Molecular detection of genetically modified organisms in seeds from local Iraqi markets

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Summery

91 samples were collected for both soybeans and soybean meal as well as rice cereals. Obtained during the current study period that started from December 2019 until March 2020, and samples were transferred to the Food Laboratory in the College of Veterinary Medicine University of Karbala. DNA was isolated from dry vegetable isolates (soybean and its by-products and rice cereals) for the varieties approved in the study using prepared protocol steps. When measuring the optical density (OD) of the DNA of all isolates, most purity values were found between (1.7 - 2.0). During the current study period, two molecular methods were used to investigate GM crops. The first method was the investigation using the PCR polymerase chain reaction in this method. Two primers were used, and they are commonly used in genetic modification. They are the CaMV-35S promoter and the terminator Nos terminator in the present study. The second method is detection using the douplex PCR chain. The current results showed that three genotypes out of seventeen genotypes belonging to the soybean meal were genetically modified containing the catalyst and terminator for the same sample of healthy Feed No. 1 soybean samples, Healthy Feed No. 2, and Healthy Feed soybean meal. No.3 using both the mono PCR chain reaction and the douplex PCR chain reaction. While all samples tested for rice grains using the same primers for the current study showed that they are free from genetic modification using the Conventional polymerase chain reaction (PCR) technique.

Keywords, Feed, GM products, local markets, GMOs , PCR.

Introduction

Biotechnology crops were marketed for the first time in 1996 and the annual cultivated area increased from 1.71 million hectares in 1996 to 181.5 million hectares in 2014 as they were cultivated in 28 countries by 18 million farmers. The United States is the pioneer country to grow GM crops with an area One million hectares, followed by Brazil, Argentina and India, then Canada, and the most important crops cultivated include corn, cotton, soybeans, canola, papaya and tomato, to produce varieties that are resistant to insects, diseases and herbicides [1].In general, the application and optimal use of biotechnologies in order to improve the characteristics of plants is an
essential need in genetic modification research and requires the development of successful and effective methods for regenerating vegetative growth from cultivated cells and tissues, with the availability of genetic modification methods for DNA. New methods of plant disease control are considered a necessary strategy, in which one or more genes are transferred. This gene can encode ketinase, for example, which breaks the cell walls containing chitin for many fungi, and plant resistance can be improved by expressing one or more antimicrobials in the plant [2]. Protocols rely on the use of PCR technology to detect genetically modified elements using genetic elements commonly used in genetic modification [3]. The scarcity of studies and research related to the detection of genetically modified plants in Iraq in general and the Karbala Governorate in particular, with suspicion of the presence of some genetically modified plants, so we wanted to verify their presence and source for the purpose of detection and determine their proportions in a manner consistent with the laws and legislation in Iraq. The was Study Aimed to detect of genetically modified soybean products and rice yield in samples tested in the Iraqi market, using common elements used in genetic modification such as the catalyst of the CaMV-35S promoter and the catalyst of the terminator Nos terminator in samples studied using the polymerase chain reaction method (PCR).

Materials and Methods

Sample collection

91 food and feed samples (soybeans- soybean feed, rice cereals) were collected randomly from different sources, which are Karbala, Najaf, Baghdad, and the Ministry of Agriculture / Crops and Seeds Department. The research plane was conducted at the Food Laboratory of the College of Veterinary Medicine, Kerbala University. Samples Obtaining during the study period that started from December 2019 until March 2020.

DNA Isolation from Plant Samples

The DNA was isolated from dry vegetable isolates (soybeans - soybean meal, rice grains), where the samples were ground by a ceramic mortar and liquid nitrogen was added. After that, I-genomic DNA extraction kit equipped by Korea / intron company was used for the varieties approved in the study, according to the manufacture company. The DNA extracted from the samples was revealed using a special device to calculate the concentration and purity for each sample, the spectrophotometer (American Thermo Corporation). The purity of the extracted DNA samples is determined by the absorbance reading of the spectrophotometer at two
wavelengths (260 and 280) nm, where the extracted DNA is considered pure when the absorbance ratio is 1.7 - 2.0 [4].

