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DETECTION OF GM CONTAMINATION IN IRRI-6 VARIETY OF RICE (*ORYZA SATIVA* L.) GROWN IN PAKISTAN

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ABSTRACT

The aim of this study was to detect genetically modified (GM) contamination in random samples of IRRI-6 rice from Pakistan. DNA was extracted by modified cetyltrimethyl ammonium bromide (CTAB) method. The polymerase chain reaction (PCR) assay was conducted with three primers to amplify the 35S promoter of cauliflower mosaic virus (CaMV-35S), nontranslated region of the nopaline synthase (T-NOS) gene and neomycin-phosphotransferase II (NPT-II) gene. Primer of sucrose phosphate synthase SPS gene was used as internal control of rice. SPS primer amplified specific single band while the other three primers failed to produce any band in any of the samples and confirmed that the analyzed commercial samples of IRRI-6 rice variety were free from GM contamination. Further, these primers (except SPS) used for genetically modified (GM) cotton to cross check; all primers showed amplification and produced band of specific size which confirm the presence of GM in cotton.

Key-words: GM contamination, IRRI-6, rice varieties, Pakistan.

INTRODUCTION

Rice (*Oryza sativa*L.) is an important cereal crop of Poaceae family, which feeds almost 2.7 billion people throughout the world (Hussain, 2012). Pakistan has an agriculture- based economy. Rice is also called the "Golden Grain of Pakistan" because the 23% of foreign trade depends on rice (Shah *et al.*, 1999). The increase in rice production could contribute towards malnutrition reduction and poverty alleviation.

Pakistan ranked 5th in the world, among the rice exporting countries list. It has exported around 3.16 million tons of rice in the fiscal year 2013-2014 (July 2013-June 2014) (Pakbiz.com, 2016) According to Pakistan Bureau of Statistics, in 2014 Rice is cultivated on an area of 2789.2 hectares and its production is 6798.1 tons (PBS, 2014). The coarse rice such as IRRI-6 is being cultivated mostly in the Sindh province of Pakistan. The surplus amount of IRRI-6 is being exported to many countries around the globe.

The genetically modified (GM) technology has increased progressively round the globe for socio-economic, environmental and human fitness benefits. During 2013 biotech crops were grown on 175.2 million hectares worldwide, which is a record for the GM cultivated area. The year 2013 was the 18th year of GM technology exploitation and it is supposed to make biotech crops as a more quickly accepted crops technology in recent years. Approximately 60% of the world population lives in the 27 countries and farming biotech or Gm crops (James, 2013).

Transgenic crops developed by genetic modification technology containing beneficial qualities such as resistance to diseases, pests and herbicides, improved flavour and delayed ripening (Hurst *et al.*, 1999). Pakistan has developed Bt (*Bacillus thuringiensis*) transgenic rice and conducted various field trials (Bashir *et al.*, 2004) but has yet to commercialize any GM rice variety. In Pakistan, transgenic cotton is being cultivated since 2007 (Ali *et al.*, 2010). From last two years, transgenic crop evaluation trials were under study, but because of insufficient data on risk assessment/biosafety assessment the commercial cultivation of cotton is not yet allowed.

At present, unauthorized GMOs (Genetically modified organisms) are prohibited into the world market for the reason that they have not been accepted for food safety and may cause a hazard to human health (Song *et al.*, 2011). The risk of contamination through a GM substance is increasing constantly. As reported by GM Contamination Register, from 1997 to 2010, 318 events of GM contamination have appeared in 57 countries relating more or less all components of the food web. In the middle of these, 94 cases, or 30% of the entire, are concerned with GM rice contamination. The presence of GM material in feed and food products is controlled by directive (EC No 1829,

2003), which keep trying to impose labeling standard for GM contamination. (Marmiroli *et al.*, 2008). Several countries have developed standard methods for the analysis of particular GMO content in foodstuffs (García-Cañas *et al.*, 2004).

