FOOD COMPOSITION AND ADDITIVES

Qualitative and Event-Specific Real-Time PCR Detection Methods for *Bt* Brinjal Event EE-1

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***Bt* brinjal event EE-1 with *cry1Ac* gene, expressing insecticidal protein against fruit and shoot borer, is the first genetically modified food crop in the pipeline for commercialization in India. Qualitative polymerase chain reaction (PCR) along with event-specific conventional as well as real-time PCR methods to characterize the event EE-1 is reported. A multiplex (pentaplex) PCR system simultaneously amplifying *cry1Ac* transgene, *Cauliflower Mosaic Virus* (*CaMV*) 35S promoter, *nopaline synthase* (*nos*) terminator, *aminoglycoside adenyltransferase* (*aadA*) marker gene, and a taxon-specific *β-fructosidase* gene in event EE-1 has been developed. Furthermore, construct-specific PCR, targeting the approximate 1.8 kb region of inserted gene construct comprising the region of *CaMV* 35S promoter and *cry1Ac* gene has also been developed. The LOD of developed EE-1 specific conventional PCR assay is 0.01%. The method performance of the reported real-time PCR assay was consistent with the acceptance criteria of Codex Alimentarius Commission ALINORM 10/33/23, with the LOD and LOQ values of 0.05%. The developed detection methods would not only facilitate effective regulatory compliance for identification of genetic traits, risk assessment, management, and postrelease monitoring, but also address consumer concerns and resolution of legal disputes.**

Fruit and shoot borer (FSB) resistant *Bt* brinjal event EE-1 expressing *Bacillus thuringiensis* (*Bt*) *cry1Ac* gene is the first genetically modified (GM) food crop in the pipeline for commercialization in India. Event EE-1 was developed by Maharashtra Hybrid Seeds Company Ltd (Mahyco, Jalna, India), which has transferred this *Bt* brinjal technology to the Tamil Nadu Agricultural University, Coimbatore; University of Agricultural Sciences, Dharwad; and Sungro Seeds Pvt. Ltd, New Delhi (1). *Bt* brinjal would provide an effective built-in control for brinjal FSB as an insect resistance management strategy.

In India, the regulation of all activities related to GM organisms (GMOs) and products derived from GMOs is governed under the provision of the Environment Protection Act, 1986, and by Rules, 1989, through the Ministry of Environment and Forests (MoEF), Government of India. The Rules, 1989, primarily implemented by MoEF and the Department of Biotechnology, Ministry of Science and Technology, essentially take care of all activities, products, and processes related to or derived from biotechnology, including foods derived from biotechnology, thereby making the Genetic Engineering Appraisal Committee the competent authority to approve the release of GM foods into the marketplace (2). Several countries have implemented labeling thresholds for unintentional mixing of GM crops defined as 0.9% in the European Union and Russia; 3% in Korea; and 5% in Japan, Indonesia, Thailand, and Taiwan (3). So far, no labeling threshold has been defined in India. However, as per the implications of the subject judgement of the Honorable Supreme Court of India, before the GM material is taken for the field trials, a protocol for testing the GM event at an LOD of 0.01% has to be established (http://www.envfor.nic.in/divisions/csurv/geac/decision-jul-95.pdf).

Since event EE-1 is already in the pipeline for commercialization in India, it is of utmost importance to develop protocols for screening and detection of *Bt* brinjal for regulatory compliance, legal requirements, addressing consumers' concerns, and post-release monitoring. At present, the most widely used detection method for compliance with regulatory obligations and labeling policies is PCR because of its simplicity, specificity, and sensitivity (4). To date, TaqMan-based real-time PCR has been the method of choice for GMO quantification, especially for compliance with labeling laws (4, 5). With commercialization of GM crops carrying the same traits produced by different developers, event-specific quantitative PCR methods are required to differentiate between different events of the same trait. Event-specific real-time PCR detection methods have been developed in the past few years for a range of GM crops/events such as Widestrike transgenic cotton event 281-24-236/3006-210-23 (6), StarLink maize event CBH-351 (7), GM maize MON863 event (8), and MON15985 and MON88913 events of GM cotton (9).

