Development and Utilization of Analytical Methods for Rapid GM Detection in Processed Food Products: A Case Study for Regulatory Requirement

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Abstract

Genetically modified (GM) food crops for desired traits have been approved in some of the countries. The approval status of a GM event varies from country to country. In India, *Bt* cotton is the only approved GM crop. So far, no GM food crop has got regulatory approval in the country, which may be considered as unauthorized GM (UGM) event in the Indian context. The entry of UGM events of food crops needs to be checked in the imported consignments as well as in the marketplace. In the present study, screening elements were identified based on the genetically modified organism (GMO) matrix developed as decision support system for 22 GM food crops approved globally. Three sets of multiplex PCR assays were developed and validated for GM detection in food crops and products: triplex PCR targeting control elements [*Cauliflower Mosaic Virus* 35S promoter (*P-35S*), *Figwort Mosaic Virus* promoter (*P-FMV*), nopaline synthase terminator (*T-nos*)], triplex PCR targeting marker genes [aminoglycoside-3'-adenyltransferase (*aadA*), neomycin phosphotransferase (*nptII*), phosphinothricin-N-acetyltransferase (*pat*)] and duplex PCR targeting *Bt* genes (*cry1Ab/Ac* and *cry2Ab2*). Limit of detection (LOD) ranged from 0.5 – 0.05% for different targets, which is in compliance with the labelling threshold of many countries. The developed assays were utilized to check the GM status of apple and maize products along with an additional test for *ctp2-cp4epsps* for herbicide tolerance in maize. These procedures could be efficiently employed as a part of GMO testing to trace GM contamination, if any, in both the imported as well as domestic food products.

Introduction

With the approval of first genetically modified (GM) food crop FLAVR SAVR™ tomato with delayed fruit ripening in 1990s, a number of GM food crops and derived products have got approval presently in some of the countries such as the United States and Canada. Since last 25 years, GM food crops have been developed for imparting resistance to insect pests, to better withstand salinity, drought and other abiotic stresses, and for improving different characteristics including color, flavor, texture and shelf life of foods along with the nutritional enhancement. The approval of GM food products is regulated in different countries by respective regulatory bodies. Labelling of genetically modified organism (GMO) is voluntary in some of the countries as in the United Sates; whereas it is mandatory in several countries as in the European Union (EU) with labelling threshold of 0.9% (Randhawa et al. 2016; Querci et al. 2010; https://ec.europa.eu/jrc/en/research-topic/gmos).

In India, *Bt* cotton is the only approved GM crop for planting and GMO labelling threshold has not yet been implemented. In the country, regulation of GMO and GM-derived products is being implemented under the "Rules for the manufacture, use, import, export and storage of hazardous microorganisms, genetically engineered organisms or cells, 1989" (Rules 1989) notified under the Environment (Protection) Act, 1986 (EPA 1986). In accordance with the Food Safety and Standards Authority of India (FSSAI) Order No. 1-1764/FSSAI/Imports/2018 (Part-1) dated 21 August 2020 and 1-1764/FSSAI/Imports/2019 (Part-1) dated 08 February 2021, every imported consignment of 24 selected crops should be accompanied with the non-GMO certification effective from 1 March 2021 and the tolerance limit of adventitious presence of GMOs at 1 per cent is permissible in the consignments of imported food crops (FSSAI 2021).

Many food products are being imported in India from the countries where the GM events of respective food crop are approved for use as food or feed. Therefore, it is necessary to check the unauthorized entry of GM products in the supply chain as well as in the marketplace as GM food products are regulated in the country. Systematic GMO testing is essential for checking the GM status of food products for verification for their non-GM status.

DNA-based methods are preferred over protein-based methods for GMO testing of processed food products or derivatives as there may be the chances of degradation of proteins. Moreover in processed food products, quality of DNA may be hampered during processing steps or due to exposure to high temperature and pressure. Addition of certain ingredients or flavors may also considerably affect the quality of DNA, thereby inhibiting the amplification (Ramos-Gómez et al. 2014). Therefore, DNA extraction has been considered as a crucial step for GMO testing in food derivatives. DNA extraction methods from food products using chemical, thermal, enzymatic, or mechanical lysis, or a combination of these, or through use of column-based purification have been reported (Rantakokko-Jalava and Jalava 2002; Reischl et al. 2000; Van Tongeren et al. 2011; Singh et al. 2021).

Selection of common targets employing GMO matrix approach could facilitate cost- and time-efficient GMO screening by eliminating the need to test for every possible GM event of a particular crop (Waiblinger et al. 2010; Randhawa et al. 2014). The selected targets can be tested using polymerase chain reaction (PCR), multiplex PCR or real-time PCR assays. Multiplex PCR enables simultaneous detection of multiple targets in a test sample. Hexaplex PCR simultaneously detecting six marker genes was developed to check the GM status of seeds (Randhawa et al. 2009). GMO matrix combined with multiplex PCR was reported for GMO screening of globally approved GM events of cotton and maize (Singh et al. 2016).

Few reports on the utilization of GM diagnostics for GMO testing of food products from the markets in Brazil, Georgia and Turkey are available (Avsar et al. 2020; Cardarelli et al. 2005; Datukishvili et al. 2015). Cardarelli et al. (2005) checked the GM status of maize and soybean derived food products including raw soybeans, dehydrated and canned soups, powdered soy milk, cookies, french fries, sausages, products of maize meal, canned corn, soy beverages, soy protein, pasta, and pet-food, procured from the Brazilian market using PCR assays for Roundup Ready soybean, Bt176 and MON810 events of GM maize, and some of the soybean derived products were found GM. Datukishvili et al. (2015) utilized multiplex PCR simultaneously targeting *Cauliflower Mosaic Virus* 35S promoter (P-*35S)*, nopaline synthase terminator (*T-nos*), 5-enolppyruvylshikimate-phosphate synthase (*cp4-epsps*), for GM detection in soy, and targeting *P-35S* and *cry1Ab* for GM detection in maize food products. Avsar et al. (2020) checked the presence or absence of GM ingredients in 123 food and feed samples derived from maize, rice, soybean and wheat from retail shops in Turkey. The GM status of samples was checked by the multiplex real-time PCR simultaneously detecting *P-35S*, *T-nos*, *P-FMV* and *bar* gene, None of the wheat or rice products were found GM positive whereas, several maize and soybean derived samples were found positive for GM elements tested.

In the present study, based on the information available in the GMO databases, common elements were identified for screening of 80% of globally approved GM events (> 130 single or non-stacked GM events) of 22 food crops. Published matrix of maize was considered. Based on the GM coverage and ease of reactions, three sets of multiplex PCR assays were developed and validated for acceptable specificity and limit of detection (LOD) ranging from 0.5 – 0.05% for different assays/ targets. These assays were utilized to test the GM status of food derivatives of apple and maize collected from the retail shops and markets.

