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Qualitative and Quantitative Molecular Testing Methodologies and Traceability Systems for Commercialised *Bt* Cotton Events and Other *Bt* Crops Under Field Trials in India

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Abstract Qualitative and quantitative PCR assays were developed for detection of commercialised *Bt* cotton events, i.e. MON531, MON15985 and other *Bt* crops, which are under different stages of field trials in India, i.e. *Bt* brinjal, *Bt* rice, *Bt* cauliflower, *Bt* potato and *Bt* okra. Multiplex PCR assays simultaneously detecting specific *cry1Ac*, *cry1Ab*, *cry2Ab* genes, *Cauliflower Mosaic Virus* 35S promoter, *nptII* marker gene along with species- or taxon-specific endogenous gene in these *Bt* crops have been developed. The quantitative real-time PCR assays were also reported for *cry1Ac* gene using designed primers and TaqMan probe. The sensitivity of developed assays for detection of specific transgene was established up to 0.01%. The analytical methods developed in the present report will be of immense use for qualitative screening and detection of *Bt* crops along with the quantitative analysis of inserted *cry1Ac* gene to meet the threshold level for regulatory compliance.

Keywords *Bt* Crops · GM Detection · Multiplex PCR · Real-time PCR · Traceability

Introduction

The global area under genetically modified (GM) crops has continued to grow remarkably in 2009 reaching 134 million ha (James 2009). Despite the high adoption rate and many benefits pertaining to GM crops, the concerns related to their impact on environment and food safety issues along with socio-ethical issues also need to be addressed effectively. Hence, the novel traits incorporated in GM crops need to be evaluated and detected for environmental and food safety, social and ethical issues.

As 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, GFM with fused *cry1Ac-cry1Ab* gene, Event 1 with *cry1Ac*, Event 9124 with synthetic *cry1C* gene and Dharwad event (Bikaneri Nerma-Bt variety) with truncated *cry1Ac* gene have been commercialised, which are being cultivated in an area of 8.4 million ha (James 2009; Karihaloo and Kumar 2009). Other *Bt* crops, i.e. *Bt* brinjal with *cry1Ac/cry1Ab* genes, *Bt* cauliflower, *Bt* rice, *Bt* okra with *cry1Ac* gene, *Bt* potato with *cry1Ab* gene are under different stages of confined field trials. The *Bt* crops encoding delta-endotoxins from *Bacillus thuringiensis* provide protection against a wide range of lepidopteron and dipteran insect pests throughout the growing season of the plant.

Precisely defined procedures for the validation of GM detection methods, along with performance and acceptance criteria, are important for meeting the scope of accreditation, which enables the testing laboratories to cope with the large number of new methods, which have to be introduced in the laboratory for efficiently addressing consumer's demands (Žel et al. 2008). Recent advances in analytical systems for the detection, identification and quantification of genetically modified organisms (GMOs) and the importance of standardised/validated methods and future techno-

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logical trends have been discussed by (Hernández et al. 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 et al. 2008). Therefore, it is imperative to develop and validate GM detection tools/methods for range of *Bt* crops, some of which are also food crops such as *Bt* brinjal, *Bt* cauliflower, *Bt* rice, *Bt* okra and *Bt* potato. 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. A hexaplex PCR method simultaneously targeting the commonly used marker genes, *viz*., *nptII*, *aadA*, *hpt*, *bar*, *pat* and *uidA* has been developed as an efficient tool for screening of GM crops (Randhawa et al. 2009a). Multiplex PCR assays have also been successfully employed for detection of various GM crops that are under different stages of testing in containment or field trials in India such as insect resistant cotton with (*vip*) 3A-type gene (Singh et al. 2008), GM potato expressing *AmA1* gene for better protein quality (Randhawa et al. 2009b, c), GM tomato with *osmotin* gene for salinity and drought tolerance (Randhawa et al. 2009d). Real-time PCR is a precise, robust and accurate quantification method (Bonfini et al. 2002; Zhang et al. 2003). Real-time PCR assays have been reported for quantitative detection of several GM crops such as maize (Lee et al. 2006a, b; Aguilera et al. 2009), cassava (Beltrán et al. 2009), rapeseed (Wu et al. 2007), and wheat (Li et al. 2004).

The present study reports on the development of qualitative and quantitative PCR assays for detection of commercialised *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.

Material and Methods

Planting Materials Seeds of commercialised events of *Bt* cotton, i.e. MON531 (Bollgard®I) with *cry1Ac* gene and MON15985 (Bollgard®II) with *cry1Ac* and *cry2Ab* genes along with non-transgenic cotton seeds and lyophilised leaf tissue of *Bt* brinjal, *Bt* rice and *Bt* okra with *cry1Ac* gene were provided by Maharashtra Hybrid Seeds Company Ltd. (Mahyco), Jalna. Seeds of *Bt* brinjal with synthetic *cry1Ab* gene was provided by National Research Centre on Plant Biotechnology, New Delhi, and plantlets of *Bt* potato with *cry1Ab* gene were provided by Central Potato Research Institute (CPRI), Shimla. Seeds of MON531, MON15985, non-transgenic cotton, *Bt* brinjal (*cry1Ab* gene) and non-transgenic brinjal were grown in National Containment Facility, National Bureau of Plant Genetic Resources, New Delhi, under optimum conditions. Leaf samples of *Bt* cauliflower and *Bt* brinjal with *cry1Ac* gene were provided by Sungro Seeds Pvt. Ltd., New Delhi.