**Oligonucleotide primer**

In this study, special Primers were used to detect genetically modified genes in the genotypes of soybeans and their derivatives (soybean feed) which is the primer CaMV35S and terminator NOS, sizes 195 bp and 118bp respectively [5]. Because most GM products contain either the CaMV- 35S mosaic virus initiator or the Nos terminator, or both, most PCR screening methods rely on the detection of these sequences in the product [6]. Also in the present study, special primers were used to detect genetically modified genes in the genotypes of rice grains, which is the primer CaMV35S and terminator NOS, of sizes 123 bp and 118 bp, respectively [7], as shown in Table (1).

| PRIMER NAME | ORIGIN SEQUENCE | TARGET SEQUENCE | AMPLICON (BP) | (5’→ 3’) SEQUENCE | REFERENCE |
|-------------|----------------|----------------|---------------|-------------------|-----------|
| Detection Primer | **Cauliflower mosaic virus** | 35S1 Forward | 195 bp | F-5’- GCTCCTACAAATGCCATC A-3’ | [5] |
| | 35S2 Reverse | | | R-5’- GATAGTGGGAATTGCGTCA A-3’ | |
| Detection Primer | **Agrobacterium tumefaciens** | HANos-118 Forward | 118bp | R-5’- GCATGACGTATTTATGAGATGGG A-3’ | |
| | HANos-118 Reverse | | | R-5’- GACACCCGCACCCGCATTA TTTATCC A-3’ | |
Table (2) Primers utilized and designed for detection and identification in PCR.

| PRIMER NAME       | ORIGIN SEQUENCE       | TARGET SEQUENCE | AMPLICON (BP) | (5'→ 3') SEQUENCE       | REFERENCE |
|-------------------|-----------------------|-----------------|----------------|-------------------------|-----------|
| Detection Primer  | Cauliflower mosaic virus | P35S-cf3 Forward | 123 bp         | FGCTCTACAAATGCCATCA-3'  | [7]       |
|                   |                       | P35S-cr4 Reverse |                | RGATAGTGATTTGCGTGCGTA-3' |           |
| Detection Primer  | Agrobacterium tumefaciens | HANos-118 Forward | 118 bp         | RGATAGTGATTTGCGTGCGTA-3' |           |
|                   |                       | HANos-118 Reverse |                | RGACACCGCAGCGATAATTTATCC-3' |           |

**PCR Condition and Amplification Analysis**

**Conventional Polymerase Chain Reaction**

Prepare daily work solutions for PCR interactions. And that is by using the components of the PCR kit and the primers solutions, taking into account the cooling by placing the kit in an ice box. PCR reaction mixture including: 5 μl of genomic DNA were added in a sterile 0.5 ml tube marked with the name of the plant examination, the green master mix(The composition 20 mM Tris-Hcl PH 8.9, IGEPAL CA-630 0.05 , 22 mM KCL, 1.8 mM Mgcl2, 20Tween 0.05%, 0.4 mM dNTPs, 1 units/μl Ampliqon Taq DNA polymerase). 1 μl of each primer, and 5.5 μl sterile free ions distill water The concentration of primers for all target gene was 10 Pmol. Finally, PCR assays were performed in a volume of 25 μl. table (3).
Table (3) : Conventional PCR reaction components for CaMV-35S Promoter and NOS terminator:

| PCR reaction mixture      | Final Concentration | Volume in one reaction (μl) |
|---------------------------|---------------------|----------------------------|
| Sterile dd H2O            |                     | 5.5 μl                     |
| Green master mix          | 12.5 μl             |                            |
| Forward Primer            | 10 Pmol             | 1 μl                       |
| Reverse Primer            | 10 Pmol             | 1 μl                       |
| DNA                       |                     | 5 μl                       |
| Final Volume for reaction |                     | 25 μl                      |

**PCR program**

The experiment was performed in a Thermocycler for Soybean Isolates - as a soybean meal, to initiate a special program multiplier reaction for each set of primers as shown in Table (4):

Table (4): The Conventional PCR interaction program for gene (CaMV 35S promoter and -118 HANos) for soybean isolates and soybean meal [5].

| PRIMER      | NO. OF CYCLES | STEPS         | TEMPERATURE | TIME |
|-------------|---------------|---------------|-------------|------|
| 35S1        | 35S2          | 1             | Initial Denaturation | 95C˚ | 1min |
|             |               | 35 Cycles     | Denaturation  | 94C˚ | 30 sec |
| HANos-118 F | HANos-118 R   | 1             | Annealing    | 62C˚ | 45 sec |
|             |               |               | Alongation   | 72C˚ | 55 sec |

Table (5): The Conventional PCR interaction program for gene (CaMV 35S promoter and Nos terminator) for Rice isolates [7].