Among conventional PCR technologies, multiplex PCR is less time-consuming and cost-effective, and it can detect multiple target sequences of inserted gene construct in a single reaction. Several reports are available for the use of multiplex PCR for diagnosis of GM crops, which are under different stages of field trials in India, such as *Bt* cauliflower with *cry1Ac* gene (10); GM tomato with o*smotin* gene (11); GM potato with *AmA1* gene (12); *Bt* potato with *cry1Ab* gene (13); *Bt* rice hybrid (MRP 5401 *Bt*) with *cry1Ac* gene (14); and GM cotton with *vip3A* gene (15). A decaplex PCR has also been reported to identify and differentiate MON531 and MON15985 events of *Bt* cotton commercially cultivated in major cotton growing

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Table 1. Details of primer pairs used for qualitative and quantitative PCRs

Primer	Primer sequence (5′-3′)	Amplicon size, bp	References
Cry2F/R	F-CAGCGGGCCCGAGTTTACCTT R-CGGATGCGATGATGTTGTTGAA	475	Present study
AadA2F/R	F-CCGCGCTGTAGAAGTCACCATTGT R-CGGGCGGCGAGTTCCATAGC	284	Present study
SP-F/R	F-GAAGGTGGCTCCTACAAATGCC R-GTGGGATTGTGCGTCATCCC	199	Present study
Nost F/R	F-GAATCCTGTTGCCGGTCTTGCG R-GCGGGACTCTAATCATAAAAACCC	127	Present study
Pomtom F/R	F-CTGCCTCCGTCAAGATTTGGTCACT R-CTCTTCCCTTTCTTGATGG	141	20
Cry1Ac transgene construct	F- GAAGGTGGCTCCTACAAATGCC R- CGGATGCGATGATGTTGTTGAA	~1.8 kb	Present study
EE-1 F/R	F-GCTGGGATCAGATAGTCG R-ATGCGGTGATAATTGAATGC	168	Present study
EE-1-TM (probe)	FAM-TGTTACCAAAAGTGCTGTCAATAAACACT-TAMRA	–	Present study

regions of India (16). For characterization of GM crops, several construct-specific PCR methods have been developed to detect GM maize (17), GM cotton expressing *vip3A* gene (15), *Bt* rice (14, 18), and GM tomato Huafan No 1 with long shelf-life (19).

Brinjal is grown on nearly 550 000 hectares in India, making it the second largest producer after China with a 26% world production share. It is an important cash crop for more than 1.4 million small, marginal, and resource-poor farmers. Brinjal, being a hardy crop that yields well even under drought conditions, is grown in almost all parts of the country (1). Along with event EE-1, several other events of *Bt* brinjal, such as event 142 with *cry1Fa1* gene, *Bt* brinjal with *cry1Ab* gene and other *Bt* crops with *cry1Ac* gene/similar gene construct such as *Bt* cauliflower, *Bt* cabbage, and *Bt* okra, are also under different stages of field trials in India. So event-specific conventional/quantitative PCR methods are required to identify and differentiate different events of the same trait or similar gene construct. Keeping this in view, this paper reports the development of qualitative assays based on multiplex (pentaplex) and construct-specific PCR, and event-specific conventional as well as real-time PCR assays for detection of *Bt* brinjal event EE-1.

Experimental

Planting and Reference Materials

Lyophilized leaf tissue of *Bt* brinjal event EE-1 along with non-*Bt* counterpart were provided by Mahyco. For specificity experiments, leaf tissue of *Bt* brinjal event 142 with *cry1Fa1* gene (initially developed by the National Research Centre on Plant Biotechnology, New Delhi, India, and subsequently transferred to M/s Bejo Sheetal Seeds Pvt. Ltd for introgression into the parental lines of the company and conducting biosafety studies as per the regulatory requirement; http://www.envfor.nic.in/divisions/csurv/geac/decision-may-100.pdf), *Bt* brinjal with *cry1Ab* gene (developed by National Research Centre on Plant Biotechnology), *Bt* cabbage and *Bt* cauliflower with *cry1Ac* gene (developed by Sungro Seeds Pvt. Ltd); *Bt* okra with *cry1Ac* gene (developed by Mahyco); and seed samples of commercialized events of *Bt* cotton, viz., MON531, MON15985, GFM-cry1A, Event 1 and Dharwad Event, were used as test crops. For the preparation of reference material to establish the LOD of reported event-specific conventional PCR assay, 20 ng/µL DNA sample extracted from lyophilized leaf tissue of *Bt* brinjal (100% GM) was serially diluted with 20 ng/µL of DNA sample (0% GM) isolated from the respective counterpart non-GM leaf tissue to obtain aliquots with different percentages of GM event, i.e., 100, 10, 1.0, 0.1, 0.05, 0.01, and 0.001%. For LOD and LOQ evaluation of developed event-specific real-time PCR assay, DNA samples with 100, 50, 10, 1.0, 0.5, 0.1, and 0.05% of GM event were used as reference materials.

