Research Article

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Detection and identification of genetically modified EE-1 brinjal (*Solanum melongena*) by single, multiplex and SYBR® real-time PCR

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Abstract

BACKGROUND: Brinjal is an important vegetable crop. Major crop loss of brinjal is due to insect attack. Insect-resistant EE-1 brinjal has been developed and is awaiting approval for commercial release. Consumer health concerns and implementation of international labelling legislation demand reliable analytical detection methods for genetically modified (GM) varieties.

RESULTS: End-point and real-time polymerase chain reaction (PCR) methods were used to detect EE-1 brinjal. In end-point PCR, primer pairs specific to 35S CaMV promoter, NOS terminator and nptII gene common to other GM crops were used. Based on the revealed 3′ transgene integration sequence, primers specific for the event EE-1 brinjal were designed. These primers were used for end-point single, multiplex and SYBR-based real-time PCR. End-point single PCR showed that the designed primers were highly specific to event EE-1 with a sensitivity of 20 pg of genomic DNA, corresponding to 20 copies of haploid EE-1 brinjal genomic DNA. The limits of detection and quantification for SYBR-based real-time PCR assay were 10 and 100 copies respectively.

CONCLUSION: The prior development of detection methods for this important vegetable crop will facilitate compliance with any forthcoming labelling regulations.

Supporting information may be found in the online version of this article.

Keywords: genetically modified organisms; cry1Ac gene; event-specific PCR; *Bt* brinjal

INTRODUCTION

Brinjal or eggplant (*Solanum melongena* L.) is an important solanaceous crop of the subtropics and tropics.[1] It is grown extensively in India, Bangladesh, Pakistan, China and the Philippines. The species *S. melongena* has been known in India from ancient times.[2] The global area under brinjal cultivation is 1.67×10^6 ha, yielding about 42.94×10^6 t of brinjal fruit.[3] India accounts for about 10.37×10^6 t, with an area of 0.6×10^6 ha under cultivation, making it the second largest producer of brinjal in the world after China.[3] Brinjal is primarily consumed as a cooked vegetable in various ways. It is a good source of minerals and vitamins and is rich in total water-soluble sugars, free reducing sugars and amide proteins among other nutrients.[4] Brinjal is subject to attack by many pests, with the fruit and shoot borer *Leucinodes orbonalis* being the most destructive. The estimated damage caused ranges from 50 to 70% of the crop, corresponding to around $221 million in economic terms.[5,6]

Efforts have been made to develop insect-resistant brinjal varieties by incorporating cry1Ac gene from *Bacillus thuringiensis*, commonly known as *Bt* brinjal. A *Bt* brinjal developed by M/s Maharashtra Hybrid Seeds Company Limited contains cry1Ac gene (event EE-1). In view of the lack of safety data concerning the long-term impact on human health and the environment, the regulatory authorities in India, adopting a cautious, precautionary principle-based approach, have imposed an indefinite moratorium on the commercial release of *Bt* brinjal. Analytical methods for the detection and identification of *Bt* brinjal (event EE-1) are required for use in the future.

Polymerase chain reaction (PCR) is currently the method of choice for the rapid, sensitive and specific detection and quantitation of genetically modified (GM) crops. Our laboratory has previously reported PCR systems for the detection of Roundup Ready (RR) soya, MON810 maize and wheat.[7,8] Soya, maize and cotton being the principal GM crops, various PCR-based methods are available for their detection.[9–11] The analytical methods reported for the detection of insect-resistant crops target common genetic elements such as cry1Ac, nptII, CaMV 35S, etc.[12] Although useful for screening, these assays are limited by the fact that several genetically modified organisms (GMOs) have the same detectable elements. The method does not differentiate between *Bt* crops such as cotton, brinjal, rice, cauliflower, potato and okra.[12] The focus of the present study was to establish a very specific detection method for insect-resistant EE-1 brinjal harbouring cry1Ac. An

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event-specific PCR based on the 3′ flanking region for the unequivocal identification and quantification of insect-resistant brinjal EE-1, employing both a conventional and a real-time PCR format, is described. Furthermore, a multiplex PCR was developed to detect multiple genetic elements simultaneously, using a primer pair specific to event EE-1 and two primer pairs specific to the construct-specific region. This multiplex PCR format, which is very specific to event EE-1, is expected to speed up routine analysis in quality control laboratories.

MATERIALS AND METHODS

Materials

EE-1 brinjal (*S. melongena* L.) fruits and insect-resistant cauliflower florets were a kind gift from Sungro Seeds Pvt Ltd (Delhi, India). *Bt* cotton (MRC-6918 *Bt*) seeds and non-transgenic brinjal were purchased from a local market. Certified reference materials (CRMs) used in this study were 50 g kg^{-1} RR soya (IRMM-410), *Bt*176 maize (IRMM-411), *Bt*11 maize (IRMM-412), MON810 maize (IRMM-413) and TC1507 maize (ERM-Bf 418C). These CRMs were purchased as dry powders from Fluka (Buchs, Switzerland). *Taq* PCR core kit and RNase A were obtained from Qiagen (Hilden, Germany). Agarose was from Amresco (Solon, OH, USA). Ethidium bromide and GenElute™ Plant Genomic DNA Miniprep extraction kit were procured from Sigma Aldrich (Bangalore, India). Maxima™ SYBR Green/ROX qPCR Mastermix kit, mass loading dye (6×) and 100 bp DNA ladder were obtained from Fermentas (Glen Burnie, MD, USA), while 1 kb DNA ladder was purchased from Bangalore Genei (Bangalore, India).

