Iraqi Journal of Biotechnology, May 2019, Vol. 18, No. 2, 160-168

Detection of CaMV-35S Promoter and NOS Terminator in Genetically Modified Tomato Seed in Iraqi Markets

Shurook M.K. Saadedin[1] , Mohammed Riyadh Abbas[2] , Ahmed A. Suleiman[3]

[1]Institute of Genetic Engineering and Biotechnology / University of Baghdad / Baghdad / Iraq.
[2]Directorate of seed Testing and certification / Ministry of Agriculture / Baghdad / Iraq.
[3]College of Science / University of Anbar / Anbar / Iraq.

Received: February 17, 2019 / **Accepted:** April 7, 2019

Abstract: The present study was focused to detect leading elements that control gene expression in genetically modified tomato by using conventional PCR technique.These common elements in all GM. plants are CaMV-35S promoter isolated from *cauliflower mosaic virus* and T-Nos terminator from the *Agrobacterium tumefaciens*. Seventy eight tomato genotypes were collected from Iraqiinstitutions and markets. The experiment was conducted in the Institute of Genetic Engineering/University of Baghdad/ Iraq and Directorate of Seeds Testing and Certification/Ministry of Agriculture/ Iraq. The tomato DNA samples were extracted manually by C- hexadecyl- Trimethyl-Ammonium-Bromide (CTAB) method. When measuring the optical density (OD) of the tomato samples, most purity values were found to be between (1.7-1.9).Two specific primers of CaMV-35S promoter, Nos terminator supplied by Canadian Alpha DNA Company, AccuPower®PCR Pre mix PCR supplied by Korean Bioneer Company and positive control (plasmid) supplied by Dr. ShathaAyidYousif/ Directorate of Agricultural Research/ Ministry of Science and Technology/ Iraq, were used in this study. Results showed that twenty four tomato genotypes were genetically modified. The primer specific of CaMV-35S promoter recorded a PCR product of 195 bp in 15 GM tomato and 13 GM tomato genotypes contain Nos terminator were a PCR product of 180 bp which as match with results of positive control (plasmid) which contains promoter and terminatorand that four tomato genotypes contain major components CaMV-35S promoter and Nos terminator together in the same sample.

Keywords: Tomato, Conventional PCR, CaMV-35S promoter and T-Nos terminator.

Corresponding author: (Email: ssaadedin@gmail.com).

Introduction:

A genetically modified plant is a plant whose genetic formation has been modified by means of the recombinant DNA technology(1). The genetic modification commonly involves inclusion of a piece of DNA (the insert) taken from other naturally occurring organisms, through the execution of genetic transformation (2). With the aim of ameliorate the agricultural practices and nutritional quality, plant breeding techniques have been developed to produce genetically modified (GM) crops expressing interesting advantages such as herbicide tolerance, insect resistance, and abiotic stress resistance(3). Despite such clear successes GM crops have been plagued by consumer refusal, especially in the European Union(4). Modification on the expression level of natural components of the manipulated organism can exacerbate sensitivity. In addition, the modified gene may encode an enzyme

implicated in other natural metabolic pathways of the modified organisms. Such changes might alter the levels of other metabolites, including toxic ones, at some "metabolic distance" from actual metabolic disorder. Another screenplay of potential risk is that the inserted gene might disrupt the safety of existing genomic information in the plant, leading to inactivation, or other modulation, of endogenous genes (5). Also, antibiotics are frequently employed, typically as selection markers, to distinguish successful transformed bacteria from those in which the transecting genes did not take contract. Thus, the mechanism to genetically modify an organism carries the risk of transferring the genes of antibiotics resistance into the benign bacteria include the microflora of human and animal gastrointestinal tracts, or, worse, yet, to pathogenic bacteria harbored by the consumer of GM a food(6). Thus, the development and application of an authoritative and specific simultaneous analytical detection method is essential in order to guarantee the consumer's access to information as well as to enforce food labeling by the competent authorities(7). Many DNA-based methods, targeting simple transgenic integrated sequences, have been widely developed. The PCR protocols used for GMO detecting are based on the detection of the known gene, such as 35s promoter in the *Cauliflower mosaic virus* (CaMV) and the *Agrobacterium tumefaciens* nopaline synthase (nos) terminator sequences, and in some statuses the reporter gene neomycin phosphotransferase (nptII) (8). Aim of this study was to screen the existence of the genetic modification of (CaMV-35S promoter and Nos terminator) in imported tomato by using conventional PCR technique.

