Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol14-issue4-fulltext-12 *RESEARCH ARTICLE*

Detecting adventitious transgenic events in a maize center of diversity

Luis Fernando Rimachi Gamarra[1] · Jorge Alcántara Delgado[1] · Yeny Aquino Villasante[1] · Rodomiro Ortiz[2] ✉

1 Instituto Nacional de Innovación Agraria, Lima, Perú

2 Martín Napanga 253, Apt. 101, Miraflores, Lima, Perú

✉ Corresponding author: rodomiroortiz@gmail.com
Received December 15, 2010 / Accepted May 9, 2011
Published online: July 15, 2011

Abstract

Background: The genetic diversity of maize in Peru includes several landraces (within race clusters) and modern open pollinated and hybrid cultivars that are grown by farmers across various regions, thereby making this country a secondary center of diversity for this crop. A main topic of controversy in recent years refers to the unintended presence of transgenic events in locally grown cultivars at main centers of crop diversity. Peru does not yet have biosafety regulations to control or permit the growing of genetically modified crops. Hence, the aim of this research was to undertake a survey in the valley of Barranca, where there were recent claims of authorized transgenic maize grown in farmers fields as well as in samples taken from feed storage and grain or seed trade centers. **Results:** A total of 162 maize samples (134 from fields, 15 from local markets, eight from the collecting centers of poultry companies, from the local trading center and four samples from seed markets) were included for a qualitative detection by the polymerase chain reaction (PCR) of *Cauliflower Mosaic Virus* (*CaMV*) 35S promoter (*P35S*) and *nopaline synthase* terminator (*Tnos*) sequences, as well as for six transgenic events, namely *BT11*, *NK603*, *T25*, *176*, *TC1507* and *MON810*. The 134 maize samples from farmers fields were negative for Cry1Ab delta-endotoxin insecticidal protein and enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) using lateral flow strips. The PCR analysis did not detect any of the six transgenic events in samples from farmers fields, local markets, seed trading shops and the local collecting center. There were four transgenic events (*T25, NK603, MON810* and *TC1507*) in grain samples from the barns of poultry companies. **Conclusions:** This research could not detect, at the 95% probability level, transgenes in farmers' fields in the valley of Barranca. The four transgenic events in grain samples from barns of poultry companies were not surprising because Peru imports maize, mainly for animal feed, from Argentina and the United States that are known for growing transgenic maize.

Keywords: biosafety, corn, sampling, transgenes, Zea mays

INTRODUCTION

A lot of maize genetic variation occurs in Peru (Grobman et al. 1961), which may be regarded as an important center of diversity for this crop. Sevilla (2005) indicates that there are about 55 Peruvian races of maize that played an important role in the development of modern maize cultivars, particularly in the highlands Table 1. Maize races have been extensively studied and classified using specific ear and kernel traits (Grobman et al. 1961). This maize germplasm clustering was further confirmed with modern numerical taxonomy methods (Ortiz et al. 2008a; Ortiz et al. 2008b). Highland farmers grow distinct races in their maize fields that led to cultivar mixtures due among other causes, to gene flow through pollen, close cropping of diverse landraces or formation of seed banks. Maize races are, however, easily distinguished by farmers, particularly when “foreign genes” are brought from modern hybrids.

Table 1. Maize races from Peru (Sevilla, 2005).

Races	Coast	Highlands	Jungle
Primitive		Confite Morocho Confite Puntiagudo Confite Puneño Kully	Enano
Derived from primitive races	Mochero Alazan Pagaladroga Rabo de Zorro Chapareño Iqueño	Chullpi Huayleño Paro Morocho Huancavelicano Ancashino Shajatu Piscorunto Cusco Cristalino Amarillo Cusco Blanco Granda Uchuquilla	Sabanero Piricinco
From second derivation	Huachano Chancayano	San Gerónimo San Gerónimo Huancavelicano Cusco Gigante Arequipeño	Chimlos Marañón
Introduced	Pardo Arizona Colorado		Alemán Chuncho Cuban Yellow
Emerging	Jora Coruca Chancayano Amarillo Tumbesino Morochillo	Morado Canteño Morocho Cajabambino Amarillo Huancabamba Allajara Huarmaca Blanco Ayabaca Huanuqueño	
Not defined		Sarco	Perlilla

Gene flow is not something peculiar to transgenic plants. It happens at any time one organism breeds with a related species, thus passing along their combined DNA to the offspring. A main issue, which led to strong debates elsewhere, is the adventitious presence of transgenic events in main centers of crop diversity (Quist and Chapela, 2001; Christou, 2002; Kaplinsky et al. 2002; Metz and Fütterer, 2002; Quist and Chapela, 2002; Celis et al. 2004; Ortiz-García et al. 2005; Raven, 2005; Mercer and Wainwright, 2008; Piñeyro-Nelson et al. 2009; Schoel and Fagan, 2009). For example, the potential genetic and ecological impacts of gene flow from transgenic cultivars to landraces, weedy relatives and wild species are mainly related to the genetic integrity of landraces and crop wild relatives, and to developing plants with enhanced invasiveness or weediness in ecosystems (Cleveland et al. 2005; Engels et al. 2006; Scurrah et al. 2008; Warwick et al. 2009; Sahoo et al. 2010). Farmers' behavior and crop husbandry may significantly influence transgene spread in native germplasm. However, the perceptions of farmers and consumers that the transgenes are “polluting” and that landraces or local cultivars containing transgenes are “contaminants” could cause that these landraces or local cultivars may be rejected, which would mean a direct loss of agro-biodiversity (Bellon and Berthaud, 2006). The global spread of transgenic crops has also significant implications for organizations involved in germplasm conservation and genetic enhancement. In this regard, Mezzalama et al. (2010) describes a protocol used for monitoring unintentional transgene flow in maize gene bank and breeding plots. Their protocol is based on polymerase chain reaction (PCR) markers for detecting specific recombinant DNA sequences in bulked samples collected from sentinel plots.