DNA Extraction

Genomic DNA was extracted from fresh and lyophilized leaf tissue using a DNeasy Plant Mini Kit (Qiagen, Hildon, Germany) as per the manufacturer's instructions with minor modifications. The quality of the extracted DNA was measured by UV spectrophotometer (DU 650; Beckman, Corona, CA) absorption at 260 nm, and the purity was evaluated by the A260/A280 ratio. A final concentration of 20 ng/µL was used for GM analysis.

Primers and Probes

The primer pairs used for amplification of *cry1Ac* transgene, *CaMV* 35S promoter, *nos* terminator, and *aad*A marker gene were designed using Primer Select Version 5.05 of (DNASTAR Inc., Madison, WI). For the amplification of species-specific *β-fructosidase* gene, published primer pair was used (20). This reported primer pair pomtom F/R amplified the specific region of *β-fructosidase* gene in four *Solanaceae* members, viz., potato, tomato, eggplant, and pepper, and all four members amplified the specific amplicons with different product length. Event-specific primer pair and TaqMan probe labeled with the fluorescent 6-carboxyfluorescein (FAM) and the quench dye 6-carboxytetramethylrhodamine (TAMRA) at the 5′ and 3′ ends, respectively, for event EE-1 were designed using

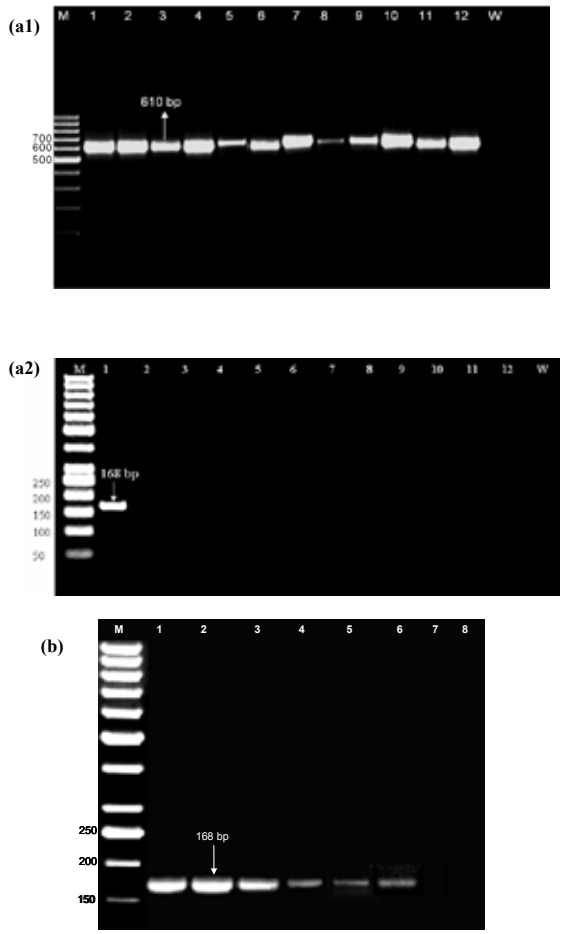

Figure 1. PCR amplification (a) to check the amplification using plantA1/plantA2 primer pair and (b, c) to test the specificity (b) and sensitivity (c) of event-specific EE-1 F/R primer of the *Bt* brinjal event EE-1 (a, b); Lane M: 50bp ladder; Lane 1: *Bt* brinjal event EE-1; Lane 2: *Bt* brinjal with *cry1Fa1* gene; Lane 3: *Bt* brinjal with *cry1Ab* gene; Lane 4: *Bt* cabbage (*cry1Ac* gene); Lane 5: *Bt* cauliflower (*cry1Ac* gene); Lane 6: *Bt* okra (*cry1Ac* gene); Lane 7: MON531; Lane 8: MON15985; Lane 9: Event 1; Lane 10: GFM-cry1A; Lane 11: Dharwad event; Lane12: Non-*Bt* brinjal; Lane W: Water control (b) Lane M: 1kb ladder; Lanes 1–7: Serial dilutions of *Bt* brinjal with 100, 10, 1.0, 0.1, 0.05, 0.01 and 0.001% of GM trait; Lane 8: Non-*Bt* brinjal.

