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Full Length Research Paper

Detection of genetically modified maize (*Zea mays* L.) in seed samples from Nepal

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Maize is the second major cereal in Nepal; its food biosafety and ecological conservation is an important concern. To address this issue, it is necessary to detect genetically modified (GM) maize and establish a monitoring and regulatory system in Nepal. Currently, Nepal does not have legal regulations or labeling directives for GM maize. Therefore, the authors aimed to survey the current status of GM maize seeds in Nepal. First, they performed multiplex polymerase chain reaction (mPCR) to detect 8 GM maize lines in 46 maize seed samples from different locations in Nepal. Suspected samples were then verified by real-time PCR (RT-PCR) and screen-specific PCR. Based on current evidence, they can not identify any GM maize in the seed samples. This first report may formulate and implement a baseline for quality regulation and biodiversity conservation of maize seeds in Nepal.

Key words: Genetically modified crops, maize, seeds, Nepal, multiplex polymerase chain reaction, real-time PCR.

INTRODUCTION

For the increasing world population, conventional breeding of crop varieties can not meet the demand for food supply. To fulfill the needs, agricultural biotechnology had developed various genetically modified (GM) crops for insect or herbicide resistance, abiotic stress tolerance, or nutritional improvement. GM crops were intended to satisfy industrial needs and consumers' desires for food security, conserving biodiversity, alleviating greenhouse gases, biofuels production and sustainable economic benefits. To date, 24 GM crops have been approved and grown worldwide. The total global areas planted with GM crops increased rapidly from 1.7 - 166 million ha during the period 1996 - 2008 (James, 2008; AGBIOS). Among them, soybean (60%), maize (24%), cotton (11%) and rapeseed (5%) are four main GM crops. Currently, 54 GM maize lines are grown in the European Union and other countries occupying 30% (37.3 million ha) of the global maize production area (James, 2008; AGBIOS). Most maize is used as animal feed; the remainder is used for human consumption, ethanol (biofuel) and other industrial purposes (FSAI, 2003; James, 2006). With the increase of various GM maize lines and cultivated areas, the risk of the introduction of GM maize or product into the environment or food chain has become an important issue of bioecology and biosafety concern (Eastham and Sweet, 2002). For example, India has approved trials of stacked maize hybrids containing insect-resistant MON 89034 and glyphosate-tolerant NK603 (Poupard, 2008). In addition, China has approved the importation of 12 GM maize lines for food and feed use (AGBIOS). To ensure the safety of GM crops and/or seeds, GM crops must be thoroughly evaluated during their growth and post-harvesting before their commercialization. Thus, the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) have established general safety assessment and regulation guidelines for GM crops and/or seeds. Many countries have also implemented threshold and labeling requirement of GM food and derived products (Gruere and Rao, 2007).

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Abbreviations: GM, Genetically modified; **PCR,** polymerase chain reaction; **mPCR,** multiplex polymerase chain reaction; **RT,** real-time; **FAO,** food and agriculture organization; **WHO,** world health organization; **GMOs,** genetically modified organisms; **GRMs,** genomic reference materials; **CRMs,** certified reference materials; **CTAB,** cetyltrimethylammonium bromide; **BLAST,** basic local alignment search tool; **MGBNFQ,** minor-groove-binding non-fluorescent quencher dye.

Non-GM seeds and/or foods can be contaminated with their GM counterparts in the local or international trade market. The contamination of GM seeds may arise accidentally from the blending of seed lots, cross-pollination from GM crops or their wild relatives, seed flow and voluntary mixing (Brookes et al., 2004; Chilcutt and Tabashnik, 2004; Lee et al., 2009). As an example, StarLink maize, approved for restricted use as animal feed, was found in taco shells produced by Kraft (Bhat, 2008). As well, in Serbian food market with a total of 347 soybean products, 11% of them were proved to be GM positive and 3% of these examined foods contained more than 0.9% of GM soybean (Nikolic et al., 2009). Two types of unapproved GM maize, GA21 and DBT418, were present in 4 supermarket-brand tortilla chips in the United Kingdom (FoE, 2002). In addition, although not approved for cultivation, some GM soybeans were found in local markets in China (Zhou et al., 2007). Also in Hungary, 38% of tested food-market samples were found contaminated with Roundup Ready (RR) soybean (Ujhelyi et al., 2008).