Materials And Methods

Reference Material for Method Validation

Certified reference material (CRM) of GM maize events, namely, Bt11 (SYN-BTØ11 – 1), Bt176 (SYN-EV176-9), NK603 (MON-ØØ6Ø3–6), TC1507 (DAS-Ø15Ø7 – 1), GA21 (MON-ØØØ21 – 9), MIR604 (SYN-IR6Ø4–4), MON810 (MON-ØØ81Ø-6), MON863 (MON-ØØ863-5), 3272 (SYN-E3272-5), 59122 (DAS-59122-7), 98140 (DP-Ø9814Ø-6), DAS 68416-4 (Blank), and GM soy events, namely, DAS 40278-9 (DAS-4Ø278-9), DAS 81419-2 (DAS-81419-2), 356043 (DP-356Ø43 – 5), 305423 (DP-3Ø5423-1), 40-3-2 (MON-Ø4Ø32 – 6), were procured from the European Reference Material (ERM), Joint Research Centre-European Commission, through Sigma Aldrich. Seed samples of approved *Bt* cotton events, MON531 (MON-ØØ531-6) and MON15985 (MON-15985-7) of Maharashtra Hybrid Seeds Company Limited along with the non-GM counterpart were used. DNA samples of GM maize events, MON89034 (MON-89Ø34 – 3), GM cotton events, MON88913 (MON-88913-8), MON15985 x MON88913 (*Bt* Roundup Ready Flex), MON15985 x Cot102 (Bollgard®III), MON15985 x MON88913 x Cot102 (Bollgard®III Roundup Ready Flex), 281-24-236 x 3006-210-23 (DAS-24236-5 X DAS-21Ø23 – 5) imported for research purposes through the ICAR-National Bureau of Plant Genetic Resources, New Delhi, were used. Seeds of non-GM maize and soybean were purchased from the National Seeds Corporation, New Delhi.

Food Products/ Derivatives

Total of 14 food products of maize and apple were analyzed using developed multiplex PCR assays. As GM maize has been approved for consumption as food or feed in many countries, the samples of six food products were used, which included Corn flour (Brown & Polson), Natural Roasted Puff (Cool Crunchies), Corn Chips (Makino). Masala Munch (Kurkure), Sweet Corn Vegetable Soup (Knorr), and Plain Flour All Purpose (Organ). GM apple has been approved in the United States and Canada, therefore, five samples of apple fruit and three samples of apple products, *viz*., Apple Pie Cookies (Merba), Puffs Cereal Snack (Strawberry Apple) (Gerber), and Apple Fruit Drink (Amul Tru) were also subjected to the GMO testing. The samples were purchased from the retail shops/ grocery stores/ supermarkets based at Delhi or nearby location.

Sample Preparation

Genomic DNA from CRM/ reference material (RM) was extracted using DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) protocol (as per the manufacturer's instructions). The contents of whole packet of each food product were homogenized to fine powder for representing whole sample using a Knife Mill Grindomix GM-200 (Retsch). DNA from food derivatives was extracted using modified CTAB method or DNeasy® Mericon Food Kit (Qiagen) protocol (Singh et al. 2021). Quantity and quality of DNA extracts were estimated using a UV Spectrophotometer (Eppendorf, Hamburg, Germany). DNA extracts were diluted to a concentration of 40 ng/µl for further use. Before method validation, DNA extracts of CRM/ RM were validated using respective event- or gene-specific protocols.

Gmo Matrix, Selection Of Targets And Multiplex Pcr Assays

The screening targets for GM detection in 22 GM food crops including alfalfa, apple, Argentina canola, common bean, chicory, cowpea, eggplant, flaxseed, melon (*Cucumis melo*), papaya, Polish canola, plum, potato, rice, safflower, soybean, squash, sugarbeet, sugarcane, sweet pepper, tomato and wheat, were identified employing GMO matrix approach based on the information compiled from open access GM crop databases, namely, http://bch.cbd.int/database/organisms, http://www.isaaa.org/gmapprovaldatabase/. Selected genetic elements, which can cover most of the GM events of these crops are shown in Supplementary Information 1 and Fig. 1. Previously reported crop-specific GMO matrix was referred for GM maize (Singh et al., 2016). Triplex PCR targeting two promoters (*P-35S*, *P-FMV*) and a terminator (*T-nos*); triplex PCR targeting three marker genes (*aadA*, *nptII*, *pat*) and duplex PCR targeting *cry1Ab/Ac* and *cry2Ab2* transgenes were developed and validated. Details of primers used are given in Table 1.

Table 1
Primers used in the study

Target	Primer Code	Sequence	Product size	Reference
P-35S	p35S-cf3	CCACGTCTTCAAAGCAAGTGG	123 bp	Lipp et al., 2001
	p35S-cr4	TCCTCTCCAAATGAAATGAACTTCC		
P-FMV	pFMV-F	CAAAATAACGTGGAAAAGAGCT	78 bp	ISO/TS 21569-5: 2016, 2016
	pFMV-R	TCTTTTGTGGTCGTCACTGC		
T-nos	NOS-1	GAATCCTGTTGCCGGTCTTG	180 bp	Lipp et al., 1999
	NOS-3	TTATCCTAGTTTGCGCGCTA		
aadA	AadA-1-F	TCCGCGCTGTAGAAGTCACCATTG	406 bp	Randhawa et al., 2009
	AadA-1-R	CCGGCAGGCGCTCCATTG		
nptII	APH2 short	CTCACCTTGCTCCTGCCGAGA	215 bp	ISO21569:1–69, 2005
	APH2 reverse	CGCCTTGAGCCTGGCGAACAG		
Pat	Pat-f	GAAGGCTAGGAACGCTTACG	262 bp	Permingeat et al., 2002
	Pat-r	GCCAAAAACCAACATCATGC		
cry1Ab/Ac	Bt-F1	GAGGAAATGCGTATTCAATTCAAC	74 bp	Grohmann et al. 2015
	Bt-R	TTCTGGACTGCGAACAATGG		
Cry2Ab2	Cry2Ab-F	CAGCGGCGCCAACCTCTACG	260 bp	Randhawa et al. 2010
	Cry2Ab-R	TGAACGGCGATGCACCAATGTC		
ctp2-cp4epsps	GT73-TmF	GGGATGACGTTAATTGGCTCTG	88 bp	Grohmann et al., 2009
	GT73-TmR	GGCTGCTTGCACCGTGAAG		
Adh1	Adh-FF3	CGTCGTTTCCCATCTCTTCCTCCT	135 bp	Mazzara et al., 2013
	Adh-RR4	CCACTCCGAGACCCTCAGTC		
cp-tRNA	PlantA1	CGAAATCGGTAGACGCTACG	~ 615 bp	Taberlet et al. 2001
	PlantA2	GGGGATAGAGGGACTTGAAC		

Multiplex PCR was performed in Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in 20 µl volume with the composition as follows:

Component	Set1	Set 2	Set 3
DNA (40 ng/µl)	200 ng	200 ng	200 ng
Muliplex PCR Master Mix (Qiagen® Multiplex PCR Kit)	1x	1x	1x
Primers (F/R)	*P-35S*: 0.1 µM each	*aadA*: 0.25 µM each	*cry1Ab/Ac*: 0.25 µM each
	P-FMV: 0.4 µM each	*nptII*: 0.25 µM each	*cry2Ab2*: 0.25 µM each
	T-nos: 0.3 µM each	*pat*: 0.25 µM each	

The cycling profile comprised of initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 50 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR products were analyzed on 4% (w/v) metaphor agarose gels or on 2% (w/v) agarose gels in 1x TAE buffer, and visualized using UV Gel Imaging System (Alpha Innotech, USA).