Lightcycler Probe Design software 2.0 (Roche Applied Science, Mannheim, Germany). The right border sequence and adjacent flanking sequence of brinjal genomic DNA of EE-1 elite event from sequence ID No. 7 of Mahyco's patent WO/2007/091277 (21) was used. Primers and probe were synthesized by Pivotal Marketing (Genaxy Scientific Pvt. Ltd, New Delhi, India). The details of nucleotide sequences of primers and probes are given in Table 1.

Qualitative PCR

To test the specificity of designed event-specific primers, conventional PCR was performed in 20 µL volume reactions in a PTC 200 programmable thermal cycler (MJ Research Inc., Ramsey, MN) with 20 ng/µL template DNA samples of test crops, 1x Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTP mix, 0.3 µM each forward and reverse primers, and 1 unit Taq DNA polymerase (MBI Fermentas, Inc., Hanover, MD). The following amplification conditions were used: initial denaturation for 5 min at 95°C and followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min with a final extension step of 72°C for 8 min. To test the sensitivity of event-specific conventional PCR assay, the amplification was carried out using the serial dilutions by mixing *Bt* brinjal DNA with non-*Bt* counterpart on a volume/volume basis to have different GM content, i.e., 100, 10, 1.0, 0.1, 0.05, 0.01, and 0.001 ng/µL. To evaluate the specificity and amplification efficiency of designed primer pairs for *cry1Ac* transgene, *CaMV* 35S promoter, *nos* terminator, and *aad*A marker gene, simplex PCR was performed (data not shown).

The multiplex (pentaplex) PCR assay was performed in 25 µL volume reactions with 1x Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 µM dNTP, and 1 unit Taq DNA polymerase (MBI Fermentas, Inc.) using primer concentrations of 0.4 µM for *cry1Ac*, 0.2 µM for *CaMV* 35S promoter, 0.3 µM for *nos* terminator, 0.4 µM for *aadA*, and 0.25 µM for *β-fructosidase* gene. The PCR cycling conditions were adjusted to have an initial denaturation step at 95°C for 10 min followed by 40 cycles, which involved 95°C for 1 min annealing at 59°C for 50 s and extension at 72°C for 1 min followed by final extension at 72°C for 8 min.

Construct-specific PCR amplification was carried out using forward primer of *CaMV* 35S promoter and reverse primer of *cry1Ac* transgene with a 25 µL reaction volume comprising 2.0 mM $MgCl_2$, 0.4 µM dNTPs, 0.3 µM of each primer, and 2 units of recombinant Taq DNA polymerase (MBI Fermentas, Inc.). The PCR cycling program was used as follows: an initial denaturation step at 95°C for 5 min; followed by 10 cycles with 95°C for 20 s, 59°C for 50 s, and 68°C for 3 min; followed by 30 cycles with 95°C for 20 s, 59°C for 50 s, and 68°C for 3 min with autoextension at 2 s/cycle. Final extension was performed at 68°C for 10 min. The extension time was ascertained by calculating the length of construct-specific amplicon. To confirm the integrity of the construct-specific PCR product, the amplification product of approximately 1.8 kb was further utilized for nested PCR using SP-F/R primer pair.

The amplified products were analyzed on 2.0% (w/v) agarose gel and 4% (w/v) metaphor@ agarose gel (Cambrex Bioscience Rockland, Rockland, ME) using 1X TAE running buffer stained with ethidium bromide for horizontal electrophoresis and then visualized using a UV Gel Documentation System (Alpha Innotech Corp., San Leandro, CA).

Event-Specific Real-Time PCR

The real-time PCR product amplified with event EE-1 specific primer pair was integrated using the TOPO TA cloning kit (Invitrogen Life Technologies Inc., Carlsbad, CA) to construct a standard plasmid on the basis of a pCR2.1-TOPO vector (Invitrogen Life Technologies Inc.). The recombinant plasmid was used to transform *Escherichia coli* strain TOP10 cell (Invitrogen Life Technologies Inc.). This recombinant plasmid's DNA was extracted using the Qiagen Plasmid Midi Kit, which was then digested with *Hind*III restriction endonuclease. The linearized plasmid DNA was purified from 2% agarose gel by a Qia Quick Gel Extraction Kit (Qiagen). The concentration

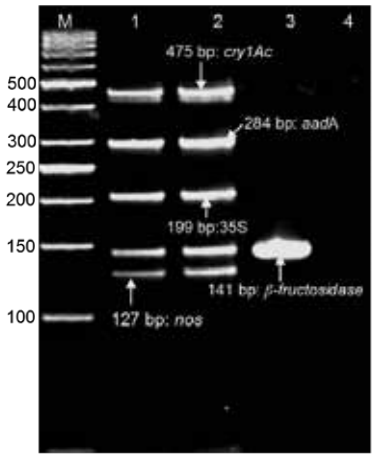

Figure 2. Pentaplex PCR for the simultaneous amplification of *cry1Ac, aadA, CaMV* 35S promoter, and *nos* terminator along with the endogenous *β-fructosidase* gene in *Bt* brinjal, M: 50 bp ladder; Lanes 1, 2: samples of *Bt* brinjal event EE-1; Lane 3: sample of non-*Bt* brinjal; Lane 4: water control.