The awareness for cultivation of genetically modified crops is rising in developing countries contrary to European Union (EU), which are surrounded by opposing attitudes, from the time when GM crops were released initially in 1990's till now (Skaria *et al.*, 2011). However, educated people specially women have more concerns about GM crops and view positively towards organic Food (OF) (Pervaiz *et al*., 2010).

The accessibility of analytical control method is an essential requirement to trace small amount of GM contamination in samples. PCR-based technique is generally accepted as a method of choice due to its high sensitivity, specificity, reliability, applicability and universality (Forte *et al.*, 2005). The detection of GM contamination depends on PCR based strategy, in order to select suitable oligonucleotide primers complete knowledge of DNA sequence that introduced into the host is prerequisite. If there is a slight or no knowledge about genetic modification, then GM detection is done by using frequent tests of cauliflower mosaic virus (CaMV) 35S promoter, nopaline synthase (NOS) terminator and for neomycin phosphotransferase (nptII) or kanamycin-resistance marker gene (Michelini *et al.*, 2008). Presented study is designed with an aim to optimize a reproducible and reliable protocol for detection of GM contamination in IRRI-6 rice variety samples commercially available in the market of Pakistan.

MATERIALS AND METHODS

Sample collection

For present study a total of 300 commercially available IRRI-6 rice samples were collected from different Districts of Sindh and Punjab. The samples were properly labeled in Zipper plastic bags and codes were given to avoid any confusion during results compilation.

DNA Extraction

DNA was isolated using modified cetyltrimethyl ammonium bromide CTAB (Khan *et al.*, 2015). Rice samples 1g of each grounded using clean autoclaved mortar and pestle followed by addition of 4 mL of extraction buffer containing (100 mMTris-Hcl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 0.2% Mercaptoethanol). After that, a mixture of chloroform and isoamyl alcohol (25:1) was added and centrifuged for 10 minutes at 10,000 rpm. Upper surface was separated and equal volume of ice cold isopropanol were added. After centrifugation the obtained DNA pellet washed with 5 mL washing buffer (76% ethanol, 24 mL water, 10 mM Ammonium Acetate) for 20 minutes. Centrifuge washed DNA at 10,000 rpm for 10 min and air-dried the pellet and dissolved in 100 *u*L of PCR grade water.

DNA Quantification

Concentration and quantity of DNA was determined using spectrophotometer (JENWAY, Model Genova, Serial No. 1489) at 260/280 nm wavelength. Furthermore, the quality of isolated DNA was checked by agarose gel electrophoresis using 0.8% agarose gel and stained by ethidium bromide (0.5 *u*g/mL). Add Electrophoresis was done in 1x TBE buffer of pH 8.0 (89 mM Tris Base, Boric acid 8 mM, EDTA 1 mM), at 60 volts and 500 mA, DNA bands visualized under gel documentation system and gel images were captured and saved.

Polymerase Chain Reaction

The sequences of oligonucleotide primers used in this study is mentioned in Table 1. Oligonucleotide primers synthesized by Bio Basic Canada Inc. Primers were diluted to working concentration of 5pmol/*u*L. Amplification reactions were carried out in a 25 *u*L total volume of PCR reaction. DNA amplification was carried out in a Hi-Temp 96 wells thermo cycler (Mastercycler gradient, Eppendorf®, Germany). The pre-denaturation was done for 5 min. At 95^0C followed by 35 cycles of 95^0C for 35 sec, annealing at 55^0C for 30 sec and extension was done at 72^0C for 30 sec. A final extension step for 5 min. at 72^0C was also included and hold at 4^0C till recovery.

Agarose gel electrophoresis

PCR product of each sample was loaded on 1.2% agarose gel in a 1X TBE buffer (10 mM Trise-base, 2.75 g/L boric acid, 1 mM EDTA). Electrophoresis was done for 90 min at 65 volts and 500 mA; visualized in UV light gel documentation system (UV Tech TM, UK) after staining with 10 *u*L (5 *u*g/mL) of ethidium bromide. Ladder of 250 bp was used to measure the size of amplified PCR product.

