PROTOCOL EXCHANGE | *COMMUNITY CONTRIBUTED*

Assessment of cry1Ab transgene cassette in commercial Bt corn MON810: gene, event, construct and GMO specific concurrent characterization

Chandra K. Singh , Abhishek Ojha , Suchitra Kamle & Devendra N. Kachru

Food Toxicology Division

Introduction

Abstract

The cultivation of transgenic maize (MON810, Monsanto) is widespread globally & exponentially on increase. The international safety regulations mandate use of authentic and stable transgenics and encourage detection methodologies for assessment of the transgenic material, its labeling and rapid identification of spurious GM seed/produce. This study was aimed to develop simpler detection assays with universal assay conditions to characterize the entire transgene cassette in Bt corn (MON810) and verify the insert integrity.
Two distinct PCR assays were developed, employing variable, multiple combinations of primer sets & assay conditions. The PCR assays; one in a standard array & the other in a multiplex format, facilitated concurrent detection of cry1Ab gene sequence (gene specific); maize genome – P-e35S adjoining sequence (event specific); hsp-cry1Ab sequence (construct specific), npt-II marker gene & 02 regulatory (P-e35S promoter & T-nos terminator) sequences- (GMO specific). The standard simplex assay also enabled amplification of maize specific hmgA gene (internal control) and plant specific chloroplast t-RNA gene (positive control). The Limit of detection for cry1Ab gene could be determined simultaneously, by multiplex PCR assay (gene, event and construct specific) and it was 0.1% MON810. A longrun cry1Ab transgene cassette specific PCR assay developed with variable combinations of primer sets, facilitated assessment of structural integrity & stability of the entire transgene cassette of the GM maize. The reported protocol, hence, is an efficient and simple one to provide transgenic maize (MON810) detection under unified assay conditions and elicit all basic information pertaining to GM miaze.

Introduction

Since GMOs entered the food chain, public and scientific debate concerning their detection, labeling, safety, traceability, gene flow, fate of GM protein, resistance development & regulatory compliance continues unabated. Syngenta (USA) had inadvertently sold unapproved genetically modified corn seed Bt-10 as approved Bt-11, to US farmers between 2001-2004[1]. A genetically engineered corn, Starlink, contains a special protein that takes longer than normal to break down in the digestive system and as a consequence it has not been approved for human consumption. Despite the ban, Starlink maize was discovered in the human food chain in the year 2000 (“http://www.greenpeace.org.uk/MultimediaFiles/Live/FullReport/7926.pdf”:http://www.greenpeace.org.uk/MultimediaFiles/Live/FullReport/7926
Owing to such consumer concerns regarding GM food, there is a pressing need of food producers, retailers and authorities to have access to appropriate rapid and rugged GMOs detection methods. In the European Community (EC), foods require labeling if >1% of any ingredients originate from GM material[2].
Bt corn (MON810, Monsanto, USA - Yieldgard®) – is a corn genetically engineered to resist European corn borers (Ostrinia nubilalis) by producing its own insect control protein, Cry1Ab (“http://www.agbios.com/dbase.php?action=Submit&evidx=9”:http://www.agbios.com/dbase.php?action=Submit&evidx=9). Bt corn was developed by transferring the cry1Ab gene from Bacillus thuringiensis into corn cultivar Hi-II by gene gun transformation. Global production of Bt maize during the 2006 growing season was estimated at 11.1 million hectares, representing 14% of all transgenic crops grown that year[3]. As estimated 20,000-30,000 different types of food contain corn or soybean ingredients.
There are several conflicting reports on transgene stability in MON810 maize[4-6]. Though PCR assays for detection of MON810 have been reported catering to either specific gene or select associated regulatory sequences of the transgene cassette, but none of them is comprehensive enough[7-10]. Our present study provides a complete assessment of GM maize – MON810. Our standard PCR array assay format comprises of gene, construct, event, GMO and maize specific (house keeping), assays for detection & characterization of MON810. Additionally, for structural stability and integrity analysis, a construct, a gene and three event-specific PCR assays were developed. Also, for amplification of whole transgene cassette, using forward primer of P-e35S and reverse primer of T-nos, one long run construct specific PCR assay was developed. The developed PCR assays can be effectively used for routine detection & monitoring of trait stability, integrity of MON810 GM maize. The reported assays can be of immense use for breeders, buyers and trans-border regulatory agencies.

Subject terms: Nucleic acid based molecular biology Model organisms Genetic analysis Plant biology Spectroscopy Structural biology

Keywords: MON810 GMOs cry1Ab gene PCR detection and stability of transgene

Reagents

oligonucleotide primer pairs (MWG, Germany),
QIAGEN DNeasy Plant Mini Kit (QIAGEN, Germany),
QIAGEN Multiplex PCR kit,
QIAGEN QIAquick Gel Extraction Kit,
Longrun PCR amplification kit (Fermentas, USA)
Agarose,
0.5X TBE buffer pH 8.0 and
Ethidium bromide
CAUTION Toxic; avoid contact with skin, eyes or mucous membranes

Reference material:

The target DNA was sourced from transgenic maize – certified reference material MON810, RR Soya (gift, Dr. Lalita, Central Food Technological Research Institute, Mysore, India), Bt-cotton (gift, Dr. S. Vasanthi, National Institute of Nutrition, Hyderabad, India), VipCot (courtesy, Dr. R. K. Bhatanagar, International Centre for Genetic Engineering & Biotechnology, New Delhi) and native maize (from local market).