Method Validation Of Developed Multiplex Pcr Assays

Specificity of multiplex PCR assays was confirmed using defined set of targets and non-targets for respective transgenic elements (Table 2). Sensitivity was determined in terms of LOD using 100% test sample with respective targets. The 100% GM sample was diluted with non-GM counterpart to obtain the samples with 50, 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01% GM content (v/v) for respective target. For sensitivity test, DNA sample of MON89034 was used for triplex PCR targeting control elements. An equivalent mix of DNA samples of two GM events of cotton, namely MON15985 and 281-24-236 x 3006-210-23 was used for triplex PCR targeting marker genes. Sample of MON15985 was used for duplex PCR targeting *Bt* genes.

Table 2
Reference material used in validation of Multiplex PCR for food testing

GM event	GM % in RM/ CRM	Crop	Triplex PCR (control elements)						Triplex PCR (marker genes)						Duplex PCR (*Bt* genes)			
			P-35S		*P-FMV*		*T-nos*		*aadA*		*nptII*		*pat*		*cry1Ab/Ac*		*cry2Ab2*	
			S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
MON531	100%	Cotton	+	nd	-	nd	+	nd	+	+	+	+	-	-	+	+	-	-
MON15985	100%		+	nd	-	nd	+	nd	+	+	+	+	-	-	+	+	+	+
MON15985 x Cot102	100%		+	nd	-	nd	+	nd	+	+	+	+	-	-	+	+	+	+
MON15985 x MON88913	100%		+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
MON15985 x MON88913 x Cot102	100%		+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+
Widestrike	100%		-	-	-	-	-	-	-	-	-	-	+	+	+	nd	-	nd
Non-GM cotton	0		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bt11	10%	Maize	+	+	-	-	+	+	-	-	-	-	+	+	+	+	-	-
Bt176	1%		+	nd	-	nd	-	nd	-	nd	-	nd	+	nd	+	+	-	-
GA21	5%		-	-	-	-	+	+	-	-	-	-	-	-	-	nd	-	nd
MIR604	10%		-	nd	-	nd	+	nd	-	-	-	-	-	-	-	nd	-	nd
MON810	5%		+	nd	-	nd	-	nd	-	-	-	-	-	-	+	nd	-	-
MON863	10%		+	+	-	-	+	+	-	nd	+	nd	-	nd	-	-	-	-
MON89034	100%		+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+
NK603	5%		+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-
TC1507	10%		+	+	-	-	-	-	-	nd	-	nd	+	nd	-	-	-	-
98140	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	nd	-	nd
Non-GM maize	0		-	-	-	-	-	-	-	-	-	-	-	-	-	nd	-	nd
DAS 68416-4 (Blank)	0	Soy	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DAS 40278-9	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	nd	-	nd
356043	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
305423	10%		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40-3-2	10%		+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-
DAS 81419-2	5%		-	-	-	-	-	-	-	nd	-	nd	+	nd	+	nd	-	nd
Non-GM soy	0		-	-	-	-	-	-	-	nd	-	nd	-	nd	-	-	-	-

S: Theoretical data based on the information available in GM databases, namely, http://bch.cbd.int/database/organisms, http://www.isaaa.org/gmapprovaldatabase/

R: Verified experimentally using specificity tests with developed multiplex PCR assays.

nd: not tested

Gmo Testing Of Food Derivatives

Amplifiability of DNA extracts of food products was confirmed by PCR and real-time PCR targeting endogenous *Adh1* gene of maize and for apple products, PCR assay targeting chloroplast tRNA (*cp-tRNA*) gene was employed (Mazzara et al. 2013; Taberlet et al. 2001). All the three sets of multiplex PCR were used to test the GM status of food derivatives using same PCR conditions as mentioned in method development and validation. All the samples were tested in replications with appropriate positive and negative controls to reduce the chance of errors due to false negatives or positives. An additional PCR test targeting commonly employed *ctp2-cp4epsps* region for herbicide tolerance was conducted in case of maize products.

Results And Discussion

DNA-based methods, including PCR and real-time PCR may be preferred over protein-based methods for GM detection in food products due to stability of DNA. Commonly present transgenic elements such as *P-35S* and *T-nos* are the targets of choice for GMO screening (Lipp et al. 2001; Waiblinger et al. 2010; Holden et al. 2010; Bak and Emerson 2019). However, the inclusion of more screening targets with respect to approved GM events of a particular crop, may enhance the GM coverage in GM detection, for which GMO matrix approach can be applied for selection of suitable targets. In the present study, multiplex PCR assays targeting common transgenic elements identified in GM food crops approved globally using matrix approach were developed and validated. These assays were adopted for preliminary GMO screening in food derivatives of maize and apple as an initiative for GMO testing of food products in India. The screening assays give a fair idea for the GMO status of a sample and further confirmation could be done using event-specific tests in case of positive screening results.

Selection Of Screening Targets

GMO matrix has been utilized as a cost-effective strategy for screening of authorized and unauthorized GMOs (Holst-Jensen et al. 2012; Kralj Novak et al. 2009; Van den Bulcke et al. 2010; Waiblinger et al. 2010). Randhawa et al. (2014) reported an Indian GMO screening matrix for detection of 141 GM events of 21 crops including commercialized *Bt* cotton events and other GM events, under field trials or imported for research purposes. Ten targets, namely, *P-35S, T-nos, Os-Msca1, cry1Ab, cry1Ac. cry1C, cry2Ab*, GA20 oxidase1, *nptII*, *bar* were identified for screening of these events.

In accordance with the FSAAI (2021), 24 food crops have been identified for assurance of non-GM status in the imported consignments. Among these, information of more than 130 GM events 22 GM food crops including alfalfa, apple, Argentina canola, common bean, chicory, cowpea, eggplant, flaxseed, melon, papaya, plum, Polish canola, potato, rice, safflower, soybean, squash, sugarbeet, sugarcane, sweet pepper, tomato and wheat, was compiled from the GM crop databases. While analyzing the GMO matrix, only non-stacked GM events were considered as the genetic information of stacked GM events is derived from individual events comprising the stack. Nine elements were identified, which can detect 80% of globally approved GM events of 22 GM food crops, as shown in Fig. 1 and Supplementary Information 1. For maize, previously reported crop-specific GMO matrix was referred (Singh et al. 2016) where more than 90% of globally approved GM events could be covered using the selected targets. Based on the elements selected, triplex PCR targeting two promoters (*P-35S*, *P-FMV*) and a terminator (*T-nos*); triplex PCR targeting three marker genes (*aadA*, *nptII*, *pat*) and duplex PCR targeting *cry1Ab/Ac* and *cry2Ab2* transgenes were developed and validated. Assay for *ctp2-cp4epsps* was also selected for GMO teating of specific food crops with Roundup Ready® trait such as maize. One of the crops, namely, pineapple included in FSSAI (2021) was not covered with these elements and single approved event can be individually tested for this.

