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Multiplex PCR-Based Simultaneous Amplification of Selectable Marker and Reporter Genes for the Screening of Genetically Modified Crops

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The development and commercialization of genetically modified (GM) crops with enhanced insect and herbicide resistance, abiotic stress tolerance, and improved nutritional quality has expanded dramatically. Notwithstanding the huge potential benefits of GM crops, the perceived environmental risks associated with these crops need to be addressed in proper perspective. One critical concern is the adventitious presence or unintentional mixing of GM seed in non-GM seed lots, which can seriously affect the global seed market. It would therefore be necessary though a challenging task to develop reliable, efficient, and economical assays for GM detection, identification, and quantification in non-GM seed lots. This can be systematically undertaken by preliminary screening for control elements and selectable or scorable (reporter) marker genes. In this study, simplex and multiplex polymerase chain reaction (PCR) assays individually as well as simultaneously amplifying the commonly used selectable marker genes, i.e., *aadA*, *bar*, *hpt*, *nptII*, *pat* encoding, respectively, for aminoglycoside-3′-adenyltransferase, *Streptococcus viridochromogenes* phosphinothricin-*N*-acetyltransferase, hygromycin phosphotransferase, neomycin phosphotransferase, *Streptococcus hygroscopicus* phosphinothricin-*N*-acetyltransferase, and a reporter gene *uidA* encoding β-D-glucuronidase, were developed as a reliable tool for qualitative screening of GM crops. The efficiency of the assays was also standardized in the test samples prepared by artificial mixing of transgenic seed samples in different proportions. The developed multiplex PCR assays will be useful in verifying the GM status of a sample irrespective of the crop and GM trait.

KEYWORDS: Genetically modified crops; multiplex PCR; reporter genes; selectable marker genes; qualitative screening

INTRODUCTION

The global planted area under GM crops has increased dramatically from 1.66 mha in 1996 to 125 mha in 2008 (*1*). However, the employment of recombinant DNA techniques in GM crops has led to public concern pertaining to their potential risks to the environment and human health (*2*). Hence, novel traits incorporated in GM crops need to be diagnosed and evaluated for environmental and food safety along with addressing socio-ethical issues before their approval for commercialization. The use of appropriate qualitative methods for GM screening is the need of the hour. A qualitative GM screening procedure is based on the testing of control elements such as promoters and transcription terminators, and selectable and scorable marker genes (*3*).

Polymerase chain reaction (PCR) is the most widely used method for GM detection and is also accepted worldwide to meet the regulatory obligations (*3*, *4*). PCR-based assays are sensitive and are frequently used to detect the inadvertent contaminations of GM with non-GM seed lots (*5*). Multiplex PCR assays have also been developed to simultaneously amplify two or more gene elements in a single reaction (*6*). In GM crops such as soybean, maize, and canola, a multiplex PCR system has been developed to detect multiple target sequences using simultaneous amplification profiling (*7*). Multiplex PCR simultaneously detecting five lines of GM maize by employing sequence-specific primers was also developed (*8*). A sensitive and specific triplex nested PCR assay was developed for the detection of housekeeping gene (*lectin*) and inserted elements of Roundup Ready soybean, i.e., constitutively expressed *CaMV 35S* promoter, *cp4epsps* gene encoding for 5-enol-pyruvyl-shikimate-3-phosphate for herbicide tolerance, *nos* terminator, and a chloroplast transit peptide (*ctp*) facilitating transport of epsps protein, in highly processed products (*9*). A multiplex PCR assay for the detection of *vip-s* transgene encoding vegetative insecticidal protein along with other GMO-specific sequences, i.e., *nptII* marker gene, *CaMV* 35S promoter, and *nos* terminator, was validated in GM cotton and tobacco with a *vip-3A* type gene (*10*). Recently, multiplex PCR-based assays have also been developed in GM vegetable crops, simultaneously detecting *cry1Ac* gene for