Isolation of genomic DNA

Insect-resistant cauliflower florets and non-transgenic and EE-1 brinjal fruits (without stalks) were diced using a sterile scalpel and dried at 37 °C to a moisture content below 100 g kg^{-1}. Samples were then ground to fine powders, sieved (aperture size 450 μm) and stored at −20 °C until use. DNA was extracted from the individual powders, including CRMs (100 mg), using the GenElute™ Plant Genomic DNA Miniprep extraction kit (Sigma Aldrich) according to the manufacturer's protocol. DNA concentration was determined from the A_{260} value (1 absorbance unit = 50 μg mL^{-1} double-stranded DNA) measured using a UV–visible spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). The purity of the isolated DNA was assessed via the $A_{260/280}$ ratio, with a value of 1.8 being considered as pure.

Oligonucleotide primers

Target genes, primer sequences and expected amplicon lengths are listed in Table 1. On the basis of the published DNA sequences and the data obtained by sequencing a portion of the gene of interest, primers were designed using Primer Express Version 2.0 (Applied Biosystems, Foster City, CA, USA). Primers synthesised by Sigma Genosys (Sigma-Aldrich, Bangalore, India) were diluted to a final concentration of 10 pmol μL^{-1} with Milli-Q water and stored at −20 °C until use.

PCR conditions

The PCRs comprised 1× PCR buffer (containing 1.5 mmol L^{-1} $MgCl_2$), 200 μmol L^{-1} dNTPs, 2.5 units of *Taq* polymerase, 20 pmol of forward and reverse primers and 100 ng of genomic DNA in a total volume of 50 μL. The thermal step cycle programme included denaturation at 95 °C for 5 min, denaturation at 95 °C for 20 s, annealing at 52 °C (nptII), 60 °C (NOS) and 62 °C (CaMV 35S promoter and T-DNA RB-plant genome) for 40 s and extension at 72 °C for 60 s. The cycle was repeated 35 times and followed by a final extension at 72 °C for 7 min. All reactions were amplified in a thermal cycler (GeneAmp® PCR System 9600, Applied Biosystems, Foster City, CA, USA). The amplifiability of the extracted DNA was examined by carrying out a eukaryote-specific PCR targeting the 18S rDNA.[13] The PCR mixture contained 1× PCR buffer, 200 μmol L^{-1} dNTPs, 0.6 units of *Taq* polymerase, 20 pmol of each primer and 100 ng of template DNA. The following cycle conditions were used: denaturation of DNA at 95 °C for 4 min 30 s, followed by 30 cycles of denaturation at 95 °C for 46 s, annealing

Table 1. Nucleotide sequences of primers used in study

Target gene	Primer	Sequence	Amplicon size (bp)	Reference
CaMV 35S promoter	35S-111 F	5′-GTGGTCCCAAAGATGGACCC-3′	111	This study
	35S-111 R	5′-CCCTTACGTCAGTGGAGATATCACA-3′		
nptII	Tn5-1	5′-GGATCTCCTGTCATCT-3′	173	15
	Tn5-2	5′-GATCATCCTGATCGAC-3′		
NOS terminator	NOS F	5′-TTAAGATTGAATCCTGTTGCCG-3′	192	14
	NOS R	5′-TAATTTATCCTATCCTAGTTTGCGCGC-3′		
18S rDNA	TRO3	5′-TCTGCCCTATCAACTTTCGATGGTA-3′	137	13
	TRO4	5′-AATTTGCGCGCCTGCTGCCTTCCTT-3′		
CaMV 35S promoter-cry1Ac	*Bt*B-35S F[a]	5′-GAGGACACGCTGACAAGCT-3′	181	This study
	*Bt*B-cry1Ac R[a]	5′-GCTGAGCAGAAACTGTGTGC-3′		
T-DNA RB-plant genome	EE-1 F[b]	5′-CGTTTCCCGCCTTCAGTTTA-3′	151	This study
	EE-1 R[b]	5′-GCGGTGATAATTGAATGCAT-3′		
nptII-NOS terminator	*Bt*B-nptII F[a]	5′-ACGCCGGCTGGATGATC-3′	126	This study
	*Bt*B-NOS R[a]	5′-AAGACCGGCAACAGGATTCA-3′		
Brinjal plant	BPlant-102 F	5′-TGCTGTCAATAAACACTTAGAAAGG-3′	102	This study
	BPlant-102 R	5′-TGATAATTGATGCATCTCTTGAAG-3′		

[a] Primers designed for multiplex PCR.
[b] Primers used in event-specific end-point and real-time PCR.

at 65 °C for 1 min 26 s and extension at 72 °C for 1 min 25 s. A final extension step was performed at 72 °C for 3 min 15 s.