Specificity And Sensitivity Of Multiplex Pcr Assays For Gmo Screening In Food Derivatives

Multiplex PCR allows simultaneous detection of multiple targets in a run thereby reducing the time and cost of GMO testing. Event-specific multiplex PCR assays have been reported by James et al. 2003, Kim et al. 2013, Eum et al. 2019. James et al. (2003) developed multiplex PCR targeting GM events of soybean (GTS40-3-2), maize (Bt176, Bt11, MON810, T14/25), and canola (GT73, HCN92/28, MS8/RF3, Oxy 235). Event-specific multiplex PCR (LOD of 0.05%) targeting six GM events of soybean, namely, A2704-12, A5547-127, DP305423-1, DP356043-5, GTS40-3-2 and MON89788, along with an endogenous gene was developed (Kim et al. 2013). This method was used to check the GM status of 30 soybean food products from South Korean and US markets and 19 samples were found positive for three GM soybean events (GTS40-3-2, A2704-12, and MON89788). Eum et al. (2019) reported multiplex PCR for detection of four GM cotton events, namely, Cot102, DAS-81910-7, MON88701 and T304-40.

As the event-specific assays are restricted to identification of particular GM event so testing a sample for all the possible GM events is cumbersome and time-consuming. GMO screening methods could reduce the time and cost of GMO testing by eliminating the need of testing for all the GM events (Randhawa et al. 2014; Waiblinger et al. 2010). Multiplex PCR- based screening assays were reported by our group to detect more than 90% of globally approved GM events of cotton and maize (Singh et al. 2016).

In the present study, based on the common screening targets identified in 22 GM food crops, three sets of multiplex PCR assays were developed and validated. While optimizing the multiplex PCR conditions, choice of DNA polymerase was considered as a key parameter for optimum PCR performance to avoid the formation of non-specific products and primer-dimer for simultaneous detection of targeted products without any primer interference (Randhawa et al., 2009). In this study, the ready-to-use master mix based on Hot Start DNA polymerase was used, Triplex PCR, simultaneously targeting control elements, *i.e.*, 123 bp region of *P-35S*, 78 bp region of *P-FMV* and 180 bp region of *T-nos*, was developed. Another triplex PCR targeting three marker genes, *i.e.*, 406 bp region of *aadA*, 262 bp of *pat*, and 215 bp of *nptII* was optimized and validated. *Bt* gene specific duplex PCR targeting *cry1Ac/Ab* and *cry2Ab2* was developed, which targeted 74 bp region of *cry1Ac/Ab* and 260 bp region of *cry2Ab2*.

Specificity was confirmed using defined set of targets and non-targets for each element included in the assay (Table 2). Amplification was detected in the samples of respective targets (Fig. 2, Table 2) whereas no amplification was detected in respective non-targets and negative controls. In triplex PCR targeting three control elements, specific product of 123 bp for *P-35S* was detected in the samples of MON15985 x MON88913, MON15985 x MON88913 x Cot102, Bt11, MON863, MON89034, NK603, TC1507, 40-3-2; 180 bp for *T-nos* in the samples of MON15985 x MON88913, MON15985 x MON88913 x Cot102, Bt11, GA21, MON863, MON89034, NK603, 40-3-2; and 78 bp for *P-FMV* in the samples of MON15985 x MON88913, MON15985 x MON88913 x Cot102, MON89034 (Fig. 2a). No amplification was detected in respective non-targets and non-template control. Sensitivity of each assay was determined using a series of dilutions with 100, 10, 5.0, 1.0, 0.5, 0.1, 0.05, 0.01% GM content. LOD was established as 0.5% for triplex PCR simultaneously detecting *P-35S*, *P-FMV* and *T-nos* (Fig. 3a),

On multiplex PCR simultaneously detecting three marker genes, amplification product of 406 bp region of *aadA* was detected in the samples of MON531, MON15985, MON15985 x MON88913, MON15985 x Cot102, MON15985 x MON88913 x Cot102, whereas no amplification was detected in respective non-targets [Bt11, GA21, MIR604, MON89034, NK603, TC 1507, 98140, 305423, 356043, 40-3-2, 68416-4 (Blank), Widestrike™, non-GM cotton) and non-template control. The amplification products of desired size, i.e., 262 bp of *pat* and 215 bp of *nptII* were detected in the respective targets and no amplification was observed in the non-targets and non-template control, as expected (Fig. 2b). This assay was found sensitive to detect as low as 0.5% for each target (Fig. 3b).

Specificity test of duplex PCR targeting *cry1Ab/Ac* and *cry2Ab2* genes showed amplification of expected product of 74 bp for *cry1Ab/Ac* in the targets, namely, MON15985, Cot102 x MON15985, MON15985 x MON88913, Cot102 x MON15985 x MON88913 and MON531 events of *Bt* cotton, and Bt11 and Bt176 events of Bt maize, whereas no amplification was detected in respective non-targets and negative control (Fig. 2c). Product of 260 bp for *cry2Ab2* gene was detected in the samples of MON15985, Cot102 x MON15985, MON15985 x MON88913, Cot102 x MON15985 x MON88913 and MON89034 and no amplification was detected in rest of samples used as non-targets (Fig. 2c), The *Bt* gene specific assay was found sensitive to detect as low as 0.05% for each target (Fig. 3c).

The results indicated that the developed multiplex PCR assays showed acceptable specificity over the range of targets and non-targets analyzed (Fig. 2 and Table 2). The established LOD of developed assays was up to 0.5 – 0.05% depending on the targets (Fig. 3) is in compliance with the labelling threshold of many countries including 0.9% in the EU (https://ec.europa.eu/jrc/en/research-topic/gmos), or the tolerance limit of adventitious presence of GMOs permissible in the imported consignments as in the case of India (FSSAI 2021). These procedures could be efficiently employed as a part of GMO testing to trace GM contamination, if any, in both the imported as well as domestic food products.

Utilization Of Developed Assays For Gmo Testing Of Food Derivatives

Few reports are available on the utilization of GM diagnostics for checking the GM status of food products from the markets in Brazil, Georgia and Turkey (Avsar et al. 2020; Cardarelli et al. 2005; Datukishvili et al. 2015). Very recently, our group has conducted a study for checking the unapproved GM event CDC Triffid flax using GM diagnostics in 123 flaxseed accessions imported from Plant Gene Resources of Canada for research purpose, as a precautionary approach (Singh et al. 2022). As an initiative for GMO testing of food products in India in a systematic manner, in the present study, developed assays were utilized to check the GM status of food products of apple and maize.

A set of 14 food products purchased from local markets or retail shops were allocated codes before subjected to testing (Table 3). DNA extraction procedures as optimized previously by our group were used for most of the matrices (Singh et al. 2021). Quality of DNA from processed products may be hampered by the processing steps when subjected to high temperature and pressure, or presence of inhibitors due to complex composition and amplifiability may get hindered to the inhibitors or complex composition (Nguyen et al. 2009; Ramos-Gómez et al. 2014; Singh et al. 2021). To address this issue and to eliminate the chances of false negatives, quality and amplifiability of DNA extracts was first ensured using PCR assays targeting respective taxon-specific assay or plant-specific marker. The amplicon of expected size of 135 bp for *Adh1* gene was detected in the DNA extracts of food samples of maize (Fig. 4-i) and amplicon of ~ 615 bp for *cp-tRNA* gene was detected in the DNA samples of fruits and products of apple (Fig. 5A-I, B), thereby confirming the amplifiability of DNA extracts.