Genomic DNA Extraction The genomic DNA from fresh (collected from 3–4-week-old seedlings) or lyophilised leaf tissue of *Bt* and non-*Bt* crops under study was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The DNA samples were quantified using DU 640 UV Spectrophotometer (Beckman, USA). Final concentration of extracted DNA was made up to 20 ng/µl.

Oligonucleotide Primers and Probe The primer pairs and TaqMan probe labelled with FAM and TAMRA at the 5' and 3' ends, respectively for *cry1Ac* gene were designed using the Primer Select 5.05 software (DNASTAR Inc., USA) or “Primer3 Online” primer designing software. The purified primers and probes got synthesised by Roche Applied Sciences, Germany, and Pivotal Marketing (Axygen Pvt. Ltd., India). The published primer pairs for detection of *CaMV* 35S promoter, *nptII* marker and endogenous genes were used. The details of the primer pairs and probe employed in the present study are given in Table 1.

Qualitative PCR for Detection of Bt Crops The qualitative PCR was carried out using PTC-200 Programmable Thermal Cycler (MJ Research Inc., USA). Simplex PCR assays were performed in 20 µl reaction volume containing 100 ng of genomic DNA as template, 1× polymerase buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl), 1.5 mM of $MgCl_2$, 200 µM of dNTPs, 0.5 U of Taq DNA polymerase (MBI Fermentas) and 0.25 µM each of forward and reverse primers. The PCR conditions were as follows: temperature of 94°C for 10 min, and 40 cycles of 94°C for 30 s, 59°C for 1 min, 72°C for 1 min, and 72°C for 8 min. An annealing temperature of 55°C was used for amplification of *cry1Ab* gene and amplification for control elements and marker genes were also standardised at 55°C.

Multiplex PCR assays were carried out using a final volume of 25 µl with the following reagent concentrations: as template, 100 ng of genomic DNA; 1× HotStart Taq PCR buffer; 2.0 mM of $MgCl_2$; 200 µM of dNTP mix and 0.4 U of HotStart Taq DNA Polymerase (MBI Fermentas Inc., USA). The primer concentration for each

Table 1 Primer pairs and TaqMan probe employed in the study

Primer name	Primer sequence (5'-3')	Target	Amplicon size observed in the present study	Source
For qualitative PCR				
Cry1Ac-1-f/r	f-ATTCAGCGGGCCCGAGGTTTA r-CAGCGGATGGCAAGTTAGAAGAGG	*cry1Ac*	219 bp in *Bt* cauliflower	Present study
Cry1Ac-2-f/r	f-TGACCGCTTACAAGGAGGGATACG r-CACGGAGGCATAGTCAGCAGGAAC	*cry1Ac*	230 bp in *Bt* cotton	Present study
Cry1Ac-3-f/r	f-TCCACCTTGTACAGAAGACCCT r-GGTCTCCACCAGTGAATCCTGG	*cry1Ac*	336 bp in *Bt* brinjal	Present study
Cry1Ac-4-f/r	f-TCCAAGGAGGCGACGATGTGTTC r-GCGTGTCCGTCTTGGGTCTTGA	*cry1Ac*	461 bp in *Bt* rice and *Bt* okra	Present study
Cry2Ab-f/r	f-GGACCTACCGCGACTACCTGAAGA r-TGAACGGCGATGCACCAATGTC	*cry2Ab*	453 bp in MON15985 event of *Bt* cotton	Present study
Cry1Ab F/R	f-TCTCCAACTACGACAGCAGGACCT r-GGTGAATCCACGAGAACATGGGAG	*cry1Ab*	620 bp in *Bt* brinjal and *Bt* potato	CPRI, Shimla
Npt II F/R	f-CTCACCTTGCTCCTGCCGAGA r-CGCCTTGAGCCTGGCGAACAG	*nptII*	215 bp in *Bt* brinjal with *cry1Ab* gene	Singh et al. 2008
nptII F/R	f-GGGCGCCCGGTTCTTTTTG r- ACACCCAGCCGGCCACAGTCG	*nptII*	515 bp in *Bt* rice and *Bt* okra	Randhawa et al. 2009b
SP1 F/R	f-TTGCTTTGAAGACGTGGTTG r-ATTCCATTGCCCAGCTATCT	*CaMV* 35S promoter	196 bp in *Bt* brinjal and *Bt* potato	Designed in the laboratory
35 S-F/R	f-GCTCCTACAAATGCCATCA r-GATAGTGGGATTGTGCGTCA	*CaMV* 35S promoter	195 bp in *Bt* okra, *Bt* cauliflower	Lipp et al. 1999
fsACP-2F/2R	f-CAAACAAGAGACCGTGGATAAGGTA r-CAAGAGAATCAGCTCCAAGATCAAG	*fsACP* (endogenous gene for cotton)	116 bp in cotton	Lee et al. 2007
Pomtom F/R	f-CTGCCTCCGTCAAGATTTGGTCACT r-CTCTTCCCTTTCTTGATGG	*β-fructosidase* (endogenous gene for brinjal)	141 bp in brinjal	Chaouachi et al. 2008
Rice1/rice2	f-GCG CCC AAC CTA CAC CAA CC r-GGC CTT CTC CGC AGA GAT CAC T	*α-tubulin* (endogenous gene for rice)	295 bp in rice	Peterhänsel et al. 2004
SRK1,2	f-TTGGGAGGCTATTCGACACAGAGG r- CAAGCCAATCCATGAAAGCAGTCC	*SRK* (endogenous gene for cauliflower and other *Brassicaceae* members)	311 bp in cauliflower	Present study
UGP-af7/af8	f-GGACATGTGAAGAGACGGAGC r-CCTACCTCTACCCCTCCG	*UGPase* (endogenous gene for potato)	88 bp in potato	European Commission Protocol 2006
PlantA1/plantA2	f-CGAAATCGGTAGACGCTACG r-GGGGATAGAGGGACTTGAAC	*Chloroplast specific tRNA gene*	610 bp in okra	Taberlet et al. 2001
For real-time PCR				
Cry1Ac-f/r	f-TTGCCTGAGTTGTCCGTGATC r-GACGGAACGCTGATTGTTCTGT	*cry1Ac*	–	Present study
Cry1Ac-TM (probe)	FAM-ACGGTGACTTCAACAATGG CCTCA-TMR	*cry1Ac*	–	Present study

