Detection of genetically modified rice: Collaborative validation study of a PCR based detection of genetically modified rice *Oryza sativa* commercially available in Saudi Arabia

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Abstract

A collaborative trial study has been conducted for validation of an extraction method and a subsequent PCR for the detection of transgenic rice sold in Saudi Arabia. The tests were carried out in Saudi Arabia using Real-Time PCR and the positive samples were validated in another lab in Malaysia using PCR and agarose gel visualization. The samples were tested for the existence of the NOS Terminator. A total of 150 samples were tested out of which three samples tested positive as GM-rice which were retested in Malaysia. The presence of GMO rice in Saudi Arabia supports the necessity of developing precise quantitative and qualitative ways for routine analyses and detection of GMO products in the Saudi Arabian market. With the discovery of GM products in the Saudi Arabian market it would be of no surprise that other Middle Eastern nations also knowingly or unknowingly import GM crops.

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Introduction

Oryza sativa L. is a major food crop consumed by half of the world's population. 40% more rice will need to be produced by the year 2030 in order to satisfy growing demand, according to various estimates (Khush 2005). Particularly in Asia, rice is the most important crop grown, both with regard to consumption and in a cultural context. During the growth period, disease, insect pest and abiotic stress (drought, heat, cold, salt etc.) are main factors causing the yield reduction. To solve the above-mentioned problems, modern biotechnology is applied in rice breeding which introduces improved agronomic characteristics. Transgenic rice lines manifesting agronomically useful traits, elevated quantities of beta-carotene (provitamin A) (Ye et al. 2000), disease and insect resistance (Datta et al. 1998; Irie et al. 1996; Lin et al. 1995), virus resistance (Hayakawa et al. 1992), and herbicide resistance (Datta et al. 1992) are just a few examples of products that have been successfully developed via transgenic

techniques. Although some of these transgenic rice breeding lines or varieties have been released into the World for assessments of environmental risk (Huang et al. 2002; Jia & Peng 2002; Messeguer et al. 2001), no transgenic rice varieties have thus far been periodically released into the environment for large-scale commercial production in Asia which is the largest consumer of rice. This is, in part, due to the fact that rice, especially rice grains with the outer layers removed, are used principally for direct human consumption.

In this research, a molecular based method of screening from the basic DNA extraction to the PCR stage is used for detecting GMO in rice grains which are found in Saudi markets, supermarkets and grocery shops.

A commonly recommended or required approach is to screen for any biotech crop or material derived thereof by the detection of genetic elements that are shared between virtually all commercialized agricultural GMOs. The two best- known examples are the 35S promoter from Cauliflower Mosaic Virus (CaMV) and the NOS terminator from Agrobacterium tumefaciens. These are widely used, well-characterized elements, which direct the expression of the biotechnology genes they reside in. These tests will work on most matrices: corn, feed pellets, soybeans, corn starch, maltodextrin, seasonings, vitamins, beverages and all finished food.

Materials and Methods

The independent tests were carried out in Saudi Arabia using the *SureFood PREP Plant kit* (Art no. S1002) and the *Quick Protocol of Wizard® Magnetic DNA Purification System for Food* in Malaysia. The SureFood GMO35S/NOS Screening Real Time PCR was used for screening of GMO in the Saudi Arabian lab.

In the Malaysian lab, the PCR protocol was designed in-house with the NOS Primer pairs ordered from Eurogentic EIt,Singapore. Standard TAE Buffers and Agorose from Promega were used for the gel. Go TAq Flexi DNA Polymerase Kit from Promega was used in the PCR master mix. Eppendorf Mastercycler was used for PCR.

DNA extraction

Standard protocol from the R-biopharm surefood PREP Plant Kit (ART S1002) was used for the DNA extraction in the Saudi Arabian lab while the Quick Protocol of ®Magnetic DNA Purification System for Food (Promega) was used according to manufacturer instruction (Bitner et al., www.promega.com) in the Malaysian lab.

R-biopharm sure food PREP plant kit

100 mg of crushed rice samples were placed in 1.5 ml tubes. 400 µl of Lysis Buffer and 20 µl of Proteinase K were added and vortexed. 40 µl of RNase was also added to the homogenous sample during lysis. The samples were incubated at 65°C for 30 min in a thermomixer. The sample lysate was then centrifuged at 12.000 rpm for 2 min and the liquid supernatant transferred into the spin filter prepared earlier in a receiver (2 ml). The receiver tube with the spin filter was centrifuged at 12.000 rpm for 2 min and the spin filter discarded. 200 µl of binding buffer was added to the filtrate and vortexed thoroughly. A new spin filter into a new receiver tube and the filtrate transferred. The tubes were then centrifuged at 12.000 rpm for 2 min and the filtrate discarded. 550 µl of pre-wash buffer was added into the spin filter and centrifuged at 12.000 rpm for 1 min. The filtrate was discarded and the spin filter placed back into the receiver tube. The process was repeated with wash buffer twice. The spin filter was then centrifuged for 2 min at 12.000 rpm to remove the wash buffers completely. The spin filters were then placed into a new receiver tube and 100 µl of pre-heated (65°C) Elution Buffer was added and centrifuged for 2 min at 10.000 rpm. The eluted DNA was used for PCR.