Table 3
Food products/ derivatives of maize and apple used in the study

S. No.	Product Name	Brand/ Company	Country/ Origin	GMO Label	No. of replicates (samples) tested	Transgenic elements detected on multiplex PCR
	Maize Products					
1.	Corn Flour	Brown & Polson	India	na	2 (1)	None
2.	Natural Roasted Puff (Cool Crunchies)	The Mumum Co. (Faraway Foods Private Limited)	India	na	2 (1)	None
3.	Corn Chips (Makino)	Recorn® Foods Private Limited	India	Non-GMO	2 (1)	None
4.	Masala Munch (Kurkure)	Mfg. Daawat Foods Private Limited; Marketed by PepsiCo India Holdings Private Limited	India	na	2 (1)	None
5.	Sweet Vegetable Corn Soup (Knorr)	Unilever Food Solutions India	India	na	2 (1)	None
6.	Plain Flour All Purpose	Orgran (Roma Food Products)	Australia	GMO free	2 (1)	None
	Apple Fruits					
7.	SMB		India	na	3 (1)	None
8.	Red	Red Delicious	USA	na	3 (1)	None
9.	NZ Queen	New Zealand Queen	New Zealand	na	3 (1)	None
10.	MSR		India	na	3 (1)	None
11.	LWSTAR	Taifeng	China	na	3 (1)	None
	Apple Products					
12.	Apple Pie Cookies	Merba	Netherlands	na	3 (3)	None
13.	Puffs Cereal Snack (Strawberry Apple)	Gerber	USA	Non-GMO	3 (2)	None
14.	Apple Fruit Drink	Amul Tru	India	na	3 (1)	None

na: information on GM status not available on the pack

Samples of food products of maize and apple were analyzed in replications and appropriate positive and negative controls were run along with the samples. On multiplex PCR assays, none of the tested samples of maize were found positive for the transgenic elements (*P-35S, P-FMV, T-nos, aadA, nptII, pat, cry1Ab/Ac* and *cry2Ab2*) included in the assays (Fig. 4-ii-iv). An additional PCR test for *ctp2-cp4epsps* was also conducted for more GM coverage in case of maize and none of the samples were found positive for this specific element (Fig. 4-v). Amplification was detected in respective positive controls of each target, indicating the accurate working of the assay used. Based on the tests conducted using multiplex PCR assays, none of the samples of apple were found positive for the targeted transgenic elements (Fig. 5A-ii-iv, C-E).

All the samples were tested in replications along with appropriate positive and negative controls for each assay to avoid type I (false positives) and type II (false negatives) errors. Before GMO testing, the DNA samples were first tested for ensuring quality and amplifiability using endogenous gene specific assays: *Adh1* for maize products and *cp-tRNA* for apple products, in order to minimize the chances of type II error. The acceptance criteria in accordance with the Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing, European Network of GMO Laboratories were referred (JRC Technical Report, 2015). In all the replications, none of the food samples were found positive for selected transgenic elements, whereas specific amplicon was detected in respective positive control for each target. The statistical interpretations confirmed that the GMO analysis results complied scientifically and not by any chance factor.

The results indicated that the developed diagnostics can be efficiently utilized to check the GM status of food products as a measure for controlled distribution and use of GM foods based on the approval status of particular GM food crop/ product in a country.

Conclusions

More than 20 GM food crops have been approved in different countries (https://www.isaaa.org/gmapprovaldatabase/). The approval status of GM food crops in a country has demanded the need for checking the possible UGM event(s) in the derived food products of respective crops for regulatory compliance as well as for addressing consumers' apprehensions to an extent. So far, no GM food crop has been approved for commercial cultivation or

for use as food in India, though several GM food crops have been developed or imported for research purposes or were under field trials (https://geacindia.gov.in/meetings.aspx, Singh and Randhawa 2016). *Bt* eggplant event EE1 is a classical example, which has been developed by Maharashtra Hybrid Seeds Co. (Mahyco), Jalna and under field trials in the country, in spite of this the event has not got regulatory approval though it was commercialized in the neighboring country of Bangladesh. Since 2006, the Food Safety and Standards (FSS) Act of 2006 came into act in the country, empowering the FSSAI under the Ministry of Health & Family Welfare, as the single authority for establishing and implementing science-based standards for food safety, including that of GM foods (FSSA 2006). Every imported consignment of 24 selected crops (including alfalfa, apple, Argentina canola, common bean, chicory, cowpea, eggplant, flaxseed, maize, melon, papaya, pineapple, Polish canola, plum, potato, rice, safflower, soybean, squash, sugarbeet, sugarcane, sweet pepper, tomato and wheat) need to be accompanied with the non-GMO certification and the tolerance limit of adventitious presence of GMOs at 1 per cent is permissible in the consignments of imported food crops (FSSAI 2021).

In view of existing regulation or policy for GM crops or derived products in India and other countries, three sets of multiplex PCR assays for GMO detection in food products were developed and validated with the LOD range of 0.5 – 0.05% depending upon the target, which fulfils the requirement of the labelling threshold of many countries or the tolerance limit of adventitious presence of GMOs permissible in the imported consignments. These assays targeted the common transgenic elements including *P-35S*, *P-FMV*, *T-nos, aadA*, *nptII*, *pat*, *cry1Ab/Ac* and *cry2Ab2* that can be utilized for GM detection in food products derived from 22 food crops including alfalfa, apple, Argentina canola, common bean, chicory, cowpea, eggplant, flaxseed, maize, melon, papaya, Polish canola, plum, potato, rice, safflower, soybean, squash, sugarbeet, sugarcane, sweet pepper, tomato and wheat.

These procedures could be efficiently employed as a part of GMO analysis to trace the UGM contamination, if any, in both the imported as well as domestic food products.