Equipment

UV spectrophotometer (Spectronic Unicam, Genesys, USA),
epigradient Mastercycler (Eppendorf, Germany),
Horizontal gel electrophoresis,
Alphalmager 2200 Gel documentation system (San Leandro, California, USA)

Procedure

a. DNA extraction:

1. Isolate DNA from the test material (i.e. GM maize CRM-MON810 5.0, 2.0, 1.0, 0.5, 0.1 %) using QIAGEN DNeasy Plant Mini Kit (QIAGEN, Germany).

2. Evaluate the DNA concentration of the extracted DNA by UV absorption at 260nm and the purity by A260/280nm ratio using a UV spectrophotometer (Spectronic Unicam, Genesys, USA).

3. Dilute the isolated DNA to a final concentration of 50ng/µl and use as stock solution for the PCR analysis.

b. Oligonucleotide primers:

1. The oligonucleotide primer pairs for cry1Ab transgene (gene and 3' event specific) were designed with the help of PrimerSelect program of "DNA STAR" software (DNASTAR Inc.,USA). The primer pairs for event (maize genome – P-e35S), construct (hsp70 – cry1Ab)8-9, CaMV 35S promoter (P-e35S), Nopaline synthase terminator (T-nos), Neomycin phosphotransferase (npt-II) marker gene11-12 and housekeeping gene – High mobility group (hmgA)13 gene were chosen from published literature. To confirm the plant origin of extracted DNA and its suitability for PCR, chloroplast t-RNA (trnl intron) amplification, with universal primer for three noncoding regions of chloroplast DNA was done14. The amplification of trnl intron served also as a positive control. The characteristic features of in house designed & other primers are given in Table 1.

2. Dilute desalted oligonucleotide primers (MWG, Germany), to a final concentration of 10pmole/ µl with milli-Q water and store at -20°C until use.

c. PCR conditions:

1. Programme the PCR assay in an epigradient Mastercycler (Eppendorf, Germany). The PCR reaction comprises of 25µl of reaction mixture using 1X QIAGEN Multiplex PCR Master mix solution which contains- HotStarTaq DNA polymerase, dNTP mix, multiplex PCR buffer with a final concentration of 3.0 mM MgCl2.

2. Prepare a typical optimal assay containing primer 20pmole each, 10% QIAGEN Q solution and 100ng template DNA. Set the standard PCR cycle conditions as: initial denaturation at 95°C for 15 min. followed by 35 cycles, each cycle should have a denaturation step at

94°C for 30 s, annealing at 60°C for 90 s and extension at 72°C for 60 s. followed by a final extension at 72°C for 7 min.

3. Perform Multiplex PCR assay using equimolar primer concentration for each primer set and maintain the annealing temperature at 64°C. The optimal annealing temperature of 64°C was ascertained by a gradient Multiplex PCR assay (data not shown). The reaction composition and other assay conditions are same as that of standard PCR detailed above.

4. Carry out the Longrun construct specific PCR amplification by a long run PCR assay. A typical reaction assay contains long PCR enzyme mix, 1X buffer, 1.5 mM MgCl2, 200 µM dNTP and primer 0.2 µM each (Fermentas, USA). Set the PCR cycling program as: initial denaturation step at 95°C for 2 min. followed by 30 cycles, each cycle involving a denaturation step at 95°C for 30 s, annealing at 55°C for 30 s and extension at 68 °C for 2.0 min. for npt-II transgene cassette but 4.0 min. for cry1Ab and vip-s transgene cassette with auto-extension of 2 s per cycle, followed by a final extension at 68°C for 10 min. The extension time was ascertained by calculating the length of construct specific amplicon.

5. For small construct specific assay, different combinations of primer sequences were designed and amplified (Figure 5 a & b) using same assay condition and PCR programme with minor modifications (elongation time 2.0 min.).

6. For nested PCR assay, purify the construct-specific PCR amplicons with QIAGEN QIAquick Gel Extraction Kit and use it as template DNA. The nested PCR program and assay conditions set same as that of the standard PCR assay.

d) Agarose gel electrophoresis:

Analyze the amplified products by electrophoresis on 2.0% (w/v) agarose gel in a buffer containing 89mM Tris, 89mM Boric acid, 2.0mM EDTA (0.5X TBE buffer, pH 8.0) and visualize after Ethidium bromide staining, on a Alphalmager 2200 Gel documentation system (San Leandro, California, USA). For multiplex PCR product, use 2.5% (w/v) agarose gel, while for DNA quality checks and long run PCR amplicon, use 1.0% (w/v) agarose gel. Set the running conditions as constant voltage at 80V for 2 hours.