Peru does not yet have biosafety regulations to control or permit the growing of genetically modified crops, and their introduction is a source of lively debate in the Peruvian media (Laursen, 2011). Very recently, Gutiérrez-Rosati et al. (2008) indicated that 1/3 of 42 samples of yellow maize grains from the valley of Barranca (north of Lima, Peru) were positive for transgenic events SYN-BTØ11-1 (*BT11*) and MON-ØØ6Ø3-6(*NK603)*, which provide host plant resistance to insect and tolerance to glyphosate herbicide, respectively. Their reports refer to both grains from harvests in this valley as well as from stores of animal feed. The 1999 Peru's Law 27104 (Prevention of risks from the use of biotechnology) and the 2002 Supreme Decree No. 108-2002 (regulating this law) empowers the Instituto Nacional de Innovación Agraria (INIA) as the sectoral body in agriculture to enforce provisions under national and international policy, to regulate, manage and control risks arising from the contained use and environmental release of living (LMO) or genetically (GMO) modified organisms. INIA formally asked for more information to Gutierrez-Rosati on the location of the fields where the samples were obtained, and the submission of the respective counter samples to validate their claim. Unfortunately, further details or the respective counter samples were not provided. The main goal of our research was therefore to assess qualitatively the presence of promoter *P35S* and sequence of *Tnos* terminator, to detect 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and Cry1Ab delta-endotoxin from *NK603* and *BT11* respectively, using immune-assays, as well as six commercial transgenic events, namely *BT11, NK603,*ACS-ZMØØ3-2(*T25*)*,* SYN-EV176-9 (*176*)*,* DAS-Ø15Ø7-1 (*TC1507*) and MON-ØØ81Ø-6 (*MON810*), with the aid of PCR amplification using event specific primers in maize samples taken from farmers fields, local markets, seed trade centers or barns of poultry farms in the valley of Barranca and neighboring locations following proper sampling and screening methods.

MATERIALS AND METHODS

The sampling area of maize fields was mainly along the Pativilca River Basin Figure 1, which is the main river of the valley of Barranca. Four sites from the neighboring Fortaleza River Basin were also added to this field sampling. There were additional grain samples from one local maize collecting center, eight poultry farm barns, four private seed dealers and 15 local markets.

Each maize field was regarded as an experimental unit, thereby estimating the sample size (*n*) for a categorical variable (presence or absence) with a finite population size as follows (Cochran, 1977):

$$n = \frac{N * {Z_{1-\alpha}}^2 * p * q}{d^2 * (N-1) + {Z_{1-\alpha}}^2 * p * q}$$

Where *N* is the population size; *i.e.*, the total number of maize fields (2100), *p* the prevalence (0.1), *q* equals 1 – *p* (0.9), *d* is the precision, *α* is the significance level (0.05), 1- *α* is the confidence level, and $Z_{1-\alpha}$ a pre-established value. The sample size used was 130 maize fields taking into account the above sampling equation and the putative transgene frequency (33.3 to 62%) in the valley of Barranca (Gutiérrez-Rosati et al. 2008). The sub-sampling within each location used the probability of detection (*Pd*) as follows (Remund et al. 2001; Lockwood et al. 2007):

$$Pd = 1 - (1 - p_{GM})^{m.s}$$

Where p_{GM} is the uniform frequency of a genetically modified organism (GMO), m is the number of fields or seed lots sampled, and s is the number of individuals or 2*n* alleles sampled per field or seed lot. This protocol could allow, with a 95% probability, detecting transgenic events with a frequency equal or greater than 0.05% ensuing from unauthorized GM-seed imports when sampling at least 50 plants in each location. The probability of detection of GMO with a frequency equal or greater to 0.05% (using a PCR assay) will be 96.13% following above equations used for this two-step sampling approach of 130 maize fields and 50 plants per field.

The number of maize fields sampled was determined according to their relative number in each irrigation district Table 2. A zigzag walk was used for leaf sampling in a minimum of 1 ha and taking one leaf per plant from at least 50 plants. Some farmers maize fields included in the field survey were larger than 1 ha and due to logistics only 1 ha was taken randomly for sampling. The leaf samples had

between 5 to 10 cm in length and preferably from the middle part of healthy tender leaves. Two thousand grains were collected from each of the maize fields that were at harvest time; *i.e.*, 100 randomly selected ears were selected from each field, and 20 grains were obtained from two rows per each ear. This grain sampling provides a 99% certainty to detect the adventitious presence of transgenic events with $P \geq 0.005$. Grain samples (500 g ≈ 2100 grains) from local markets were purchased from the main wholesalers and retailers. They were grouped into three subsamples of approximately 700 seeds each. Based on the binomial probability, if the 3 sub-samples showed negative results in the PCR analysis, there would be a 95% certainty that the transgenic event frequency was below 1%. Similar approach was used for grain samples (of same weight) from the local maize collecting center and private seed dealers. Seed samples of hybrid maize cultivars Agroceres 003 and Agroceres 1596 were kindly provided by a local dealer. Four samples (≈ 2 kg) were taken from two grain lots in each of the poultry farm barns.

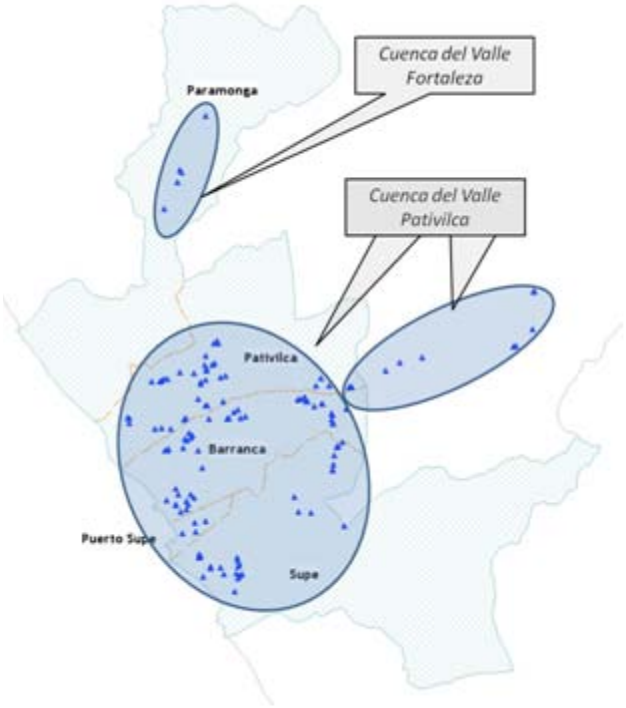

Fig 1. Sampling areas of maize plants and grains in the Fortaleza and Pativilca river basins. Each blue dot shows the collecting locations.

The analysis of all field samples were carried out in two stages, the first called scanning or screening used qualitative PCR detection for *P35S* and *Tnos* sequences, which are present in most transgenic maize events. The second stage involved the identification of specific transgenic events that were indicated as grown in Peru by previous reports (Gutiérrez-Rosati et al. 2008). Three of them have *P35S* and *Tnos* sequences (*BT11*, *NK603* and *MON863*) and one (*T25*) only has *P35S*.