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Table 1. Details of Nine GM Events Employed in the Present Study

event	transgenes (trait)	marker genes	importer	source	PCR format
Cotton					
MON531*	*cry1Ac* (insect resistance)	*nptII*, *aadA*			simplex, multiplex
MON15985*	*cry1Ac*, *cry2Ab* (insect resistance)	*nptII*, *aadA*, *uidA*			simplex, multiplex
Widestrike**	*cry1Ac*, *cry1F* (insect resistance)	*pat*	Dow AgroSciences Pvt. Ltd., Mumbai	Dow AgroSciences, USA	simplex, multiplex
Twinlink**	*cry1Ab*, *cry2Ae* (insect resistance)	*bar*	Bayer BioSciences Pvt. Ltd., New Delhi	Bayer Crop Sciences, USA	simplex
MON88913**	*cp4 epsps*, *cry1Ac*, *cry2Ab* (herbicide tolerance)	*nptII*, *aadA*, *uidA*	Monsanto India Ltd., Mumbai	Monsanto, USA	simplex
Rice					
Bt rice**	*cry1Ca* (insect resistance)	*bar*	Bayer BioSciences Pvt. Ltd., Hyderabad	Bayer BioScience NV, Belgium	simplex, multiplex
GM rice**	*ferritin* (improved nutrition)	*hpt*	Rice Research Station, West Bengal	International Rice Research Institute, Phillipines	simplex, multiplex
Maize					
Bt11***	*cry1Ab* (insect resistance)	*pat*			simplex, multiplex
Bt176***	*cry1Ab* (insect resistance)	*bar*			simplex

* Commercialized in India ** Imported GM seed materials used, are the integral part of mandatory molecular testing, on the instructions of Review Committee on Genetic Manipulation (RCGM), Department of Biotechnology, Govt. of India *** IRRM 5% Certified Reference Materials (CRMs from Fluka Biochemica)

insect resistance, *CaMV* 35S promoter and endogenous *SRK* (S-locus Receptor Kinase) gene in Bt cauliflower (*11*); *osmotin* gene for salinity and drought tolerance, *CaMV 35S* promoter and endogenous *LAT52* (late anther tomato) gene in GM tomato (*12*); *cry1Ab* gene for insect resistance, *CaMV 35S* promoter; *npt II* marker gene and endogenous *UGPase* (uridine diphosphate glucose pyrophosphorylase) gene in Bt potato (*13*); *AmA1* gene for improved nutritional quality; and endogenous *ST-LS1* gene in GM potato (*14*). These studies demonstrate that the multiplex PCR system is a precise, reliable, cost-effective, and efficient assay for GM detection.

The GM events commercialized globally and in India or other GM events under different stages of field trials in India mostly contain *aadA* encoding aminoglycoside-3′-adenyltransferase for spectinomycin resistance, *bar* encoding phosphinothricin-*N*-acetyl transferase for bialaphos resistance, *hpt* encoding hygromycin phosphotransferase for hygromycin resistance, *nptII* encoding neomycin phosphotransferase for kanamycin resistance, *pat* encoding phosphinothricin-*N*-acetyltransferase for bialaphos resistance as selectable marker genes; and *uidA* encoding β-D-glucuronidase as reporter gene. Hence, in the present study, a precise, robust, and sensitive method based on simplex and multiplex PCR has been developed for qualitative detection and identification of six commonly used marker genes, viz., *aadA*, *bar*, *hpt*, *nptII*, *pat*, and *uidA*, individually as well as simultaneously in a single run using the test samples prepared by artificial mixing with different proportions of GM seed materials. The developed assays will be of immense use to test unintentional mixing of GM seeds with non-GM seed lots, which would further help by certification of GM-free status for export the facilitation of global seed trade.