Materials and Methods:

Collection and Germination of tomato seeds:

Seventy eight genotypes of tomato seeds were collected randomly from Iraqi Institutions and markets in the period from December 2016 to January 2017. Tomato genotypes were planted in incubator germination and growth conditions were 23ºC, 55% humidity and continuous light and after two weeks plants with 2-5 young leaves were cut and sterilized with ethanol 70%. Then (CTAB) method was used for DNA extraction.

50-mg of the young leaf tissues were ground to a fine powder in 700 µl 2% CTAB extraction buffer [20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB, plus 0.4% b-mercaptoethanol were added just before use], inside 1.5 ml eppendorf(9). Tubes were then incubated at 65ºC for 1 h, softly mixed every 15 min for adequate homogenization.500 µl of chloroform-isoamylalcohol (24:1) was then added, mixed softly for 1 min, and centrifuged at 8,000 rpm for 10 min (10). 500 µl of the supernatant was then transferred to a fresh tube with 700 µl of cold isopropanol (-20ºC); samples were softly mixed by inversion and centrifuged at 12,000 rpm for 10 min. The mixture is then placed in the freezer for one hour, and so it was possible to visualize the DNA adhered to the bottom of the tube. The liquid solution was then released and the DNA spherule washed with 700 µl of 70% ethanol to remove salt residues adhered to the DNA, and set to dry for

approximately 2 h, or until the next day, with the tubes inverted over a filter paper, at room temperature (9). The spherule was then resuspended in 100 µl TE buffer (10 mMTris-HCl pH 8.0, 1 mM EDTA pH 8.0) plus 5 mL ribonuclease (RNAse 10 mg mL–1) in each tube; this solution was incubated at 37ºC for 1h, and then stored at -20ºC(10).

Purity and Concentration of DNA:

DNA quality is characterized in terms of purity using UV Nanodrop, presence of inhibitors, using a PCR inhibition assay and intactness, using gel electrophoresis. UV Nanodrop was used to esteem DNA purity by measuring the sample's absorbance(11). DNA samples diluted 100 fold with TE buffer solution before measure the concentration by Nanodrop.

Agarose Gel Electrophoresis:

To check the DNA integrity, samples were migrating through1% agarose gel electrophoresis.

Detection of primers for GM Tomato:

Because most GM products contain either the cauliflower mosaic virusCaMV- 35S promoter or the nopaline synthase (Nos) terminator, or both, for GM products are based on detecting these sequences(12). Accordingly, the European Commissionhttps://ec.europa.eu/commission/index_enwhich carries registration codes for the genebank databasehttp://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotideSequence primers was illustrated in (Table 1).

Table (1): Primers utilized for detection and identification.

Primer Name	5'to3' Sequence	Amplicon [bp]	Primer source	Dilution for 100µM
CaMV P35S Promoter	**F**-5'-GCTCCTACAAATGCCATCA-3'	195bp	QL-ELE-00-005	944µl dd.W
	R-5'-GATAGTGGGATTGTGCGTCA-3'			850 µl dd.W
NOS terminator	**F**-5'-GAATCCTGTTGCCGGTCTTG-3'	180bp	QL-ELE-00-007	261µl dd.W
	R-5'-TTATCCTAGTTTGCGCGCTA-3'			265µl dd.W

AccuPower® PCR PreMix:

The final reaction mixture volume 20µl of basic mixture interaction PreMix was conducted. PCR reactions were set in Ice.2µl primer concentration 10 pmol\µl were added to 5 µl lyophilized AccuPower® PCR tube (1 µl Forward primer, 1 µl Reverse primer). 11 µl of distilled water were added to the AccuPower® tubes.2 µl templates DNA (tomato DNA) were added to AccuPower® PCR tube to a total volume of 20 µl.Dissolved the lyophilized blue pellet by microcentrifuge, and brieflied spin down. Negative control sample that contains all the basic components of the reaction mixture without DNA and the volume was complete into 20µl with free ions distilled water.