specific primer pair was standardised, which varied from 0.2 to 0.4 μM, depending upon the intensity and visibility of amplification products of each primer on agarose gel. The primer concentration of 0.2 μM for *CaMV* 35S promoter and endogenous genes, 0.3–0.4 μM for *nptII* marker gene and 0.3 μM for *cry1Ac* gene and 0.25 μM for *cry1Ab* gene were used. The amplification conditions for multiplex assays with *cry1Ac* gene specific primers were: initial denaturation at 95°C for 10 min, 40 cycles consisting of denaturation at 95°C for 50 s, primer annealing at 59°C for 50 s, primer extension at 72°C for 50 s; and final extension at 72°C for 5 min. Whereas for multiplex assays with *cry1Ab* gene specific primers, PCR cycling conditions were adjusted to have an initial denaturation step at 95°C for 10 min, followed by 40 cycles, which involved 94°C for 50 s, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by final extension at 72°C for 8 min.

The PCR amplified products were resolved on 2.0% (*w*/*v*) agarose gel (Lonza, Rockland, ME, USA) or 4.0% (*w*/*v*) metaphor® agarose gel (Cambrex Bio Science Rockland, Inc. Rockland, ME, USA) stained with ethidium bromide using 1× TAE as running buffer on horizontal electrophoresis. The amplification pattern of products was then visualised under UV light and photographed using Gel Documentation Imaging System (Alpha Innotech, USA).

Reference Molecule for Real-Time PCR As a reference molecule, a standard plasmid was constructed on the basis of a pCR®2.1-TOPO® vector (Invitrogen Life Technologies Inc.), in which the real-time PCR product amplified with the designed primer pair specific for *cry1Ac* gene was integrated using the TOPO TA cloning kit (Invitrogen Life Technologies Inc.). This recombinant plasmid was used to transform *Escherichia coli* strain TOP10 cell (Invitrogen Life Technologies Inc.). The cloned plasmid was selected by restriction digestion with *Eco*RI (Roche Applied Sciences, Germany). The cloned plasmid's DNA was extracted by the Qiagen Plasmid Midi kit (Qiagen, Germany), which was digested with *Hind*III restriction endonuclease. The linearized plasmid DNA was purified from 2% agarose gel by the QIA Quick Gel Extraction kit (Qiagen, Germany). The concentration of the plasmid DNA was measured using a DU 640 UV Spectrophotometer (Beckman, USA). The standard plasmid was serially diluted to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 copies/µl, which were then used as calibrant for quantitation.

Real-time PCR for Quantitative Analysis of Bt Crops Real-time PCR assay was performed using Light cycler®480 system (Roche Applied Science, Germany). In each well of a 96-well plate, 20 µl volume of reaction mixture was composed of 100 ng of genomic DNA as template, 0.4 µM of primer pair, 0.1 µM probe and 10.0 µl of universal master mix (Roche Applied Science, Germany). The PCR conditions were as follows: denaturation at 95°C for 7 min, 55 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 1 min and extension at 72°C for 1 s.

For the generation of standard curve, eight serial dilutions of constructed standard plasmid DNA with 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 copies/µl were used as standards. Repeatability of the standard plasmid's copy numbers was estimated from the data of triplicate reactions. Accuracy and precision of the developed assays were determined by calculating the standard deviation and relative standard deviation values.

Traceability for Specific cry Gene in Bt and Non-Bt Crops For the development of efficient traceability system, the assays for detection of specific *cry* gene with the detection limit as low as 0.01% were developed.