DNA was extracted from each leaf sample following a modified CTAB method (Doyle and Doyle, 1990). The DNA from grains was taken according to the manual of the EU for detecting GMO in food samples (Querci et al. 2006). DNA extraction was from 1 cm^2 each in groups of 10 leaves due to the number of samples; i.e. a total of five DNA sub-samples every field with its own duplicate. DNA was quantified automatically on the Nanodrop 2000, by the standard spectrophotometric relations to 260 nm, 260/280 nm and 260/230 nm. The quality of DNA was visualized by electrophoresis on 0.8% agarose gels (Sambrook and Russell, 2001). All samples were standardized at a concentration of 10 ng ml^{-1} before mixing the five sub-samples for their later use in PCR amplification.

Table 2. Sampling of maize fields per irrigation district in Pativilca and Fortaleza basins.

Irrigation district	Fields (#)	Area sown (ha)	Sampled field
Arayaz	152	281.39	9
Chacarita Puerto	145	379.17	9
Galpón	103	101.35	8
Huanchay	4	5.00	2
Huarangal Antival	115	54.22	7
Huayto	239	503.83	17
La Vega-Otopongo	163	147.89	7
Llamachupan	51	55.56	3
Paramonga	274	255.46	9
Paycuán	107	244.53	6
Potao	212	447.33	11
Purmacana	144	346.53	17
San Nicolás	143	249.11	15
Santa Elena	45	111.63	3
Venado Muerto	38	77.84	1
Vinto	166	362.52	6
Valle Fortalezaz			4
Total	2101	3623.36	134

[z]Leaf and grain samples were taken in fields from both, whereas only leaf samples were taken from the remaining irrigation districts.

Protocols and programs for qualitative PCR amplification were standardized following known protocols Table 3. New multiplex PCR assays were standardized for the analysis of two or three primers per reaction Table 4 and Table 5 with the aim of reducing costs and time. The primers were synthesized by Invitrogen (São Paulo, Brazil) and IDT (Coral Ville, Iowa, USA) whereas other reagents used in PCR amplification (10 x PCR Buffer, dNTP, MgCl2, *Taq* polymerase) were from QIAGEN (Hamburg, Germany). The controls for the PCR amplification assays and for the analysis of amplification products by electrophoresis were *BT11*, *NK603*, *MON810*, *TC1507*, *176* and *T25* [positive checks provided by the Instituto Nacional de Tecnología Agropecuaria of Argentina (INTA)], a 329 bp *zein* gene (DNA amplification check), DNA from hard yellow maize cultivar INIA 611 (negative check), and a PCR master mix without DNA ("blank" check). Amplified products were separated by electrophoresis on 2% agarose gels (120 volts x 80 min) and visualized by ethidium bromide staining (0.3 mg ml^{-1}) and photo-registered with ChemiDocTM XR. The amplified product size of the samples analyzed for each of the primers and positive controls (at a 5% weight/weight in the working GM/non-GM samples provided by INTA) were compared with the ladder of DNA fragments of 100 bp (Invitrogen: 1500 to 100 bp) and 50 pairs bases (Fermentas/Gen Lab del Peru S.A.C., Lima, Peru): 1031 to 50 bp). Eye scoring for absence or presence of transgenic constructs was used for recording into a database.

Immunoassay for detecting GMO using lateral flow strips was conducted in farmers' fields. The kits for the detection of Cry1Ab and CP4 EPSPS proteins of transgenic events *Bt11* and *NK603*, respectively, were purchased from Estrategic Diagnostic (Newark, Delaware, USA). Other kits to verify the detection of the same proteins from transgenic events *Bt11* and *NK603* were also kindly provided by AGDIA (Elkhart, Indiana, USA). The methodology for using both sets of kits was described in the respective company manuals.

RESULTS

There were 127 maize leaf DNA samples from the sampled fields (94.77%), whose concentrations ranged from 20 to 150 ng ml^{-1}. The DNA obtained from grain samples had a concentration of 40 to 130 ng ml^{-1}. The seven samples with non-amplifiable DNA for PCR analysis were collected from fields at harvest time. The degree of leaf deterioration did not allow to obtain quality DNA and to get appropriate concentrations. Hence, the probability of detecting a GMO with a frequency greater or equal to 0.05% was adjusted to 95.82%.

Table 3. Primers used for PCR analysis of maize samples from Barranca.

Primer type	Primer	Sequence	Product size (bp)	Target	References
Endogenous	ZEIN01	TGCTTGCATTGTTCGCTCTCCTAG	329	*Zein* gene specific	Chiueh et al. 2002; Rahman et al. 2007; GMDD, 2010
	ZEIN02	GTCGCAGTGACATTGTGGCAT			
General screening of transgenes	P35S F	ATTGATGTGATATCTCCACTGACGT	101	*Cauliflower Mosaic Virus (CaMV) 35S* promoter sequence (*P35S*)	Lee et al. 2004; GMDD, 2010
	P35S R	CCTCTCCAAATGAAATGAACTTCCT			
	P35SL	GATAGTGGGATTGTGCGTCA	195	*P35S*	Lin et al. 2000; GMDD 2010
	P35SU	GCTCCTACAAATGCCATCA			
	Tnos F	GTCTTGCGATGATTATCATATAATTTCTG	151	*Nopaline synthase* terminator sequence from *Agrobacterium tumefaciens* (*Tnos*)	Lee et al. 2004; GMDD, 2010
	Tnos R	CGCTATATTTTGTTTTCTATCGCGT			
Specific screening of transgenes	VW01	TCGAAGGACGAAGGACTCTAACG	170	Between maize DNA and *CaMV* promoter DNA in *MON810* maize	GMDD, 2010
	VW03	TCCATCTTTGGGACCACTGTCG			
	QTC1507-1F	GACGTCTCAATGTAATGGTTAACGA	83	Between *Pat* gene and maize genomic DNA in TC1507 maize	Yang et al. 2007
	QTC1507-1F	CCTAGTATATGAAAGAATGAAAAGGTGCTT			
	Cry1Ab event 176-F	CGGCCCCGAGTTCACCTT	420	*Cry1Ab* transgene in 176 maize	Cardarelli et al. 2005; Zaulet et al. 2009; Dinon et al. 2010
	Cry1Ab event 176-R	CTGCTGGGGATGATGTTGTTG			
	E176 1-5-F	GTAGCAGACACCCCTCTCCACA	189	Construct specific between *PEPC* promoter and *Cry1Ab* transgene in 176 maize	Matsuoka et al, 2001; Onishi et al. 2005
	Cry1A1-3-R	TCGTTGATGTTKGGGTTGTTGTCC			
	T25R3	TGAGCGAAACCCTATAAGAACCC	209	Construct specific between *Tnos* and *PAT* gene in T25 maize	GMDD, 2010
	T25F7	ATGGTGGATGGCATGATGTTG			
	IVS2	CTGGGAGGCCAAGGTATCTAAT	189	Construct specific between IVS2 intron and *PAT* gene of BT11 maize	GMDD, 2010
	PATB	GCTGCTGTAGCTGGCCTAATCT			
	NK-R393	GAGAGATTGGAGATAAGAGATGGGTTC	231	Construct specificbetween protein 70 gene and peptide 2 gene from the chloroplast (for NK603)	Lee et al. 2004
	NK-F163	CCTCCTGATGGTATCTAGTATCTACCAACT			

Table 4. PCR amplification conditions for multiplex assays using 25 µL as final volume.

