.

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INTRODUCTION

GM crops, although are known to be beneficial in most cases, still consumers have the right to know and choose between GM and Non - GM products, they buy. Consumer concerns related to development and marketing of GMO and derived food products have resulted in increased awareness regarding the food labelling all over the world. As far as India is concerned no GM crop (Food/products) is allowed in India to be marketed except Bt - cotton which is a feed crop. Under Jurisdiction of Supreme Court on May8, 2007 GEAC was instructed to lay down the protocols for ensuring 0.01% of contamination by GM crops in field trials. In the year 2003 China had faced problems while accepting and implementing the labeling norms because of the lack of proper infrastructure to detect 1% GM levels and they had no option but to procure very expensive detection kits from international market (Hepeng Jia, 2003). Cotton is of the genus Gossypium, of the tribe Gossypieae, and of the family Malvaceae. Worldwide, four species of cotton are of agronomic importance: the two diploid Asiatic species, *G. arboreum* and *G. herbaceum,* and the two-allotetraploid New World species, *G. barbadense* and G. *hirsutum.* In India, cotton is primarily grown in dry tropical and sub tropical climates at mean temperatures between 11°C and 25ºC with a rainfall of 250-1500 mm. It is cultivated in India from sub Himalayan region of Punjab in the North to Tamilnadu in South and dry regions in the East. Cotton (*Gossypium* sp.) is an important fiber crop of India covering an area of 88.20 lakh hectares with production of 242.50 lakh bales and productivity of 467.00 kg lint per hectare. In Karnataka, it is grown on an area 3.80 lakh hectares producing 7.00 lakh bales with productivity of 312.00 kg lint per hectare. The most commonly cultivated species of cotton in the world include *Gossypium hirsutum.* Thus, one can define a GM food/crop/organism as a functional foreign genes, incorporated by genetic engineering that are not present naturally in them, are termed as Transgenic organisms or Genetically modified organisms (GMOs) and foreign gene insert is called a Transgene. A common example of genetic engineering is the insertion of *Bacillus thuringiensis* genes into corn to make Bt-cotton *Bacillus thuringiensis* is a bacterium that naturally produces a protein that is lethal to insect larvae. By transferring the genes that encode this protein into cotton balls, scientists have created a type of cotton that produces its own pesticides, making it resistant to insects such as the Bt-cotton event Bollgard II. Bollgard II cotton, which has two modes of action for improved lepidopteran control and increased

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spectrum of activity over Bollgard cotton, is expected to provide significant benefits to cotton production including the reduction in pesticide use, improved control of target insect pests, improved yield, reduced production costs, and improved profitability for cotton growers. Detailed food, feed, and environmental safety assessments confirm the safety of this product. Cotton (*Gossypium hirsutum L.*) is not a crop mainly consumed by humans as food, it is an important economic crop in the world because its boll can be used as fiber, its seed oil as a cooking oil or snack food, and its meal and hulls as important protein concentrates for livestock (Lin.xi Jiang, 2009). TheMON15985 event was produced based on a previously commercialized Lepidopteran resistant cotton variety known as MON531, expressing a chimeric form of a Cry1A insect resistant protein. In the MON15985 event, one copy of complete t-DNA containing the Cry2Ab gene was integrated at a single site in host cotton genomic DNA.

Bollgard II cotton event 15985 was developed by Monsanto Company to produce the Cry2Ab insect control protein, which provides effective season-long control of key lepidopteran insect pests. This product was produced by re-transformation of Bollgard® cotton event 531, which produces the Cry1Ac insect-control protein and the nptII selectable marker protein. Therefore, Bollgard II cotton produces two proteins for effective control of the major lepidopteran insect pests of cotton, including the cotton bollworm, tobacco budworm, pink bollworm, and armyworm. Bollgard II cotton also produces the ß-D-glucuronidase (GUS) marker protein. In addition, Bollgard II cotton provides a more effective insect resistance management program compared to single gene products. Bollgard cotton has been grown globally on more than 32 million acres since commercial introduction in 1996 (James, 2002). The primary benefits that have resulted from the use of Bollgard cotton are reduced insecticide use, improved control of target insect pests, improved yield, reduced production costs, and improved profitability for cotton growers.