Abbreviations

aadA: Aminoglycoside-3'-adenyltransferase

cp4-epsps: 5-enolppyruvylshikimate-phosphate synthase

CRM: Certified reference material

CTAB: Cetyltrimethyl ammonium bromide

DNA: Deoxyribonucleic acid

EU: European Union

FSSAI: Food Safety and Standards Authority of India

GM: Genetically modified

GMO: Genetically modified organism

LOD: Limit of detection

nptII: Neomycin phosphotransferase

P-35S: Cauliflower Mosaic Virus 35S promoter

pat: Phosphinothricin-N-acetyltransferase

PCR: Polymerase chain reaction

P-FMV: Figwort Mosaic Virus promoter

RM: Reference material

T-nos: Nopaline synthase terminator

UGM: Unauthorized genetically modified

Declarations

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Figures

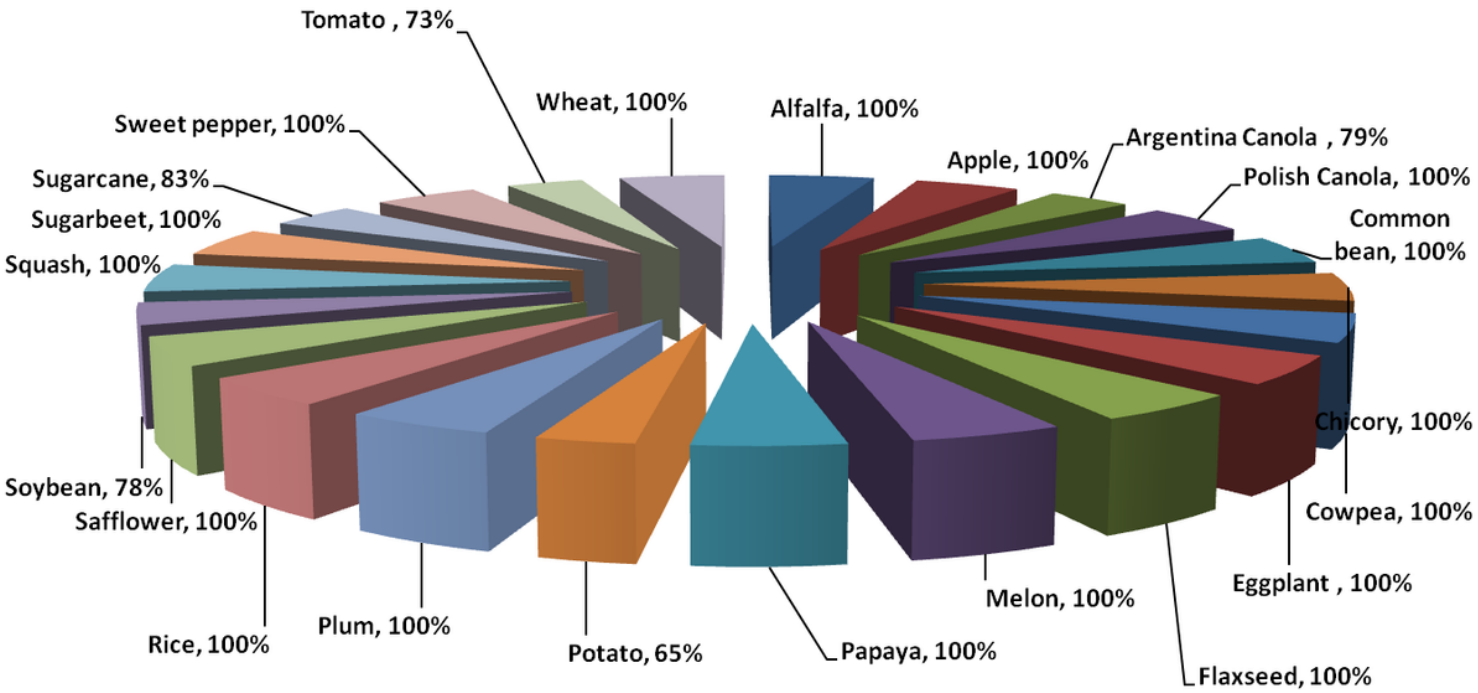

Figure 1

Coverage for detection of GM events of different food crops using selected targets. Approximately 80% of globally approved GM events of 22 GM food crops (alfalfa, apple, Argentina canola, common bean, chicory, cowpea, eggplant, flaxseed, melon, papaya, plum, Polish canola, potato, rice, safflower, soybean, squash, sugarbeet, sugarcane, sweet pepper, tomato and wheat) are covered using selected targets

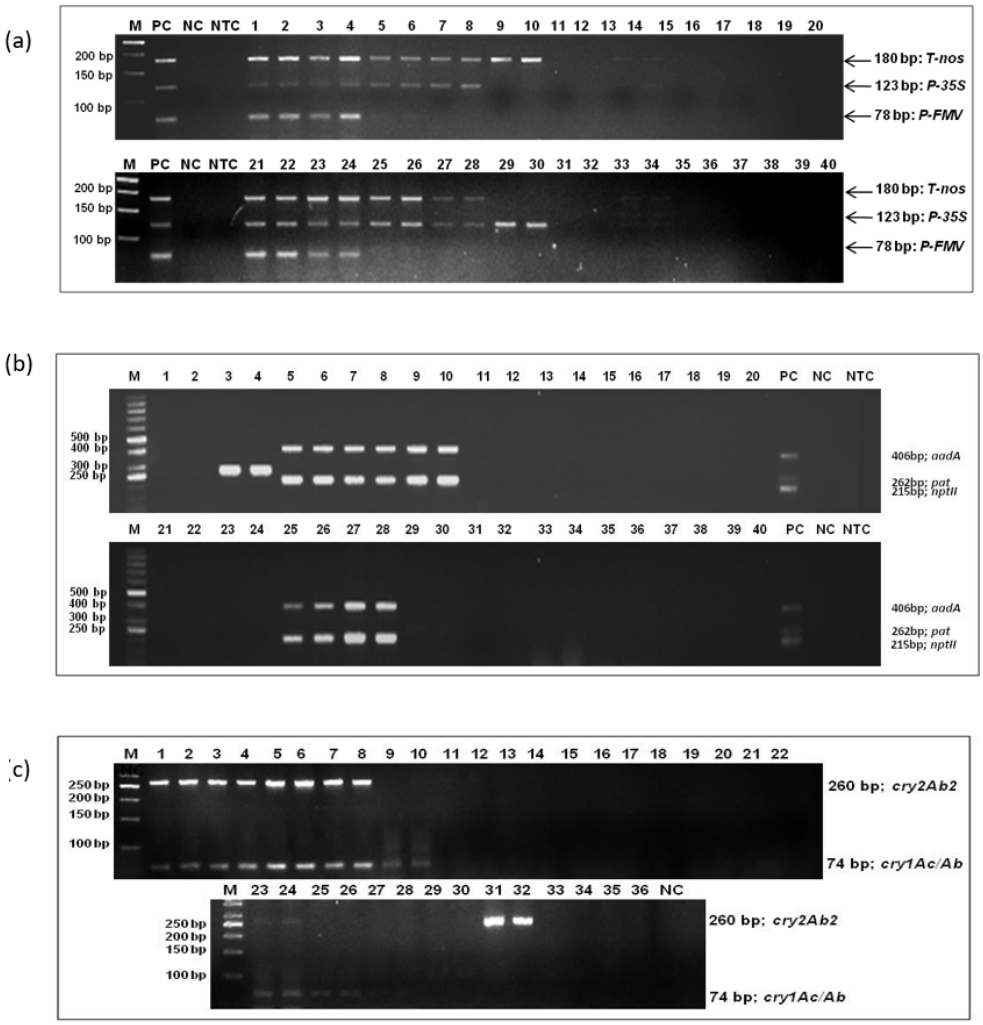

Figure 2

PCR profiles for specificity test of multiplex PCR assays

(a) Triplex PCR targeting *P-35S, P-FMV, T-nos* (M: 50 bp DNA ladder; **1-2:** MON15985 x MON88913 x Cot102, **3-4:** MON15985 x MON 88913, **5-6:** NK 603, **7-8:** Bt11, **9-10:** GA 21, **11-12:** DP-98140, **13-14:**356043, **15-16:** DAS 81419-2, **17-18:** Non-GM cotton, **19-20:** Non-GM maize, **21-22**: MON89034, **23-24**: MON15985 x MON88913 (repeated), **25-26**: MON863, **27-28**: 40-3-2, **29-30**: TC1507, **31-32**: DAS 68416-4 (Blank), **33-34**: 40278-9, **35-36:** 281-24-236 x 3006-210-23, **37-38:** Non-GM soybean; **39-40:** 305423, PC: Positive control; NC: Negative control, NTC: Non-template control