The sensitivity for developed simplex PCR assays with primer pairs specific for *cry1Ac* and *cry1Ab* genes was assessed using the serially diluted DNA samples of *Bt* crops as reference materials. The reference samples were prepared by mixing the 20 ng/µl DNA sample of *Bt* crop (100% GM) with DNA of its non-*Bt* counterpart to obtain different percentages of GM trait, i.e. 100%, 10%, 1.0%, 0.1%, 0.05% and 0.01%. A volume of 5 µl of the serially diluted DNA was used for PCR.

Similarly, serial dilutions with different percentages of transgene, i.e. 100%, 50%, 10%, 5.0%, 1.0%, 0.1%, 0.05% and 0.01% were used as reference samples to assess the sensitivity of developed quantitative real-time PCR assay for detection of *cry1Ac* gene. The sensitivity of real-time assays was evaluated by comparing the experimental mean value with the theoretical value of the GM content.

Results and Discussion

For ensured food safety and quality of GM crops and to address consumer concerns, it is necessary that the efficient analytical methodologies are available for GM testing (Rodríguez-Lázaro et al. 2007). The present study reports on the development of robust, cost-effective and sensitive PCR methods, which can be used to qualitatively and quantitatively detect different *Bt* crops to meet the regulatory obligations, to address the consumers concerns and to solve the legal disputes, if arise.

Qualitative Detection of Bt Crops The simplex and multiplex PCR assays were developed for amplification of specific *cry* genes (*cry1Ac*/*cry1Ab*/*cry2Ab*) individually as well as simultaneously with marker gene, *CaMV* 35S promoter and endogenous gene in different *Bt* crops, i.e. MON531 and MON15985 events of *Bt* cotton with *cry1Ac* and *cry2Ab*, *Bt* brinjal events with *cry1Ac* and *cry1Ab* genes, *Bt* rice, *Bt* cauliflower and *Bt* okra with *cry1Ac* gene, *Bt* potato with *cry1Ab* gene. To check the specificity of each primer pair for *cry* genes, simplex PCR was carried out. The amplicons of desired size were detected for specific *cry* genes, i.e. 230 bp for *cry1Ac* gene in MON531 and MON15985 events of *Bt* cotton, 336 bp for *cry1Ac* gene in *Bt* brinjal, 461 bp for *cry1Ac* gene in *Bt* rice and *Bt* okra, 219 bp for *cry1Ac* gene in *Bt* cauliflower, 453 bp for *cry2Ab* gene in MON15985 event of *Bt* cotton, and 620 bp for modified/truncated *cry1Ab* gene in *Bt* brinjal and *Bt* potato. However, no amplicons were detected in non-*Bt* counterparts and water control.

Reliable, cost-effective and efficient multiplex PCR assays in duplex, triplex and tetraplex formats were developed for qualitative detection of these *Bt* crops. The developed assays allowed simultaneous amplification of the

multiple target sequences of the inserted gene construct, i.e., *cry* gene, control element (*CaMV* 35S promoter), marker gene along with endogenous reference gene. In all the developed multiplex PCR assays, the amplification of endogenous gene for each crop was included as an internal control. The detection of endogenous reference gene provides an efficient method to evaluate DNA quality and PCR efficiency, thus, reducing the risk of false negatives.

Bt Crops with *cry1Ac* Gene

1. *Bt* cotton MON531 and MON15985 events: A triplex PCR assay was developed simultaneously amplifying the *cry1Ac* and *cry2Ab* genes along with the endogenous fibre-specific acyl carrier protein (*fsACP*) gene for differentiating the MON531 and MON15985 events of *Bt* cotton. The desired amplicon of 230 bp for *cry1Ac* gene was amplified in both the events, whereas the amplicon of 453 bp for *cry2Ab* gene was detected only in MON15985 event, and no amplification for these two genes were detected in non-*Bt* cotton and water control (Fig. 1a). However, 116-bp sized amplicon for endogenous *fs-ACP* gene, used as internal control, was amplified in *Bt* as well as non-*Bt* cotton.
2. *Bt* brinjal with *cry1Ac* gene: On triplex PCR, the desired amplicons of 336 bp for *cry1Ac* gene, 196 bp for *CaMV* 35S promoter and 141 bp for endogenous *β-fructosidase* gene were detected simultaneously in *Bt* brinjal, and amplicon for *β-fructosidase* gene was also detected in non-*Bt* brinjal being an endogenous (Fig. 1b).
3. *Bt* rice: Using triplex PCR, the desired amplicons of 461 bp for *cry1Ac* gene, 515 bp for *nptII* marker gene