| Primer      | No. of Cycles | Steps     | Tempreture | Time |
|-------------|---------------|-----------|------------|------|
| 35S1        | 35S2          | 1         | Initial Denaturation | 94C˚ | 5min |
|             |               | 35 Cycles | Denaturation  | 94C˚ | 1min |
| HANos-      | HANos-        |           | Annealing    | 60C˚ | 40 sec |
Polymerase Chain Reaction

Duplex Polymerase Chain Reaction

Daily work solutions were prepared to conduct PCR Duplex reactions. The PCR Duplex molecular method was used to detect GM crops and their soybeans as shown in Table (6).

| PCR reaction mixture                  | Final Concentration | Volume in one reaction (μl) |
|--------------------------------------|---------------------|----------------------------|
| Sterile dd H2O                       |                     | 15 μl                      |
| Green master mix                     |                     | 25 μl                      |
| Forward CaMV-35S Primer              | 10 Pmol             | 1 μl                       |
| Reverse CaMV-35S Primer              | 10 Pmol             | 1 μl                       |
| Forward HANos Primer                 | 10 Pmol             | 1 μl                       |
| Reverse HANos Primer                 | 10 Pmol             | 1 μl                       |
| DNA                                  |                     | 6 μl                       |
| Final Volume for reaction            |                     | 50 μl                      |

PCR program

The thermal recycling program in a Duplex PCR gene (35S and 118HANos) reaction for soybean isolates, soy feeds as shown in the Table (7).

Table (7) shows the thermal circulation program in the Duplex PCR reaction for a gene (35S and 118HANos) for soybean isolates and soy feeds as shown in the table.

| No. of Cycles | Steps         | Temperature | Time  |
|---------------|---------------|-------------|-------|
| 1             | Initial Denaturation | 95C’        | 5min  |
| 35            | Denaturation   | 94C’        | 30 sec|
|               | Annealing     | 61C’        | 45 sec|
|               | Alongation    | 72C’        | 55 sec|
| 1             | Extension     | 72C’        | 5min  |
Electrophoresis on agarose gel

The electrical migration process started by preparing the gel by dissolving 1 g of agarose in 100 ml of buffer TBE (IX). The melting is done by heating it in the wave oven until all the agarose is dissolved, then left to cool at 50°C. The solution of an ethidium dye bromide was added with a final concentration of 0.5 μl / ml, 5 μl of the Polymerization reaction mixture was added in the gel well, Also, DNA Marker was added by 5μl in the gel well, The electrodes reached and the models traveled electrically with a voltage of (100) volts for one hour [8].

Results and Discussion

The ratio between the reading of the wave of 260 nm and the wave of 280 nm (280 OD / 260 OD) helps in estimating the purity of the DNA as this ratio should range between 1.7-2.0 [4].

Table (8) shows the concentration and purity of genotypes of soybean isolates and soybean feed.

| NO. | Sample       | A$_{260/280}$ | Concentration μg/ml |
|-----|--------------|---------------|---------------------|
| 1   | Healthy Feed No.1 | 1.92          | 162.5               |
| 2   | Healthy Feed No.2 | 1.79          | 100                 |
| 3   | Healthy Feed No.3 | 1.76          | 292.5               |
| 4   | Hassan        | 1.92          | 130                 |
| 5   | Lee74         | 1.80          | 132.5               |
| 6   | Qt5           | 1.94          | 345                 |
| 7   | Tn12          | 2.01          | 422.5               |
| 8   | Hawija101     | 1.86          | 282.5               |

When measuring the purity of the soybean DNA, most purity values were found between 1.7-1.9 Table (8), which confirms the good purity of the extracted DNA and this is shown by previous studies [4], that the DNA has a high Quality ratio A260 / A280 from 1.7-2.0.
Investigation of potential genes in soybean isolates

1. Detection of the CaMV-35S encoder gene and nos-terminator in soybeans gain and soybean mail and rice gain using the Conventional polymerase chain reaction technique

The results were observed when using specialized primers in the detection of P-35S promoters for the presence of a package resulting from the inflation process in the fourth, fifth and sixth well, respectively, at the molecular weight corresponding to 195 base pairs using the same primer P-35S promoter with a soybean meal sample. Healthy Fead No. 1 (S1) in the fourth well, and with a sample of healthy Fead No.2 (S2) in the fifth well, as well as with a healthy Fead No. 3 (S3) soybean sample, while the rest of the samples gave results Negative is known to be free of genetic modification as shown in Figure (1).

