Research Article

Molecular Identification of Transgenic Tomato in Iran by P35S Promoter-Based PCR

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ABSTRACT

Background: With the increasing population of the world, we should face food shortages in the near future. In this regard, agriculture of transgenic crops becomes very important. However, ensuring of GM products labeling is of great importance. Tomato is one of the most important food crops and for this reason it has undergone many genetic changes. This study aims to introduce a rapid, sensitive, and accurate method to identify non-labeled transgenic tomatoes in Iran market.

Results: In this study, after optimization of PCR test based on P35S promoter, the amplicon was cloned in the plasmid PTZ57R in Escherichia coli JM107 to confirm and make positive control. After optimized PCR test of 50 tomatoes it was found that 4% of the Iranian markets’ tomatoes contain P35S promoter and are probably transgenic while none of the samples have been labeled in this regard.

Conclusion: PCR technique is a suitable, available, fast and accurate method for screening transgenic products such as tomatoes.

Keywords: Polymerase Chain Reaction, GMO, Tomatoes, P35S Promoter

INTRODUCTION:

An explosive increase of the human population of the Earth and the disappearance of arable land, have raised many concerns about the world's ability to produce enough food for a growing population. So that it is predicted that in the not too distant future we will face a challenging food crisis. On the other hand, environmentalists are steadily concerned about the overuse of pesticides and herbicides and their long-term detrimental effects on the environment. However, the science of biotechnology has come to help us to find a response to problems of this type. In this regard, the cultivation of transgenic crops is becoming more important and more arable land area is dominated by GMO, so that since confirmation and entry of the first transgenic plant (Flavr savr tomato) in 1996, the recombinant DNA technology has been widely used in the modern agriculture. 184 transgenic species have been approved in 59 countries for food products and the areas of cultivation on a commercial scale reached to 170 million hectares by 2012 as the 17th year of commercialization of biotech crops. Most transgenic crops are cultivated in the United States of America (69.50%), Brazil (36.60%), Argentina (23.90%), Canada (11.60%) and India (10.80%). Genetic changes compose a variety of properties including resistance to herbicides and insects damage. With globalization of the business of genetically modified ingredients, many countries
Increasingly tend to be aware of their presence in the food, despite the low wide acceptance of biotechnology in the field, their acceptance by consumers is still uncertain. Also, low acceptance of GMOs in Europe and other countries led to the introduction of strict rules for the entry of these products into the consumer markets and their labeling. In addition, with regard to the controversial issue of food safety, environmental risks and ethical concerns, more countries and regions have deemed it necessary to label the transgenic crops. In the late 90s some countries asked the implementation of mandatory labeling systems and considered the labeling as an important tool and a bridge of confidence between producer and consumer. As a result, to meet requirements of the new rules of labeling and tracking, reliable and sensitive methods are required for detection of transgenic organisms in foods and crops. In addition, with increasing international food and crops business, international integration of GMO detection methods seems necessary. Therefore, development of reliable and standardized methods in order to identify, track, detect and quantify the GMOs is the key step in their development and commercialization. However, differences in laws from one country to another put obstacles in the way of achieving this goal. An important agreement on international trade of live transgenic organisms was obtained by the Cartagena Protocol on Biosafety and it has been tried a common system of unique indicators to be developed. According to the International Cartagena Protocol, countries exporting transgenic goods must record and declare related genetic events (genetic traits developed in the manipulated products). As a result, evaluation of GM crops is performed randomly and based on the declared traits in order to control the amount of transgenes.

GM crops and their products can be detected by both identification of the introduced genes at the DNA level and the resulting protein or the created phenotype. For this reason, many analytical methods have been developed including methods based on polymerase chain reaction (PCR) to detect the DNA inserted, immunological assays to detect the resulting protein or biological test to detect the phenotype created. ELISA is one of the main methods of immunochemical analysis and disadvantages such as low antibody affinity to the antigen, the low quantity of transgenic protein and change of the protein structure during food processing and production reduce the efficiency of this method. Currently, PCR method is the most widely used technique to detect and measure the amount of GM ingredients in food which is based on the amplification of the desired DNA by the enzyme DNA polymerase to millions times of its original value.