The development and commercialisation of GM crops is increasing at a faster pace, to develop qualitative and quantitative methods for detection of GM crops has become even more challenging. In India, till date, six events of Bt cotton, i.e. MON531 with cry1Ac gene, MON15985 with cry1Ac and cry2Ab genes. Recent advances in analytical systems for the detection, identification and quantification of genetically modified organisms (GMOs) and the importance of standardized/validated methods and future technological trends have been discussed by (Hernández, 2005). The suitability of an analytical method for its specific purpose is determined by the process of validation. Based on the results of a validation study, a method can be considered as reliable and robust (Bellocchi, 2008). Polymerase chain reaction (PCR) is the most widely used and accepted analytical method for GM detection. Multiplex PCR, a derivative of conventional PCR, is reliable, efficient and cost-effective qualitative assay, as fewer reactions are required to test the transgenic nature of a crop by simultaneously detecting the target sequences of the inserted gene construct, i.e. specific transgene, marker genes, promoter and terminator gene sequences in a single PCR assay (Randhawa, 2009a). The present study reports on the development of qualitative and quantitative PCR assays for detection of commercialized Bt - cotton events, which are being widely cultivated in the North, Central and South zones, i.e. MON531 and MON15985 and other Bt crops, i.e. Bt brinjal, Bt cauliflower, Bt potato, Bt rice and Bt okra, which are under different stages of field trials in India. The simplex as well as quantitative real-time PCR assays for detection of specific cry gene up to 0.01% have also been developed. (Randhawa, 2009b).

MATERIALS AND METHODS

Plant Material

BT-Cotton (BG-II) event MON 15985 was given By Dr. V. Kumar, Main Cotton Research Station Navasari Agricultural University, Athwa Farm, Ghod Dod Road, Surat, Gujarat.

DNA Extraction

Genei Pure Column kit was used for DNA isolation. Genei Pure Column kits provide a fast and simple way to isolate DNA from seeds. Up to 25mg of tissue can be processed using Genei Pure Column kits Purification was done as per the suggested protocols with slight modification in the incubation time from 15 min to one hour at the first step to get better yield of DNA as experienced in our earlier attempts. Genomic DNA of Bt-Cotton were run on 0.8% agarose gel electrophoresis The gel was run for one 1hr in 1X TBE buffer at 100V. The gels were visualized by ethidium bromide staining under UV light (254–366 nm) and pictures were captured using UV Gel Documentation System.

Qualitative Polymerase Chain Reaction (PCR)

All PCR reactions were performed in a final volume of 50 µl, using 2.5 units proof reading Taq DNA polymerase (Sigma), 50 µM of each forward and reverse primers, 200 mM of each dNTP and 3 µl DNA. PCR was carried out in DNA Engine MJ Research, PTC-200 (Peltier Thermal Cycler) using Sigma PCR mixtures. Thermal cycling conditions were 94°C for 15 minutes followed by 30 cycles of 94°C for 1minute.,55°C for 45sec., And 72°C for 10 minute , with a final extension step of 10min.at 72°C.As a result were obtained PCR products were kept at 4°C till gel electrophoresis. An aliquot of the PCR product was analyzed on a 2% agarose gel. The gel was run for one 1hr in 1X TBE buffer at 100V. The gels were visualized by ethidium bromide staining under UV light (254–366 nm) and pictures were captured using UV Gel Documentation System. 100 bp DNA Ladder Plus (MBI Fermentas) consisting of DNA fragments ranging in size from 50 to 1000bp, was used as DNA molecular weight marker.PCR involves two primers, usually, (one reverse and one forward) for detection of transgene in transgenic cotton. For detection of transgene Cry1A(c) specific set of primers (sequence FTGACCGCTTACAAGGAGGGATACG, **R**CAC GGAGGCATAGTTCAGCAGGAAC. While detection of transgene Cry2Ab specific set of primers F-GGACCTACCGCGACTACCTGAAGA R- TGAACGGCGA TGCACCAATGTC

RESULTS

DNA extracted from different sample was evaluated for suitability in quantitative analysis. In order to select for the best applicable DNA isolation procedure we tried Bangalore

GeneiPure Column kit method. Isolated DNA was checked on 0.8% agarose gel electrophoresis. After isolation and quantification of DNA from different Bt and non Bt cotton seed samples (I, II, III, IV, V, VI) they were put to PCR amplification using specific set of primers of 26Sr-RNA (Sequence ***F-*** CAC AAT GAT AGG AAG AGC CGA ***R*** - CAA GGG CTT GGC AGA ATC).PCR product so obtained was checked on 2% Agarose gel electrophoresis, with a 100bp ran parallel to check the size of amplicon. A product of 516 bp was observed which confirms the presence of 26S r-RNA in all samples as shown in figure 1. The primers to detect 26S r-RNA for cotton as an internal control already published primers were adapted to compare the two for suitability. (Tuli 2009). By using specific set of primers of Cry1A(c) having 230bp (Sequence *F -TGACCGCTTACAAGGAGGGATACG R-CACGGAGGCATAGTTCAGCAGGAAC).* PCR products so obtained was checked on 2% Agarose gel Electrophoresis, with a 100 bp ladder ran parallel to check the size of amplicon. A product of 230 bp was observed only in Bt-cotton samples but not present in non-Bt cotton samples.