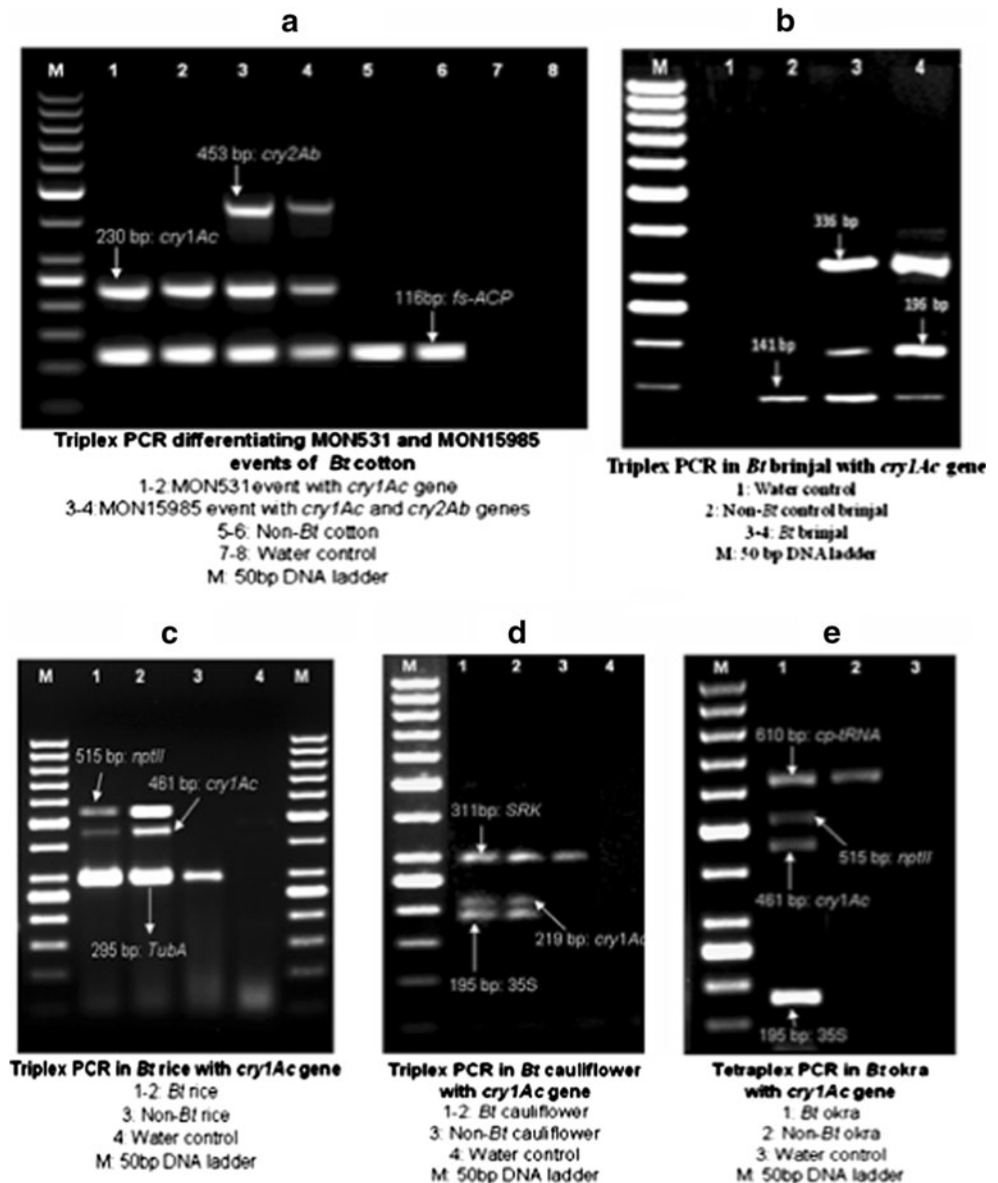

Fig. 1 Multiplex PCR assays for qualitative detection of *Bt* crops with *cry1Ac* gene

and 295 bp for endogenous *α-tubulin* gene were simultaneously detected in *Bt* rice, and amplicon for *α-tubulin* gene was also detected in non-*Bt* rice (Fig. 1c).

4. *Bt* cauliflower: The desired amplicons of 219 bp for *cry1Ac* gene, 195 bp for *CaMV* 35S promoter and 311 bp for endogenous *SRK* (*S*-locus receptor kinase) gene were simultaneously detected in *Bt* cauliflower on triplex PCR and 311-bp specific to *SRK* gene was also detected in non-*Bt* cauliflower (Fig. 1d).
5. *Bt* okra: Tetraplex PCR assay was developed for simultaneous amplification of 461 bp for *cry1Ac* gene, 515 bp for *nptII* marker gene, 195 bp for *CaMV* 35S promoter and 610 bp for plant specific *chloroplast-tRNA* gene (*cp-tRNA*) in *Bt* okra. So far, no endogenous gene for okra has been validated, hence, in the present study, the plant specific *cp-tRNA* (Taberlet et al. 1991) was included as internal control, which was also detected in non-*Bt* okra (Fig. 1e).

Bt Crops with *cry1Ab* Gene

6. *Bt* brinjal with *cry1Ab* gene: Duplex PCR assays simultaneously amplifying (a) 620 bp for *cry1Ab* gene and 196 bp for *CaMV* 35 S promoter; (b) 620 bp for *cry1Ab* gene and 141 bp for endogenous *β- fructosidase* gene in *Bt* brinjal were developed, whereas desired region of endogenous *β-fructosidase* gene was also amplified in non-*Bt* brinjal. (Fig. 2a).
7. *Bt* potato: Using tetraplex PCR, the desired amplicons of 620 bp for *cry1Ab* gene, 215 bp for *nptII* marker gene, 196 bp for *CaMV* 35S promoter and 88 bp for endogenous *UGPase* gene were detected in *Bt* potato. Desired amplicon for *UGPase* gene was also detected in non-*Bt* potato (Fig. 2b).