MATERIALS AND METHODS

Seed Materials. The seeds of three GM crops, i.e., cotton, rice, and maize, with nine transgenic events having different marker genes were used. The GM crops/events under study, as detailed in **Table 1**, include five GM cotton events, i.e., MON 531, MON 15985, MON 88913, Widestrike, and Twinlink; two GM rice events, i.e., Bt rice with *cry1Ca* gene and GM rice with *ferritin* gene; and two GM maize events, i.e., Bt11 and Bt176, and all of these were used in simplex PCR assays. Out of these, six GM events, i.e., MON 531 of cotton, MON 15985 of cotton, Widestrike cotton, Bt rice with *cry1Ca* gene, GM rice with *ferritin* gene, and Bt176 maize were employed for the preparation of test samples to be used in multiplex PCR assays, along with non-GM seeds of cotton, maize, and rice as controls. National Bureau of Plant Genetic Resources, New Delhi (our institute) is the nodal agency to issue import permits and undertake the quarantine of transgenics for research purposes as per Government of India Notification No. GSR 1067 (E) dated 05.12.1989 and Plant Quarantine (Regulation of Import into India) Order, 2003 vide Government of India Notification No. S. O. No. 1322, dated 18.11.2003. Besides quarantine processing, all of the imported transgenic seed/planting materials are being tested regularly by our laboratory for terminator technology and for all selectable/reporter marker genes, promoters, and specific transgenes since 1998 before releasing to the indentors. Hence, the experiments for the imported transgenic seed material used in the present study have been carried out during the molecular testing of imported transgenic seed materials of MON 88913 cotton, Widestrike cotton, Twinlink cotton, Bt rice with *cry1Ca* gene, and GM rice with *ferritin* gene.

Preparation of Test Samples and Genomic DNA Extraction. To determine the applicability and specificity of multiplex (hexaplex) PCR assays, test samples were prepared by the strategy employed by Germini et al. (*15*) for simultaneous detection of four GM maize events (MON810, Bt11, Bt176, and GA21) and one GM soybean event (Roundup Ready) by mixing blind samples of different GM events in random proportions.

In the present study, seven different test samples (sets A−G) were employed to assess the efficiency of hexaplex PCR. Out of these, six test samples (sets A−F) were prepared by artificial random mixing of the seed powder of six different GM events, i.e., three GM cotton events (MON 531, MON 15985, and Widestrike), two GM rice (Bt rice and GM rice with *ferritin* gene), one GM maize event Bt11, and three non-transgenic samples, i.e., cotton, rice, and maize. Set G was purely non-GM containing seed powder of three different non-transgenics, i.e., cotton, rice, and maize to be used as negative amplification control. Initially, different percentages of six GM events, i.e., MON 531 of cotton, MON 15985 of cotton, Widestrike cotton, Bt rice, GM rice with *ferritin* gene, and Bt176 maize with their respective non-GM seed material, were prepared, e.g., for set A, after preparing 0.5% MON 15985 cotton, 0.5% Widestrike cotton, 2% Bt rice, and 2% GM rice with *ferritin* gene, equal amounts of these 4 GM events were mixed to get 500 mg content, i.e., 125 mg of 0.5% MON 15985 cotton + 125 mg of 0.5% Widestrike cotton + 125 mg of 2% Bt rice + 125 mg of 2% GM rice with the *ferritin* gene. The composition of all test samples has been given in **Table 2**.

For random mixing, all of the test samples (sets A−G) were homogenized separately in an electric mixer, and 500 mg of each set was distributed into five equal parts of 100 mg each. DNA was extracted from five aliquots of each sample using modified SDS extraction protocol (*16*). Also, genomic DNA was extracted from the purified seed powder of

Table 2. Details of GM Events Used for Artificial Mixing and Composition of Test Samples (Sets A−G)

	In Terms of the Percentage of GM Seed Powder Used								
artificial samples	MON 531 cotton	MON 15985 cotton	Widestrike cotton	Bt rice	GM rice	Bt 176 maize	non-GM cotton	non-GM rice	non-GM maize
A	–	0.5%	0.5%	2%	2%	–	–	–	–
B	5%	5%	5%	0.5%	–	–	–	–	–
C	–	2%	1%	–	–	–	–	traces	–
D	2%	–	–	–	–	2%	–	–	–
E	1%	–	–	–	–	–	–	–	traces
F	–	–	–	1%	–	–	traces	–	–
G	–	–	–	–	–	–	traces	traces	traces
selectable/ reporter genes	*npt II*, *aadA*	*npt II*, *aadA*, *uidA*	*pat*	*bar*	*hpt*	*bar*	–	–	–