PCR program:

Several million copies of DNA can be generated from a single molecule by only few cycles of PCR. The PCR reaction conditions ofCaMV-35S promoter wereinitial5min denaturation at 95°C, 1 cycle, (45sec denaturation at

95°C,45sec annealing at 52°C and 1min elongation at 72°C,40 cycles) and10min final extension at 72°C, 1 cycle, and the PCR program of Nos terminator wereinitial5min denaturation at 95°C, 1 cycle, (35sec denaturation at 95°C, 45sec annealing at 55.1°C and 1 min elongation at 72ºC, 40 cycles) and 10 min final extension at 72ºC, 1 cycle.

Detection and analysis of PCR products by agarose gel electrophoresis:

Gel electrophoresis is a common technique used to visualize proteins and DNA. Gel electrophoresis has allowed the visualization of DNA and RNA with the use of markers (13). This process is achieved by sorting of a sample by size and charge. PCR products were analyzed according to the method of(14).

Results and Discussion:

Germination:

The results showed that eight tomato varieties did not germinate because the seed vitality was low.

DNA Extraction:

The C- hexadecyl- Trimethyl- Ammonium- Bromide (CTAB) based method and its modifications have been used to obtain good quality DNA for PCR-based downstream applications (15). And this extraction method was widely used in the enforcement laboratories for GMO detection(16). DNA was extracted for all tomato genotypes using this method and after measuring the purity and concentration the results showed that the highest concentration obtained were(2696.2, 2378.5, 2304 and 1750 ng/µl), and less concentration obtained were (85.7, 112.7, 132.4 and 148.8 ng/ µl), and that the highest purity (OD) of DNA(A260/A280) obtained were (2.19, 2.11, 2.06 and 2.03), the lowest purity recorded were (1.64, 1.66, 1.68 and 1.70). When measuring the optical density of the tomato DNA, most purity values were found to be between (1.7-1.9), thus confirming the good purity of extracted DNA. Good-quality DNA should have an A260/A280 ratio of 1.7–2.0 (17). DNA samples were tested through electrical migration in 1% agarose gel electrophoresis. Gel imaging results show that the DNA of tomato genome was not broken. It was not exposed to the denaturation by endo and exonucleases enzymes, as shown in (Figure 1).

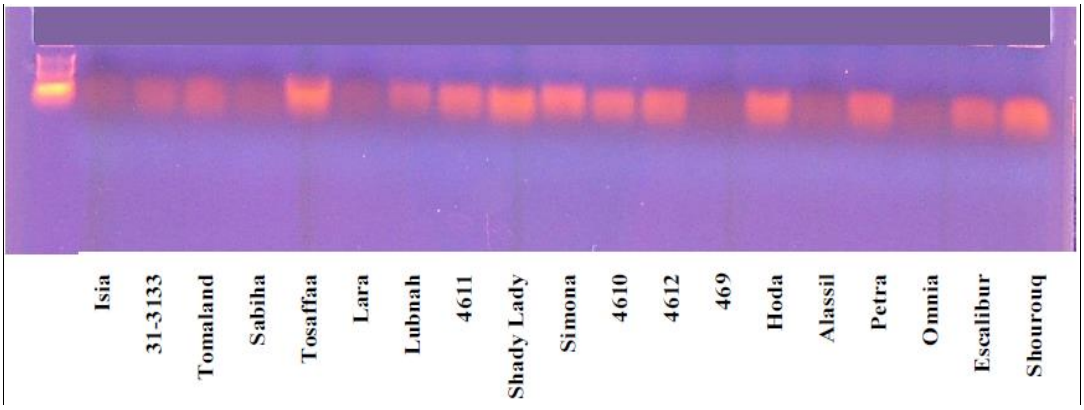

Figure (1): Genomic DNA bands of nineteen tomato genotypes on 1%agarose gel at 90 volt for 20 min, DNA samples were extracted by the CTAB method.

Detection of CaMV-35S promoter:

Conventional PCR technique used to determine GM tomato content and detect CaMV-35S promoter for PCR product a gradient PCR includes six temperatures (50, **52**, 54, 56, 58, and 60), there was no product at all temperatures except 52°C annealing temperature. The Melting Temperature (Tm) equation was used to determine the annealing temperature for CaMV-35S promoter. The results of the equation applicated show that 52°C is ideal temperature. The primers specific to CaMV-35S promoter recorded a PCR product of 195 bpFigur2 and 3. The 52°C annealing temperature reaction program was applied to all DNA tomato genotypes. The results showed that 15 GM tomato genotypes contained CaMV-35S promoter, the proportion of genetically modified genotypes was 15 out of 78genotypes (19.23%), and this finding was expected since most GM tomato genotypes contain the control component CaMV-35S promoter. An Iraqi study concerning GMO conducted by (18) found 1 out of 86 genotypes (1.2%) of the rice genotypes were genetically modified for primers specific to CaMV-35S promoter.