Maize is the second major cereal in Nepal, and the total maize area is about 870,166 ha, representing 30% of the total cultivated land (MOAC, 2007/08). Many unique varieties of maize are grown in Nepal. Conservation of maize diversity in Nepal is important for maize seed-bank management and maize cultivar adoption, selection and improvement. Currently, 86% of the maize area is cultivated with improved seed varieties. Farmers are keen to upgrade or obtain superior varieties from seed trade in local markets or companies. There are more than 100 private-sector seed companies and 60% of them trade maize seeds in Nepal (Paudyal et al., 2001). In Nepal, non-GM maize seeds and grains are allowed to be imported from other countries (MOAC, 2007/08; Shrestha and Wulff, 2007; Paudyal et al., 2001). However, only 10% of the seed supply of cereal crops in Nepal is from formal channels, with the remaining seeds supplied by informal channels (Sthapit and San, 2001). The disorganized seed market or trade with either smallholders or small enterprises in Nepal is a serious problem (Shrestha and Wulff, 2007). It is highly possible that the contamination of local maize seed with GM maize occur through informal channels. Especially, 2 neighbouring countries of Nepal, India and China, have adopted the use of GM crops for years, the risk of GM maize contamination in local market of Nepal should not be completely ignored.

Although, guidelines for biosafety assessment have been approved in Nepal, no separate legislation exists for risk assessment, regulation and management of GM organisms (GMOs), biotechnology, and biosafety (Shrestha and Wulff, 2007). As well, GM crops for field cultivation and human and livestock consumption have not been defined and officially prohibited from import into Nepal. Accordingly, to provide feature information of GM maize regulation and traceability, the authors aimed to monitor the current status of GM maize seeds in Nepal. Specifically, they used multiplex polymerase chain reaction (mPCR), real-time PCR and screen-specific PCR to survey maize seeds from different trading sites of Nepal.

MATERIALS AND METHODS

Seed and grain materials

Forty-six seed samples of maize were collected from different sites in Nepal (that is, seed markets, the National Maize Research Programmed and farmers' saved seeds) during August and September 2008 (Figure 1 and Table 1). Maize samples from the eastern part of Nepal were not included because of flooding problems. All of the tested seed samples were directly imported from Nepal.

Standard DNA samples

Standard genomic DNA reference materials (GRMs) for GM maize were prepared as previously described (Shrestha et al., 2008). In brief, 100 ng/μL (v/v) of genomic DNA from Event176, Bt11, TC1507, NK603, T25, MON863, MON810, and GA21 maize lines were prepared as positive control samples for single PCR (100% GM maize) and mPCR (0.25% GM maize). DNA extracted from the leaves of a local Taiwan maize cultivar (cv. Tainan No.5) was used as a non-GM control. In addition, certified reference materials (CRMs) for the NK603, T25, MON810 GM, DAS-59122-7 and MIR604 maize lines were purchased from the Institute for Reference Materials and Measurements (IRRM, Geel, Belgium; http://www.erm-crm.org) and used for real-time and screen specific PCR analysis.

Isolation and quantification of genomic DNA

Large-scale genomic DNA was extracted from 300 - 700 g of maize seeds representing 2,000 maize grains by calculating 100 grain weight. Samples were ground by an electric Oster mill (Sunbean Products, Inc., BocaRaton, FL. USA) then sampled by the spoon method (SDQCSS, 2000; ISTA, 2004; Holst-Jensen, 2007). Two ground samples, 15 and 20 g each, were used for DNA isolation and mPCR and real-time PCR analysis. All small-scale genomic DNA samples and reference materials were extracted from 100 mg of maize seeds by the cetyltrimethylammonium bromide (CTAB) method (Lipp et al., 1999; Somma and Querci, 2004) with minor modification. The 100 mg maize seed was ground into fine powder by TissueLyser (Retsch QIAGEN, Germany). Then, 500 uL CTAB buffer was added, mixed and heated at 65°C for 30 min. After centrifugation, the supernatant was extracted with chloroform. Then, 2 volumes of CTAB precipitation solution were added to 500 μL of supernatant and incubated at room temperature for 1 h. The supernatant was discarded after centrifugation, then 350 μL NaCl (1.2 uM) and a 0.6 volume of isopropanol were added. The DNA pellet was precipitated, washed with 70% ethanol, air-dried and dissolved again in 50 μL queeensland water (QH_2O). The DNA concentration and quantity were measured fluorometrically with the use of a NanoDrop, ND-1000® Spectrophotometer (Thermo Fisher Scientific; Wilmington, DE.). Each DNA sample was diluted to 100 ng/μL for further analysis.