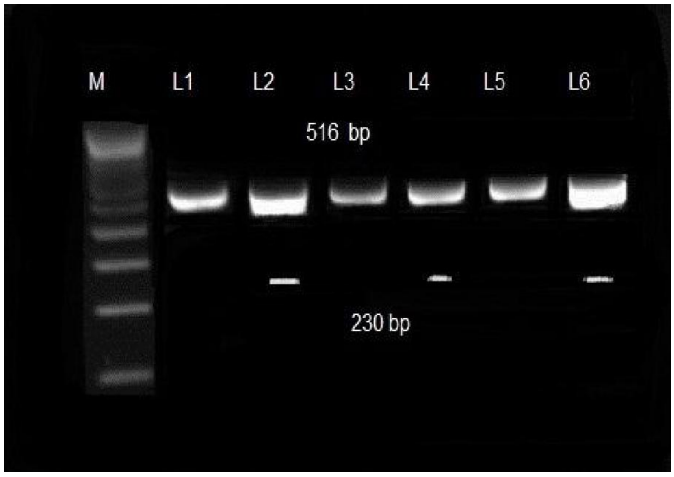


Figure 1. Duplex PCR having internal control in all cotton samples and Transgenes in Bt-cotton samples

M-100 bp Marker
Lane 1-Gujarat Cotton Hybrid-6(Non Bt) showing 516 bp amplified product
Lane 2-Gujarat Cotton Hybrid-6(BG-II) showing 516 bp and Bt 230 bp amplified product
Lane 3-Gujarat Cotton Hybrid-8(Non Bt) showing 516bp amplified product
Lane 4-Gujarat Cotton Hybrid-8(BG-II) Bt showing 516bp and 230 bp amplified product
Lane 5-Gujarat Cotton 10(Non- Bt) showing 516 bp amplified product
Lane 6-Gujarat Cotton 10(Bt) showing 516 bp and 230 bp amplified product.

PCR of another transgene i.e. Cry2A(b) having453 bp by using specific set of primers GGACCTACCGCGACTACCTGAAGA R- TGAACGGCGATGCACCAATGTC. PCR product so obtained was checked on 2% agarose gel electrophoresis, with a 100 bp ladder ran parallel to check the size of amplicon. A product of 453 bp was observed in Bt-cotton samples i.e. Cry2A(b) was observed. Duplex PCR for internal control 26Sr-RNA and transgene Cry2Ab in MON 15985 Figure 2.

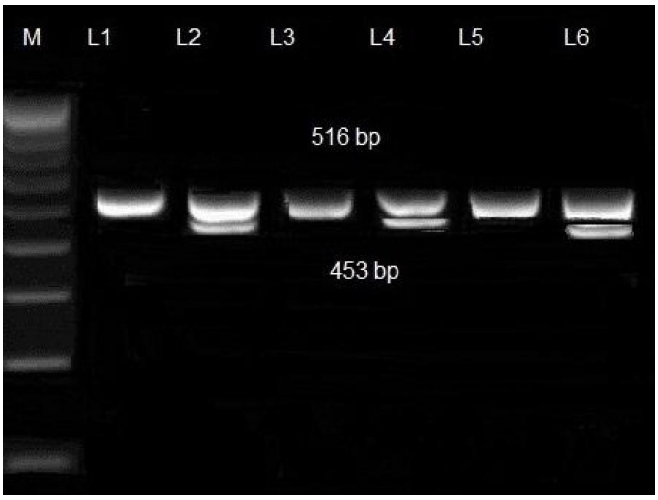
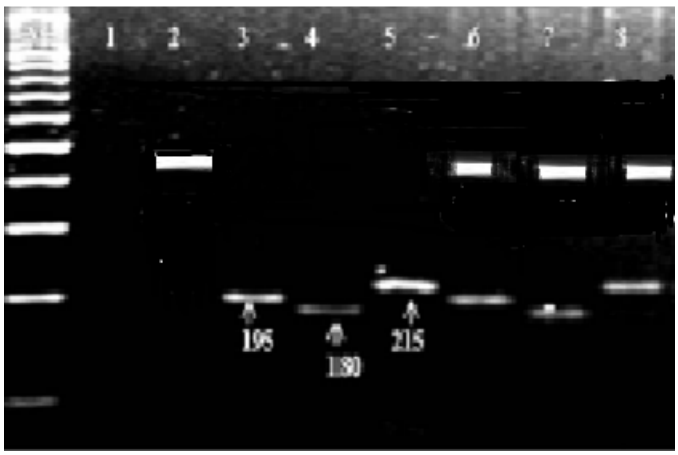