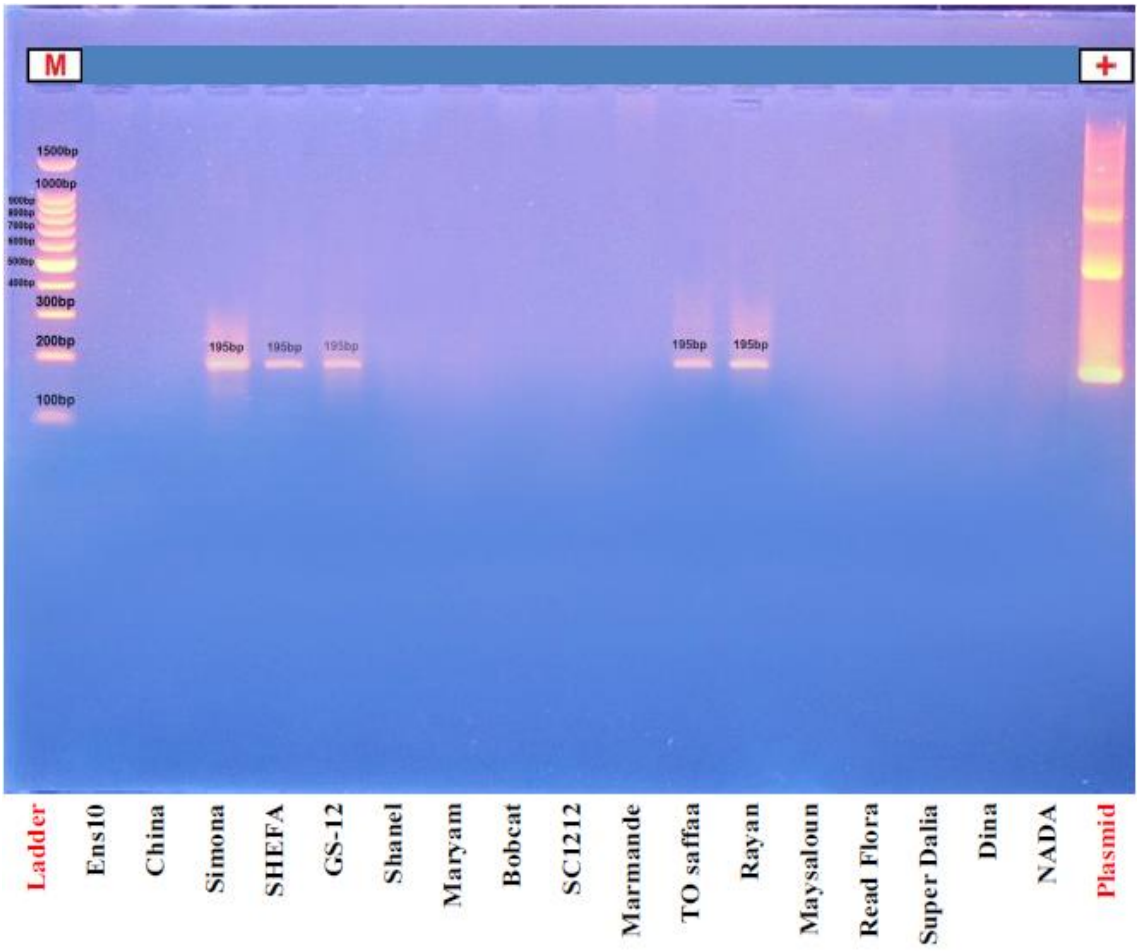

Figure (2): Detection of CaMV-35S promoter by Conventional PCR for 17 tomato genotypes using 100 bpLader and positive control (plasmid containing target gene), on 2% agarose gel at 75 volt for 90 minutes.

Figure (3): Detection of CaMV-35S promoter by Conventional PCR for 17 tomato genotypes using 100 bpLader and positive control (plasmid containing target gene) on 2% agarose gel at 75 volt for 90 minutes.