Primers	PCR buffer 10 x	dNTP	MgCl2	Primer concentration	*Taq* Hot Start
ZEIN01-ZEIN02 P35S F-P35S R Tnos F-Tnos R	1.0 x	0.22 mM	1.5 mM	0.25 µM	0.6 U
ZEIN01-ZEIN02 VW01-VW03 T25R3-T25F7	1.0 x	0.22 mM	1.5 mM	0.25 µM	0.6 U
ZEIN01-ZEIN02 IVS2-PATB NK-R393-NK-F163	1.0 x	0.2 mM	1.5 mM	0.22 µM	0.6 U
ZEIN01-ZEIN02 P35SL-P35SU	1.0 x	0.22 mM	1.0 mM	0.25 µM	0.6 U
ZEIN01-ZEIN02 QTC1507-1F QTC1507-1F	1.0 x	0.2 mM	1.5 mM	0.3 µM	0.6 U
ZEIN01-ZEIN02 Cry1Ab event 176-F Cry1Ab event 176-R E176 1-5-F Cry 1A 1-3-R	1.0 x	0.2 mM	1.5 mM	0.4 µM	0.6 U

Table 5. Programs for multiplex PCR amplification assays.

Assay type	Primers	Pre-denaturation		Denaturation		Annealing		Extension		Final extension	
		Temp.(ºC)	Time	Temp. (ºC)	Time	Temp. (ºC)	Time	Temp. (ºC)	Time	Temp. (ºC)	Time
Screening	ZEIN01-ZEIN02 P35S F-P35S R Tnos F-Tnos R	95 1 cycle	7 min	94 40 cycles	30 sec	60	45 sec	72	30 sec	72 1 cycle	7 min
	ZEIN01-ZEIN02 P35SL-P35SU	95 1 cycle	7 min	94 42 cycles	30 sec	60	45 sec	72	30 sec	72 1 cycle	7 min
Transgenic event	ZEIN01-ZEIN02 VW01-VW03 T25R3-T25F7	95 1 cycle	7 min	94 40 cycles	30 sec	63	45 sec	72	30 sec	72 1 cycle	7 min
	ZEIN01-ZEIN02 IVS2-PATB NK-R393-NK-F163	95 1 cycle	7 min	94 40 cycles	30 sec	63	45 sec	72	30 sec	72 1 cycle	7 min
	ZEIN01-ZEIN02 QTC1507-1F QTC1507-1F	95 1 cycle	7 min	95 38 cycles	30 sec	63	30 sec	72	30 sec	72 1 cycle	7 min
	ZEIN01-ZEIN02 Cry1Ab 176-F Cry1Ab 176-R E176 1-5-F Cry 1A 1-3-R	95 1 cycle 95 1 cycle	10 min 10 min	95 10 cycles 95 28 cycles	30 sec 30 sec	64 62	60 sec 60 sec	72 72	60 sec 60 sec	72 1 cycle 72 1 cycle	7 min 7 min

Sixteen out of 127 field samples that amplified the endogenous maize gene region were positive thrice for *P35S* (101 bp), but none of these samples was positive for *Tnos* Figure 2a. Four of the 15 samples from local markets were positive thrice for *P35S* but they were negative for *Tnos* Figure 2b. The eight grain samples from the poultry farm barns amplified for both *P35S* and *Tnos* sequences Figure 2c, whereas the grain sample from the local maize collection center or the local seed dealers did not amplify for either. The 16 field samples and four samples from local markets showed faint bands for *P35S*, compared to well-defined bands from samples of the poultry farm barns.

None of the 127 field samples, including the 16 samples that amplified the *P35S* sequence, showed positive results for the presence of transgenic constructs *BT11*, *NK603*, *T25*, *176*, *TC1507* and *MON810* in the three repetitions used Figure 3. There were no positive results for the presence of the same transgenic constructs in the three repetitions for tests on 15 grain samples from local markets, including the four samples that amplified the sequence *P35S* Figure 4. Five of the eight grain samples from the poultry farm barns amplified the transgenic construct *T25*, whereas eight samples amplified from the transgenic constructs *NK603* and *MON810* Figure 5. The transgenic constructs *176* and *BT11* were not found in any of the grain samples. The grain sample from the local collecting facility did not amplify any of these six transgenic constructs

The immunoassays using lateral flow trips for Cry1Ab-delta endotoxin and EPSPS with field samples were negative. Samples from positive (NK603 and Bt11) and negative (INIA 611) cultivar checks were used to validate the functionality of these lateral flow strips.

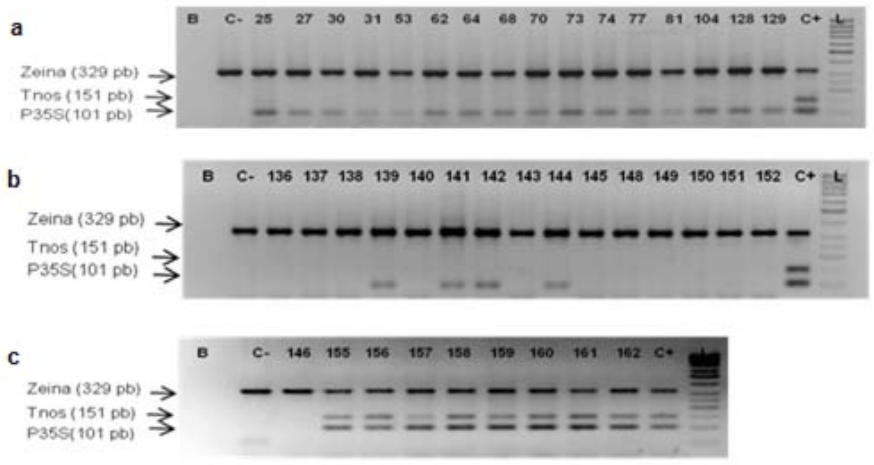

Fig. 2 Electrophoretic profiles for the detection of *P35S* and *Tnos* sequences in maize samples from fields (a), local markets (b), and poultry farm barns (c). Numbers indicate testing samples (25 to 129 from fields, 155 to 162 from poultry farm barns and 146 for the local collecting center), C (-) and C (+) are the negative (INIA 611 maize cultivar) and positive (BT11 maize) checks, B is the “blank” check, and L shows the 50 bp ladder.