Table 1. GM Primers Sequences used in this study.

S.No	Primer	Sequence	Gene Specificity	Amplicon (bp)
1	35S (cf3) F 35S (cf3) R	5'-CCA CGT CTT CAA AGC AAG TGG-3' 5'-TCC TCT CCA AAT GAA ATG AAC TTC-3'	Promoter Gene	123
2	Taxon-target(s) F Taxon-target(s) R	5'-TTG CGC CTG AAC GGA TAT-3' 5'-GGA GAA GCA CTG GAC GAG G -3'	Sucrose Phosphate Synthase	277
3	NPT II-F NPT II-R	5'-GGA TCT CCT GTC ATC T-3' 5'- GAT CAT CCT GAT CGA C-3'	Neomycine Phosphate Tranferase	173
4	T-NOS-F TNOS-R	5'-GAA TCC TGT TGC CGG TCT TG-3' 5'-TTA TCC TAG TTT GCG CGC TA-3'	Terminator	180

RESULTS AND DISCUSSION

The genetically modified (GM) screening methods are of great importance for routine analyses. However, in order to detect genetically modified organisms (GMOs) in food and feed products, most of the time scientists used two GMO screening methods which are commonly based on the detection of T-NOS 3'nontranslated region of the nopaline synthase gene and the 35S promoter of the cauliflower mosaic virus (CaMV-35s) (Reiting *et al*,. 2007). The 35S promoter is used as a universal molecular marker for the testing of 95% of commercially available GMO plants in Europe (EU). CaMV-35 is a functional, well-defined and constituently expressed promoter. It has been integrated into several constructs and used in lot of genetically modified crops which are growing commercially, such as maize, soy, canola, papaya (Holden *et al*., 2010) and rice (Zdjelar *et al*., 2013). Similarly, the polyadenylation site of the T-NOS sequence from *Agrobacterium tumefactions* acts as a polyadenylation site in some of the similar constructs (Holden *et al*., 2010).

The NPT-II gene code for an enzyme Neomycine phosphotransferase (NPT-II), commonly used as selectable marker for transformation in bacterial and eukaryotic cells. NPT-II is a bacterial enzyme, which resist to some aminoglycoside antibiotics by phosphorylation of the 3'-hydroxyl group of the aminoglycoside (Wood *et al*,. 1995). However, in order to estimate the quality of DNA and PCR efficiency, rice-specific internal control sucrose phosphate synthase (SPS) has been used as a reference gene at the international level, which also helps to eliminate the chances of possible contamination or improper/inappropriate DNA quality and handling. It also reduces the risk of false negative results and increases the reliability.

In order to detect genetically modified (GM) rice using PCR technique, the first step to be carried out is DNA isolation. Although variety of DNA extraction protocols have been established. Only for the reason of high quality and high yield of DNA, we used CTAB based method, which provided good quality DNA from rice grains. Dried seed embryos of IRRI-6 rice were used for DNA extraction. CTAB as a main detergent used. DNA degradation must be prevented and contamination must be avoided to get purified DNA.

The specific primers for the marker gene Neomycine-3'-Phosphotransferase (NPT-II), NOS-terminator (NOS-T), Cauliflower mosaic virus(CaMV-35S) promoter and Sucrose Phosphate Synthase (SPS) shown in (Table 1) were used in this study. The ladder of 250 bp were used to check the size of resulting amplified PCR product of the SPS gene (277 bp), NPT-II (173 bp), CaMV-35S (123 bp) and T-NOS (180 bp). Negative PCR control was also used in which milli-Q water was added instead of template DNA. As no DNA band was observed in negative control, the present results confirmed that the studied rice samples were free from GM contamination. Only SPS primer showed amplification in all PCR reactions (Fig. 1). Further, in order to check the specification of GM primers the GM cotton variety namely Trend-1 was subjected to PCR and cross-checked the PCR amplicon. The PCR amplification products of NPT-II (173 bp), NOS-T (180 bp) and CamV-35S-promoter (123 bp) were successfully amplified and (Fig. 2) and PCR product gave band of specific size.