Standard Plasmid as a Reference Molecule for Real-Time PCR and Quantitative Analysis of Bt Crops The standard plasmid was constructed by the integration of PCR product specific for *cry1Ac* gene pCR®2.1-TOPO® vector (Fig. 3). As a reference molecule, eight levels of standard plasmids for *cry1Ac* gene were set to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 copies per reaction for the quantitative real-time PCR. In the eight levels of the standard samples, the repeatability of the standard plasmid's copy numbers was calculated from the data of triplicate reactions. The values of relative standard deviation (RSD) of the triplicate reactions ranged from 1.71% to 12.2% (Table 2). All of the RSD values were found less than 20% indicating that variation in this range was not significant; hence, the standard plasmid was confirmed to be a stable and reliable reference molecule.

Standard curve was generated using the serial dilutions of the known standard with 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 copies/μl. The linearity of the standard curve for *cry1Ac* gene was confirmed in the quantitative PCR using the designed primer pair, probe and the standard plasmid, and the correlation coefficient (r^2) of the regression line was 0.99 with the $Y = -3.435 \times +38.12$ regression equation (Fig. 4a). Good linearity between copy number and fluorescence values (Ct) as visualised in the calibration curves for *cry1Ac* indicated that the developed real-time PCR assay combined with reference molecule in this study were suitable for further quantitative measurements. Hence, the standard curve generated can be utilised for the quantitative detection of *Bt* crops by estimating the copies of *cry1Ac* gene present in the unknown samples.

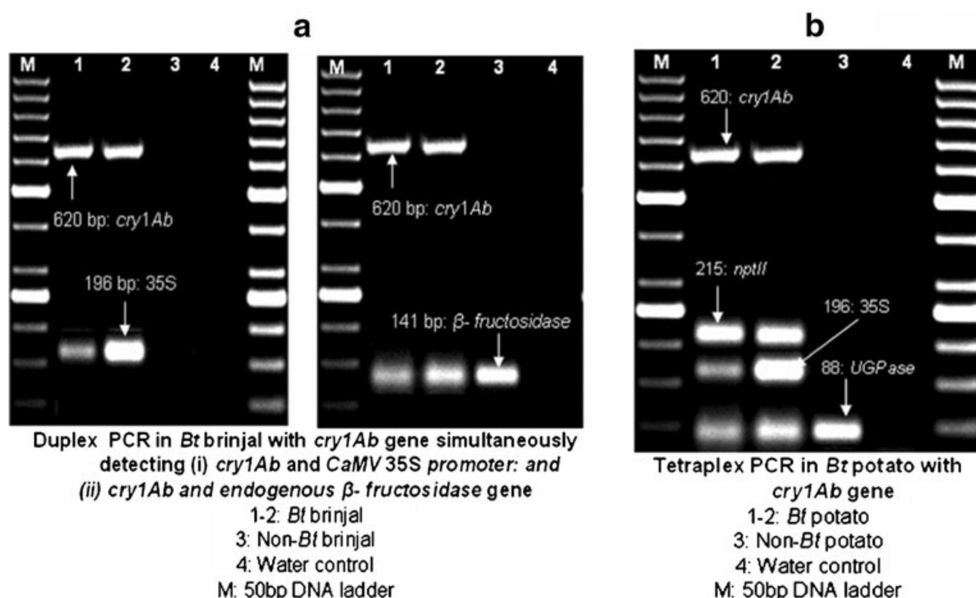

Fig. 2 Multiplex PCR assays for qualitative detection of *Bt* crops with *cry1Ab* gene