For multiplex PCR the reaction mixture comprised 1× PCR buffer, 2.5 mmol L^{-1} $MgCl_2$, 200 µmol L^{-1} dNTPs, 2.5 units of *Taq* polymerase, 20 pmol each of *Bt*B-nptII F, *Bt*B-NOS R, *Bt*B-35S F and *Bt*B-cry1Ac R, 10 pmol each of EE-1 F and EE-1 R (Table 1) and 100 ng of template DNA. The thermal cycling parameters were as described for CaMV 35S promoter.

The amplified products were resolved by electrophoresis on 20 g kg^{-1} agarose gel (supplemented with 0.5 µg mL^{-1} ethidium bromide) in 0.5× TBE buffer for 30 min at 150 V and visualised using the Gel Doc™ 2000 documentation system (Bio-Rad, Hercules, CA, USA). Amplicons obtained by multiplex PCR were resolved on 25 g kg^{-1} agarose gel. All samples were analysed in triplicate.

DNA sequencing

The PCR amplicons were purified with the Genelute™ PCR clean-up kit (Sigma Aldrich) and subjected to DNA sequencing using the respective forward and reverse primers and the BigDye Terminator® Version 3.1 cycle sequencing kit (Applied Biosystems). The BigDye terminators were removed with the DyeEx 2.0 spin kit (Qiagen), and the DNA sequences were determined on a 310 Genetic Analyzer using Sequence Analysis Version 3.7 (Applied Biosystems, Foster City, CA, USA).

SYBR Green real-time PCR (RT-PCR)

Real-time PCR assays were performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in a 25 µL reaction volume containing 5 µL of tenfold serially diluted EE-1 brinjal genomic DNA (10^5 copies down to 10 copies) in nuclease-free water, 2× Maxima™ SYBR Green/ROX qPCR Mastermix and 200 nmol L^{-1} EE-1 F/EE-1 R primers. The thermal cycling programme comprised a single cycle of DNA polymerase activation for 10 min at 95 °C followed by 40 amplification cycles of 15 s at 95 °C and 1 min at 60 °C. Subsequent to PCR amplification, melting curve analysis of the amplicons was performed using Dissociation Curve Analysis Version 1.0 (Applied Biosystems, Foster City, CA, USA). The PCR products were heated to 95 °C for 15 s, cooled to 60 °C for 20 s and then slowly heated back to 95 °C at a rate of 0.2 °C s^{-1}. The melting temperature (T_m) of the amplicons was calculated by plotting the negative derivative of fluorescence over temperature *versus* temperature ($-dF/dT$ *vs* T). The fluorescence reporter signal was normalised against the internal reference dye (ROX) signal, and, after manual adjustment of the baseline and the fluorescence threshold line, threshold cycle (*Ct*) values were determined using SDS Version 2.1 (Applied Biosystems, Foster City, CA, USA). The *Ct* value of each real time PCR was plotted against the logarithm of the estimated copy number, and the linear regression was calculated. All reactions were performed in triplicate.

Determination of limits of detection and quantification

To determine the limits of detection (LOD) and quantification (LOQ), five different haploid genomic DNA concentrations (100 000, 10 000, 1000, 100 and 10 copies) of EE-1 brinjal were used.

RESULTS AND DISCUSSION

Screening PCR for detection of EE-1 brinjal

Screening PCR assays are usually based on the promoter and terminator genetic elements used in numerous GMO constructs. Owing to their presence in several GMOs, such assays do not allow identification of a GMO event, yet are useful as routine GMO screening methods. These simple PCR methods allow a low-cost and rapid first-level screening. Using the promoter (CaMV 35S), *Agrobacterium tumefaciens* nopaline synthase terminator (T-NOS) and antibiotic-resistant marker neomycin phosphotransferase II (nptII) gene sequences available in the public domain, the following screening methods were optimised. The primer pair NOS F/NOS R specifically amplifies a 192 bp fragment of the nopaline synthase terminator gene derived from *A. tumefaciens*.[14] The positive amplification of the 192 bp fragment only in EE-1 brinjal (Fig. 1(a), lane 4) and its absence in native brinjal (Fig. 1(a), lane 3) confirm the presence of the NOS terminator in EE-1 brinjal. The nptII gene in GMOs is often used as a selectable marker. Expression of the nptII gene confers resistance to kanamycin and allows for the selection of positive transformations.[15] The presence of the nptII gene in EE-1 brinjal was demonstrated using the primer pair Tn5-1/Tn5-2. A 173 bp fragment of the nptII gene was amplified with EE-1 brinjal DNA (Fig. 1(a), lane 6), confirming the presence of nptII. The primer pair 35S-111 F/35S-111 R (Table 1) was designed to amplify a 111 bp fragment from the 35S promoter of cauliflower mosaic virus (Accession No. EU 477376.1) for the general screening of GM foods. Owing to the presence of an enhanced promoter, a 374 bp amplicon in addition to the 111 bp amplicon was observed in EE-1 brinjal (Fig. 1(a), lane 8). The 374 bp PCR product was purified and subjected to dideoxy sequencing, which confirmed the presence of an enhanced 35S promoter. The presence of a complementary annealing site for the forward primer 35S-111 F (Fig. 1(b)) resulted in the amplification of this 374 bp fragment. These results are similar to those reported for transgenic cotton[16] and potato.[17] Amplification of the CaMV 35S promoter in these two transgenic crops resulted in a 195 bp and an approximately 450 bp fragment corresponding to E-35S. In transgenic RR soya and MON810 maize a single 111 bp amplicon was observed (Fig. 1(a)), indicating the absence of the E-35S promoter. No amplicon was observed when DNA from conventional non-transgenic brinjal was used as the template (Fig. 1(a), lane 7). These results are concurrent with the data on an enhanced version of the 35S promoter (E-35S) used in EE-1 brinjal.