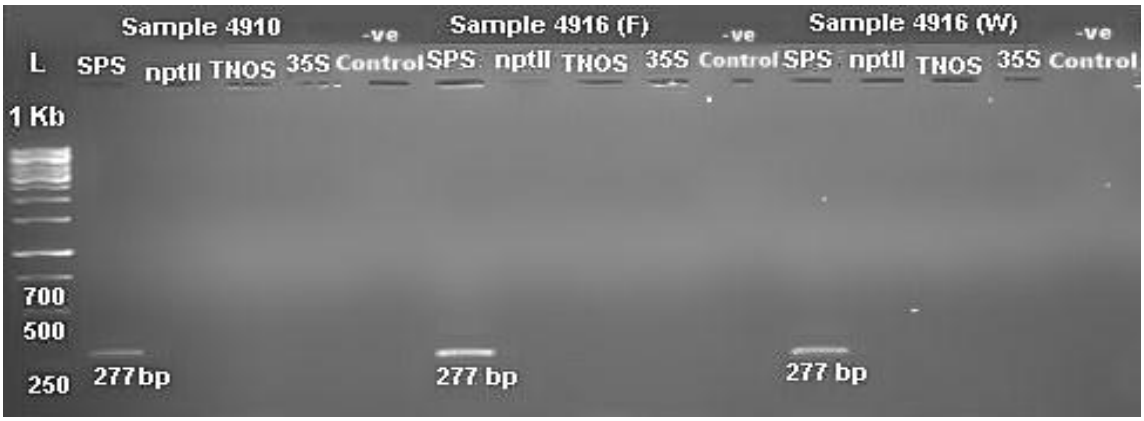

Fig. 1. Lane 1 SPS primer, Lane 2 NPT-II primer, Lane 3 T-NOS, Lane 4 35S and Negative control.

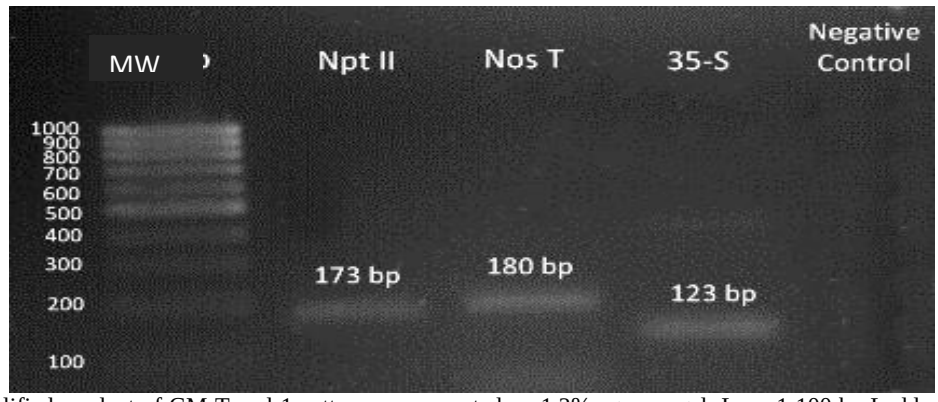

Fig. 2. The amplified product of GM Trend-1 cotton was separated on 1.2% agarose gel. Lane 1 100 bp Ladder, Lane 2 NPT-II (173 bp), Lane 3 NOS-T (180 bp), Lane 4 CamV-35S-promoter (123 bp) and in Lane 5 negative control.

Conclusion

This is so far the first reported attempt on detection of GMO in IRRI-6 commercial rice variety in Pakistan. All rice samples showed no amplification with GM specific primers except the SPS primer. There was no GMO/transgenic contamination detected from any of the randomly collected IRRI-6 rice samples with respect to above mentioned three commonly used genes. Our results confirm that no GM IRRI-6 rice variety is grown in the country.

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