Tomato is the most important food crops so that after potato, tomato shows the world's largest vegetable production and it is one of the most important antioxidants in the food chain that has undergone many genetic changes. Therefore, in this study we try to introduce a rapid, sensitive, accurate, and economical method to identify non-labeled transgenic tomatoes in the market of Iran. It is worth mentioning the present study is the first study in Iran to identify transgenic tomatoes in Iran market by PCR and using transgenic indicator P35S.

**Experimental State:**

**Preparation of the Samples:**

Totally 50 samples were collected from the fruit market in Tehran, Alborz, Isfahan, Khorasan Razavi, Fars, Eastern Azerbaijan, Ilam, Zanjan, Khuzestan, Hamedan, Mazandaran, Kurdistan, Kerman and Hormozgan Provinces. Samples collected, labeled with the number and location of collection, were kept in the freezer. The interior flesh of tomatoes were mixed by a mortar and 300 micro liters of the tomato juice and its interior flesh were poured into 1.5 ml tubes and then numbered and stored to be ready for DNA extraction.

**DNA Extraction:**

To obtain sufficient quantities of template DNA, in the first step, DNA was extracted from the collected samples by the method Cetyl Trimethyl Ammonium Bromide (CTAB) in accordance with EN ISO 21571: 2005; ISIRI 10763 1st edition as follows:
750 ml extraction buffer CTAB was heated in the block heater in a temperature 65 °C for 5 minutes to be warm and uniform under vortex and then it was added to the 300 µl microtube containing the sample and then 40 micro-liters of protein kinase K was added and put in 65 °C for 60 minutes and the samples were under vortex every 10 minutes.

Then the mixture was centrifuged for 10 min at 12000 rpm, the supernatant was transferred to a new tube and 500 µl of chloroform was added and it was flipped for 10 times. Then it was centrifuged for 15 min at 12000 rpm. The supernatant was transferred to a new tube and again 500 µl of chloroform was added to it. Then it was centrifuged for 5 min at 12000 rpm and the supernatant was transferred to a new tube. Then two volumes of the sediment buffer CTAB was added to the mixture and then flipped by pipettage technique, and left for an hour at room temperature. Next, the mixture was centrifuged at 12000 rpm for 15 minutes, the supernatant was discharged and the sediment left was solved in 350 ml 1/2 M NaCl. Then 350 ml chloroform was added to it and flipped several times and then centrifuged for 10 min at 12000 rpm. Then the aqueous phase was separated and transferred to a new tube and the same volume of cold isopropanol was added. Then the tube was flipped several times and left in freezer for 20 minutes. Next, it was centrifuged at 12000 rpm for 15 minutes and the supernatant was discharged. Then 1000 micro-liters of cold 70% ethanol were added to the tube that completely removes CTAB. The mixture was centrifuged for 10 minutes at 12000 rpm, the supernatant was discarded and the sediment was dried at 65 °C for 5 to 10 minutes to completely remove ethanol. Then 50 µl water or buffer TE was added to the tube.

**Selection of Primer:**

Primer is a transformation index based on 35 Cauliflower virus promoter (ISO24276: 2006, ISIRI9613). This genetic element exists in almost all transgenic plants. The primer sequence is transformation parameter based on the trigger 35S of cauliflower mosaic virus and has a 195 bp product for the P35S index. The sequence of primers used in this research is as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ →3′)</th>
<th>Size of the amplified piece (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35S1</td>
<td>GCT-CCT-ACA-AAT-GCC-ATC-A</td>
<td>195 bp</td>
</tr>
<tr>
<td>35S2</td>
<td>GAT-AGT-GGG-ATT-GTG-CGT-CA</td>
<td></td>
</tr>
</tbody>
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**Polymerase Chain Reaction: (PCR)**

The method used in this research to screen transgenic tomato is the fast, accurate and widely used polymerase chain reaction. The reaction happened in the volume of 25 microliters containing 2.5 µl of buffer 10XPCR, 0.5 µl of dNTP mixture, 0.4 µl of the enzyme Taq DNA polymerase and 0.75 µl of MgCl2, 1 µl of primers, 5 µl of template DNA, and 14 µl of double deionized distilled water.