Quick protocol of magnetic DNA purification system for food

200 mg of crushed rice samples were placed in a 2 ml tubes. The tubes were tilted

on its side so the dry sample is on the sides of the tube. 500 µl of Lysis Buffer A and 5 µl of RNase A were added. The tubes were capped and vortexed vigorously. 250 µl of Lysis Buffer B were added and vortexed for 10-15 seconds. The tubes were laid on its side and incubated for 10 minutes at room temperature (22-25 °C). 750 µl of Precipitation Solution was added and vortexed vigorously. The tubes were centrifuged for 10 minutes in a microcentrifuge at maximum speed (13.000 × g). The supernatants were transferred (liquid phase) to a new 2 ml tube 50 µl of MagneSil™ PMPs was pipetted into the supernatant and vortexed. 0.8 volume of isopropanol was added. The tube was inverted 10-15 times and incubated for 5 minutes at room temperature with occasional mixing. The tubes were placed onto the MagneSphere® Magnetic Separation Stan for 1 minute. Liquid phase was discarded. The tube was removed from the stand and 250 µl of Lysis Buffer B was added. The tubes were inverted 2-3 times and placed back in the stand. The MagneSil™PMPs were allowed to separate for 1 minute and the liquid phase was removed. 1ml of wash solution was added and the tubes were placed on the stand for 1 minute. The liquid waste was discarded and repeated twice for a total wash of 3 washes. Any liquid left was pipetted out of the tubes. The particles were dried (15-30 minutes at room temperature or 10 minutes at 65 °C). 100 µl of Nuclease-Free Water was added, vortexed and incubated at 65 °C for 5 minutes. The tubes were placed onto the magnetic stand and the liquid transferred into a clean tube for PCR.

Real-Time Polymerase Chain Reaction

The screening method used here for the Real-Time PCR system conform to the official German method L-00.00-31 for the detection of genetically modified DNA sequences as described in the national German Food Law under act 35. In this test, the SureFood GMO real-time PCR kit was used. Two reactions each (for 35S and NOS) with the 'positive control DNA' and one reaction as a no-template control were prepared apart from the DNA from the rice samples. For each reaction, 17 µl of the 35S/NOS reaction mix with 1.0 µl FDE and 0.1 µl Taq Polymerase was added. The required number of reaction tubes for the 35S/NOS tests were arranged and 18 µl of the 35S/NOS were pipetted respectively. The PCR protocol was programmed as follows: Initial denaturation of 1 min at 95 °C and subsequent denaturation for 5 s at 95 °C, annealing for 10 s at 60 °C, elongation for 15s at 65 °C with a total repetition of 45 cycles and finally cooling for 10 s at 40 °C.

Polymerase chain reaction (detection of NOS terminator)

Primers for the detection of the Nos Terminator was synthesized using Primer 3 Software (http://frodo.wi.mit.edu/primer3/) from the NPTII gene sequence, which are NOS Ter forward (5'-GAATCCTGTTGCCGGTC TTG - 3') and reverse (5'-TTATCCTAGTTT GCGCGCTA-3') (Spoth and Strauss, www. promega.com). PCR was set up using 1 µl from 100 µl elution from rice seeds sample. The PCR master mix was obtained from the Go TAq Flexi DNA Polymerase Kit from Promega.. For sample consistency, a master mix was assembled containing all reagents for the amplification reaction with the exception of the template DNA. The master mix was dispensed into 0.5 ml reaction tubes on ice. The concentration of the PCR reaction components are as follows: DNA template (5–10 ng/µl), Forward Primer, (50 pmol/µl), Reverse Primer 2 (50 pmol/µl), 10X Reaction Buffer, MgCl2 (25 mM Solution), PCR Nucleotide Mix (10 mM), *Taq* DNA Polymerase (b) (5 u/µl); Nuclease-Free Water . The optimum condition was 94 °C for 2 min for initial denaturation, then 29 cycles were performed using incubations of 94 °C for 30 sec, 60 °C for 45 sec and 72 °C for 120 sec followed by a final extension step of 72 °C for 5 min. The positive control was taken from genetically modified tobacco (in house

transformed using pHellsgate 8 vector, CSIRO, Australia). The PCR product was analyzed on 1.7% gel at 80V for one and half hour and observed under UV light (fig. 1).