Event-specific PCR for qualitative detection of EE-1 brinjal

Four different strategies, i.e. screening, gene-specific, construct-specific and event-specific, are currently used to detect GMOs. Among these, the best and most specific PCR method is the event-specific strategy, amplifying integration of the host genome and transgenic construct. An event is identified as the integration locus between the exogenous DNA and host genome, which is the only unique signature of a transformation event.[18] So far, several event-specific qualitative and quantitative PCR methods have been established for RR soybean,[19] MON863,[20] genetically modified potato,[21] MON810 maize,[22] *Bt*11 maize,[23] GA21 maize[18] and *Bt*176 maize.[24] An event-specific PCR method to identify EE-1 brinjal is not available. Sequences specific for the transgenic event EE-1 brinjal were retrieved from the European Commission Joint Research Centre (EC-JRC) database using the MRS search engine (http://mrs.cmbi.ru.nl/mrs-web/). The molecular description of EE-1 brinjal encompassing several patents in conjunction with *in silico* analysis resulted in the design of two event-specific primers EE-1 F and EE-1 R (Table 1) targeting the 3′ transgene integration site (Accession No. CS 803547, EC-JRC database). These primers were initially used to establish a qualitative PCR assay specific for EE-1 brinjal. The EE-1 F primer sequence targets the right border of

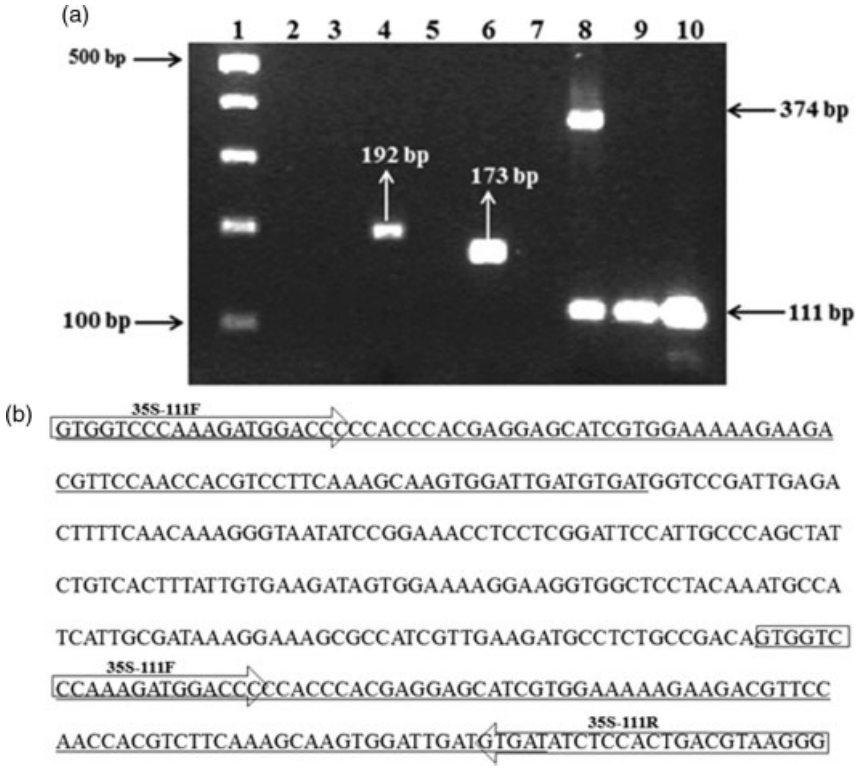

Figure 1. (a) Agarose gel electrophoresis of PCR amplification products from genomic DNA. Lane 1, 100 bp DNA ladder; lane 2, premise control; lanes 3, 5 and 7, non-transgenic brinjal; lanes 4, 6 and 8, EE-1 brinjal; lane 9, RR soya; lane 10, MON810 maize. The lengths of the amplified products are 192 bp (NOS F/NOS R), 173 bp (Tn5-1/Tn5-2) and 111 bp (35S 111 F/35S 111 R) for NOS terminator, nptII and CaMV 35S promoter respectively. (b) DNA sequence of 374 bp amplicon of CaMV 35S promoter observed in lane 8. Duplicated sequence in bold is underlined. Arrows indicate the forward and reverse primer sequences used to amplify the 111 bp amplicon of CaMV 35S promoter.

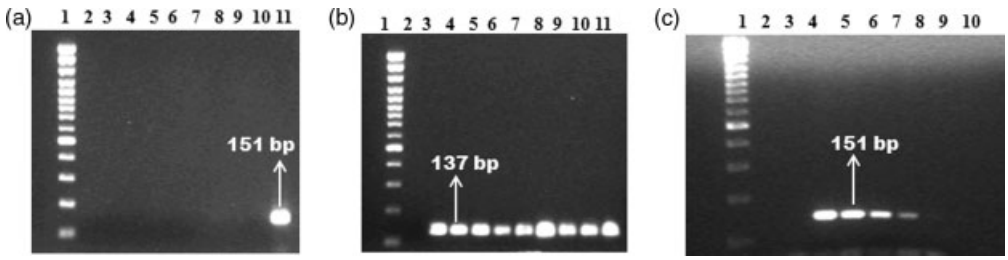

Figure 2. Qualitative event-specific PCR assay for EE-1 brinjal. (a) Specificity of primer pair EE1 F/EE1 R designed for event-specific PCR. (b) Amplifiability of genomic DNA using eukaryote-specific primer pair TRO3/TRO4. Lane 1, 100 bp DNA ladder; lane 2, premise control; lanes 3–11, *Bt*11, *Bt*176, MON810, MON863, TC1507, RR soya, *Bt* cotton, *Bt* cauliflower and EE-1 brinjal respectively. (c) Sensitivity of EE-1 F/EE-1 R event-specific PCR system. Lane 1, 100 bp DNA ladder; lane 2, premise control; lane 3, non-transgenic brinjal; lanes 4–9, 20 ng, 2 ng, 200 pg, 20 pg, 2 pg and 0.2 pg of EE-1 brinjal DNA respectively.

T-DNA, while the EE-1 R primer sequence targets the 3′ transgene brinjal integration site (Fig. 2(a)). The specificity of the designed primer pair EE-1 F/EE-1 R was evaluated by qualitative PCR using genomic DNA extracted from different GMOs. DNAs extracted from *Bt*11, *Bt*176, MON810, MON863, TC1507 and RR soya CRMs were used as representative transgenic lines. DNAs extracted from *Bt* cotton and *Bt* cauliflower were also used. A product of 151 bp was observed exclusively in EE-1 brinjal (Fig. 2). The absence of this amplification product in all other transgenic lines indicates the specificity of the primer pair EE-1 F/EE-1 R. In order to reconfirm that the absence of amplification is not induced by PCR inhibitors, amplification of a highly conserved repeat unit within 18S rDNA was carried out in parallel using the primer pair TRO3/TRO4. A 137 bp amplicon was observed for all GMOs tested (Fig. 2(b)). The absence of amplification using EE-1 F/R was due neither to PCR inhibitors nor to DNA quality. The integration sequence between the host plant genome DNA and transgene are unique and specific to a GMO event.[23] The DNA sequence of the 151 bp PCR amplicon corresponded to the brinjal event EE-1 with a nucleotide homology of 100%. To further demonstrate that the flanking region is indeed that of brinjal DNA, PCR was carried out using BPlant-102 F/BPlant-102 R, which was designed to target the brinjal genomic sequence (Accession No. CS 803547, EC-JRC database) flanking the transgene integration site. A 102 bp amplicon was observed in both transgenic and non-transgenic brinjal genomic DNAs (see 'Supporting information 1'). This confirmed that the 151 bp fragment comprised the T-DNA right border sequence followed by a flanking brinjal genome DNA sequence.

In addition to specificity, another important performance criterion, sensitivity, was also assessed. DNA extracted from EE-1 brinjal