Polymerase chain reaction was run under thermal cycles of 95 °C for 10 min “First Denaturation”, 94 °C for 20 seconds “Denaturation”, 50 to 60 °C for 40 seconds “Annealing”, 72 °C for 60 seconds “Extension” and then the fragments for the ultimate reproduction were put at 72 °C for 3 minutes (the cycle was repeated for 40 times).

**Agarose Gel Electrophoresis:**

To see the 195-bp PCR product (in the length of P35S) 2% agarose gel and marker “50 bp DNA Ladder” (THERMOSCIENTIFIC) or marker “Low range DNA Ladder SM1103”, made by Fermentas Co., and Sybr safe (Sinaclon) dye were used.

**Cloning:**

Next, cloning of the 195 bp fragment of CaMV35S promoter was performed after purification of PCR product using the T / A Cloning kit, Thermo Scientific™ (Cat: k1214) by vector pTZ57R of this kit in E. coli JM107. In order to verify the cloning process, bacteria was cultured on Agar + ampicillin media containing X-GAL and IPTG LB and after 24 hours of incubation, white and blue colonies grown to verify the cloning were examined.

**RESULTS:**

**Extraction and quality control of the samples DNA:**

Extraction of the genomic DNA was performed according to CTAB standard procedure. Then
quantity and quality of DNA extracted by spectrophotometry were determined at wavelengths of 260 and 280 nm. In the low spectrum of the extracted DNA by increasing the sample mass to 200 mg, DNA was extracted again. Samples with high purity were used as a positive control in the later stages.

**Limit of detection (LOD):**
In order to express the results and quality assurance, limit of detection (LOD) should be stated in good conditions that the value can be expressed as a certain amount of genomic DNA solution. Absolute LOD has not been determined, but fluorometry showed that this method can reproduce a piece of DNA of at least 0.1 ng of DNA extracted from fresh tomatoes (ISIRI 9617).

**Optimization of the PCR test:**
Optimization of PCR tests was done to avoid false negative and positive results of contamination during DNA extraction from the samples. Standardization of thermal profile and mixture of PCR reaction was done with primers P35S1, P35S2. To ensure no contamination, 5µL of double-ionized and sterile water was used in negative control instead of template DNA. The result of optimized PCR test of P35S gene on 2% agarose gel is shown in Figure 1:

**Screening of Samples:** All samples were analyzed for P35S GM index. Of all the 50 samples studied, 2 samples (2%) were positive (Figure 2).

**DISCUSSION:**
This study aimed to assess the presence or absence of transgenic tomatoes in the consumer market and Iranian citizens’ baskets. According to the results of this study, 4% of the tomatoes in the markets of Iran are transgenic but none of them are labeled. However, this is contrary to policies intended to rights of consumers stipulated in the Cartagena Protocol.

Qualitative nucleic acid based methods to detect GM products include screening methods, methods for structure, methods for target species, and procedures for special transgenic event. In the screening method, for detection of DNA of genetically-engineered plant, the DNA sequence can be detected with a variable number of copies derived from the CaMV35S, nos, nptII.