Timing

Three days or 30hrs

Critical Steps

I. Isolation of good quality DNA

II. Individual PCR setups for standard array type assay,
multiplex and long run PCR assays with adequate controls.

III. Agarose gel electrophoresis.

Troubleshooting

o Little or no product
~ HotStarTaq DNA polymerase not activated – ensure the activation step 15min at 95°C.
~ Quality of DNA – DNA template should be pure, evaluated by A260/280nm ratio using a UV spectrophotometer and chloroplast DNA (trnl intron) amplification.
~ Pipetting error or missing reagent
~ PCR assay conditions not optimal.
~ Problem with thermal cycler.

o Some products are missing
~ Primers degraded or of low quality
~ Annealing temperature too high

o Additional products detectable
~ Too many PCR cycle
~ Primer concentration not optimal
~ Too high starting template concentration

Anticipated Results

Standard PCR assay for MON810

To characterize each segment of MON810 GM gene cassette, different single standard PCR assays were designed (Fig. 1a). Two different types of event specific and three types of construct specific PCR assays are feasible. The construct specific cry1Ab- T-nos junction amplification is not possible since T-nos has moved from 3' region of cry1Ab gene to elsewhere in genome 5. Hence, only two types of construct specific (P-e35S – hsp70 & hsp-cry1Ab) assays were possible. However, only construct hsp-cry1Ab assay was carried out due to suitability of assay. Amplification of event (from 5' region) maize genome – P-e35S border sequences was successfully carried out. The amplification from 3' region is not feasible due to absence of T-nos.
The standard PCR assays were performed in an array format, where all gene segments corresponding to primer sets were clearly and individually amplified using a common PCR programme and assay conditions (Fig. 1a & b). The amplicon 170bp represents MON810 event (maize genome – P-e35S); amplicon 113bp – represents cry1Ab construct specific (hsp-cry1Ab), amplicon 599bp represents – gene specific (cry1Ab), amplicons 215, 195 & 180bp represent amplification of GMO specific elements – nptII, P-e35S & T-nos respectively, amplicon 175bp represents high mobility group (hmgA) gene, which is the characteristic specific feature of maize and amplicon 550 represents the amplification of trnl intron of chloroplast t-RNA which is used in this study as a positive control. Four different controls were used for the specificity and validation of the assay – (a) environment control (no DNA) (b) negative control – DNA isolated from 0.0% CRM MON810; (c) internal control- Housekeeping gene hmgA & (d) positive control – trnl intron of chloroplast t-RNA. The amplification of house keeping gene hmgA and cry1Ab gene reflects the presence of maize origin and presence of cry1Ab gene respectively.

Multiplex PCR assay

The compatibility of primer sets is the key factor for success of a multiplex PCR assay. Multiple combinations of primer sets were considered, based on compatibility and amplicon size, for the distinct amplification. Finally, two batches of triplex PCR assays were designed, optimized and amplified (Fig. 2). The batch one; MON810 specific triplex PCR assay could easily demonstrate the amplification and characterization of cry1Ab transgene (gene specific), junction sequences of maize genome – P-e35S (event-specific) and hsp-cry1Ab sequence (construct-specific). The event specific PCR assays involve the overlapping regions of endogenous and inserted DNA. The event, construct and gene specific assays provide more accurate determination, since same gene and / construct are used in the creation of several GM plants, and only few of these may eventually be approved for commercialization.
The second batch of triplex PCR assay is for amplification of GMO specific sequences (npt-II, P-e35S & T-nos)11-12. Although, the generated amplicons are in close proximity, they can still be distinguished by a 2.5% agarose gel run. Non-appearance of bands in the respective negative and environmental controls, confirms the specificity of the assay. The GMO specific triplex PCR assay could, as a stand alone assay, be used for the screening of genetically modified plants. These GMOs specific sequences are important for the selection and expression of transgene and are present in more than 80% GM plants. The detection of these elements indicates the presence of GM material.

Limit of detection (LOD)

The estimation of the accuracy and precision of the PCR assay is important for labeling norms in the EU that considers 1% of transgenic material as the threshold limit for compulsory labeling[2, 13]. MON810 specific triplex PCR assay was employed to establish the LOD for MON810. The DNA samples isolated from certified reference material of MON810 (5%, 2%, 1%, 0.5%, 0.1% & 0.0%) were used as template. The assay yielded a clear distinct gel picture, demonstrating significant amplification at all test concentrations of CRM samples. As expected, 0% sample showed no band (Fig. 3). The uniqueness of this assay is the ability to establish LOD, with greater confidence, being supported by concurrent generation of gene, event and construct specific amplicons of MON810. Our data led us to infer that as low as 38 copies of transgene cassette could easily be detected (Table 2). The copy no. of GMO genome was calculated using nuclear DNA content of the Zea mays15. The gel picture is tempting enough to suggest that even 0.05% sample detection is feasible. However due to non-availability of 0.05% MON810 CRM, the assay was not performed. The LOD of 0.1% is adequate enough for regulatory compliance on GMO's. The specificity of event, construct & gene specific triplex PCR assay for MON810 was confirmed by testing genomic DNA sourced from other GMOs i.e. RR Soya, Bt-Cotton, transgenic cotton carrying vip-s gene and native maize. None of the templates except that of MON810 elicited any amplification, confirming the specificity of the assay (data not shown).