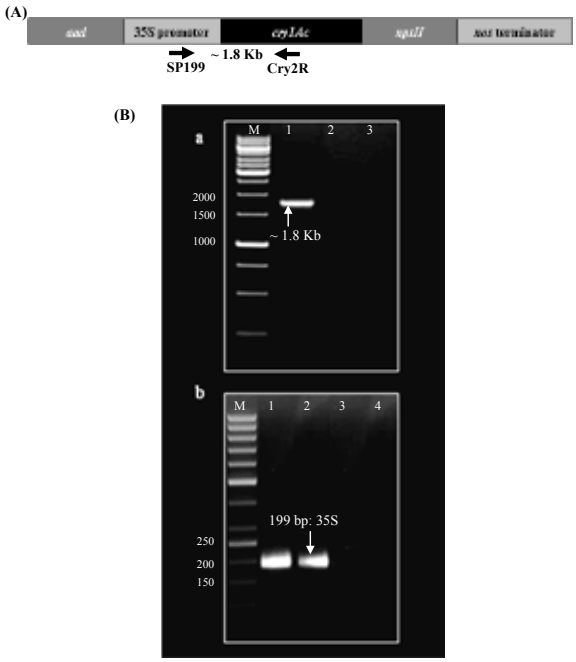

Figure 3. Construct-specific PCR followed by nested PCR for detection of a part of inserted gene construct in *Bt* Brinjal for confirming construct PCR results: (A) representation of Bt brinjal *cry1Ac* transgene construct indicating the location of construct specific product size; B(a) construct-specific PCR amplified amplicon of ~ 1.8 kb using forward primer of promoter and reverse primer of transgene, i.e., SP F/ cry2R, M: 1kb Ladder, Lane1: Sample of *Bt* brinjal, Lane 2: Sample of non-F*Bt* brinjal; B(b) nested PCR assay to confirm the long-run PCR product using primer pair SP-F/R for 35S promoter gene; M: 50 bp ladder; Lanes 1–2: samples of *Bt* brinjal (in duplicate); Lane 3: sample of non-*Bt* brinjal; Lane 4: water control.

of plasmid DNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). 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 calibrants for quantification.

Real-time PCR was performed on a light cycler® 480 system (Roche Applied Science) in a 96 well plate. Each reaction was run in triplicate during 45 cycles in a total volume of 20 μL in each well containing 100 ng sample template DNA, 0.4 μM primer pair, 0.2 μM Taqman probe, and 10.0 μL universal master mix (Roche Applied Science) with the PCR conditions as follows: denaturation at 95°C for 10 s, annealing at 60°C for 1 min, and extension at 72°C for 2 s. For the generation of a standard curve, eight serial dilutions of the constructed standard plasmid DNA 10^8 to 10 copies/μL were used as calibrants. The repeatability of the standard plasmid copy numbers was estimated from the data of triplicate reactions. The method acceptance criteria and method performance requirements as set by the Codex Alimentarius Commission report in 2010 (22) were considered. The squared correlation coefficient (R^2) was calculated as the correlation coefficient of the standard curve obtained by linear regression analysis. The average value of R^2 has been considered as suitable when not lower than 0.98. The PCR performance and LOD of real-time PCR assay were tested using serial dilutions of *Bt* brinjal event EE-1 DNA with non-*Bt* counterpart to have 100, 50, 10, 1.0, 0.5, 0.1, 0.05 ng/reaction. The parameters including accuracy in terms of precision and trueness, LOD, and LOQ of the method were evaluated by comparing the experimental mean value with the theoretical value of the GM content.