Table 3. Details of Primer Pairs Employed for the Study

selectable marker/ reporter gene	GenBank accession no.	primer	primer sequence (5′-3′)	expected amplicon size (bp)	reference
nptII	AM887683	NPT II F/R	F-CCGCCACACCAGCCGGCC R-CCGACCTGTCCGGTGCCC	508	present study
aadA	M86913	AadA-1-F/R	F-TCCGCGCTGTAGAAGTCACCATTG R-CCGGCAGGCGCTCCATTG	406	present study
uidA	U12639	Gus F/R (uidA)	F-TTTCTTTAACTATGCCGGAATCCATC R-CACCACGGTGATATCGTCCAC	82	*16*
bar	X17220	barF2/R2	F-GCACAGGGCTTCAAGAGCGTGGTC R-GGGCGGTACCGGCAGGCTGAA	177	*9*
pat	M22827	patF2/R2	F-GAAGGCTAGGAACGCTTACG R-GCCAAAAACCAACATCATGC	262	*17*
hpt	AF007759	B1/B2	B1-CGCCGATGGTTTCTACAA B2-GGCGTCGGTTTCCACTAT	839	*18*

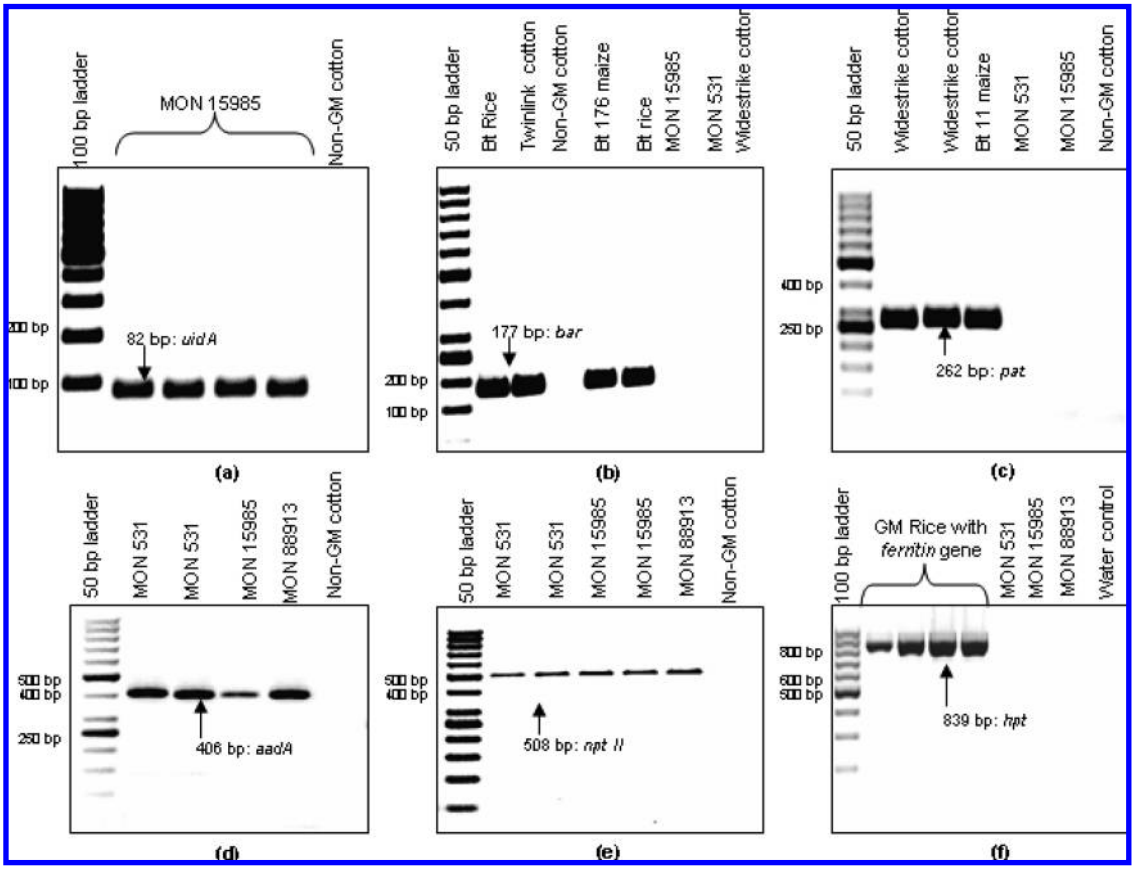

Figure 1. Simplex PCR amplicons in different transgenic seed materials for their respective selectable marker genes using the specific primer pairs for the detection of (**a**) *uidA*; (**b**) *bar*; (**c**) *pat*; (**d**) *aadA*; (**e**) *npt II*; and (**f**) *hpt* genes.

reference materials, i.e., 5% Bt11 maize CRM, 5% Bt176 maize CRM, and seeds of GM events, i.e., MON 531 of cotton, MON 15985 of cotton, MON 88913 of cotton, Widestrike cotton, Twinlink cotton, Bt rice, and GM rice with the *ferritin* gene, and non-transgenic cotton, rice, and maize using modified SDS extraction protocol to carry out simplex PCR assays.

DNA concentration was measured by UV absorption at 260 nm, while DNA purity was evaluated on the basis of the UV absorption ratio at 260 nm/280 nm ranging from 1.6 to 1.9 using DU 640 UV Spectrophotometer (Beckman, USA). Final concentration of extracted DNA was made to 30 ng/μL and used as stock solutions for the PCR analysis.

Designing of Oligonucleotide Primers. For the amplification of *nptII* and *aadA* marker genes, primer pairs were designed using Primer Select 5.05 software (DNASTAR Inc., USA). For the amplification of *bar*, *uidA*, *pat*, and *hpt* genes, published primer pairs (*7*, *17*, *18*) were used (**Table 3**). Oligonucleotide primers in purified and lyophilized form were synthesized by M/s Pivotal Marketing Ltd., the dilutions were made for a final concentration of 20 μM with Milli-Q water, and 4× concentration of primer mix was made by mixing 1.6 μM of each primer pair.

PCR Conditions. PCR analyses were carried out on PTC-200 Programmable Thermal Cycler (MJ Research Inc., USA). The efficiency of the primer pairs to amplify the target sequences was tested by simplex PCR using the corresponding target genomic DNA with different marker genes.

For testing of primer-dimer formation in multiplex PCR, 5 primer mixes in 4× concentration comprising two, three, four, five, and six primer pairs were prepared to reduce the analysis-to-analysis variability as per the methodology used by Germini et al. (*15*).

To test the efficiency of Taq Polymerase to be employed in PCR assays, comparative tests were made with several Taq polymerases (data not shown). All simplex as well as multiplex PCR assays were performed in a final volume of 25 μL with the following reagent concentrations: 150 ng of template DNA, 1× Hot Start PCR buffer, 3.5 mM of $MgCl_2$, 0.4 mM of dNTPs, 1× primer mix, and 0.15 U/μL of Hot Start Taq DNA polymerase (MBI Fermentas Inc., USA). The final working concentration of each primer in the primer mix was 0.4 μM. The amplification conditions were initial denaturation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 50 s, primer annealing at 59 °C for 50 s, primer extension at 72 °C for 50 s, and final extension at 72 °C for 5 min.

Analysis of PCR Products. PCR products were analyzed on agarose gel electrophoresis. The gel was prepared with 2.0% Seakem LE agarose (Lonza, Rockland, ME USA) and 4.0% Metaphor agarose (Cambrex Bioscience Rockland, Inc., Rockland, ME USA) for simplex and multiplex PCR, respectively.

RESULTS AND DISCUSSION

With the dramatic expansion of global area under cultivation of GM crops, there is an urgent need to step up the development of robust, efficient, and reliable methods for GM detection. In the present study, an efficient and reliable qualitative simplex and multiplex PCR system based on the detection of commonly used six selectable marker/reporter genes has been developed for the screening of GM crops and for unintentional mixing of GM seeds with non-GM seeds.

Simplex and Multiplex PCR for the Screening of Marker Genes. The choice of DNA polymerase is very important for the optimum performance of the PCR, and therefore, several DNA polymerases such as Taq DNA polymerase (MBI Fermentas), Taq-Red DNA polymerase (SBS Genetech), and Hot Start DNA polymerase (MBI Fermentas) were used for the study. The Hot Start DNA polymerase, coupled with a preoptimized primer mix for different multiplex reactions, gave the best results both in terms of reproducibility and robustness (data not shown). The use of Hot Start DNA polymerase prevents the formation of misprimed products and reduces primer-dimer formation. As the number of primers increases, the possible sequence dependent interactions between primers of different primer pairs also increase, which results in the formation of primer-dimers. Small differences in amplification efficiencies for the different primer pairs might result in the preferential amplification of some of the PCR products, leaving other PCR products at subdetectable levels. Hence, primer design, PCR cycling conditions, and the concentration of each reaction component need to be cautiously optimized in order to avoid the formation of primer-dimers and to detect all DNA targets simultaneously without any primer

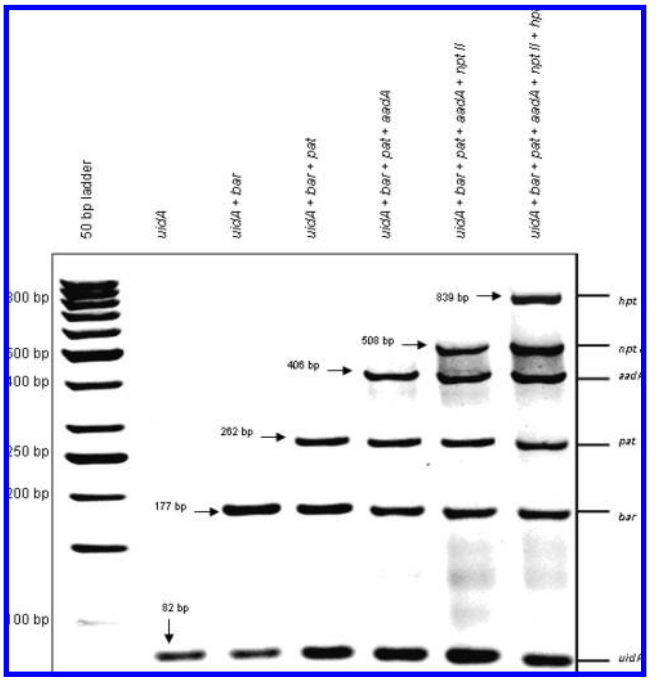

Figure 2. Multiplex PCR assay for testing of primer interference using equivalent DNA mix of six different GM events, i.e., MON 531 of cotton, MON 15985 of cotton, Widestrike cotton, Bt rice, GM rice with the *ferritin* gene and Bt176 of maize.

interference. Preliminary experiments to test the efficiency of the primer pairs using single templates of different transgenic seed materials in combination with single primer pairs were carried out. The simplex PCR with each primer pair, i.e., GusF/R (*uidA*), barF2/R2, patF2/R2, Aad-1-F/R, NPTII-F/R, and B1/B2 (*hpt*) produced a single specific amplicon of the expected size only in the target GM crops/events, i.e., 82 bp for the *uidA* gene in MON 15985 of cotton (**Figure 1a**); 177 bp for the *bar* gene in Bt rice, Twinlink cotton, and Bt 176 maize (**Figure 1b**); 262 bp for the *pat* gene in Widestrike cotton and Bt11 maize (**Figure 1c**); 406 bp for the *aadA*; 508 bp for the *nptII* genes in GM cotton events MON 531, MON 15985, and MON 88913 (**Figure 1d** and **e**, respectively); and 839 bp for the *hpt* gene in GM rice with the *ferritin* gene (**Figure 1f**).

An equivalent mix of the extracted DNA of six different GM events at the concentration of 30 ng/μL was amplified in six runs initially using only one pair of primer starting with the smallest amplicon (*uidA* gene-specific primer, amplicon size 82 bp), followed by the addition of a second primer pair, until the sixth primer pair. Specific products of expected sizes were amplified in the following PCR formats (**Figure 2**): simplex (82 bp for *uidA*), duplex (82 bp for *uidA* and 177 bp for *bar*), triplex (82 bp for *uidA*, 177 bp for *bar*, and 262 bp for *pat*), tetraplex (82 bp for *uidA*, 177 bp for *bar*, 262 bp for *pat*, and 406 bp for *aadA*), pentaplex (82 bp for *uidA*, 177 bp for *bar*, 262 bp for *pat*, 406 bp for *aadA*, and 508 bp for *nptII*), and hexaplex (82 bp for *uidA*, 177 bp for *bar*, 262 bp for *pat*, 406 bp for *aadA*, 508 bp for *nptII*, and 839 bp for *hpt*). The main aim of such multiplexing was to investigate if there was any primer interference that could result in primer sequestration, thus hindering the specificity of a specific primer. A similar study for the detection of primer interference has also been reported by Germini et al. for simultaneous amplification of five events of GM maize and soybean using a seven-target multiplex PCR for the amplification of sequences specific for different events, viz., MON 810 (110 bp), Roundup Ready soybean (125 bp), Bt11 (189 bp), Bt176 (209 bp), and GA21

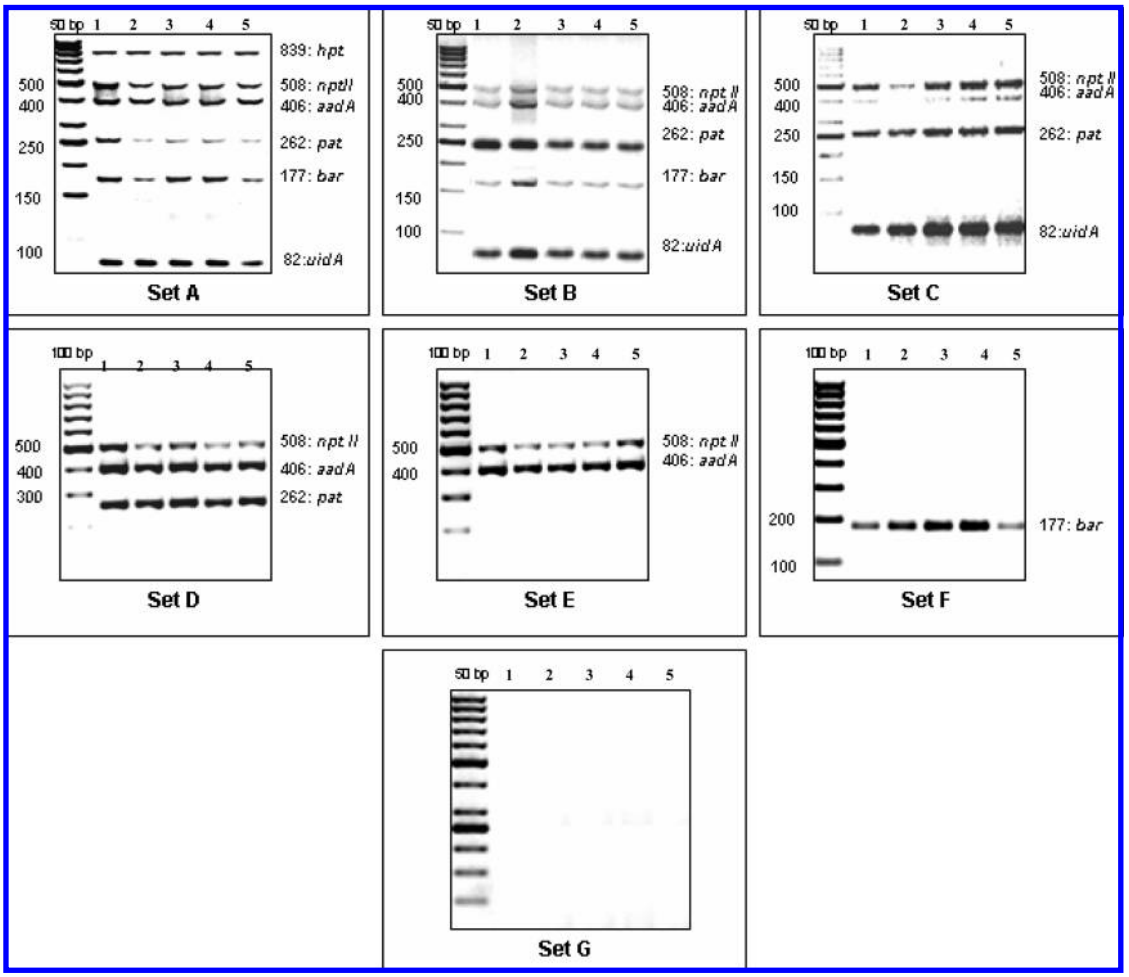

Figure 3. Agarose gel electrophoresis of the PCR amplification products of subsets (100 mg each) of artificially prepared test samples (sets A−G) containing different transgenic/non-transgenic seed materials having varying compositions (using a primer mix of six primer pairs) as described in **Table 2**. Subsets of sets A−G: A1−A5; B1−B5; C1−C5; D1−D5; E1−E5; F1−F5; and G1−G5.

(270 bp), and for endogenous genes, viz., maize zein gene (139 bp) and soybean lectin gene (157 bp) (*15*).

Applicability of Developed Multiplex PCR Assays for the Screening of GM Seed Material. Multiplexing provides a cost-effective diagnostic assay for GM detection with higher throughput and less consumption of samples and reagents as compared to simplex assays. The efficiency of the multiplex assays developed in the present study was tested in the samples prepared by artificial mixing with different proportions of transgenic samples. The multiplex PCR was thoroughly validated for specificity, repeatability, and reproducibility using six sets A−G with artificially mixed GM seed samples and one non-GM set G as negative amplification control as detailed in **Table 2**. The multiplex (hexaplex) PCR system has been developed, and the specific amplicons of desired size were detected in the corresponding sets (**Figure 3**). The hexaplex PCR simultaneously amplified the six specific amplicons for *uidA*, *bar*, *pat*, *aadA*, *nptII*, and *hpt* marker genes in set A replicates. Similarly, the five, four, three, two, and one amplicon of expected size in the corresponding sets of test samples, i.e., replicates of sets B, C, D, E, and F, were detected. Reproducibility of all developed assays was checked thrice. No amplification was detected in the replicates of set G comprising the non-transgenic cotton, maize, and rice. Absence of amplicons in the non-transgenic set confirms the specificity and reliability of the hexaplex assay for the screening of GM crops/events.

Pertaining to specificity, the developed multiplex system is robust since no amplification was detected in the sets negative for specific selectable marker or reporter gene, for example, in set C with no GM rice with the *ferritin* gene and *hpt* as the marker gene and Bt176 maize with *bar* as the marker gene, the specific amplicons for *hpt* and *bar* genes were not detected using hexaplex PCR (**Figure 3**, set C).

Several reports based on the simultaneous amplification profiling for GM detection are available. Multiplex PCR assays have been developed for detecting multiple target sequences in different events of GM soybean (Roundup Ready), maize (event 176, Bt11, MON810, and T14/25), and canola (GT73, HCN92/28, MS8/RF3, and Oxy 235) along with the internal control targets, i.e., *lectin* and *β-actin* genes in soybean, *invertase* gene in maize, and *cruciferin* gene in canola (*7*). Seven-target multiplex PCR for the simultaneous detection of four GM maize events (MON810, Bt11, Bt176, and GA21) and one GM soybean event (Roundup Ready) has been reported (*15*). An event- and construct-specific multiplex PCR technique has recently been established for the simultaneous detection of eight GM maize lines using nine sets of designed primers including six event-specific for Bt176, Bt11, TC1507, NK603, MON863, and MON810, two construct-specific for T25 and GA21, and one for an endogenous *zein* gene (*19*).

Besides detecting different GM events, there is a need for the development of a reliable and efficient diagnostic assay for the screening of a large number of commercialized GM crops; for monitoring the adventitious presence of the GM trait in native crop landraces; identification of deliberate or inadvertent mixing

of GM seeds in non-GM seed lots; and to meet mandatory regulatory obligations. So far, simultaneous amplification profiling and multiplexing of screening elements to test unknown samples have not been reported. To develop GM screening strategies, the present study targets multiplex PCR assays by simultaneous amplification of six marker genes commonly being employed in most of the developed GM crops. Hence, the developed multiplex PCR with most of the marker genes employed in the development of GM crops is a reliable, efficient, and economical technique for detecting and identifying the mixing of GM seed with non-GM seed lots, for effective evaluation of environmental and food safety, and also for the compliance of regulatory obligations, which will greatly help in the certification for GM-free status during export, thereby enabling standardization in global seed trade.

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