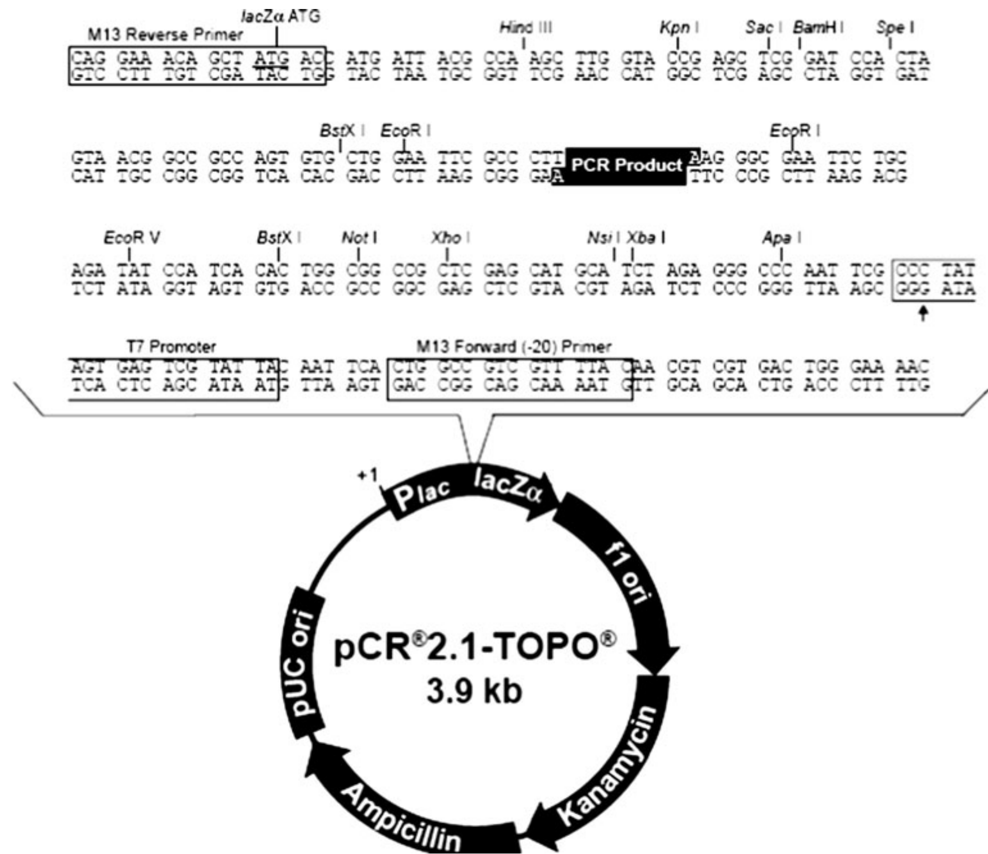

Fig. 3 Schematic presentation of pCR®2.1-TOPO® vector used as a reference molecule showing the integration site for PCR product

In real-time PCR assay, all the *Bt* crops with *cry1Ac* gene gave fluorescent signals whereas no signals were detected in non-*Bt* counterparts and water control (Fig. 4b).

Sensitivity of Developed PCR Assays To assess the sensitivity of developed simplex PCR assays for *cry1Ac* gene, the serial dilutions of DNA samples in six mixing levels with different percentage of transgene content, i.e. 100%, 10%, 1.0%, 0.1%, 0.05% and 0.01% used as reference samples were tested. In simplex PCR, using primer pair Cry1Ac-4-f/r for *cry1Ac* gene, the amplicon of desired size, i.e. 461 bp was detected in all the dilutions up to 0.01% of *Bt* rice whereas no amplicon was detected in non-*Bt* sample (Fig. 5a) and *Bt* okra. Similarly, the desired amplicon of 610 bp for *cry1Ab* gene was also detected in all the serial dilutions up to 0.01% of *Bt* brinjal (Fig. 5b) and *Bt* potato. No desired amplicon for *cry1Ac* or *cry1Ab* gene was detected in non-*Bt* counterpart.

Table 2 Repeatability of the copy number of standard plasmids for *cry1Ac* gene

True value	Mean value	Relative Standard Deviation
10	12.1	12.2
100	487	9.7
1,000	1520	1.71
10,000	13,000	4.07
100,000	159,000	3.78
1,000,000	1,450,000	6.54
10,000,000	12,700,000	3.18
100,000,000	103,000,000	2.54

In real-time PCR assay with primer pair and TaqMan probe specific to *cry1Ac* gene, all the reference samples with 100%, 50%, 10%, 5.0%, 1.0%, 0.1%, 0.05% and 0.01% transgene content showed the amplification signals whereas no signal was detected in non-*Bt* samples. The precision of the method was evaluated as the bias (percent) of the experimental mean value from the theoretical value. The accuracy was evaluated by RSD values. At low mixing levels, i.e. 1.0%, 0.5%, 0.05%, 0.01%, the biases were 7.0%, −7.8%, −2.0% and 10%, respectively, and their RSDs were 5.1%, 14.5%, 4.2% and 18.2%, respectively (Table 3). Overall, the values of RSDs of three-time repeated tests ranged from 0.33–18.2% for *cry1Ac* gene. According to the approach suggested by Codex, the limit of quantification (LOQ) should correspond to the lowest level of analyte, for which the RSD is 25% or less (Codex Alimentarius Commission 2001). In this study, the RSD value of the lowest concentration level (0.01%) was also below the 25% criteria. In conclusion, according to the Codex Alimentarius guidelines, the LOQ of this method