Detection of Nos terminator:

The Nos terminator genetic element has been widely used for the development of GM plants and it is joint in numerous authorized and unauthorized GMOs (19). As the most common terminator in genetically modified plants to control of gene expression, all tomato genotypes were tested to infer the presence of Nos terminator, recorded PCR product of 180 bp. The (Tm) equation was used to determine annealing temperature for Nos terminator. The result of applied equation showed that the ideal temperature of annealing was 55.1ºC. It is the core temperature in the amplification reaction. The primers were used specific to detect Nos terminator recorded a PCR product of 180 bp (Figure 4 and 5). The results of current study showed that 13 out of 78genotypes (16.67%) of the genotypes examined contain Nos terminator. A study by (20) found that 2 out of 13 genotypes (15.4%) of the tomato genotypes were genetically modified, and showed positive results for primers specific to CaMV-35S and NOS terminator by using PCR technique. In another study by (21) for detection of Nos terminator in 28 tomato genotypes, results did not show any indication of the presence of genetic modification element.

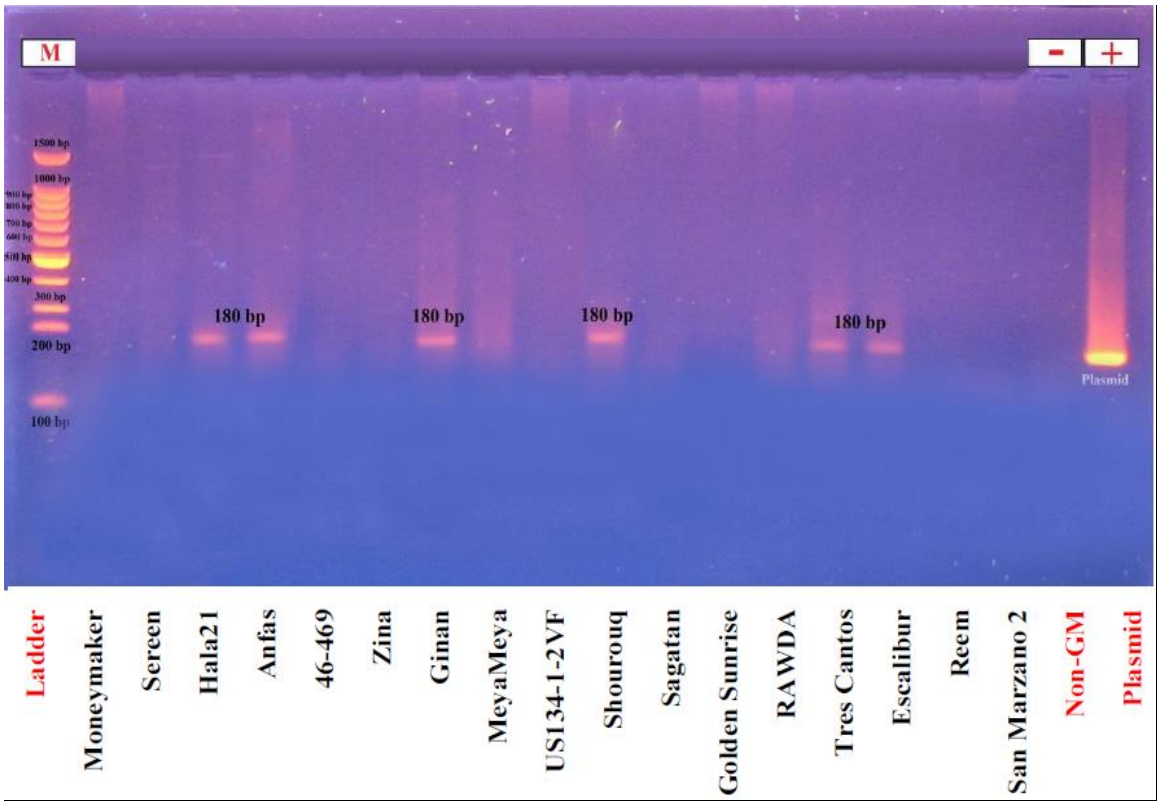

Figure (4): Detection of Nos terminator by Conventional PCR for 17 tomato genotypes using 100 bpLader and positive control (plasmid containing target gene) on 2% agarose gel at 75 volt for 90 minutes.