Figure 2. Duplex PCR with Cry2Ab and Internal control 26S r-RNA in Cotton samples

M-100 bp Marker Lane 1-Gujarat Cotton Hybrid-6(Non Bt) 516bp amplified products
Lane 2-Gujarat Cotton Hybrid-6(BG-II) Bt 516 and 453 bp amplified product
Lane 3-Gujarat Cotton Hybrid-8(Non Bt) 516bp amplified product
Lane 4-Gujarat Cotton Hybrid-8(BG-II) Bt 516bpand 453bp amplified product
Lane 5-Gujarat Cotton 10(Non- Bt) 516bp amplified product
Lane 6-Gujarat Cotton 10(Bt) 516bpand 453 bp amplified product

A Standard PCR and Multiplex PCR analysis of Cry2A(b) (MON15985) 453 bp Cry2Ab added in 1 PCR, 195bp(P-35S) Promoter in another tube, npt-II gene 215 in another tube & Duplex PCR with Cry2Ab453 bp with 195, 453bp with 180 bp and 453 bp with 215bp as shown in figure 3.



M- 100 bp DNA Ladder
Lane 1- Water control
Lane 2- Positive Cry2Ab having 453 bp
Lane 3- 35 S Promoter having 195 bp
Lane 4- NOS terminator having 180bp
Lane 5- npt-II gene having 215 bp
Lane 6- Duplex PCR with Cry2Ab and 35S Promoter
Lane 7- Duplex PCR with Cry2Ab and NOS Terminator

DISCUSSION

For PCR analysis it is essential that the average size of the DNA fragments in the template should not be significantly smaller than the target sequence (amplicon length) in the assay. The primers to detect 26S r-RNA as an internal control for cotton was already published primers were also adopted to compare the two for suitability (Tuli, 2009). Likewise respective transgenes i.e. Cry1A(c), 35S Promoter, Nos terminator and Cry2Ab were also detected by PCR based strategies adopted in the present study. We have searched for different primes in literature and selected the primers more than 200 bp so that they can catch template even in degraded DNA. Same PCR program, which was also applicable to QPCR, was optimized for all the genes. This gives us an advantage to run all the PCR assay sets together and analysis of all the genes can be done simultaneously.

The results showed that all the transgenes were repeatedly detected in all PCR assays optimized. On applying the above PCR assay in seeds sample DNA, detected in all the cotton (26 S r-RNA) as an internal control in cotton ,Cry1A(c) and Cry2Ab in Bt-cotton these are two transgenes present in MON15985 event of Bt-cotton. The presence of 195bp specific amplicon for 35S promoter, 180bp presence of Nos terminator and npt-II gene having 215bp. (Randhawa, 2010). Further to make the method cost effective the internal control and transgene of Bt-cotton were made into duplex PCR assay. The internal control 26S r-RNA primer in Bt-cotton duplex was 101 the one designed in the lab (230bp amplicon size) as the reported primer was of 516bp (amplicon size) and was not differentiable from transgene. Third duplex assay for general screening of transgenes with 35 S promoter, NOS terminator and npt-II gene was also optimized. The duplex assays were checked for the limits of detection of transgenes in them. Duplex PCR for 35S, NOS terminator and npt-II showed that the transgenes was detectable. Still duplex PCR optimized here is cost effective and time saving.

Conclusion

In the present work attempt has been made to develop and standardize different detection of transgenes Cry1Ac, Cry2Ab, npt-II, 35S Promoter & NOS terminator for Bt - cotton using PCR based strategies and applied to the chosen specific contain any GM genes. Lastly, this effort will be of relevance for timely detection of transgenes in GM foods and products (GMOs) that are still not approved to be imported in India.

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