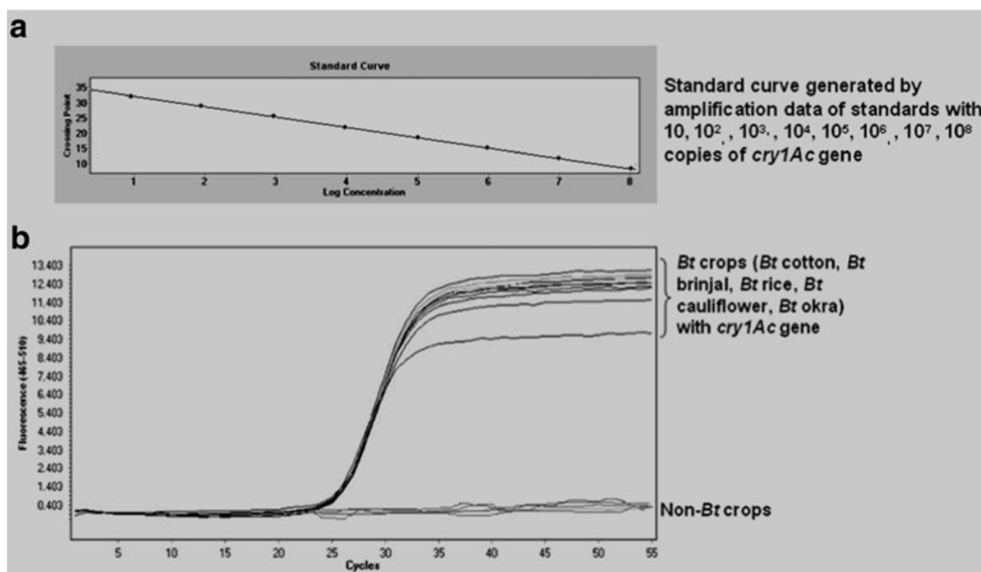

Fig. 4 Quantitative analysis of *Bt* crops on real-time PCR using primers and Taqman probe specific to *cry1Ac* gene

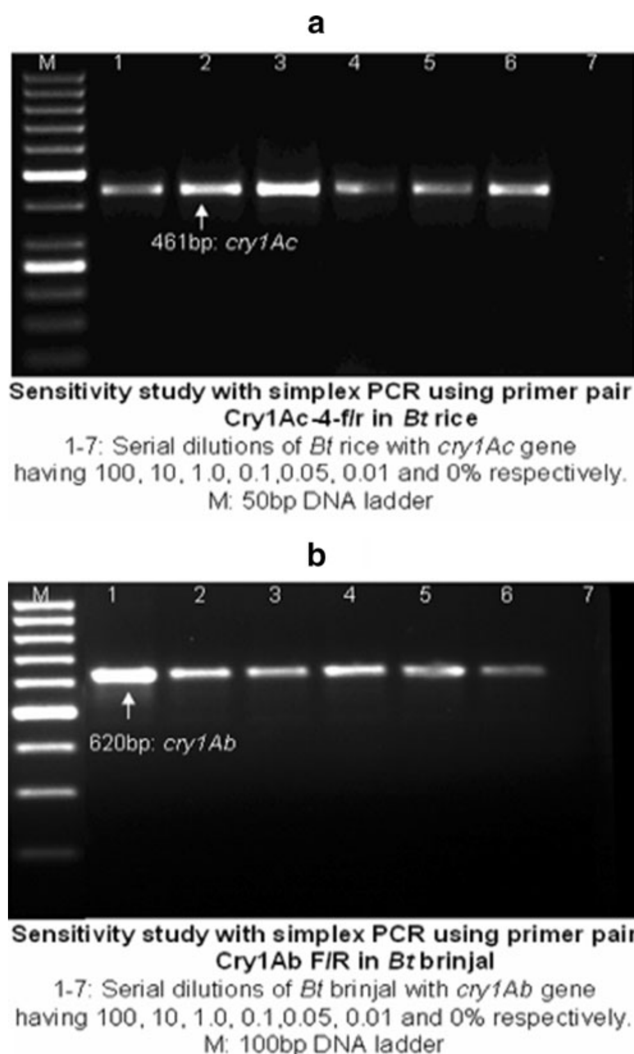

Fig. 5 Sensitivity of developed simplex PCR assays for detection of *cry* (*cry1Ac and cry1Ab*) genes

was 0.01%, which is a feasible level for detection of a particular GM crop.

The reported sensitivity of developed PCR assays may be applicable for GM detection for regulatory compliance, which will further provide an efficient traceability system for *Bt* crops.

Conclusions

In the present study, development of qualitative and quantitative PCR assays has been reported for testing of *Bt* crops, which are either commercialised or are under different stages of field trials in India. Multiplex PCR assays in duplex, triplex and tetraplex formats can be efficiently utilised in qualitative detection of *Bt* crops to meet the regulatory obligations prior to their commercial-

Table 3 Accuracy and precision statistics for quantitative real-time assay for *cry1Ac* gene

True value of transgene content (%)	Accuracy		Precision	
	Mean transgene content (%)	Bias true value (%)	Standard Deviation	Relative Standard Deviation
0.01	0.011	10.0	0.002	18.2
0.05	0.049	−2.0	0.0021	4.2
0.5	0.461	−7.8	0.067	14.5
1.0	1.07	7.0	0.055	5.1
10	9.93	−0.7	0.25	2.5
50	50.01	0.02	0.48	0.96
100	99.7	−0.3	0.33	0.33

isation and post-release monitoring studies as well as for traceability. The reported sensitivity of developed simplex PCR assays to detect *cry1Ac* or *cry1Ab* gene based on serial dilutions of the extracted DNA of *Bt* crops with the DNA of non-*Bt* counterpart was up to 0.01%, which is also in compliance with the Supreme Court of India's stipulation of developing a protocol for testing contamination to a detection limit of 0.01% prior to conducting field trials of GM crops. The validated real-time PCR assays will have immense use in estimating the copies of inserted *cry1Ac* gene and quantitative analysis of *Bt* crops with *cry1Ac* gene to meet the threshold level.

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