(b) Triplex PCR targeting *aadA, nptII, pat* (M-50 bp DNA Ladder; 1-2: Non GM Maize, 3-4: 281-24-236 x 3006-210-23, 5-6: MON531, 7-8: MON15985, 9-10: MON15985 x MON88913, 11-12: GA21, 13-14: MON89034, 15-16: DP98140, 17-18: 305423, 19-20: 40-3-2, 21-22: MIR604, 23-24: 40278-9, 25-26: MON15985 x MON88913 x Cot102, 27-28: MON15985 x Cot102, 29-30: Bt11, 31-32: 356043, 33-34: Non-GM soybean; 35-36: 68416-4 (Blank), 37-38: NK603, 39-40: Non-GM cotton; PC: Positive control; NC: Negative control, NTC: Non-template control

(c) Duplex PCR targeting *cry1Ab/Ac, cry2Ab2* (M-50 bp DNA Ladder; 1-2: MON15985, 3-4: Cot102 x MON15985, 5-6: MON15985 x MON88913, 7-8: Cot102 x MON15985 x MON88913, 9-10: MON531, 11-12: 356043, 13-14: 305423, 15-16: 40-3-2, 17-18: DAS 68416-4 (Blank), 19-20: MIR604, 21-22: TC1507; 23-24: MON15985 (1% Dilution), 25-26: Bt176 , 27-28: NK603, 29-30: MON863, 31-32: MON89034, 33-34: Non-GM cotton, 35-36: Non-GM soybean, NC: Negative control)

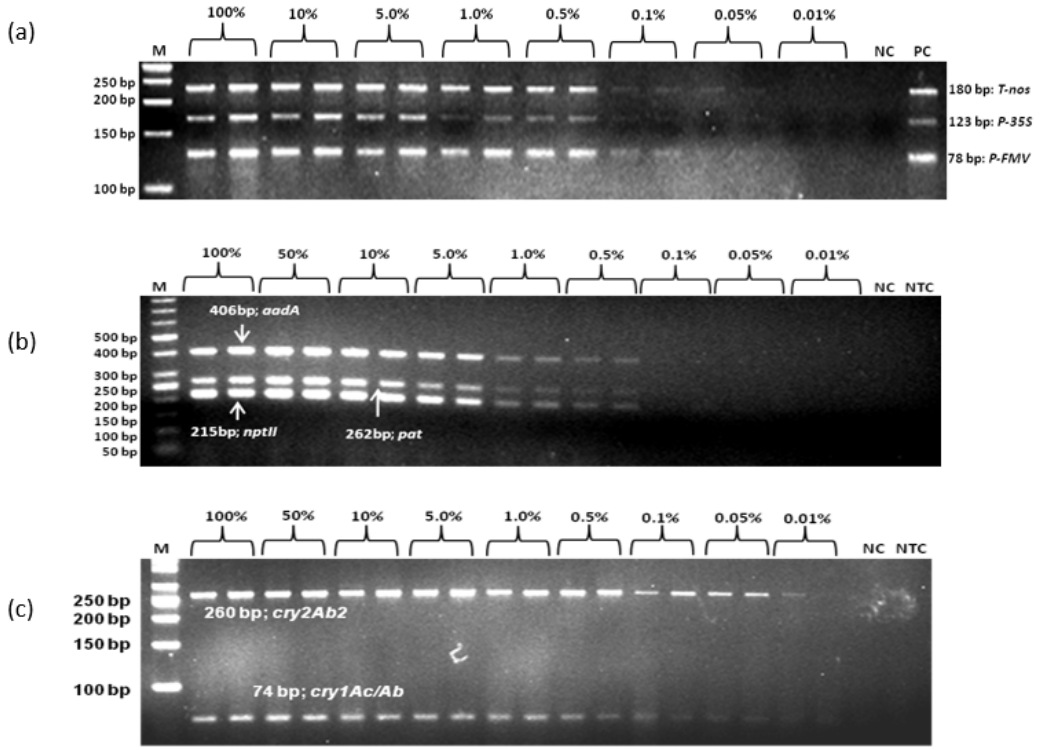

Figure 3

PCR profiles for sensitivity test of multiplex PCR assays (a) Triplex PCR targeting *P-35S, P-FMV, T-nos* (b) Triplex PCR targeting *aadA, nptII, pat* (c) Duplex PCR targeting *cry1Ab/Ac*, *cry2Ab2* (M: 50 bp DNA ladder, NC: Negative control, NTC: Non-template control)