Results and Discussion

Specificity and Sensitivity of Designed Event-Specific Primers

Specificity of designed event-specific primer pairs EE-1-F/R was tested using conventional PCR assay. The amplification in DNA samples of all test crops was confirmed using PlantA1/PlantA2 primer pair (23) targeting chloroplast-specific tRNA gene (Figure 1a). This experimental control plant-specific assay would check the possibility, in case PCR fails due to the presence of DNA impurities in the negative controls. The PCR showed amplification of expected size, i.e., 168 bp in *Bt* brinjal event EE-1, whereas no amplification was detected in other tested samples (Figure 1b) and, thus, distinguishes event EE-1 from other *Bt* brinjal events expressing *cry1F* gene (event 142) and *cry1Ab* gene and from other *Bt* crops with *cry1Ac* gene or similar transgene construct such as *Bt* cabbage, *Bt* cauliflower, *Bt* okra, and five commercialized *Bt* cotton events, i.e., MON531, MON15985, GFM-cry1A, Event 1, Dharwad event, and non-*Bt* counterpart.

To test the sensitivity of event-specific conventional PCR assay, amplification was performed using the serial dilutions of *Bt* brinjal event EE-1 DNA with non-*Bt* counterpart, mixed on the volume/volume basis, having different GM content, i.e., 100, 10, 1.0, 0.1, 0.05, 0.01, and 0.001 ng/μL. The primer pair EE-1F/R amplified 168 bp product of the desired size in all the test samples up to 0.01% (Figure 1c). The results indicated that the LOD of the developed simplex PCR assay was 0.01%, which is also in compliance with the Supreme Court of India's

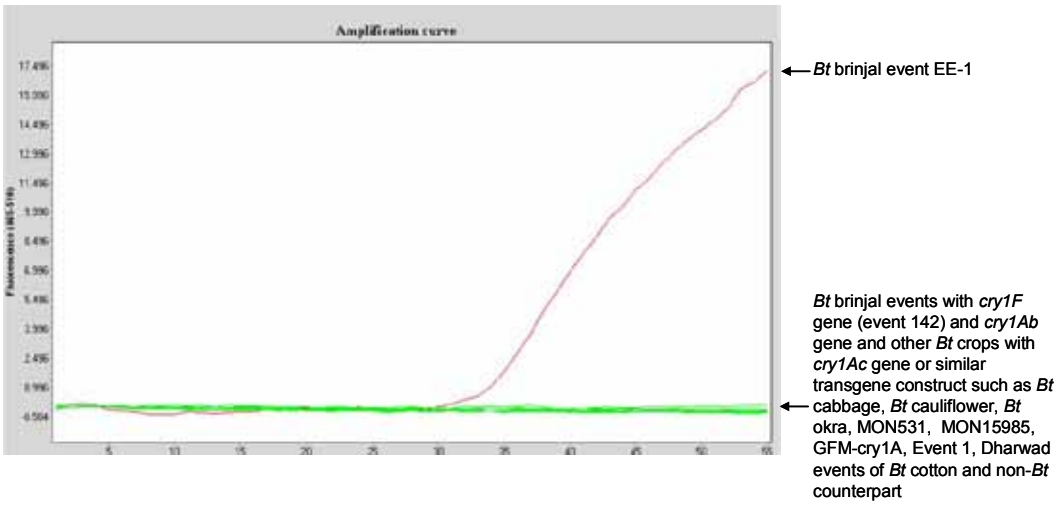

Figure 4. Amplification plots to confirm the specificity of designed event-specific primers and probe using real-time PCR for *Bt* brinjal event EE-1.

stipulation of developing an event-specific protocol for testing contamination to an LOD of 0.01% prior to conducting field trials of GM crops. Hence, the reported sensitivity level would be effectively utilized in the future to detect unintentional mixing of GM seeds in seed lots after commercialization of event EE-1 in India.

Qualitative Analysis of Bt Brinjal Event EE-1

Multiplex PCR assay was performed in pentaplex format using primer pairs targeting amplification of *cry1Ac* transgene along with *CaMV* 35S promoter, *nos* terminator, *aadA* marker gene, and endogenous *β-fructosidase* gene. The multiplex reaction was optimized by combining the primer pairs at different concentrations and setting the annealing temperature at 59°C. The multiplex PCR simultaneously amplified clearly visible amplicons of desired size, i.e., 475 bp for *cry1Ac* gene, 199 bp for *CaMV* 35S promoter, 127 bp for *nos* terminator, 284 bp for *aadA* marker gene along with the 141 bp for endogenous *β-fructosidase* gene in *Bt* brinjal (Figure 2), whereas the desired amplicon for *β-fructosidase* gene was also amplified as an internal control in non-*Bt* brinjal. The developed pentaplex PCR assay would be used for reliable, efficient, and cost-effective screening of *Bt* brinjal with *cry1ac* gene.