Longrun transgene cassette specific PCR assays

As per US patent # US2004180373, YieldGard(R) corn event MON810 was generated using plasmid pMON15772 which has two expression cassettes, at 5' end nptII marker gene and linked at a 3' end to cry1Ab transgene cassette7. Both the transgene cassettes are flanked with P-e35S promoter at 5' end and with T-nos terminator at 3' end. Maize hsp70 intron sequence is placed just between the P-e35S promoter and cry1Ab transgene. The presence of these transgenic inserts was already confirmed by standard PCR assay (Fig. 1a & b) and its stability and integrity was now evaluated through longrun construct specific PCR assays.
For assessing molecular stability of transgene cassette, long construct and event specific PCR assays were performed using existing published primer sets and also the in house designed new ones (Fig. 5a & b). The variable combinations employed in either case were unique and have not been reported so far. The longrun construct specific PCR assay was designed using forward primer of P-e35S & reverse primer of T-nos, the elongation time for amplification adjusted at 1 min./Kb of amplicon coupled with auto extension of 2

sec./cycle. The expected amplicon size for npt-II transgene cassette is ~1.5Kb and for cry1Ab transgene cassette it is ~5.0Kb, if construct remains in a stable configuration (Table 3 a & b). The PCR run yielded no amplicon for any of the transgene cassettes (Fig 4a & b). A similar PCR run for amplification of npt-II transgene cassette of Bt-cotton and vip-s transgene cassette of Vip-cotton, (two positive controls) was affirmative and confirmed the validity of the assay. The anticipated amplicons sizes of positive controls were ~1.5Kb (Bt-cotton) and ~3.1Kb (Vip-cotton) samples (Fig. 4c & d). On the basis of these observations, this long run assay confirms the structural instability of MON810 transgene cassettes. Since the cry1Ab gene in MON810 maize is truncated and T-nos being absent, the long run amplification was not expected. However, inability to amplify npt-II transgene cassette suggests migration of either one or both of its constituent components (P-e35S & T-nos). Contrary to the claims of Monsanto regarding absence of selection marker gene (npt-II) in the genome of MON810 maize4, we consistently observed the presence of both npt-II & T-nos by our standard PCR assays (Fig. 1b). There have been conflicting claims regarding the presence of T-nos in MON8108 - http://www.i-sis.org.uk/UTLI.php

Structural and functional stability of MON810

To monitor the insert integrity and structure of the existing truncated, but functional, cry1Ab transgene cassette, different combinations of primer sets were tested (Fig. 5a). Amongst number of assays evaluated, three long run event specific assays were performed. Initial two long run assays involved amplification from – 5' region, first one denoting junction region of maize genome – P-e35S end sequence, with amplicon size of 336bp, and the second assay represented border sequences of maize genome and cry1Ab transgene bridged by covering P-e35S & hsp70 gene sequence, with resultant amplicon size of 1205bp. The third assay amplified 3' region of truncated cry1Ab transgene – maize genome with amplicon size of 1533bp (Fig. 5a & b). Additionally a construct specific PCR assay was run by amplifying junction sequences of P-e35S – cry1Ab transgene bridged by hsp70 gene, resulting in 992bp amplicon. Finally, a cry1Ab gene specific PCR assay was executed, resulting in a 599bp amplicon. The amplicons generated by two event specific assays; (a) maize genome – P-e35S, (b) maize genome – cry1Ab transgene and one construct specific; P-e35S – cry1Ab transgene, were at variance from the calculated anticipated sequence sizes detailed in Table 3. Rather, the data suggested deletion in P-e35S region. Subsequent to our study, sequence of synthetic construct truncated cry1Ab gene was listed on public domain (NCBI acc. no. AY326434), which explains and confirms our findings (Fig. 5c). The long run event specific & construct specific amplicons of 5' region of cry1Ab transgene cassettes were confirmed/validated by an independent nested PCR assay (Fig. 6). For such nested PCR, long run amplicons were purified from gel & served as the template.

During transgene inheritance, deletion, duplication, rearrangement and repeated sequence recombination for transgenic loci have been reported16. The proposed longrun construct specific PCR assays are able to signal such a deletion & can simultaneously assess the truncated transgene cassette, for structural stability and integrity over succeeding generations of MON810. The functional stability of the truncated MON810 transgene, was confirmed by detection of Cry1Ab protein by a commercial Dip Stick kit (Envirologix, USA)17. In view of the observed truncation of MON810 transgene with functional stability remaining intact, it would be prudent to assess the transgene structure stability over generations and define critical regions essential for GM protein expression.