Primers and probes

Primers for mPCR were as described (Shrestha et al., 2008). For

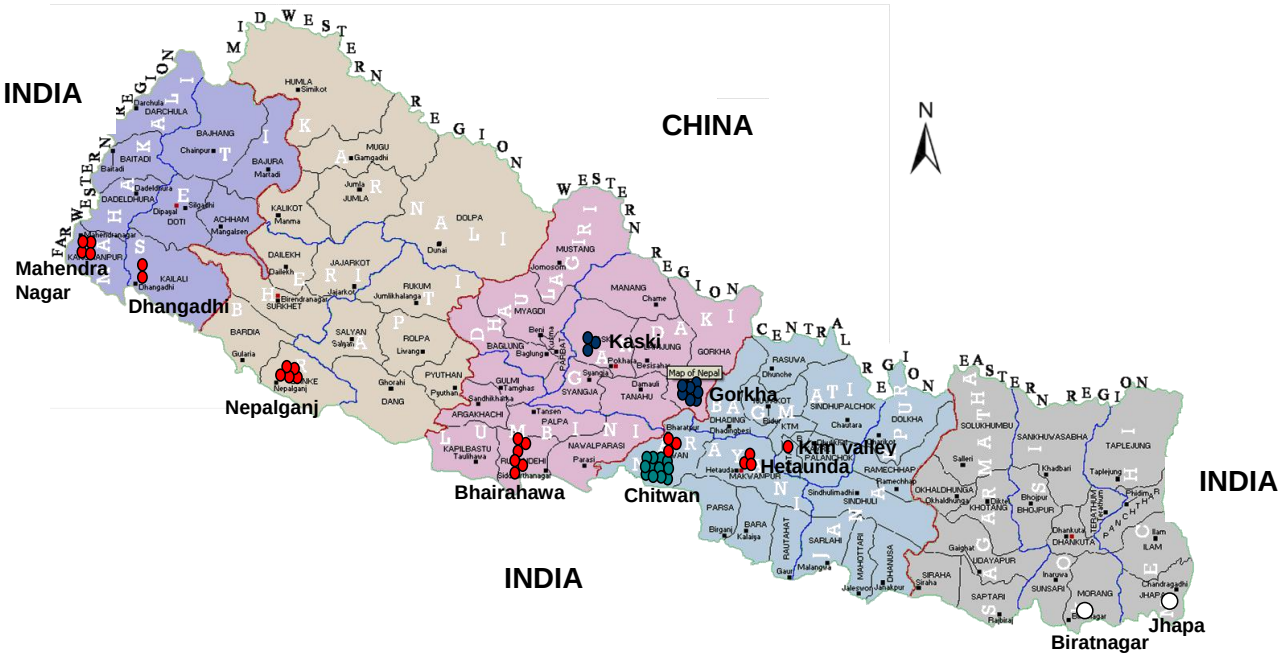

Figure 1. Map of Nepal (http://ncthakur.itgo.com/map04.htm) showing locations where maize seed samples were collected (red circles, seed traders; green circles, National Maize Research Programmed (NMRP); blue circles, farmers' seed; white circles, the place where sample was not collected because of flooding).

Table 1 Maize samples collected from locations in Nepal during August and September 2008.

Location	Seed company/research institute/farmer	No. of samples	Tested maize seed sample number
Lalitpur	Seed company	1	1. Manisha-7272 (hybrid)
Hetauda	Seed company	3	2. Subham HM-929; 3. Arun-2; 4. Arun-2
Chitwan	Seed company	3	5. Rampur Composite; 6, Godawari-989; 7. Arun-2
Bhairaha/Butwal	Seed company	6	8. Arun-2; 9. Datta, 10. 70 days, 11. Mahi-155, 12. 20 days, 13. Mahalaxmi-HB
Nepalganj	Seed company	5	14. Laxmi-HB; 15. Manisha-8181; 16. Arun-2, 17. K25; 18. Arun-2
Dhangadhi	Seed company	2	19. Arun-2; 20. Manisha-6263
Mahendra Nagar	Seed company	4	21. Murali; 22. Datta; 23. Arun-2; 24. BISCO-65HB
Chitwan	National Maize Research Programmed (NMRP)	12	25. Upahar; 26. Arun-1; 27. Sitala; 28. Deuti; 29. POP-45; 30. Manakamana-1; 31. Rampur composite; 32. Arun-2; 33. Arun-4; 34. Manakamana-3; 35. S99TLWQ-GH AB; 36. Pool-17
Kaski	Farmer	3	37. Khumal yellow; 38. Local; 39. Khumal white
Gorkha	Farmer	7	40-46, Local

real-time PCR, except for primers of *Zein* gene, a maize storage protein gene used as an internal control, primers and TaqMan MGB probes targeting the GM maize lines NK603, T25 and MON810 were newly designed with the use of Primer Express® v3.0 (Applied Biosystems, Carlsbad, CA. USA) (Figure 2 and Table 2). The specificity of primers and probes was checked by a Basic Local Alignment Search Tool (BLAST) search of the GenBank database, with no significant homology observed. In addition, specificity and efficiency were checked by single PCR (data not shown). TaqMan MGB probes used for the amplification of NK603, T25, MON810 and *Zein* were labeled with the reporter fluorescence dyes 6-carboxy-fluorescein (FAM) and VIC at the 5' ends. The 3' ends of all primers and probes were labeled with minor-groove-binding non-fluorescent quencher dye (MGBNFQ). Screen-specific primers were synthesized as described (Kuribara et al., 2002). The oligonucleotide primers were synthesized by TRI-I Biotech (Taipei, Taiwan); all TaqMan MGB probes were purchased from Applied Biosystems (Custom Oligo Synthesis Service, USA).