DISCUSSION

The Barranca Valley is an agricultural area that primarily grows yellow maize, particularly commercial hybrid cultivars (93.3%) from private seed suppliers (Agricola, Agroceres, Dekalb, Pioneer HiBred, Hortus and Inti). Those commercial maize hybrid cultivars that do no longer produce good grain yields are used for fodder (locally known as "chala") and account for 4.5% of the field samples. Only three fields, of the 134 randomly selected for sampling, had landraces or local cultivars (2.2%), including two for green maize (or "choclo" as per its vernacular name), and one purple maize (for producing the local

drink "chicha morada" or desserts such as "mazamorra morada"), whose seeds can be purchased in local markets or are kept by farmers for re-seeding at every planting.

The initial screening for adventitious transgenic events was only to assess the presence of *BT11* and *NK603*, which are widely distributed worldwide and were reported to be in maize samples from Barranca (Gutiérrez-Rosati et al. 2008). We decided to screen further other transgenic constructs (*MON810*, *T25*, *TC1507* and *176*), which possess *P35S* but lack *Tnos*, after being unable to detect *BT11* and *NK603* in the samples analyzed. The screening results from field samples were also negative Table 6. Transgenic events *GA21* and *MON863* were not included for subsequent analysis because both have the *Tnos* sequence, which was negative in the previous screenings.

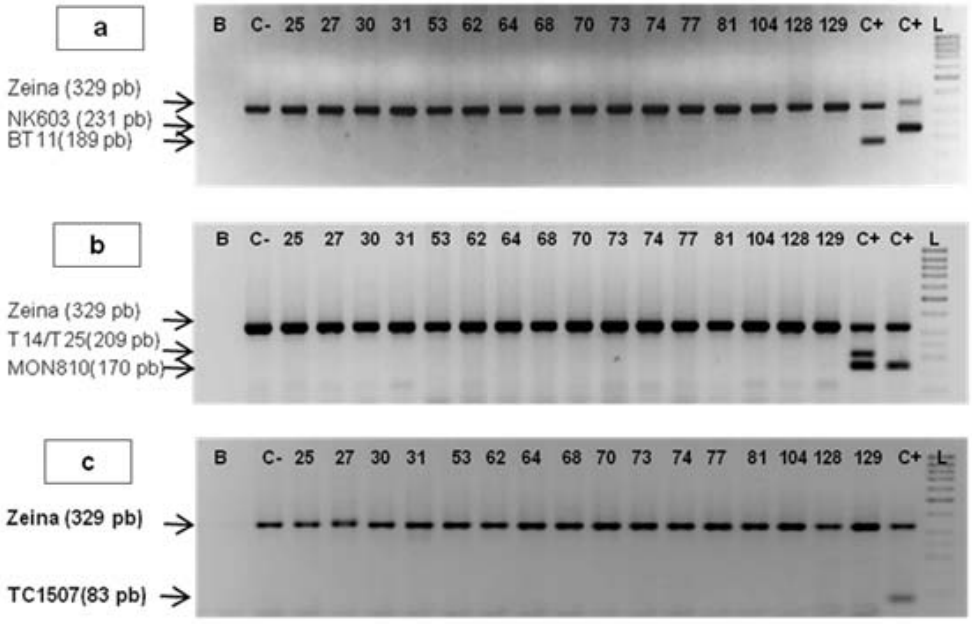

Fig. 3 Electrophoretic profiles for the detection of transgenic constructs *NK603* and BT11 (a), *T25* and *MON810* (b), and *TC 1507* (c) in maize field samples. Numbers indicate testing samples (25 to 129), C (-) and C (+) are the negative (INIA 611 maize cultivar) and positive checks, B is the "blank" check, and L shows the 50 bp ladder.

The finding of *P35S* on 16 field samples could be false positives due to the presence of the *Cauliflower Mosaic Caulimovirus* (*CaMV*) in these samples, as was also indicated by research elsewhere (Wolf et al. 2000; Holden et al. 2010). Another possible explanation would be a slight contamination in the laboratory. However, the negative checks for PCR amplification did not yield positive results in any test conducted, thereby ruling out this possibility.

The positive results for transgenic events in grain samples from poultry farm barns could be attributed to the high demand for yellow maize by the poultry industry. Peru imports about 1.5 million t (in excess of 50% of the national demand) of maize grains mainly for animal feed every year from Argentina (75% of total import of maize grains) and USA (21%), where GM-maize seeds are widely grown by their farmers and traded in export markets.

It is very important to use sound sampling protocols, analytical methods (Anklam et al. 2002) and probability models (Hernández-Suárez et al. 2008) for detecting adventitious transgenic events. We can conclude, based on our screening results with a 95% confidence level and a 95.82% probability of detecting adventitious transgenic events with a frequency equal or greater than 0.05%, that farmers do not grow transgenic maize cultivars in the valley of Barranca. Previous research about the presence of transgenes in maize samples from this valley (Gutiérrez-Rosati et al. 2008) did not indicate if they were

found in native maize cultivars. Hence, there is a lack of evidence for a possible hybridization between the landraces and GM cultivars of maize, and it seems very unlikely that such possible introgression of transgenes occurs in Peruvian maize landraces.

Pollen flow from maize hybrids to local cultivars often occurs in farmers fields of the Peruvian coast.