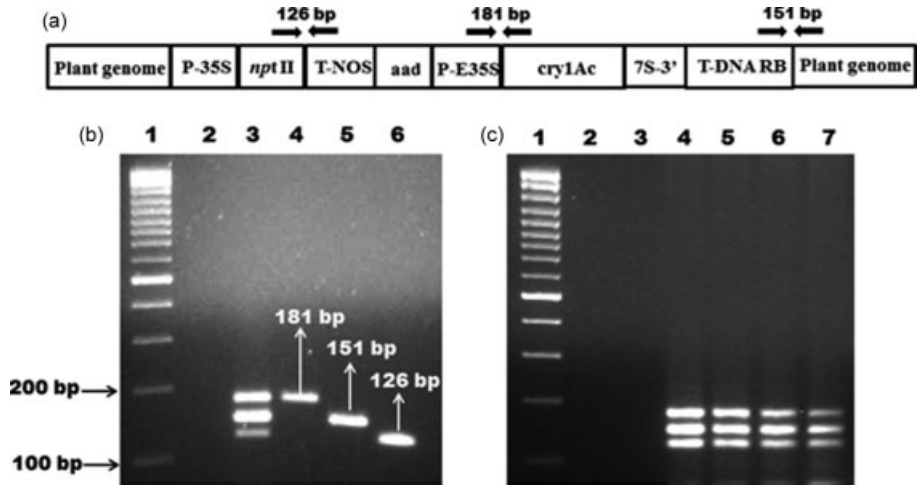

Figure 3. Multiplex PCR assay for detection of EE-1 brinjal. (a) Schematic representation of recombinant construct present in EE-1 brinjal. Arrows indicate the junction region selected for multiplex assay. (b) Agarose gel electrophoresis profile of amplification products obtained by simplex and multiplex PCR. Lane 1, 100 bp DNA ladder; lane 2, non-transgenic brinjal; lane 3, multiplex amplification; lanes 4–6, single PCR amplification using *Bt*B-35S F/*Bt*B-cry1Ac R, *Bt*B-nptII F/*Bt*B-NOS R and EE-1 F/EE-1 R primer pairs respectively. (c) Analysis of LOD of multiplex PCR. Lane 1, 100 bp DNA ladder; lane 2, premise control; lane 3, non-transgenic brinjal; lanes 4–7, 100, 50, 20 and 10 g kg^{-1} EE-1- brinjal respectively.

was serially diluted to give concentrations of 20 ng–0.2 pg μL^{-1}. A 1 μL aliquot of each of these dilutions was used as the template DNA along with EE-1 F/R to amplify the 151 bp fragment. The results indicated that as little as 20 pg of EE-1 brinjal DNA was sufficient for amplification (Fig. 2(c), lane 7). Taking into consideration the brinjal genome size of 0.98 pg per haploid genome (http://data.kew.org/cvalues/), the LOD approximates to 20 haploid genome copies. This sensitivity correlates with the previously reported detection limits for other transgenic crops.[25–27]

Multiplex PCR for detection of EE-1 brinjal

High-throughput GMO assays that provide accurate results at a reasonable cost are a necessity.[28] The application of multiplex PCR as a rapid and convenient screening assay for the detection of GMOs is well documented.[29,30] A simple and efficient multiplex PCR was developed for the simultaneous screening, identification and detection of EE-1 brinjal. Three primer pairs that generate amplification products of different size were used to detect EE-1 brinjal in a single reaction. The primers were designed to include the integration sites of the genetic elements in EE-1 brinjal as shown in Fig. 3(a). The primer pair *Bt*B-35S F/*Bt*B-cry1Ac R was designed to target the integration of 35S CaMV promoter and cry1Ac gene, which amplifies a 181 bp fragment. Similarly, the primer pair *Bt*B-nptII F/*Bt*B-NOS R was designed to amplify a 126 bp fragment of the junction region between nptII and NOS terminator gene. These primers were computed based on our sequence data of CaMV 35S-cry1Ac and nptII-NOS terminator construct regions of EE-1 brinjal (see 'Supporting information 2'). The primer pair EE-1 F/EE-1 R (Table 1) was also included in the multiplex PCR to conclusively detect the EE-1 event. The primers were designed to yield amplicon sizes of <200 bp with the aim of extending this method to detect the event in processed foods. Previously we have shown that amplification of fragments of <200 bp length is most suited for the detection of GMOs in processed foods.[7] Differences in the size of amplicons for different genes were such that differences in mobility were distinguished easily by agarose gel electrophoresis. The efficiency of a multiplex PCR is governed by identical/near identical T_m. The primers were designed such that the T_m was 66 ± 1 °C. The multiplex PCR was carried out under the same conditions as the simplex PCR. The amplicons of expected size were obtained with different intensities, indicating the need for further optimisation of the reaction conditions. Preferential amplification of one target sequence over another is not uncommon in the multiplex PCR format, leading to uneven amplification products.[27] The conditions to achieve PCR fragments of similar intensity were optimised by varying the primer and $MgCl_2$ concentrations. The multiplex PCR was optimised by combining 20 pmol each of *Bt*B-nptII F, *Bt*B-NOS R, *Bt*B-35S F and *Bt*B-cry1Ac R, 10 pmol each of EE-1 F and EE-1 R and 2.5 mmol L^{-1} $MgCl_2$ and setting the annealing temperature at 62 °C. The results are in agreement with the requirement of increased $MgCl_2$ concentration reported for the efficient detection of RR soya by multiplex PCR.[31] These conditions along with 100 ng of EE-1 brinjal DNA were used to amplify the fragments by PCR. The intensities of the three DNA fragments of sizes 126, 151 and 181 bp were similar and the fragments were well resolved (Fig. 3(b), lane 3). No amplification product was observed with non-transgenic brinjal DNA (Fig. 3(b), lane 2). In addition, the absence of non-specific amplification products suggests that the primer sets designed were compatible. The sequences of the 126, 151 and 181 bp fragments were validated by dideoxy sequencing and were identical to those available in the public databases. These results indicate the accuracy and specificity of the primers used. The simultaneous amplification of these multiple sequences in a single reaction is both cost- and labour-effective and would be of advantage in high-throughput GMO analysis.