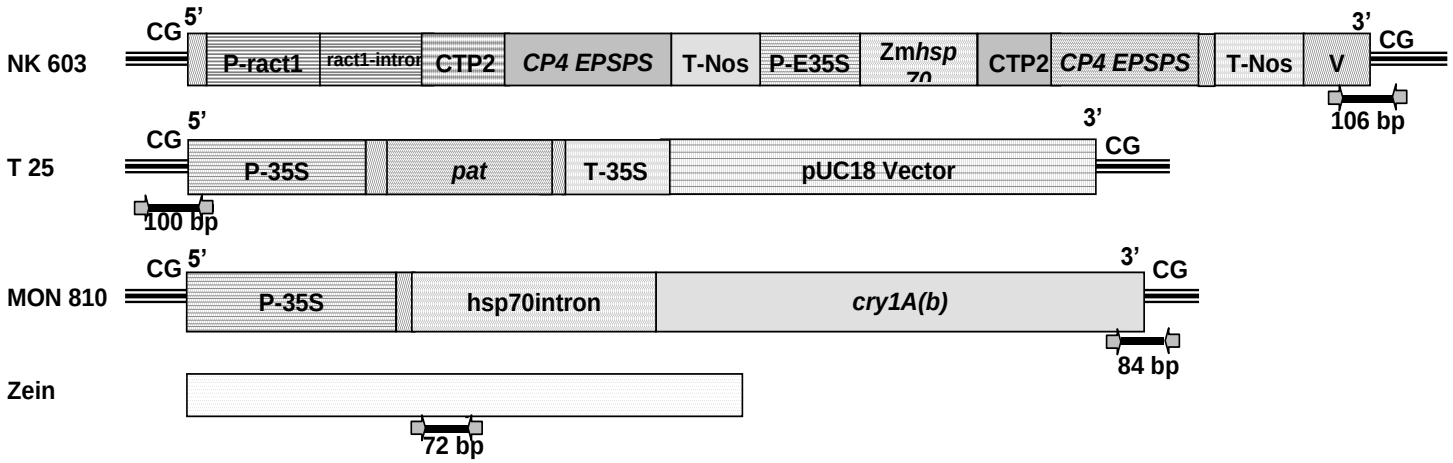

Figure 2. Construct maps of 3 genetically modified (GM) maize lines (NK603, T25 and MON810) showing the amplification region of event-specific real-time PCR primers, probes of NK603 (106 bp), T25 (100 bp), MON810 (84 bp) and endogenous *Zein* (72 bp).

Table 2 Primers and probes for detecting GM maize lines and *Zein* gene by real-time PCR.

GM maize	Primers	Sequence (5'-3')	Length (bp)	Amplicon (bp)	Orientation
NK603	NK603-08F	gacctcgagtaagcttgttaacgc	24	106	S (Vector)
	NK603-08R	cgagaagagataacaggatccactc	25		A (MG)
	NK603-08P	6FAM-taccacgcgacagact-MGBNFQ	16		P (3'-J)
T25	T25-08HF	tcgtgctccaccatgttgac	20	100	S (MG)
	T25-08R	cagctacgacatgatactccttcc	24		A (P-35S)
	T25-08P	6FAM-tcattgagtcgttccgccat-MGBNFQ	20		P (5'-J)
MON810	MON810-08F	cacttctccttggacatcgatg	22	84	S ((cry1(A) b))
	MON810-08R	gcaagcaaattcggaaatgaa	21		A (MG)
	M810-08P	6FAM-aggactttcggtagcctt-MGBNFQ	18		P (3'-J)
Zein gene	Zein 04Se	gcttgccagcttgatggcgt	20	72	S (Zein gene)
	Zein 07R	ggcatcgtctgaagcggtaagg	22		A (Zein gene)
	Zin-08P	VIC-atgctgcagcaactg-MGBNFQ	15		P (Zein gene)

Primers and probes of NK603, T25, MON810 and probe for Z*ein* were newly designed, and primers for *Zein* were from Shrestha et al., (2008); S: sense; A: antisense; P: probe.

Multiplex PCR

The mPCR analysis of the DNAs from the maize seeds for 8 GM maize lines was as described (Shrestha et al., 2008). In 25 μl of final reaction, premixed primers at different concentrations (0.2 μM for the primers for Event 176, Bt11, TC1507, T25, MON863, GA21, and *Zein*; 0.1 μM for MON810; and 0.3 μM for NK603) were used with the QIAGEN mPCR kit (Qiagen, Taiwan) according to the manufacturer's manual.