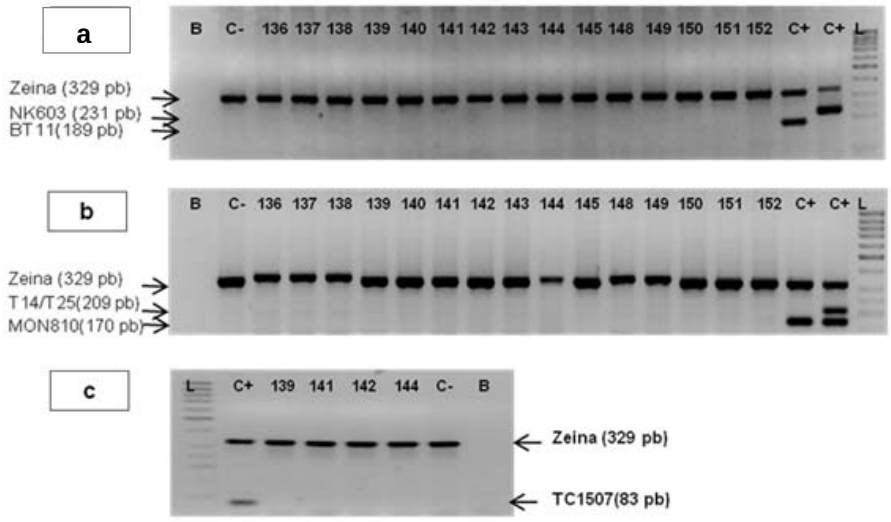

Fig. 4 Electrophoretic profiles for the detection of transgenic constructs *NK603* and BT11 (a), *T25* and *MON810* (b), and *TC 1507* (c) in maize grain samples from local markets. Numbers indicate testing samples (136 to 152), C (-) and C (+) are the negative (INIA 611 maize cultivar) and positive checks, B is the "blank" check, and L shows the 50 bp ladder.

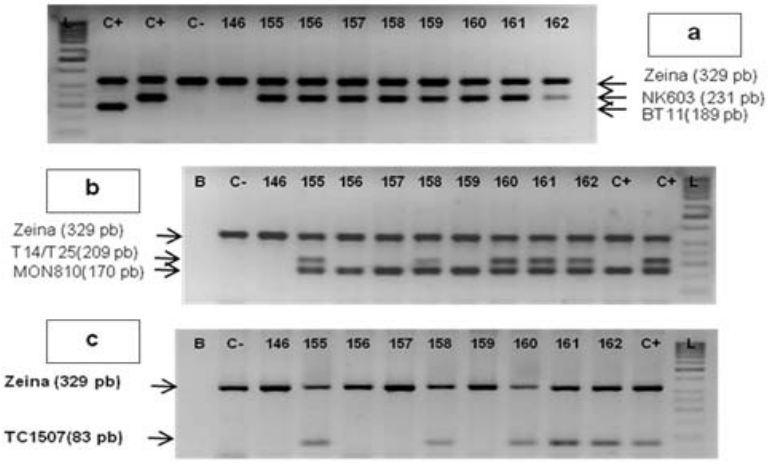

Fig. 5 Electrophoretic profiles for the detection of transgenic constructs *NK603* and BT11 (a), *T25* and *MON810* (b), and *TC 1507* (c) in maize grain samples from local collecting center (146) and poultry farm barns (155 to 162). Numbers indicate testing samples, C (-) and C (+) are the negative (INIA 611 maize cultivar) and positive checks, B is the "blank" check, and L shows the 50 bp ladder.

However, when selecting their seeds for planting, farmers retain the varietal purity of their landraces and local cultivars because the grains have special uses in drinks such as "chicha", or are freshly eaten as "choclo" (Sevilla, 2005). The seeds ensuing from the fertilization with pollen from hybrid yellow maize cultivars are easily distinguishable by the xenia effect and therefore dismissed as seed for planting by the local farmers. Furthermore, Palaudelmàs et al. (2009) found that transgenic maize volunteers had low plant vigour, rarely had cobs and produced pollen that cross-fertilized neighbour plants only at low levels.

Transgene flow raises a new set of ecological and economic issues for scientists and policymakers to consider for transgene containment (Dyer et al. 2009). Local farmer knowledge will be useful to avoid transgene flow and maintain distinct cultivars for the markets (Ortiz and Smale, 2007). Appropriate measurements should be also taken in Peru when transgenic and conventional crops of the same species will coexist in the future in the same locations if some farmers will wish to grow crops for GMO-free markets. Such regulations will also benefit from recognition of the practices farmers use to maintain the genetic integrity of their cultivars in their fields.

Table 6. Screening of transgenic sequences and constructs in maize samples from the valley of Barranca.

Sample location	Transgenic sequences		Transgenic events					
	Tnos	*P35S*	*T25*	*MON810*	*NK603*	*BT11*	*176*	*TC 1507*
Farmers' fields	0	16	0	0	0	0	0	0
Local markets	0	4	0	0	0	0	0	0
Grain collecting center	0	0	0	0	0	0	0	0
Poultry farm barns	8	8	5	8	8	0	0	5
Seed dealers	0	0	0	0	0	0	0	0

ACKNOWLEDGMENTS

The authors thanks Dr. Felipe de Mendiburu for his advice on sampling and statistical analysis, Prof. Ricardo Sevilla for reviewing the spatial distribution of local maize landraces and cultivars, colleagues from the Agencia Agraria de Barranca, Gerencia Regional de Recursos Naturales y Gestión del Medio Ambiente from Gobierno Regional de Lima and INIA for the assistance in collecting maize samples, and to the Junta de Usuarios del Valle Pativilca and her farmers for both assisting in collecting samples and allowing to take them from their fields.

REFERENCES

ANKLAM, E.; GADANI, F.; HEINZE, P.; PIJNENBURG, H. and VAN DEN EEDE, G. (2002). Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products. *European Food Research and Technology*, vol. 214, no. 1, p. 3-26. [CrossRef]

BELLON, M.R. and BERTHAUD, J. (2006). Traditional Mexican agricultural systems and the potential impacts of transgenic varieties on maize diversity. *Agriculture and Human Values*, vol. 23, no. 1, p. 3-14. [CrossRef]

CARDARELLI, P.; BRANQUINHO, M.R.; FERREIRA, R.T.B.; DA CRUZ, F.P. and GEMAL, A.L. (2005). Detection of GMO in food products in Brazil: the INCQS experience. *Food Control*, vol. 16, no. 10, p. 859-866. [CrossRef]

CELIS, C.; SCURRAH, M.; COWGILL, S.; CHUMBIAUCA, S.; GREEN, J.; FRANCO, J.; MAIN, G.; KIEZEBRINK, D.; VISSER, R.G.F. and ATKINSON, H.J. (2004). Environmental biosafety and transgenic potato in a centre of diversity for this crop. *Nature*, vol. 432, no. 7014, p. 222-225. [CrossRef]

CHIUEH, L.C.; CHEN, Y.L. and SHIH, D.Y.C. (2002). Study on the detection method of six varieties of genetically modified maize and processed foods. *Journal of Food and Drug Analysis*, vol. 10, no. 1, p. 25-33.

CHRISTOU, P. (2002). No credible scientific evidence is presented to support claims that transgenic DNA was introgressed into traditional maize landraces in Oaxaca, Mexico. *Transgenic Research*, vol. 11, no. 1, p. 3-5. [CrossRef]

CLEVELAND, D.A.; SOLERI, D.; CUEVAS, F.A.; CROSSA, J. and GEPTS, P. (2005). Detecting (trans)gene flow to landraces in centers of crop origin: lessons from the case of maize in Mexico. *Environmental Biosafety Research*, vol. 4, no. 4, p. 197-208. [CrossRef]

COCHRAN, W.G. (1977). Sampling Techniques, 3rd ed, New York, John Wiley and Sons, 428 p. ISBN 978-0471162407.

DINON, A.Z.; BOSCO, K.T. and ARISI, A.C.M. (2010). Monitoring of Bt11 and Bt176 genetically modified maize in food sold commercially in Brazil from 2005 to 2007. *Journal of the Science of Food and Agriculture*, vol. 90, no. 9, p. 1566-1569. **[CrossRef]**

DOYLE, J.J. and DOYLE, J.L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, vol. 12, no. 1, p. 13-15.

DYER, G.A.; SERRATOS-HERNÁNDEZ, J.A.; PERALES, H.R.; GEPTS, P.; PIÑEYRO-NELSON, A.; CHÁVEZ, A.; SALINAS-ARREORTUA, N.; YÚNEZ-NAUDE, A.; TAYLOR, J.E. and ALVAREZ-BUYLLA, E.R. (2009). Dispersal of transgenes through maize seed systems in Mexico. *PLoS ONE*, vol. 4, no. 5, p. e5734. **[CrossRef]**

ENGELS, J.M.M.; EBERT, A.W.; THORMANN, I. and DE VICENTE, M.C. (2006). Centres of crop diversity and/or origin, genetically modified crops and implications for plant genetic resources conservation. *Genetic Resources and Crop Evolution*, vol. 53, no. 8, p. 1675-1688. **[CrossRef]**

GMO DETECTION METHOD DATABASE (GMDD). (2010). *GMO Detection Laboratory*, Shanghai Jiao Tong University.

GROBMAN, A.; SALHUANA, W.; SEVILLA, P.R. and MANGELSDORF, P.C. (1961). Races of maize in Peru: their origins, evolution and classification. Washington, D.C: *National Academy of Sciences-National Research Council*.

GUTIÉRREZ-ROSATI, A.; POGGI, P.D.; GÁLVEZ, G.M. and CÁCERES, R.R. (2008). Investigaciones sobre la presencia de transgenes en Perú: caso maíz (*Zea mays* L.). *Revista Latinoamericana de Genética*, vol. 1, p. 89.

HERNÁNDEZ-SUÁREZ, C.M.; MONTESINOS-LÓPEZ, O.A.; MCLAREN, G. and CROSSA, J. (2008). Probability models for detecting transgenic plants. *Seed Science Research*, vol. 18, no. 2, p. 77-89. **[CrossRef]**

HOLDEN, M.J.; LEVINE, M.; SCHOLDBERG, T.; HAYNES, R.J. and JENKINS, G.R. (2010). The use of 35S and T*nos* expression elements in the measurement of genetically engineered plant materials. *Analytical and Bioanalytical Chemistry*, vol. 396, no. 6, p. 2175-2187. **[CrossRef]**

KAPLINSKY, N.; BRAUN, D.; LISCH, D.; HAY, A.; HAKE, S. and FREELING, M. (2002). Biodiversity (communications arising): maize transgene results in Mexico are artefacts (see editorial footnote). *Nature*, vol. 416, no. 6881, p. 601-602. **[CrossRef]**

LAURSEN, L. (2011). Peruvian biologist's defamation conviction overturned. *Nature*, vol. 39. **[CrossRef]**

LEE, S.H.; KIM, J.K.; PARK, Y.H.; KIM, Y.M. and PARK, K.W. (2004). Qualitative PCR method for detection of genetically modified maize lines NK603 and TC1507. *Agricultural Chemistry and Biotechnology*, vol. 47, no. 4, p. 185-188.

LIN, H.Y.; CHIUEH, L.C. and SHIH, D.Y.C. (2000). Detection of genetically modified soybeans and maize by the polymerase chain reaction method. *Journal of Food and Drug Analysis*, vol. 8, no. 3, p. 200-207.

LOCKWOOD, D.R.; RICHARDS, C.M. and VOLK, G.M. (2007). Probabilistic models for collecting genetic diversity: comparisons, caveats, and limitations. *Crop Science*, vol. 47, no. 2, p. 861-866. **[CrossRef]**

MATSUOKA, T.; KURIBARA, H.; AKIYAMA, H.; MIURA, H.; GODA, Y.; KUSAKABE, Y.; ISSHIKI, K.; TOYODA, M. and HINO, A. (2001). A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. *Food Hygiene and Safety (Shokuhin Eiseigaku Zasshi)*, vol. 42, no. 1, p. 24-32. **[CrossRef]**

MERCER, K.L. and WAINWRIGHT, J.D. (2008). Gene flow from transgenic maize to landraces in Mexico: an analysis. *Agriculture, Ecosystems and Environment*, vol. 123, no. 1-3, p. 109-115. **[CrossRef]**

METZ, M. and FÜTTERER, J. (2002). Biodiversity (communications arising): suspect evidence of transgenic contamination (see editorial footnote). *Nature*, vol. 416, no. 6881, p. 600-601. **[CrossRef]**

MEZZALAMA, M.; CROUCH, J.H. and ORTIZ, R. (2010). Monitoring the threat of unintentional transgene flow into maize gene banks and breeding materials. *Electronic Journal of Biotechnology*, vol. 13, no. 2. **[CrossRef]**

ONISHI, M.; MATSUOKA, T.; KODAMA, T.; KASHIWABA, K.; FUTO, S.; AKIYAMA, H.; MAITANI, T.; FURUI, S.; OGUCHI, T. and HINO, A. (2005). Development of a multiplex polymerase chain reaction method for simultaneous detection of eight events of genetically modified maize. *Journal of Agricultural and Food Chemistry*, vol. 53, no. 25, p. 9713-9721. **[CrossRef]**

ORTIZ-GARCÍA, S.; EZCURRA, E.; SCHOEL, B.; ACEVEDO, F.; SOBERÓN, J. and SNOW, A.A. (2005). Absence of detectable transgenes in local landraces of maize in Oaxaca, Mexico (2003-2004). *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 35, p. 12338-12343. **[CrossRef]**

ORTIZ, R. and SMALE, M. (2007). Transgenic technology: pro-poor or pro-rich? *Chronica Horticulturae*, vol. 47, no. 4, p. 9-12.