Construct-specific PCR assay involves amplification of the specific region between the sequences of any two target elements or whole transgene construct using a single primer pair. The construct-specific assay was developed for the detection of inserted gene construct using a combination of the forward primer specific to *CaMV* 35S promoter, i.e., SP-F and reverse primer specific to *cry1Ac* gene, i.e., cry2R (Figure 3a). The desired amplicon of the construct-specific region of approximately 1.8 kb was amplified only in *Bt* brinjal, and no amplification was observed in non-*Bt* brinjal and water control through long-run PCR assay [Figure 3B(a)]. Furthermore, nested PCR was used to confirm the integrity of construct-specific PCR amplicon. PCR assay was carried out using *CaMV* 35S promoter specific primer pair, i.e., SP-F/R. The specific amplicon of 199 bp was detected only in *Bt* brinjal event EE-1, but no such amplicon was detected in non-*Bt* brinjal and water control confirming the specificity of construct-specific PCR assay [Figure 3B(b)].

Performance of the Real-Time PCR System Specific to Event EE-1

Since the *Bt* brinjal event EE-1 is the first GM food crop in India, which is in the pipeline for commercialization in the near future, an event-specific PCR-based detection assay is required.

Specificity.—Specificity testing using the real-time PCR system was conducted. None of the DNA samples of test crops showed amplification for a specific event, except for the corresponding *Bt* brinjal EE-1 sample (Figure 4 and Table 2), thereby confirming that the reported real-time PCR assay was specific to test the event EE-1.

Construction of a standard plasmid as a reference molecule.—As a reference molecule, a standard plasmid was constructed by the integration of PCR product specific for event EE-1 into

Table 2. Specificity tests with the event-specific real-time PCR system

Test crop	PCR results
Non-GM brinjal	–
Bt brinjal event EE-1	+
Bt brinjal event 142	–
Bt brinjal	–
Bt cotton event MON531	–
Bt cotton event MON15985	–
Bt cotton Event 1	–
Bt cotton event GFM-cry1A	–
Bt cotton Dharwad event	–
Bt cabbage	–
Bt cauliflower	–
Bt okra	–

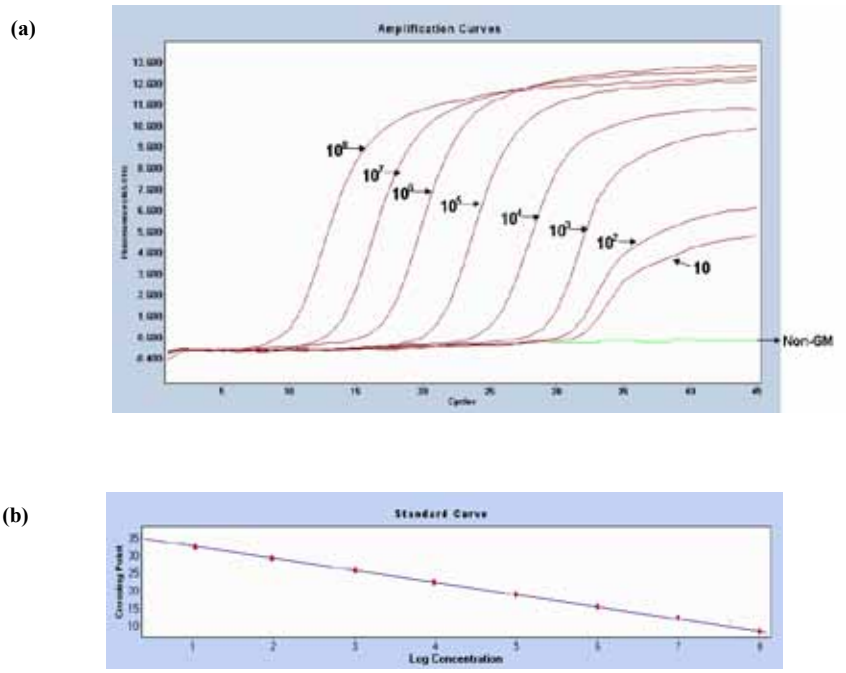

Figure 5. Amplification plots and standard curves using real-time PCR for Brinjal EE-1 event-specific gene; (a) amplification curves generated for eight serial dilutions of standard plasmid with 10 to 10^8 copies of EE-1 event, respectively and (b) standard curve generated from the amplification data for standards shown in (a).

pCR®2.1-TOPO® vector. For real-time PCR, eight levels of standard plasmid concentration were set to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10 copies/μL. The linearity of the standard curves for event EE-1 was confirmed by quantitative PCR using the designed primer pairs, probe, and standard plasmid, and the calculated R^2 value was 0.999 (Figure 5). Under the eight levels of standard plasmid, the repeatability of the standard plasmid's copy numbers was calculated from the data of triplicate reactions. The values of RSD of the triplicate reactions ranged from 1.8 to 12.1% (Table 3). The RSD values were less than 20%, indicating that there was no significant variation in this range, so the standard plasmid was confirmed to be stable and a reliable reference molecule.