Results

For the real-time PCR analysis performed in the Saudi Arabia as shown in fig. 1. Rice samples 147, 151 and 152, highlighted in blue and corresponding to their respective colors show clear signals for the amplification of the NOS which indicate the presence of GMO material. Pos 11 and 12 are the positive controls and negative controls respectively as shown in the profile also corresponding to their respective colors. The positive controls showed a clear positive result (Ct≤32), the negative controls showed a PCR negative of (Ct=45/no.Amp).

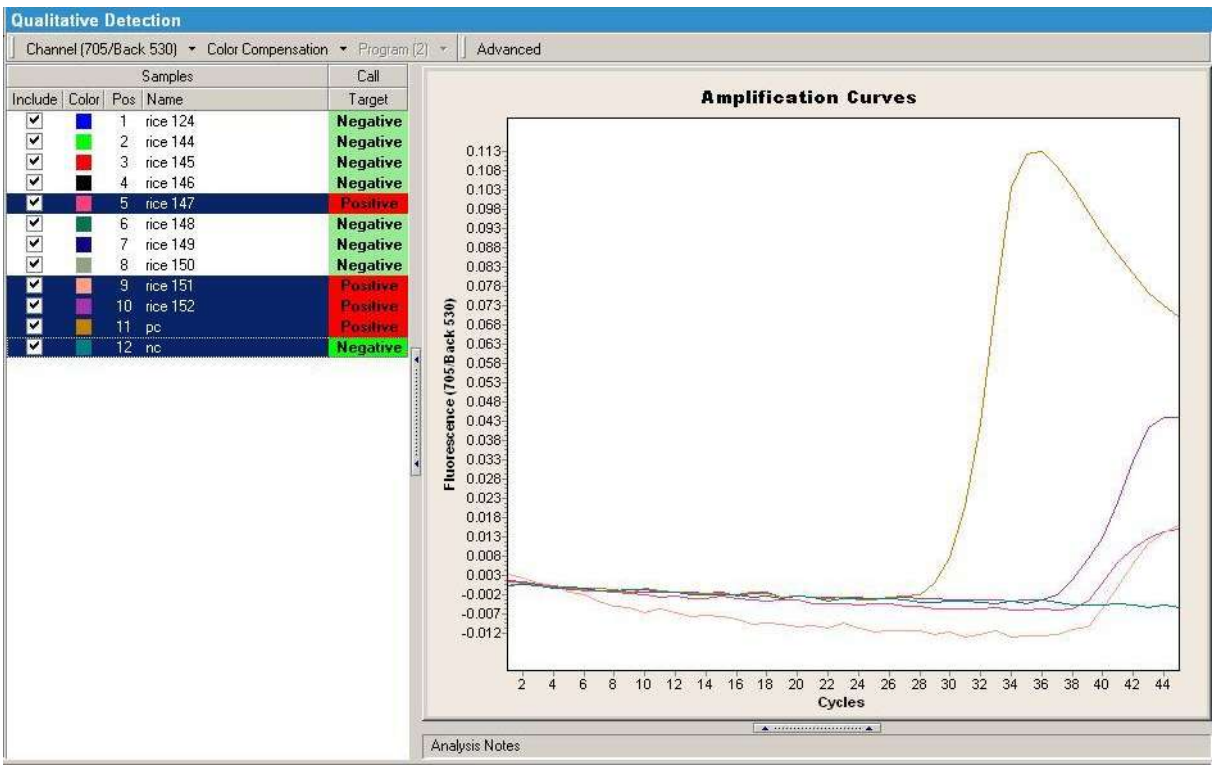

Figure 1: Profile of DNA Amplification for the presence of NOS in GMO Rice.

The positive samples were retested in the Malaysian lab for the presence of NOS using standard PCR. A 10 µl sample from each amplification reaction was mixed with 2 µl of Blue/Orange Loading Dye, 6X (promega) and was subjected to electrophoresis on a 1.7 % agarose 1X TAE gel. The 100 bp DNA Ladder (promega) was run as a size marker (lane 1). The results of the PCR is illustrated in fig. 2 where the three samples R1 (lane 5), R2 (lane 6) and R3 (lane 7) corresponding to Rice samples 147, 151 and 152 respectively showed an amplification of 180bp in line with the positive control for the presence of the NOS. Negative controls in lane 2 and 3 did not show any product while lane 4 was left empty.

Therefore the two sets of results from Fig. 1 which identified the GMO rice samples through real-time PCR and the positive samples reconfirmed by standard PCR and agarose gel electrophoresis visualization in fig. 2.