ORTIZ, R.; CROSSA, J.; FRANCO, J.; SEVILLA, R. and BURGUEÑO, J. (2008a). Classification of Peruvian highland maize races using plant traits. *Genetic Resources and Crop Evolution,* vol. 55, no. 1, p. 151-162. **[CrossRef]**

ORTIZ, R.; SEVILLA, R.; ALVARADO, G. and CROSSA, J. (2008b). Numerical classification of related Peruvian highland maize races using internal ear traits. *Genetic Resources and Crop Evolution,* vol. 55, no. 7, p. 1055-1064. **[CrossRef]**

PALAUDELMÀS, M.; PEÑAS, G.; MELÉ, E.; SERRA, J.; SALVIA, J.; PLA, M.; NADAL, A. and MESSEGUER, J. (2009). Effect of volunteers on maize gene flow. *Transgenic Research*, vol. 18, no. 4, p. 583-594. **[CrossRef]**

PIÑEYRO-NELSON, A.; VAN HEERWAARDEN, J.; PERALES, H.R.; SERRATOS-HERNÁNDEZ, J.A.; RANGEL, A.; HUFFORD, M.B.; GEPTS, P.; GARAY-ARROYO, A.; RIVERA-BUSTAMANTE, R. and ÁLVAREZ-BUYLLA, E.R. (2009). Transgenes in Mexican maize: molecular evidence and methodological considerations for GMO detection in landrace populations. *Molecular Ecology,* vol. 18, no. 4, p. 750-761. **[CrossRef]**

QUERCI, M.; JERMINI, M. and EEDE, G.V.D. (2006). The analysis of food samples for the presence of genetically modified organisms. World Health Organization-Joint Research Centre, European Commission Directorate General, Luxembourg.

QUIST, D. and CHAPELA, I.H. (2001). Transgenic DNA introgressed into traditional maize landraces in Oaxaca, Mexico. *Nature*, vol. 414, no. 6863, p. 541-543. **[CrossRef]**

QUIST, D. and CHAPELA, I.H. (2002). Biodiversity (Communications arising (reply)): Suspect evidence of transgenic contamination/Maize transgene results in Mexico are artefacts (see editorial footnote). *Nature*, vol. 416, no. 6881, p. 602. **[CrossRef]**

RAHMAN, T.; CHOWDHURY, E.H.; MONDOL, A.C.; HOQUE, M.M. and NASIRUDDIN, K.M. (2007). Detection of maize intrinsic and recombinant *Cry1Ab* gene fragment in genetically modified maize. *Plant Tissue Culture and Biotechnology*, vol. 17, no. 1, p. 103-108. **[CrossRef]**

RAVEN, P.H. (2005). Transgenes in Mexican maize: desirability or inevitability? *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 37, p. 13003-13004. **[CrossRef]**

REMUND, K.M.; DIXON, D.A.; WRIGHT, D.L and HOLDEN, L.R. (2001). Statistical considerations in seed purity testing for transgenic traits. *Seed Science Research*, vol. 11, p. 101-119.

SAHOO, L.; SCHMIDT, J.J.; PEDERSEN, J.F.; LEE, D.J. and LINDQUIST, J.L. (2010). Growth and fitness components of wild x cultivated *Sorghum bicolor* (Poaceae) hybrids in Nebraska. *American Journal of Botany*, vol. 97, no. 10, p. 1610-1617. **[CrossRef]**

SAMBROOK, J. and RUSSELL, D.W. (2001). Molecular cloning: A laboratory manual, 3rd ed. vol. 1. New York, Cold Spring Harbor Laboratory Press, 2344 p. ISBN 978-087969577-4

SCHOEL, B. and FAGAN, J. (2009). Insufficient evidence for discovery of transgenes in Mexican landraces. *Molecular Ecology*, vol. 18, no. 20, p. 4143-4144. **[CrossRef]**

SCURRAH, M.; CELIS-GAMBOA, C.; CHUMBIAUCA, S.; SALAS, A. and VISSER, R.G.F. (2008). Hybridization between wild and cultivated potato species in the Peruvian Andes and biosafety implications for deployment of GM potatoes. *Euphytica,* vol. 164, no. 3, p. 881-892. **[CrossRef]**

SEVILLA, R. (2005). Magnitud e impacto potencial de la liberación de los organismos genéticamente modificados y sus productos comerciales. Caso: Maíz. In: HIDALGO, O.; ROCA, W. and FERNÁNDEZ-NORTHCOTE, E. eds. *Magnitud e impacto potencial de la liberación de organismos genéticamente modificados y sus productos comerciales: casos algodón, leguminosas de grano, maíz y papa.* Lima, Perú, Consejo Nacional del Ambiente, p. 41-61.

WARWICK, S.I.; BECKIE, H.J. and HALL, L.M. (2009). Gene flow, invasiveness, and ecological impact of genetically modified crops. *Annals of the New York Academy of Sciences*, vol. 1168, p. 72-99. **[CrossRef]**

WOLF, C.; SCHERZINGER, M.; WURZ, A.; PAULI, U.; HÜBNER, P. and LÜTHY, J. (2000). Detection of cauliflower mosaic virus by the polymerase chain reaction: testing of food components for false-positive 35S-promoter screening results. *European Food Research and Technology*, vol. 210, no. 5, p. 367-372. **[CrossRef]**

YANG, L.; GUO, J.; PAN, A.; ZHANG, H.; ZHANG, K.; WANG, Z. and ZHANG, D. (2007). Event-specific quantitative detection of nine genetically modified maizes using one novel standard reference molecule. *Journal of Agricultural and Food Chemistry*, vol. 55, no. 1, p. 15-24. **[CrossRef]**

ZAULET, M.; RUSU, L.; KEVORKIAN, S.; LUCA, C.; MIHACEA, S.; BADEA, E.M. and COSTACHE, M. (2009). Detection and quantification of GMO and sequencing of the DNA amplified products. *Romanian Biotechnological Letters*, vol. 14, no. 5, p. 4733-4746.

How to reference this article:

RIMACHI, L.F.G.; ALCANTARA, J.D.; AQUINO, Y.V. and ORTIZ, R. (2011). Detecting adventitious transgenic events in a maize center of diversity. *Electronic Journal of Biotechnology*, vol. 14, no. 4. http://dx.doi.org/10.2225/vol14-issue4-fulltext-12