The degradation of low-quality DNA derived from GMOs often occurs in practical detection, which therefore requires a sensitive PCR detection system.[21] Admixtures of EE-1 brinjal genomic DNA and non-transgenic brinjal genomic DNA were prepared at various levels (10, 20, 50 and 100 g kg^{-1}) and subjected to multiplex PCR. The amount of template DNA was increased from 100 to 500 ng in a total reaction volume of 50 μL. All three expected amplicons were detected for all GMO levels tested, and a LOD of 10 g kg^{-1} was consistently established (Fig. 3(c)). The concentration of DNA used as template appeared to be a critical parameter affecting the efficiency of amplification by PCR. Increasing the amount of DNA from 100 to 500 ng resulted in a stronger signal of the

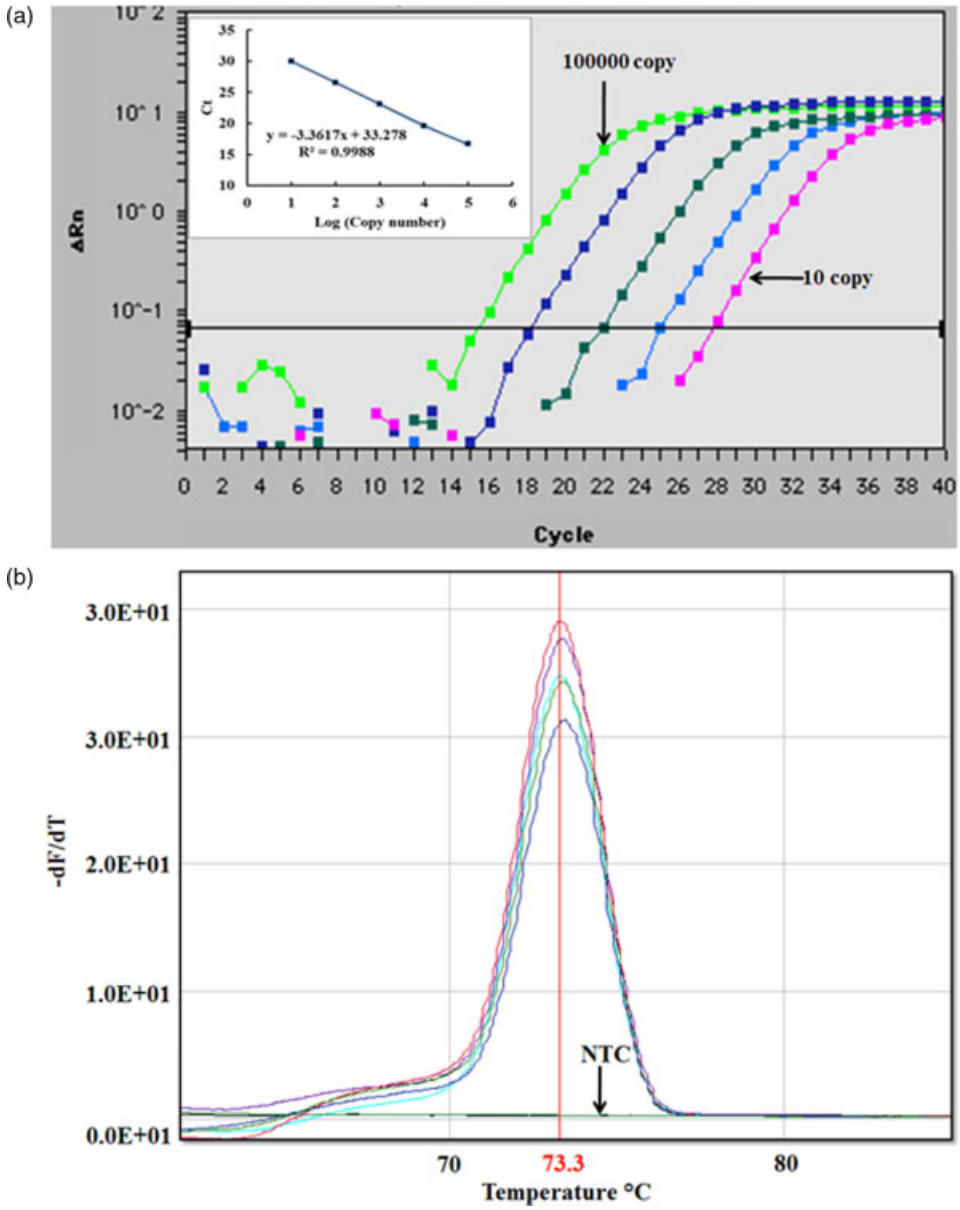

Figure 4. Event-specific RT-PCR assay for detection of EE-1 brinjal. (a) Amplification plots generated by 100 000, 10 000, 1000, 100 and 10 copies of EE-1 brinjal genomic DNA. Inset: standard curve derived from amplification. (b) Melting curves showing single peak specific to 152 bp amplicon of EE-1 brinjal with $T_m = 73.3\,^{\circ}C$.

bands visualised under UV light. These results are in accordance with the requirement of increased DNA concentration reported for the reliable detection of GMOs by multiplex PCR.[26,32] The developed multiplex PCR method is adequate for the sensitive and unambiguous identification of EE-1 brinjal.