Conclusions

Though a number of PCR based assays to detect MON810 GM crop & produce are available on public domain, none for complete characterization & structural stability analysis is available so far. The uniqueness of present study is in the experimental design, execution and interpretation of the results. In spite of the fact, that the many of our assays employed previously reported primer sets, the optimized assay conditions and use of various combinations of primers for concurrent amplification, elicited new and valuable information relating to the changes in the transgene construct.

Our analysis revealed the absence of both T-nos and part of the 3' (tail) end of the cry1Ab gene. The relocation of T-nos elsewhere in the genome indicates that it may have moved from its original position. This study also indicated a partial deletion of P-e35S and concurs with a similar recent report. The study of GMOs structure would neccessitate sensitive detection and quantification tests complying with the multiple multinational regulations. Such tests would enable monitoring of transgene stability over successive generations and characterization of different GMO cultivars produced with the same initial construct and provide information on the effect of the genomic background on the DNA insert stability. We propose that the developed assays shall fulfils the requirement of a complete molecular detection/analysis of MON810 transgene insert & its stability in any subsequent generation and also help in evaluation of safety factors like gene flow or genome fluidity.

References

1. Herrera, S. Syngenta's gaff embarrasses industry and White House. *Nature Biotechnol*. **23**, 514 (2005).

2. Ahmed, F.E. Detection of genetically modified organisms in foods. *Trends Biotechnol*. **20**, 215-223 (2002).

3. James, C. Global Status of Commercialized Biotech / GM Crops: ISAAA Briefs No.34, ISAAA, Ithaca, NY (2005) http://www.isaaa.org/

4. BATS. Genetically Modified (GM) Crops: molecular and regulatory details (2003). http://www.bats.ch/gmo-watch/GVO-report140703.pdf

5. Hernández, M., Pla, M., Esteve, T., Prat, S., Puigdomènech, P., Ferrando, A. A specific real-time quantitative PCR detection system for event MON810 in maize YieldGard® based on the 3'-transgene integration sequence. *Transgenic Res*. **12**, 179–189 (2003).

6. Decision Document 97-19: Determination of the Safety of Monsanto Canada Inc.'s YieldgardTM Insect Resistant Corn (Zea mays L.) Line MON810 http://www.inspection.gc.ca/english/plaveg/bio/dd/dd9719e.shtml#A1

7. Elaine, L.B. Corn event MON810 and compositions and methods for detection thereof Publication number US2004180373 Publication date: 2004-09-16

8. Matsuoka, T., Kawashima, Y., Akiyama, H., Miura, H., Kusakabe, Y., Isshiki, K., Goda, Y., Toyoda, M., Hino. A. A method of detecting recombinant DNAs for four lines of genetically modified maize. *J. Food Hygienic Soc. Jpn*. **41**, 137–143. (2000)

9. Bonfini, L. GMOs Methods database Report on PCR methods submitted to Ring-Trial (2004) http://biotech.jrc.it/home/ict/methods-doc/GMOmethods-Report-PCR.pdf

10. Monsanto. A Recommended Procedure for Real-Time Quantitative TaqMan PCR for YieldGard® Corn Borer Corn, MON810 Monsanto Biotechnology Regulatory Sciences (2004)
http://www.monsanto.com/products/techandsafety/technicalpubs.asp

11. Lipp, M., Bluth, A., Eyquem, F., Kruse, L., Schimmel, H., Van den Eede, G., Anklam, E. Validation of a method based on polymerase chain reaction for the detection of genetically modified organisms in various processed foodstuffs. *Eur. Food Res. Technol*. **212**, 497-504 (2001).

12. European standard document # CEN/TC 275 WG 11 N149. Food stuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Qualitative nucleic acid based methods (2001).

13. Laura, B., Petra, H., Simon, K., Van den Eede, G., Review of GMO detection and quantification techniques. European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Food Products and Consumer Goods Unit, I-21020 Ispra, Italy (2001). http://www.osservaogm.it/pdf/JRCReview.pdf

14. Taberlet, P., Gielly, L., Pautou, G., Bouvet, J. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol*. **17**, 1105-1109 (1991)

15. Arumuganathan, K., Earle, E.D. Nuclear DNA content of some important plant species. *Plant Mol Biol Report*. **9**, 208-218 (1991)

16. Yin, Z., Plader, W., Malepszy, S. Transgene inheritance in plants. *J. Appl. Genet*. **45**, 127-144 (2004).

17. EnviroLogix Inc. CrylAb/c Plate Kit. EnviroLogix Inc. 500. Riverside Industrial Parkway, Portland, Maine 0410301418, USA (2003). http://www.envirologix.com/library/ap003insert.pdf

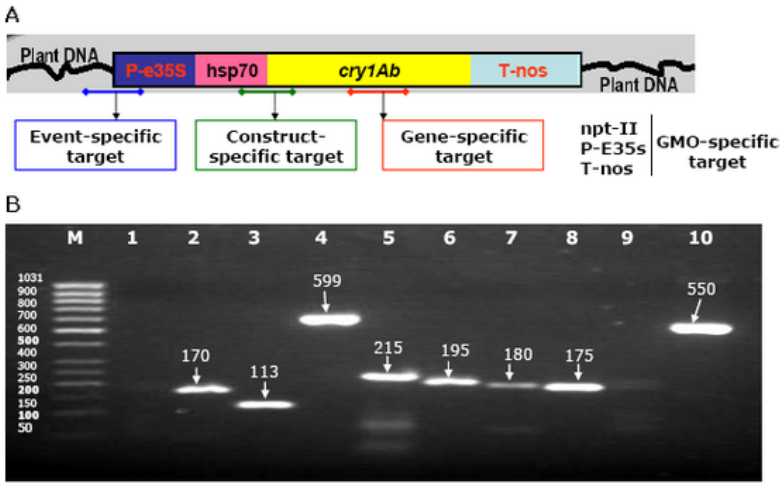
Acknowledgements

This work was supported by grants from Department of Biotechnology, India.

Figures

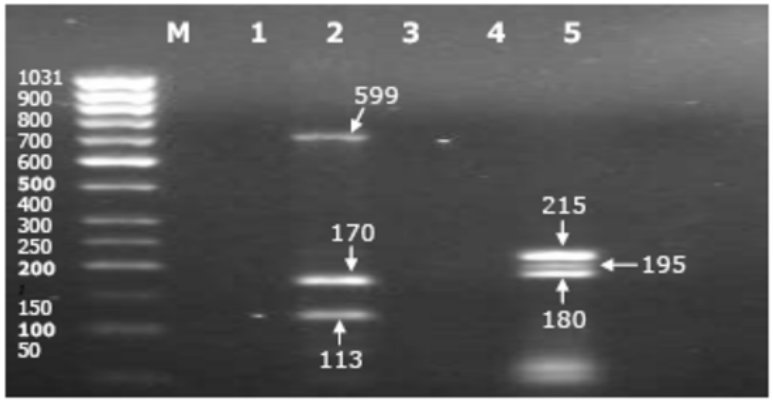
Download

Figure 1



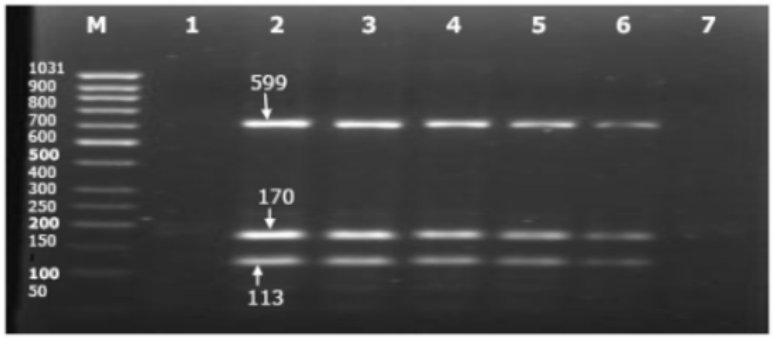
(a) Assay designing for Standard PCR assays for MON810.
(b) Molecular detection of MON810 - Standard PCR assays.
Lane M: 50bp marker, Lane 1: Env. Control, Lane 2: cry1Ab event specific (maize genome – P-e35S), Lane 3: cry1Ab construct specific (hsp-cry1Ab),Lane 4: gene specific (cry1Ab), Lane 5: npt-II, Lane 6: P-e35S, Lane 7: T-nos, Lane 8: hmgA, Lane 9: Neg. control, Lane 10: Pos. control (Chl. t-RNA)

Figure 2: Multiplex PCR assays for MON810.



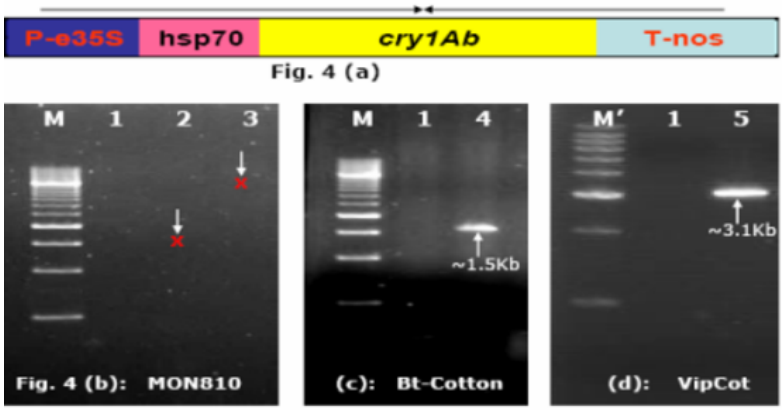
Lane M: 50bp marker, Lane 1: Env. Control, Lane 2: Multiplex PCR assay for cry1Ab transgene with gene (cry1Ab), event (maize genome – P-e35S) and construct (hsp-cry1Ab) specificity, Lane 4: Multiplexing for GMO specific sequences (npt-II, P-e35S & T-nos), Lane 3 & 5: Their respective Neg. Controls.

Figure 3 : LOD with Multiplexing for MON810 - gene (cry1Ab), event (maize genome – P-e35S) and construct (hsp-cry1Ab) specific assays.



Lane M: 50bp marker, Lane 1: Env. Control, Lane 2-7: Multiplex PCR assay for cry1Ab transgene construct with gene (cry1Ab), event (maize genome – P-e35S) and construct (hsp-cry1Ab) specificity, with 5%, 2%, 1%, 0.5%, 0.1% & 0.0% MON810 CRM respectively.

Figure 4: Universal longrun construct specific PCR assays for MON810, Bt-cotton and VipCot.



Lane M: 0.5Kb ladder, Lane M': 1.0Kb ladder, Lane 1: Env. Control, Lane 2 & 3: Longrun PCR assay utilizing forward primer of P-e35S and reverse primer of T-nos using template DNA from Mon810, Lane 4: Bt-cotton (Bollgard I), Lane 5: VipCot – vip-S bearing transgenic cotton.

Figure 5

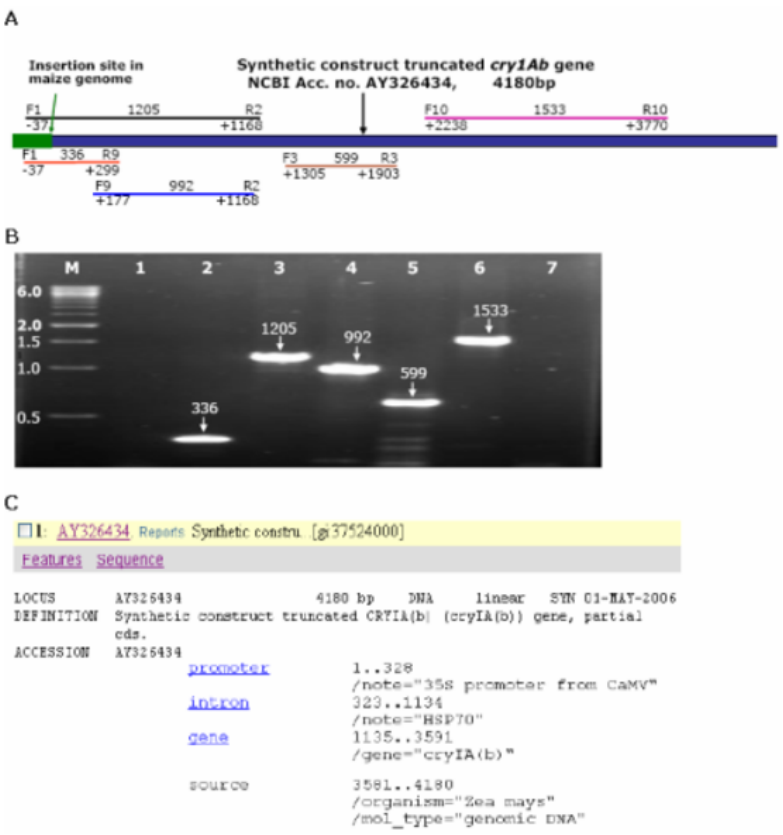
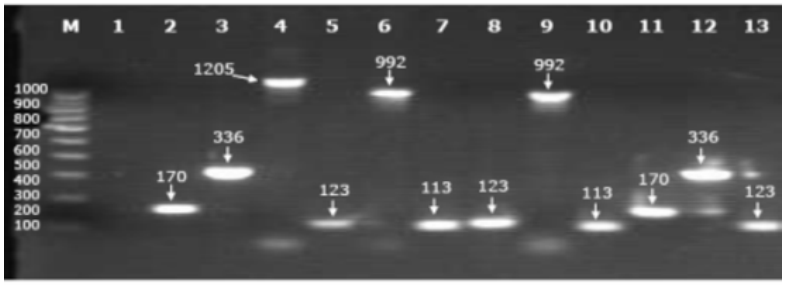


Fig. 5(a): Schematic representation of construct specific PCR assay.
Fig. 5(b): Event & Construct-specific PCR assay for MON810 - to confirm the insert integrity and stability of transgene. Lane M: 0.5Kb ladder, Lane 1: Env. Control, Lane 2: Amplification of adjoining sequences of maize genome & P-e35S (event specific), Lane 3: maize genome & cry1Ab transgene (construct specific), Lane 4: P-e35S & cry1Ab transgene (construct specific), Lane 5: cry1Ab (gene specific), Lane 6: cry1Ab – maize genome (event specific), Lane 7: Neg. Control.

Figure 6: Nested PCR assay for MON810 to confirm the transgene insert integrity.



Lane M: 100bp ladder, Lane 1: Env. Control, Lane 2-13: Amplification as given below –

Template – (1205bp) amplicon of construct-specific PCR assay

2. Maize genome F1 + P-e35S R1 - 170bp
3. Maize genome F1 + P-e35S R9 - 336bp
4. Maize genome F1 + cry1Ab R2 - 1205bp
5. P-e35S F9 & R9 - 123bp
6. P-e35S F9 + cry1Ab R2 - 992bp
7. hsp70 F2 + cry1Ab R2 - 113bp

Template – (992bp) amplicon of construct-specific PCR assay

8. P-e35S F9 & R9 - 123bp
9. P-e35S F9 + cry1Ab R2 - 992bp
10. hsp70 F2 + cry1Ab R2 - 113bp

Template – (336bp) amplicon of construct-specific PCR assay

11. Maize genome F1 + P-e35S R1 - 170bp
12. Maize genome F1 + P-e35S R9 - 336bp
13. P-e35S F9 & R9 - 123bp

Table 1: Primer sets used in this assay

Primer pair		NCBI Acc. No.	Sequence (5'–3')	Amplicon (bp)	Ref.
Event specific (maize genome – P-e35S)		AY326434	F1= 5'- tcg aag gac gaa gga ctc taa cg -3' R1= 5'- tcc atc ttt ggg acc act gtc g - 3'	170	8-9
Construct specific (hsp70 – cry1Ab)			F2= 5'-gat gcc ttc tcc cta gtg ttg a-3' R2= 5'-gga tgc act cgt tga tgt ttg-3'	113	
Gene specific (*cry1Ab*)			F3= 5'- cct ggg cct cgt gga cat cat ct -3' R3= 5'- tcg ggt agg tgc ggg agt cgt agt -3'	599	This Study
GMO specific	*npt II* gene	AF269238	F4= 5'- ctc acc ttg ctc ctg ccg aga - 3' R4= 5'- cgc ctt gag cct ggc gaa cag - 3'	215	11-12
	P-e35S	V00141	F5= 5'- gct cct aca aat gcc atc a - 3' R5= 5'- gat agt ggg att gtg cgt ca - 3'	195	
	T-nos	V00087	F6= 5'- gaa tcc tgt tgc cgg tct tg - 3' R6= 5' - tta tcc tag ttt gcg cgc ta - 3'	180	
hmgA – maize specific		AJ131373	F7= 5'-gaa atc cct gag cga gtc ggt a-3' R7= 5'-gcg atg gcc ttg ttg tac tcg a-3'	175	13
chioroplast t RNA gene – plant specific		Z00044, X15901	F8= 5'- cga aat cgg tag acg cta cg - 3' R8= 5'- ggg gat aga ggg act tga ac - 3'	550	12, 14
Additional primers used for structural stability analysis					
P-e35S		V00141	F9= 5'- cca cgt ctt caa agc aag tgg - 3' R9= 5'- tcc tct cca aat gaa atg aac ttc c-3'	123	12
cry1Ab		AY326434	F10= 5'- cgc agg ccc ttc aac atc ggt atc-3' R10= 5'- ggt cgg cgc cca aca aca aga -3'	1533	This Study

Table 2: Calculation of GMO genome copy for MON810 in a 100ng target DNA to establish limit of detection (LOD).

S. No.	MON810 CRM (%)	'GMO' genome copies for MON810
1	5.0	1930
2	2.0	772
3	1.0	386
4	0.5	193
5	**0.1**	**38**
6	0.0	0.0

- ***Zea mays*** **nuclear DNA content[19] is reported to be 4.75-5.63 pg/2C, thus 1C value would be 5.19/2 = 2.59pg.**

- **So, number of 'GMO' genome copies in 100ng of 100% MON810 DNA will be 100ng/ 2.59pg = 38,610**

Table 3: Sequence details of cry1Ab transgene cassette, initially used in making of MON8106.

S.No.	Element – name	Size (Kb)
1.	P-e35S	0.62
2.	hsp70	0.80
3.	*cry1Ab* transgene	3.50
4.	T-nos	0.24
5.	*npt-II* gene	0.79

Author information

Affiliations

1. **Industrial Toxicology Research Centre, Post Box No. 80, M. G. Marg Lucknow – 226001, U.P., India**
   Chandra K. Singh , Abhishek Ojha & Suchitra Kamle

2. **Industrial Toxicology Research Centre, Post Box No. 80, M. G. Marg Lucknow – 226001, U.P., India Tel.: +91-522-2621856 EXT 249 Fax: +91-522-2628227**
   Devendra N. Kachru

Competing financial interests

The authors declare no competing financial interests.

Readers' Comments

Comments on this thread are vetted after posting.

- *Protocol Exchange*
- ISSN 2043-0116

© 2012 Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.
partner of AGORA, HINARI, OARE, INASP, CrossRef and COUNTER