Precision, trueness, LOD, and LOQ.—Precision of the developed real-time PCR assay was evaluated as the bias (%) of the experimental mean value from the theoretical value. The serially diluted samples with different percentages of GM content, i.e., 100, 50, 10, 1, 0.5, 0.1, and 0.05, were tested using plasmid as calibrators. All the test samples with GM content up to 0.05% showed the fluorescence signals, whereas no signal was detected in the sample of non-GM counterpart. To determine the accuracy and repeatability of the results, the mean, bias true values, the SD and repeatability RSD (RSDr) were calculated. To test the reliability of the calculated RSDr values, 16 replications of each level were used, as also indicated in ISO 5725-3 (1994) by the International Organization of Standardization, i.e., estimates of repeatability should be obtained on a sufficient number of test results, at least 15 replicates (24). The calculated bias and RSDr values of the tests ranged from –7.8 to 10.0% and 0.53 to 14.5%, respectively (Table 4). According to the Codex Alimentarius Commission, the trueness should be within 25%. In this study, the bias values were also within the range, i.e., ± 25% of the assay, whereas all variations (RSDr) were within 20%; therefore, the accuracy and precision of the qualitative method were found to be reliable and effective for its practical utility and application.

LOD, as suggested by the Codex Alimentarius Commission, should correspond to the lowest level of analyte, for which the RSD for reproducibility is 33% or less, and LOQ should correspond to the lowest level of analyte for which the RSD is 25% or less (22). The RSDr value of the lowest concentration level (0.5%) in the present study was 14.5%, which is a feasible level for detection of a particular GM crop. Hence, at this level the quantitative PCR system was able to determine the transgene content with acceptable accuracy, i.e., trueness and precision. The performance of this PCR system is in accordance with criteria as established by the Codex Alimentarius Commission

Table 3. Repeatability of the copy numbers of standard plasmid targeting event EE-1

Copy No.		
True value	Mean value	RSD, %
100000000	103000000	2.54
10000000	12800000	3.22
1000000	1430000	6.41
100000	108000	4.7
10000	14000	4.2
1000	1610	1.8
100	450	9.6
10	12	12.1

Table 4. Accuracy and precision for the EE-1 specific real-time PCR system

True value of transgene content, %	Accuracy		Precision	
	Mean, %	Relative deviation from true value, %	SD	RSDr, %
100	98.3	–1.7	0.907	0.92
50	49.5	–1.0	0.264	0.53
10	9.6	–4.0	0.351	3.65
1	0.95	–5.0	0.025	2.63
0.5	0.461	–7.8	0.067	14.5
0.1	0.11	10.0	0.015	13.63
0.05	0.052	4.0	0.002	3.84

in 2010. Although this methodology is not based on relative quantification, it could be efficiently used as an event-specific protocol for testing contamination to an LOD of 0.01% prior to conducting field trials of GM crops, as per the Supreme Court of India's stipulation.

Conclusions

The present study uses qualitative and event-specific real-time PCR methods for the detection of *Bt* brinjal event EE-1. The reported multiplex, construct-specific PCR and event EE-1 specific real-time PCR assays are ideal for initial screening and characterization of *Bt* brinjal for risk assessment, management, and post-release monitoring. The event EE-1 specific conventional PCR assay reported here is sensitive enough to detect 0.01% of GM event, which is in compliance with the Supreme Court of India's stipulation of developing an event-specific protocol for testing contamination to a detection limit of 0.01% prior to conducting field trials of GM crops. Real-time PCR experiments were performed under repeatability conditions, and the results demonstrated that the method performance, including specificity, trueness, and precision, were within the limits established by the Codex Alimentarius Commission report in 2010, with an LOD and LOQ of 0.05%. The real-time PCR assays developed in this study will have immense potential for quantitative analysis of EE-1 to meet the threshold level.

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