SYBR Green real-time PCR

The EE-1 F/EE-1 R primer set used in the qualitative method for detection of EE-1 brinjal was used to optimise a quantitative method. The RT-PCR protocol for EE-1 brinjal was performed using SYBR Green I dye chemistry. SYBR Green dye chemistry was chosen as a method that would be cost-effective compared with TaqMan and other fluorescent probes. In addition, the melting temperature (T_m) curve analysis would allow post-PCR distinction of the expected target against any non-specific amplification.[33]

Specificity of the RT-PCR was assessed by using EE-1 brinjal DNA as well as DNA isolated from other GMOs. The specificity of the designed event-specific EE-1 brinjal primer observed in end-point PCR (Fig. 2(a)) was further confirmed by RT-PCR, which displayed no amplification in non-EE-1 brinjal samples (data not shown). Amplification efficiency and sensitivity of the RT-PCR were determined by analysing serially diluted EE-1 brinjal genomic DNA. The calibration curve of the EE-1 brinjal event-specific PCR assay was constructed using genomic DNA at concentrations of 100 000, 10 000, 1000, 100 and 10 copies (Fig. 4(a)). The correlation coefficient (R^2) was 0.998, indicating high correlation between copy number and *Ct* value. The slope of the calibration curve (−3.36) was used to calculate the amplification efficiency. Using the formula $E = 10^{(-1/\text{slope})} - 1$,[34] the efficiency was 98%. The high efficiency and the correlation coefficient of near unity obtained suggest that the method is well suited for quantitative estimation

Table 2. Amplification data used to determine absolute LOD and LOQ

Template copies	Signal rate (positive signals)	Mean *Ct* value	Standard deviation
100000	9/9	16.70	0.0571
10000	9/9	19.54	0.0787
1000	9/9	23.27	0.1815
100	9/9	26.55	0.2020
10	8/9	ND	ND

ND, not detected.

of EE-1 brinjal. The standard deviation (SD) of 0.05–0.31 calculated from the data indicates good reproducibility. *Ct* variations among parallel samples of the same template concentration increased with decreasing template copies. As expected, the ability to detect EE-1 brinjal decreased with decreasing genomic DNA copy number. A plot of the negative first derivative depicts a single peak with a single T_m of 73.3 °C (Fig. 4(b)). No fluorescent signal ($Ct = 40$) was observed for the no-template control (NTC) and negative control (DNA from non-transgenic brinjal), indicating the absence of both primer dimer and non-specific products. A *Ct* value above 40 is indicative that the target gene is absent. The above results demonstrate the suitability of RT-PCR for the quantitative estimation of EE-1 brinjal.

Determination of LOD and LOQ

Detection and quantification limits refer to the lowest quantity of the target that can be reliably detected and quantified with a probability of $\geq$ 95%. The absolute limit is the lowest number of initial template copies that can be detected and quantified.[35] To determine the LOD and LOQ of the established event-specific RT-PCR assay, series of DNA dilutions containing an estimated average of 100 000, 10 000, 1000, 100 and 10 copies of the EE-1 brinjal haploid genome per reaction were tested in triplicate in three parallel reactions (Table 2). As expected, the ability to detect EE-1 brinjal decreased with decreasing copy number. The EE-1 brinjal DNA could be detected in all nine reactions down to 10 copies. These results indicated that the LOD value was about 10 copies. The data also showed that the SD values of the nine reactions with the same template concentration increased with decreasing copy number, especially for the dilution with 10 copies of the haploid genome. To obtain reliable quantisation results under ideal conditions, approximately 100 initial template copies were required, and we concluded that the LOQ of the event-specific RT-PCR assay was 100 copies of the haploid genome.

Unapproved GMOs can escape from regulatory field trials and enter into commercial production, which creates problems for international trade.[36] Detection of unauthorised GMOs is the major challenge in GMO detection owing to lack of information and unavailability of reference materials. Several instances reporting the presence of unapproved GMOs in food samples have been documented. An unapproved *Bt* rice line similar to the Shanyou 63 line was detected in rice vermicelli using the junction sequence.[37] This method did not distinguish the transformation event from the lines containing the same insert. Owing to the lack of an event-specific method, the authors were unable to identify the transgenic event. Recently developed event-specific methods for Shanyou 63[38] and Kemingdao 1[39] *Bt* rice varieties could find application to detect such fraudulent use. Event-specific PCR based on the junction region between recombinant DNA insertion and adjacent host genomic DNA is a more preferred method, as this junction sequence is highly specific to a particular transformation event. The development of such methods is useful in addressing the issue of unauthorised GMOs in the food/feed chain.

CONCLUSIONS

In this study, single and multiplex PCR methods based on the event of EE-1 brinjal were developed. The detection method was based on the 3′ transgene insertion flanking sequence of a new GMO event EE-1 brinjal under trials in India. This is the first report on the development of event-specific qualitative simplex, multiplex and RT-PCR methods for EE-1 brinjal. The multiplex PCR method described here includes two construct-specific and one event-specific sequence motif for the identification of EE-1 brinjal. This method, in addition to its high sensitivity and specificity, offers the advantage of being both cost- and labour-effective. Our results show that detection strategies established in the present study allow the screening of EE-1 brinjal with a detection limit well below the regulations established by other countries. The availability of suitable detection methods for this important vegetable crop prior to its commercial release will facilitate compliance with labelling regulations.

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Supporting information

Supporting information may be found in the online version of this article.

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