Real-time PCR

For detection of GM maize in the samples, single real-time PCR was conducted in a 7500 fast real-time PCR machine (ABI, USA). The PCR products were then analyzed by use of the 7500 software v2.0.1 (ABI, 2005). Reactions were performed in 15 μL PCR reaction volume containing 2 x TaqMan fast universal PCR master mix (1x); 20 μM primers (F/R), 0.20 μM each; 20 μM TaqMan MGB probe, 0.1 μM; 1 unit/μL AmpErase®UNG (Uracil N-glycosylase), 0.1 unit; 50 ng/uL template DNA, 100 ng; and RNA free water (or QH_2O). Real-time PCR conditions were 2 min at 50°C, 20 s at 95°C, 40 cycles of 3 s at 95°C and 30 s at 60°C. Three independent replications were performed for accurate detection of the GM maize lines NK603, T25, and MON810 and *Zein*. The reference sample was a mixture of the above GM maize DNA at a final concentration of 0.67%.

Screen-specific PCR

Qualitative screen-specific PCR was as described (Kuribara et al., 2002). A 25 μL of PCR reaction solution contained 100 ng DNA, 2.5 μL of 10x buffer, 2.0 μl of 2.5 μM dNTP, 1 μL of 10 μM primers each, 0.63 μL of 1 unit/μL Pfu DNA polymerase, and 13.87 μL QH_2O. The reaction was carried out using the GeneAmp PCR System 9700 (ABI, USA) under the condition: Pre-incubation for 10 min at 95°C, 37 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 59°C and extension for 30 s at 72°C, then a final extension at 72°C for 7 min. The PCR product was analyzed by 2% agarose gel

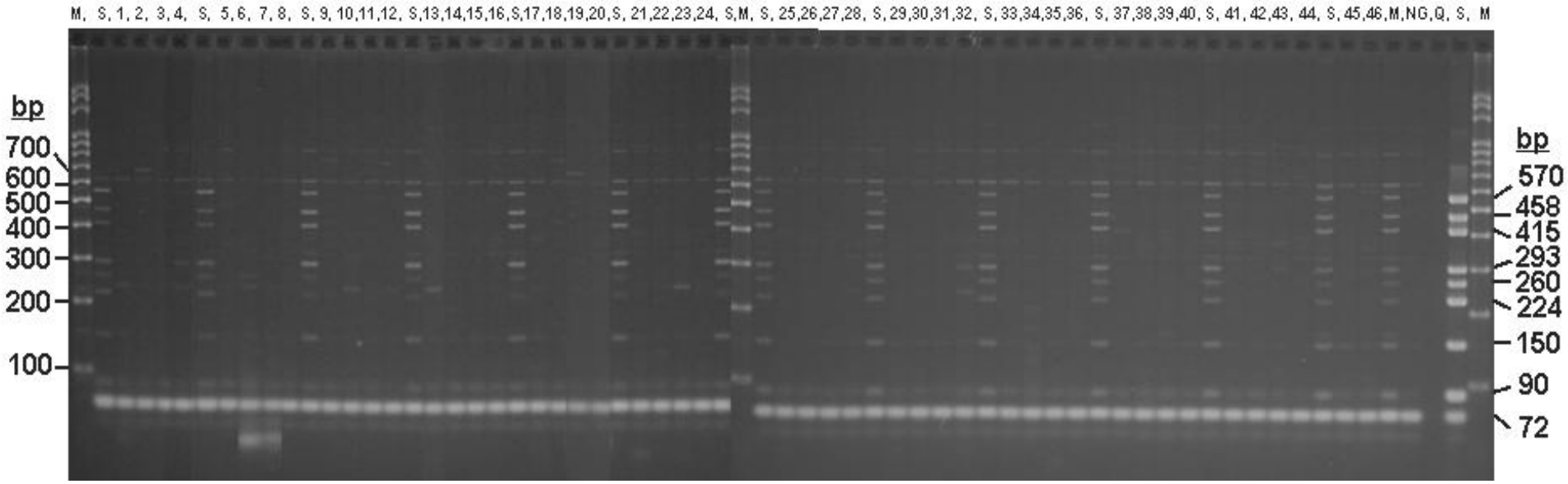

Figure 3. Multiplex PCR analysis of GM maize in 46 maize seed samples from Nepal. The numbers from 1 to 46 indicate different maize seed samples. S: 0.25% GRM prepared from genomic DNA of 8 GM maize lines (Event176, 570 bp; Bt11, 458 bp; TC1507, 415 bp; NK603, 293 bp; T25, 260 bp; MON863, 224 bp; MON810, 150bp; GA21, 90 bp) and *Zein*, 72 bp. NG: non-GM, Q: QH2O.

electrophoresis and con-firmed the presence of GM maize.