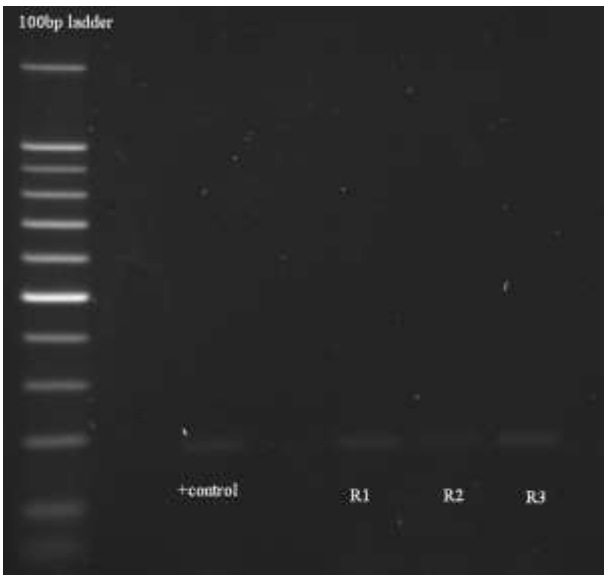

Figure 2: Results of amplification using the NOS terminator sequence primers

Discussion

Almost all commercially developed transgenic crop plants contain either the CaMV 35S promoter or the NOS terminator. The ability to detect these elements will allow detection of the vast majority of GMO plant material. A survey was conducted in Turkey to monitor the genetic modified soybean products (flour, raw bean, tofu, soy milk, soy sauce). The results showed the presence of GMO in almost all soybean products (Aril and Cakir, 2008). It should be noted that the CaMV 35S promoter or the NOS terminator may be present naturally in CaMV or *A. tumefaciens*-infected plants, respectively. A positive result therefore does not conclusively indicate the presence of transgenic material. The DG JRC Environment Institute Consumer Protection & Food Unit in collaboration with 28 different laboratories has developed and validated two protocols for DNA extraction methods and a PCR-based amplification method to identify genetically modified maize and soy products based on these sequences which is used in this paper.

To make sure that no contamination from other sources of grains, the samples were thoroughly washed with nuclease-free water and only whole grains of rice were chosen as samples. In addition the primers and protocol were from the DG JRC Environment Institute Consumer Protection & Food Unit which has designed primers specifically for GMO and not naturally present. In addition the screening methods used here for the Real-Time PCR system conform to the official German method L-00.00-31 for the detection of genetically modified DNA sequences as described in the national German Food Law under act 35.

The detection for bioengineered crops, also referred to as Genetically Modified Organisms (GMOs), has become a necessity in light of legislation imposed in member countries of the European Union, Japan, South Korea, Australia, New Zealand, and in an increasing number of other countries. labelling laws for approved bioengineered crops are established, while prohibiting the import of unapproved varieties. For approved varieties, commonly tolerance thresholds are established below which a product does not have to be labeled as containing or derived from GMOs. Two different categories of analytical methods are used to detect GMOs. One is Polymerase Chain Reaction (PCR) which is used here. It is based on the detection of genetic material (DNA). It is most versatile for detection of biotech crops and therefore the method of choice for many applications. The alternative is Enzyme-Linked Immunosorbent Assay (ELISA). It detects foreign protein(s) which most bioengineered crops contain as a result of the insertion of bioengineered gene(s). As compared to PCR, ELISA is more restricted in its applicability but can be very useful in certain raw commodities.

In today's agro food business, food and feed suppliers have to comply with regulations on traceability and labeling of their products on parameters such as GMO content, allergens, country of origin, residues, etc. In addition, end-users are demanding transparency throughout the entire supply chain.

Although analytical testing is an important risk management tool, it is not sufficient on its own to protect the value and integrity of raw materials or products. Further control, such as

traceability and segregation along the supply chain, is necessary in order to fulfill legal requirements and to reinforce consumer's confidence. Thus from the above experiments three samples of GMO rice using the PCR methodology was detected by specifically testing for the NOS terminator insert which is generally found in all GMO products.

It is very important to conclude that a national awareness is created for the presence and implications of GMO in the Saudi Arabian market like in many countries and also the establishment of a common tolerance threshold to determine whether a product has to be labelled as containing or derived from GMO. This establishment of a threshold could be a benchmark for all Middle Eastern nations. From previous research not only has GMO rice been detected but also the detection of GMO soy and porcine in many foods available in the Saudi Arabian market.

Thus with the significance of this research it is important that a National Conference and Workshop be conducted in Saudi Arabia address the issues such as food safety, security and the relevant frameworks and policies that need to be established not only for Saudi Arabia but also for the whole Middle Eastern nations as well as all the Arab World. This initiative would not only provide the much needed awareness for the Arab world but also create more jobs especially in food/crop biotechnology sectors.

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