Figure (1) Electrophoresis of the DNA chain multiplexing product using the P-35S promoter for soybean samples on a 1% acarose concentration and a potential difference of 75 and hour time. First line: DNA Marker, second line: S9, third track: S10, fourth track: S1 with promoter (P-35S promoter), fifth track: S2 with primer (P-35S promoter), sixth line: S3 with primer (P-35S promoter), seventh line: S9, eighth line: S10, The ninth line: S11, the tenth line: S12, the eleventh line: S13.

The results of this study were identical with the results of previous studies in Romania in the detection of the initiator P-35S promoter in fifty samples of soybean feeds. There were five samples out of fifty genetically modified samples with this gene, i.e. (10%) of the total of the samples in Previous study [5]. Also, In the current study when using common primers used in genetic modification, it was found that there is no genetic modification for all rice samples shown in Figure (2).

According to previous studies published in 2019 in Iran, 2 out of 81 (2.4%), tested positive for CaMV35S while no positive result was detected for the NOS terminator [7]. A previous study
conducted in China showed that one out of two rice samples that were tested were positive for CaMV 35S primer [9]. In another study, two hundred samples (including soybeans, corn and rice) were tested for genetic modification, and two pairs of control genes (p35S and NOS) were used, and this result showed that 26 and 44% of the genetically modified samples were for soy and corn samples on Respectively, by contrast, all rice samples were negative for these two components [10]. On the other hand, other researchers have announced the possibility of detecting GMOs using the PCR method with these primers [11,12]. In a previously published Iraqi study, 86 isolates of the rice crop were tested. The result was that all genotypes were free of genetically modified using the Conventional PCR technique, the rate of genetically modified (0%), by using common elements used in genetic modification [13]. Previous studies conducted in India showed that the CaMV35S stimulus of the original Agrobacterium tumefaciens was beneficial in the genetic modification of the rice crop. It mentioned a high level of gene expression with the S35 primer [14].

![Figure 2](image-url)

Figure (2) Electrophoresis of the DNA chain-multiplexing product using the P-35S promoter of the rice seed samples on 1% agarose gel with a difference of effort of 75 and time hours. First line: DNA Marker, second line: S1 with primer P-35S promoter, third line: R2, fourth line: R 4, fifth line: R 11, sixth line: R40, seventh line: R53, eighth line: R71, Ninth line: R55.

2. **Detection of P-35S promoter and NOS Terminator in Genotypes of Soybean and Derivatives Using Mono PCR and Duplex PCR.**

When examination using the electrophoresis device on the agarose gel and examined the results under UV radiation. The results are observed when using specialized primers to detect primers (35S2 and 35S1) for the presence of a package resulting from the process of inflation in the fourth track and returning to the sample (S1) as shown in Figure (3), Which shows the presence of the DNA at the expected molecular weight of this inherited match for 195 base pairs. The results were
also observed when using specialized primers in detecting the HA-nos118-terminator for the presence of a package resulting from the process of inflation in the fifth line and returning to the sample (S1) as shown in Figure (3), which shows the presence of a piece of DNA at the expected molecular weight of this legacy Corresponding to 118 base pairs.

The second method: detection using the Douplex PCR chain. In this process, two primers were used and they are commonly used in genetic modification. They are the CaMV-35S promoter and the terminator Nos terminator. The DNA chain reaction interactions were performed for all genotypes. Results were observed when using primers specialized in detecting the primer (35S2, 35S1) and the HA-nos118-terminator terminator for the presence of three samples due to the soybean gain (S1 + S2 + S3) in both the first, second and third paths genetically modified to contain both starters CaMV- 35S promoter and terminator Nos terminator.

The results of this study were identical to the results of previous studies, as the resulting DNA in our current study showed the same molecular weight using the same primer and terminator in previously published studies [5].

Figure (3) Electrophoresis of the duplex PCR DNA soybean isolate using the P-35S promoter and terminator Nos terminator in the agarose gel at a concentration of 1% and a potential difference of 75 and an hour time. line M: DNA Marker, first line: S1 with terminator HA-nos118-terminator + P-35S promote, second line: S2 with terminator HA-nos118-terminator + primer P-35S promoter, third line: S3 with terminator HA-nos118-terminator + primer P-35S promoter, fourth line: S1 with primer P-35S promoter, fifth line: S1 with terminator HA-nos118-terminator, sixth line: S4 with terminator HA-nos118-terminator + primer P-35S promote, seventh line S5: Terminator HA-nos118-terminator + primer P-35S promote, S6 with terminator HA-nos118-terminator + primer P-35S promote.
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