RESULTS AND DISCUSSION

Detection of GM maize by mPCR

The mPCR has been widely used for GM crops detection because of its accuracy, sensitivity and rapidness (Chaouachi et al., 2008; Forte et al., 2005; Huang and Pan (2004); Nikolic et al., 2009; Onishi et al., 2005; Shrestha et al., 2008). In this study, first they used mPCR method (Shrestha et al., 2008) to detect GM maize in 46 maize seed samples collected in Nepal (Table 1). The sensitivity of mPCR was reconfirmed with known GRM samples for 8 GM maize lines at 0.25% level (Figure 3). None of the maize seed samples obviously contaminated with GM maize was identified by mPCR (Figure 3). However, because of the presence of weak and nonspecific DNA bands, some samples might contaminate with very low level of NK603 (# 4, 32), T25 (#6, 43), and MON810 (#45, 46) as shown in Figure 3.

Real-time PCR analysis of samples with suspected GM maize

To rule out the possibility that very low amount of GM maize may be present, the authors further examined the above suspected seed samples by real-time PCR. Currently, real-time PCR is widely accepted as the most precise and straightforward method for quantification of GM crops (Lipp et al., 1999; 2005). Thus, they used real-time PCR to identify and quantify the GM maize lines NK603 (106 bp), T25 (100 bp) and MON810 (84 bp), as well as *Zein* (72 bp) (Figure 2 and Table 2). The authors made a composite mixture of all 3 CRM samples (GM maize NK603, T25, MON810) in a 2% volume (v/v) of final concentration 0.67%. The analyzed GM maize in selected samples (numbers 4, 6, 10, 32 and 45) by event-specific target primers and probe sets for NK603 (NK603-08F/R/P), T25 (T25-08F/R/P), MON810 (MON810-08F/R/P) and *Zein* (Zein-04Se/07R/08P) showed no signal in the non-template control or tested maize samples and only positive samples could be detected with CRMs (Figure 4). Therefore, they concluded that the suspected samples were not

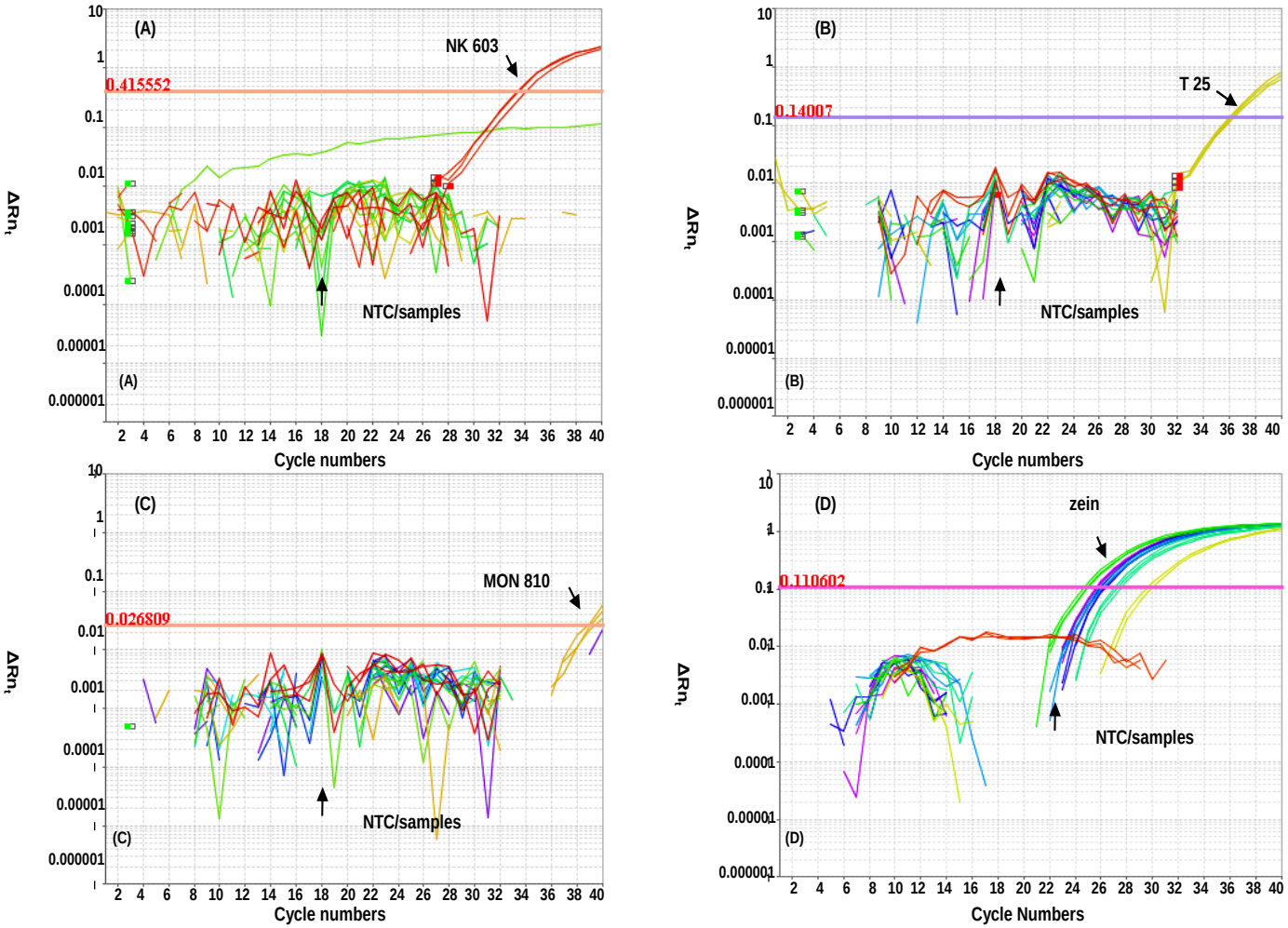

Figure 4 Real-time PCR amplification plots of suspected maize seed samples with certified reference materials (CRMs). (A) NK603 (08F/R/P), (B) T25 (08F/R/P), (C) MON810 (08F/R/P) and (D) endogenous *Zein* (04se/07R/08P). The y axis (ΔRn) represents the relative fluorescence intensities generated by the reporter at each cycle during PCR amplification. The x axis indicates the cycle numbers of PCR.

contaminated with the GM maize NK603, T25, and MON810 or *Zein* as tested by mPCR.

GM maize screening by screen-specific PCR

Although, the authors did not detect any GM maize in the Nepal maize seed samples, the samples could have been contaminated with other GM maize lines with unknown target sequences but the same constructs. Therefore, they searched the AGBIOS database for most commonly used promoters and terminators for producing GM maize. More than 91% of GM maize lines were identified to contain p35s and/or tNOS in the construct. Several reports have used the p35S and/or tNOS region in screen-specific GM maize and soybean analysis (Kuribara et al., 2002; Forte et al., 2005; Di Pinto et al., 2008; Ujhelyi et al., 2008; Lee et al., 2009; Mano et al., 2009; Nikolic et al., 2009). Therefore, they designed screen-specific primers, p35S 1-5'/1-3' (101 bp), NOS ter 2-5'/2-3' (151 bp) and *zSSIIb* 1-5'/1-3' (151 bp), to detect the presence of the *p35S*, *tNOS* and *zSSIIb.* They used 0.1% CRM samples to test MIR604 for tNOS, NK603 for tNOS/p35S and MON810 and DAS-59122-7 for p35S. In addition, they analyzed the suspected samples 6, 10, 19, 23, 32 and 45 and non-suspected samples, 4 and 15, with CRMs (Figure 5). Only the p35S-containing GM maize could be detected by the primer pair for p35S (Figure 5a). However, the primer pair for tNOS could not detect the existence of 0.1% GM maize (data not shown). This may be due to the efficiency of the primers because the range of GM maize quantification was set from 0.5 - 100% (Kuribara et al., 2002). When 1% of MIR604 and MON810 GM maize was used for further testing, they could detect positive band in the tNOS-containing MIR604 GM maize but not the remaining suspected

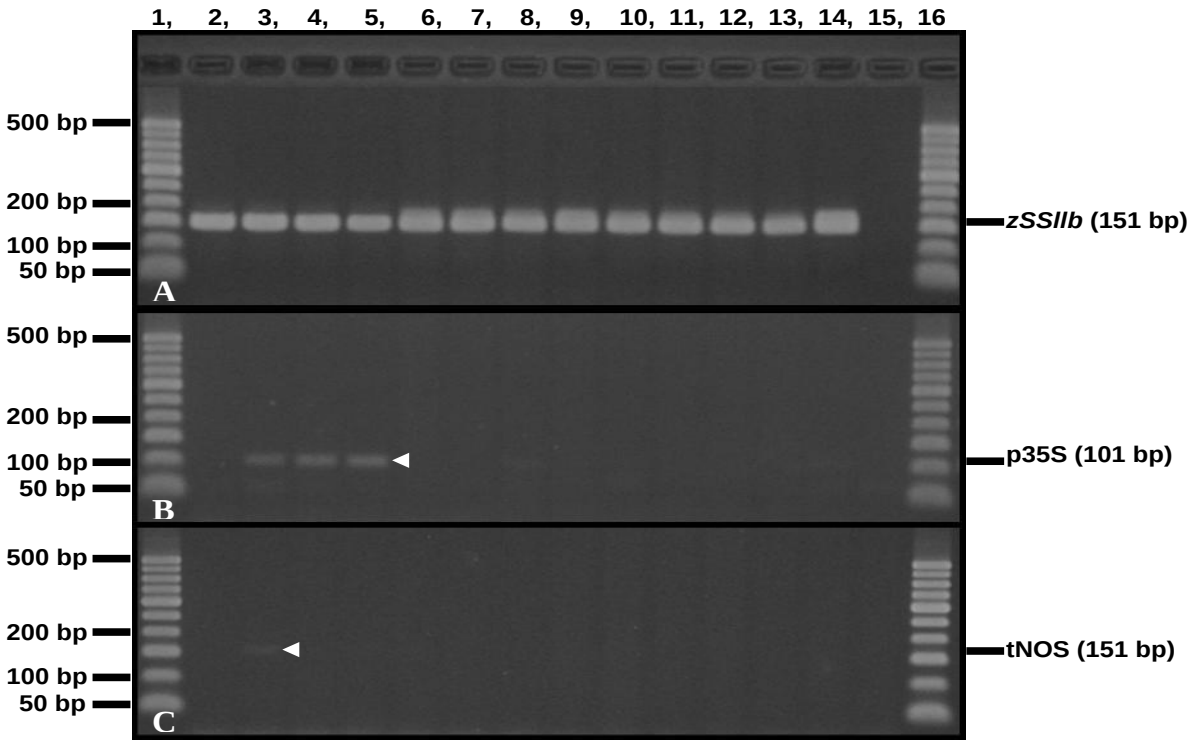

Figure 5 Screen-specific PCR of certified reference materials (CRMs) of GM maize and suspected maize samples from Nepal. In A, B and C, lanes 1 and 16 denote markers of 50-500 bp; in A and B, lanes 2-5 denote CRM of 0.1% of the GM maize lines MIR604, NK603, MON810 and DAS59122-7, respectively; lanes 6-13 denote maize samples from Nepal, numbers 6, 10, 19, 23, 32, 45, 4 and 15, respectively; lane 14 denotes non-GM maize and lane 15 no-template control (NTC) (QH_2O). Similarly, in C, lanes 2-5 denote the CRMs MIR 604 (0.1%), MIR 604 (1%), MON810 (0.1%), and MON810 (1%), respectively; lanes 6-15 are as for B and C.

maize seed samples (Figure 5c). The endogenous reference gene, *zSSIIb,* appeared in all maize samples (Figure 5a). This result again confirmed no new released or unknown GM maize lines present in the maize seed samples.

Conclusion

The escape of GM maize could be a potential risk to the local ecosystem in a country. The seed spillage from seed handling, growing, harvesting and storage may introduce GM into non-GM maize (Eastham and Sweet, 2002; Brookes et al., 2004). Thus, to maintain maize biological diversity and food biosafety, the GM maize seed status in a country must be regularly monitored and tracked. To fulfill this request, several researchers have used screen-, gene- and construct-specific PCR methods to detect GM maize (Zhou et al., 2007; Di Pinto et al., 2008; Lee et al., 2009). For example, Lee et al. (2009) used gene- and construct-specific mPCR primers for MON810, NK603, TC1507, GA21 and Bt176 to identify harvested maize products and screen-specific primers for GM maize in South Korea. However, to be effective in the detection of individual transgenic events of GM maize, it is better to include a PCR primer corresponding to the flanking sequence of genomic DNA in the integrated site by event-specific PCR (Holst-Jensen et al., 2003; Roanning et al., 2003; Yang et al., 2007; Shrestha et al., 2008). In this study, the event-specific mPCR, real-time PCR, and screen-specific PCR was adopted to monitor the current GM maize seed status in Nepal. They demonstrated that the mPCR method published before (Shrestha et al., 2008) can be practically used for detec-tion of real field maize seed samples from Nepal.

Moreover, this is the first report of the analysis of GM maize seed status in Nepal. The results suggest that GM maize may not enter into the Nepalese agricultural market. However, they are not able to completely rule out the possibility of very low presence or unintentional release of GM maize lines in Nepal. Maize in Nepal should be continually and regularly checked. This study can help in formulating and implementing quality control and regu-lation of maize seeds in Nepal. Although, the Nepal National Bio-safety Framework has been approved, no database and testing of GM crops are publicly available. Because of the shortage of human resources and faci-lities for GM crop monitoring in Nepal, this report could be a starting point for the establishment of a platform in GM crops monitoring and tracing. This survey is not compre-hensive and did not include all maize varieties. Therefore,

further testing is necessary, and a GM crop threshold in Nepal should be established. Nepal needs a legal basis for importation, processing and labeling directives for GM seed, feed and food in the near future.

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