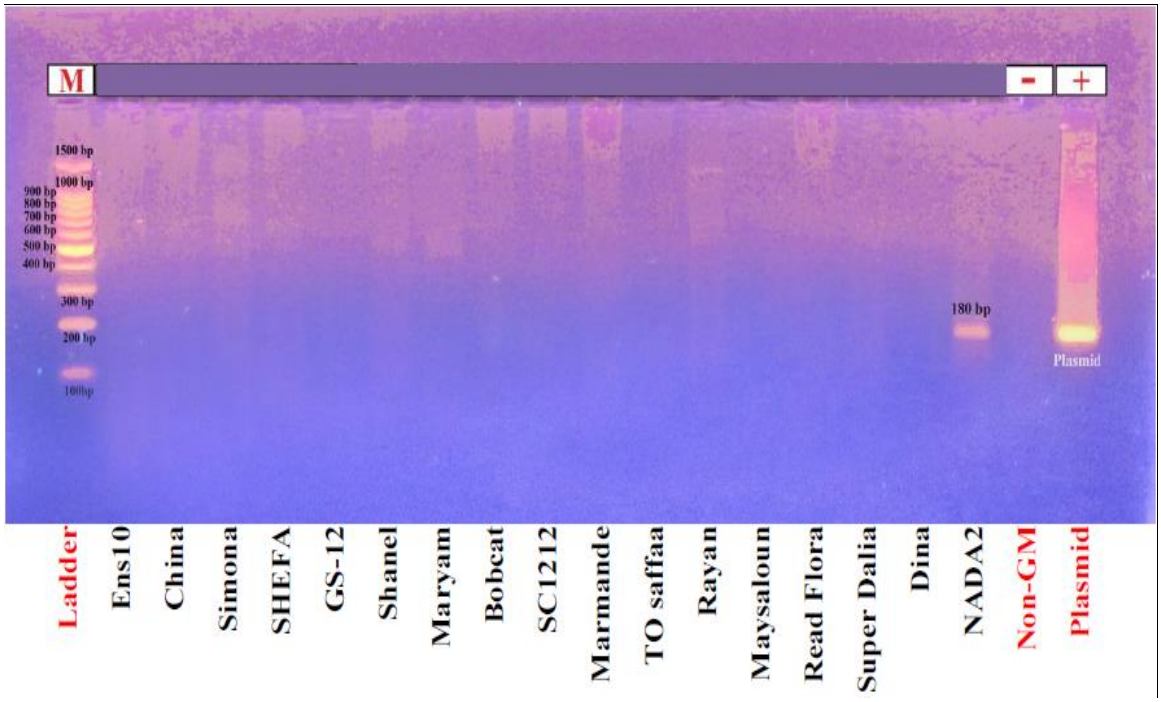

Figure (5): Detection of Nos terminator by Conventional PCR for 17 tomato genotypes using 100 bpLader and positive control (plasmid containing target gene) on 2% agarose gel at 75 volt for 90 minutes.

The results indicate that the total number of genetically modified tomato genotypes was 24 samples (30.8%) (Table 2), out of the 78 tomato genotypes that were studied. This result was expected as most genetic modification in the tomato crop involves one or both of the tools for controlling the gene expression as reported by(22).

Table (2): Results of CaMV-35S promoter and NOS terminator genes detection in DNA tomato genotypesbyConventional PCR: studied

No.	Genotypes	P35S	Nos
1	Nada	+	-
2	Sadek	-	+
3	Wogdan	+	+
4	Yamama	+	-
5	Hanine	-	+
6	Simona	+	-
7	SHEFA	+	-
8	GS-12	+	-
9	TO saffaa	+	-
10	Rayan	+	-
11	NADA2	-	+
12	Hala21	-	+
13	Anfas	-	+
14	Ginan	-	+
15	MeyaMeya	+	-
16	Shourouq	-	+
17	Tres Cantos	-	+
18	Escalibur	+	+
19	Reem	+	-
20	Flneness	+	-
21	Tomaland	-	+
22	4610	+	-
23	Omnia	+	+
24	Ban	+	+

In conclusion cultivated tomato genotypes by Iraqi farmers were not free from GM where twenty four GM tomato genotypes were detected out of eighty seven. Conventional PCR can be used for screening genetic modification in Tomato seeds by using the primers CaMv-35S Promoter and Nos Terminator.

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