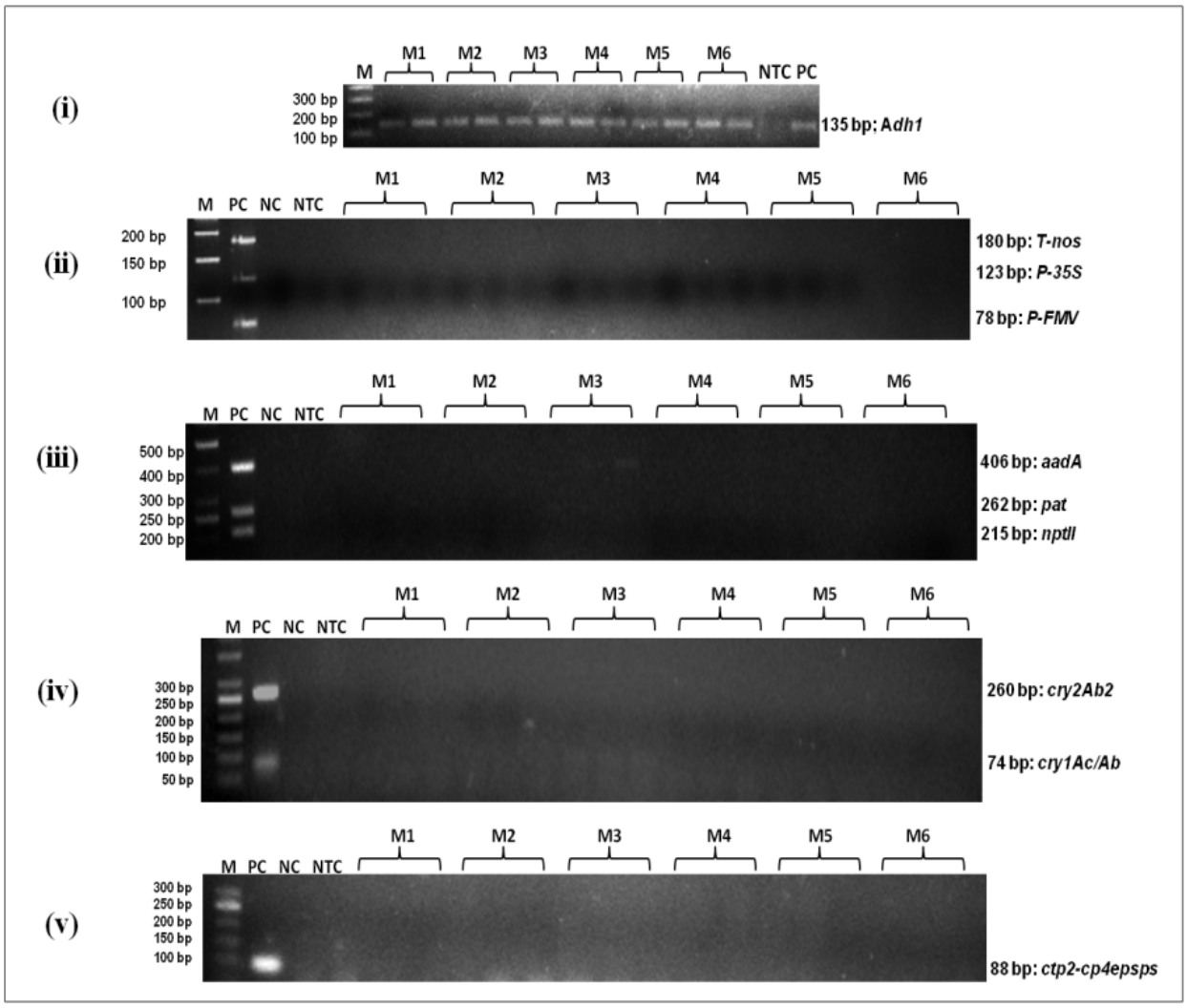

Figure 4

PCR profiles for utilization of developed GM diagnostics for checking the GM status of food products of maize. (i) Checking amplifiability of DNA extracts of food products using endogenous *Adh1*gene specific assay; (ii-v) PCR profiles for checking the GM status using developed multiplex PCR assays (ii-iv) and *ctp2-cp4epsps*specific assay (v)

Sample Details: **M1:**Corn flour (Brown & Polson), **M2:** Natural Roasted Puff (Cool Crunchies), **M3:** Corn Chips (Makino); **M4,** Masala Munch (Kurkure), **M5:** Sweet Corn Vegetable Soup (Knorr), **M6:** Organ Plain Flour All Purpose

Controls: PC: Positive control, NC: Negative control, NTC: Non-template control

Markers: (i) M: 100 bp DNA ladder, (ii-v) M: 50 bp DNA ladder

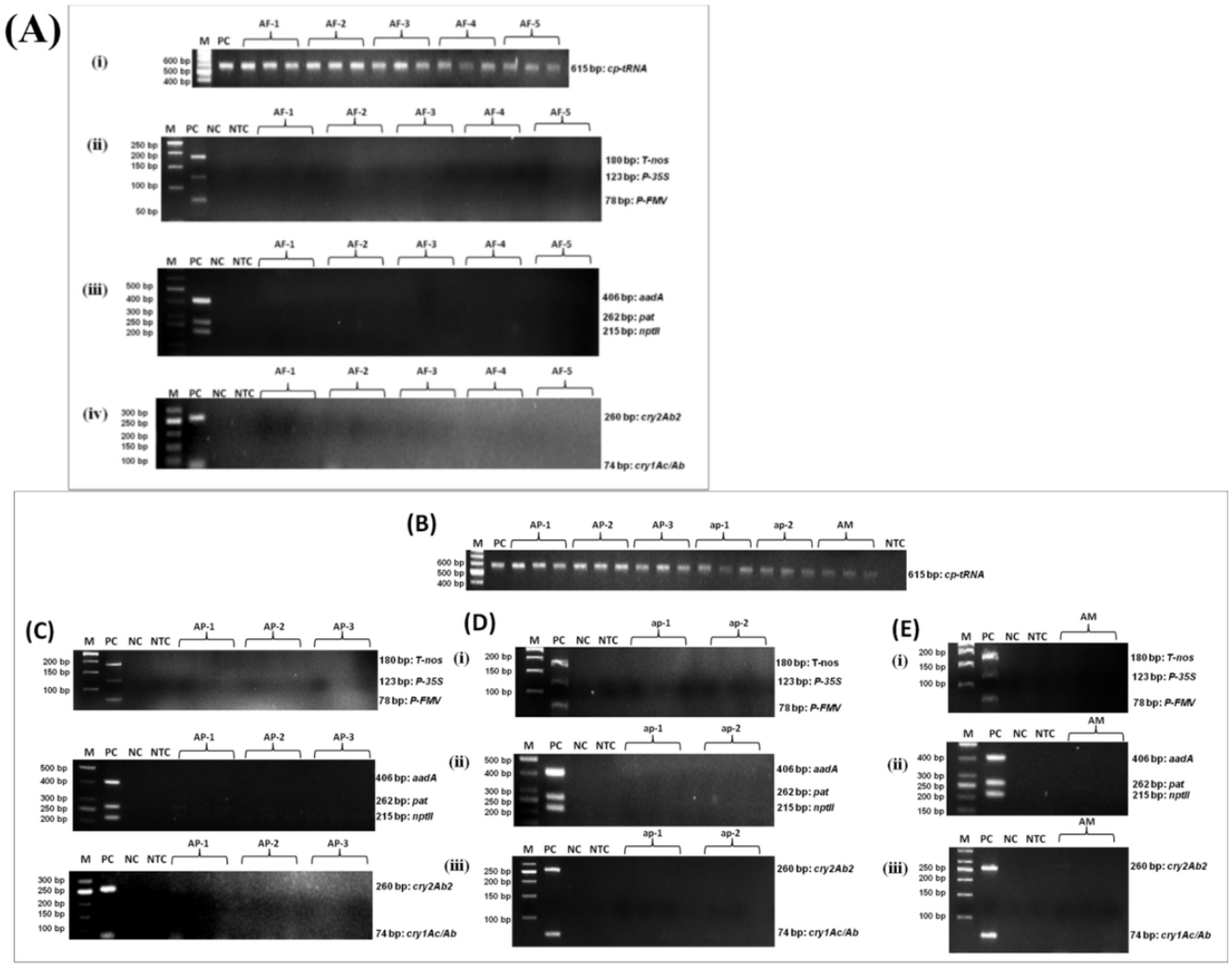

Figure 5

PCR profiles for utilization of developed GM diagnostics for checking the GM status of (A) apple fruits and (B-D) apple products.

(A) (i) Checking amplifiability of DNA extracts of apple fruits using *cp-tRNA* gene specific assay; (ii-iv) PCR profiles for checking the GM status using developed multiplex PCR assays

(B) Checking amplifiability of DNA extracts of apple products using *cp-tRNA* gene specific assay

(C-E) (i-iii)PCR profiles for checking the GM status using developed multiplex PCR assays

Sample Details: **AF1**: Fruit (SMB), **AF2**: Fruit (Red Delicious), **AF3**: Fruit (New Zealand Queen), **AF4**: Fruit (MSR), **AF5**: Fruit (LWSTAR), **AP1-AP3**: 3 samples of Apple Pie Cookies (Merba), **ap1-ap2**; 2 samples of Puffs Cereal Snack (Strawberry Apple) (Gerber)**, AM:** Apple Fruit Drink (Amul Tru),

Controls: PC: Positive control, NC: Negative control, NTC: Non-template control

Markers: (A) M: 100 bp DNA ladder (i), M: 50 bp DNA ladder (ii-v); (B) M: 100 bp DNA ladder; (C-E) M: 50 bp DNA ladder

Supplementary Files

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- SupplementaryInformation1GMOMatrix.docx