Also the method for target species which is a common procedure for tracking single-copy DNA sequence in tomatoes may be used for tracking genetically transformed tomatoes that their fruits' ripening time is longer.
Recently, in order to produce transgenic tomatoes, external genes, such as the CaMV35S, NOS, Bar, Xa21 and Cry1Ac have been widely used. In 2014 in India, using PCR method based on the gene Cry1Ab and neomycin phosphotransferase (nptII), Mr. Koul and his colleagues identified transgenic tomatoes in India, and also they could identify the gene Cry1Ab with the help of RT-PCR method for RNA replication. Cry1Ab gene is used to increase resistance to pests and the combination of these two genes was confirmed by means of Southern blotting. Almost all GM crop, commercially developed, contain the trigger CaMV35S or terminator NOS. The ability to identify these components makes it possible to identify a major part of transgenic plants, this gene or gene segments ensure and regulate proper function of the transferred gene(s). In other words, their presence is essential for transgenic plants.

There are also several methods for DNA extraction in plants including DNeasy® protocol of Qiagene Company of Germany that is used for genomic DNA extraction in plants, as well as dual extraction device “Easy Prep” and 1-PureTM solutions kit, that are used for extraction and purification of genomic DNA and both are made by Bio-Rad Company. However, CTAB method is a reliable and versatile protocol that is suitable for many different matrices. DNA extraction of samples in this study has been done by the CTAB method which is more affordable and accessible compared to methods such as use of commercial kits. Using this method, you can find sufficient quantity of pure genomic DNA while the use of the PCR reaction restrictive fragments is minimized in this method.

In different countries, different methods have been used to identify transgenic tomatoes. In 2005, Yang et al benefited from PCR and Real Time PCR for detection of genetically engineered tomatoes. In their study the internal genes LAT52 was used in PCR to detect transgenic tomato. They assessed 16 different types of tomatoes with both qualitative and quantitative PCR methods. Studying other products such as rice, soybean and ..., they could show that gene LAT52 is internal gene specific to tomatoes. The results showed that LAT52 is a good reference for internal gene of tomatoes and it is used even in the processed tomato products like ketchup. In this study, 100% of 16 samples of tomato were declared transgenic. In another study conducted by Premanandh et al in the United Arabic Emirates to check the spread of GM crops in this country in 2011, Light Cycler Real Time PCR and GMO Screening Kit were used. Also in this study, CTAB extraction method was used for DNA extraction. However, there is no legal restriction on labeling of GM products in the country, this study aimed to find the frequency of transgenic crops. The results showed that 16 out of 128 examined samples were positive.

Feng et al in 2013 used several ways to identify transgenic tomatoes. Following the law of labeling of transgenic products, they conducted a study to examine labeling of transgenic tomatoes. They used CaMV35S as a promoter, “nos” as a terminator, as well as the gene Inpt, and internal genes LAT52, mcpi, fru and apx. In addition to the PCR method, they also used “muParaflo microfluid microarray”. To this end, they designed 957 probes and finally they could examine 4 different types of tomatoes the results of which showed that micro array is the most effective method for screening.

Analytical method used in this study is the sensitive and accurate method of PCR that has proven to be a reliable method for screening of GM products among DNA-based diagnostic methods. In 2005, Leena Tripathi examined different methods to detect GMOs, including methods based on DNA and methods based on proteins and phenotypic characteristics, and stated that one of advantages of PCR to other methods is its sensitivity and accuracy in detection limit and also their qualitative and quantitative identification.

According to the documentary and research on the health and benefits of transgenic crops, the lack of their production in the country seems to be very important. To date, millions of hectares of land were planted with GM crops and any health problem caused by the consumption of transgenic crops or their products have not been
It can be predicted that concerns about GMO detection in the near future will become more complex. There are more transgenic species besides a few numbers that have already been approved by European Union, or are on the verge of approval, or already supplied in the consumer markets that are major trading partners of the European Union. As a result, to meet European regulatory requirements, the use and development of powerful diagnostic screening methods in the not too distant future will be necessary. Iran is not an exception, and since it has joined the Cartagena Protocol for a few years, it is necessary to be equipped with a GMO detection technology and to this purpose, broader studies and research should be carried out.

Acknowledgements:
We gratefully acknowledge the Iranian Gene Fanavar Institute (IGF) for their research assistance and cooperation.

REFERENCES: