ISSN: 1991-8941

Using DNA Sequencing to Detect and Verify Genetically Modified Maize (*Zea mays* L.) in Iraq.

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Received: 19/9/2012 **Accepted: 22/5/2013**

Abstract: Polymerase chain reaction (PCR) was used to detect genetically modified maize. The results revealed that 10 out of 72 maize DNA samples were genetically modified. Verification of PCR ampliconse indicated that all GM maize were of MON810 type engineered with cryIAb gene under the control of the cauliflower mosaic virus P35S promoter and NOS terminator. Direct DNA sequencing for three out of the ten detected GM maize confirm the presence of P35S promoter and NOS terminator. Also, the constructed alignment of both genetic elements in comparison with their respective sequence in the NCBI database, indicated complete identity level for P35S, whereas nucleotide mismatches was detected in two sites of NOS118 terminator both on the forward strand. Meanwhile one site was corrected by the reverse strand, the other site may be resulted from Tag polymerase mismatches. These results approved the highly conservativeness of both detected elements and the completely absence of single nucleotide mutations. Results revealed clearly that nonauthorized GM maize was entered the national agriculture sector without authorities permission, which maximize risks of spreading GM maize all around the country and need to act immediately by setting strict legislation and monitoring system.

Keywords: GM maize, DNA sequencing, NOS terminator and P35S promoter.

Introduction

Genetically-modified organisms (GMO) have the potential to solve one of the most important challenges in the world which is hunger, an issue grows and complicated day by day along with the huge increases in the world population. So, ensuring an adequate food supply for this booming population is going to be a major challenge in the next a few years. Although, GM foods promise to meet this need through producing plants tolerant to wide spectrum of biotic and abiotic stresses, uncertainty about Genetically Modified Crops (GMC) safety have limited their adoption, in addition to transgene containment which is a central concern in such crops, especially for those with out-crossing wild relatives [1].

Concern has arisen that if detrimental effects were to be detected post release of any GMC, withdrawal may be difficult, or impossible, if gene flow has already occurred. Other potential, undesirable consequences of gene flow are the evolution of increased weediness and the extinction of wild relatives [2,3,4]. Maize races are easily distinguished by farmers, particularly when "foreign genes" are brought from modern hybrids [5]. As conventional PCR used in GMO analysis, two difficulties will show up: first, different copy numbers of integrated constructs in different GMO events, and second, different GMO events containing identical transgenic construct [6]. Most of the currently available GMOs worldwide contain any of three genetic elements: the cauliflower mosaic virus (CaMV) P35S promoter, the nonpalin synthase (NOS) terminator [7]. Hence, testing for the presence of P35S and NOS sequences has been commonly used as a screening tool for detection of GM plant materials since most or all GE events or products in commerce contained one or the other or both.

An important issue related to PCR-based detection is the specificity which can be evaluated in four categories [8]. In the first

category, amplified regions are 35S promoter (CaMV), nopaline synthase terminator and/or genes encoding the resistance to antibiotics used in selections. In second category, detection is carried out by the amplification of specific-genes such as "bar" (phosphinotricin acetyltransferase) and CryIA(b). In category 3, junctions between promoter and genes and in category 4, junction between gene and its integration locus are targeted for the PCR amplification. Quantification is another criteria for GMO screening since it determines the labeling limit which is required by EU countries. This can be done by comparison of the relative ratio of copy number of a reference PCR target and genomic copies of respective sequences [9].

For the sensitivity of mPCR it has been used by [10], so this method was reconfirmed with known GRM samples for 8 GM maize lines at 0.25% level. Authors cleared that none of the maize seed samples obviously contaminated with GM maize which was identified by mPCR. While, they return the presence of weak and nonspecific DNA bands to the low contamination of some samples with very low level of NK603 and MON810.

Hernandez et al, [11] describe a qualitative PCR-based multiplex method for simultaneous detection and identification of four genetically modified maize lines. Primers were used in a single optimized multiplex PCR reaction, and sequences of the amplified fragments are reported. Their assay allows amplification of the MON810 event from the 35S promoter yielding a 468 bp amplicon.

To fulfill labeling and traceability requirement of genetically modified maize for trade and regulation, it is essential to develop an event-specific detection method for monitoring the presence of transgenes. In pursuit of this purpose, Shrestha et al, [12] systematically optimized and established a combined event- and construct-specific multiplex polymerase chain reaction (mPCR) technique for simultaneous detection of 8 GM maize lines. Altogether 9 sets of primers were designed, including six that were event-specific, one of these was for the event Mon810, two that were construct-specific for T25 and GA21, and one for an endogenous zein gene. The transgene in each GM maize line and the endogenous zein gene could be clearly detected and distinguished according to the different sizes of PCR amplicons. Nguyen et al, [13] employed PCR technology for detection and direct DNA sequencing for confirmation procedures respectively. The results demonstrated for the first time the presence P35S promoter and NOS terminator in either Malaysian or Vietnamese feed with high frequency as 20 samples out of 24 analyzed samples were positive. The P35S promoter, EPSPS gene and NOS terminator sequences obtained showed some mutations on single-stranded and double-stranded targeted sequences caused by single nucleotide insertion or single nucleotide changes.

Shrestha et al, [10] aimed to survey the current status of GM maize seeds in Nepal. First, they performed multiplex polymerase chain reaction (mPCR) to detect 8 GM maize lines in 46 maize seed samples from different locations in Nepal. Suspected samples were then verified by real-time PCR (RT-PCR) and screen-specific PCR. Based on current evidence, they can not identify any GM maize in the seed samples. This study aimed to detect and verify nonauthorized GM maize that may entered the national agriculture sector illegally or without the permission of authorities.

Materials and Methods

Maize seed samples (72 sample) were collected in 2011 from different sources; public and private sectors, imported seed stocks for human consumption, animal feeding and agriculture. Seed samples were grinded to a fine powder prepared for DNA extraction with the aid of Wizard Genomic DNA kit (Promega – USA) according to the suppliers protocol. The DNA quality was checked by mixing 2µl from each DNA sample with 7µl loading buffer (0.25g Bromphenol blue dye; 30ml glycerol) electrophoresed on a 0.8% agarose gel at a voltage equal to 5v x tank length.cm, then staining step was performed with ethidium bromide followed by gel visualization under UV light. DNA quantity was checked by using spectrophotometer at wave length of 260nm/280nm. Based on concentration values, samples were diluted to a final concentration of 200 ng/µl for amplification.

The PCR work was carried out in Medicinal and Molecular Biotechnology Dept./ Biotechnology Center/Al-Nahrain University using Eppendorf thermal cycler (Eppendorf – Germany). The PCR reaction was performed in microtubes of 200µl with final reaction size of 25µl, contained a 200ng of DNA sample, 2x (PH=8.5), dNTPs (200mM), Taq polymerase (0.3 units) and 0.2µM of *Zein* and T-*NOS* primer, and 0.1µM of *P35S* primer.

Since most of the transformed genes using in producing GM maize were under the control of either one or both of *P35S* promoter and *NOS* terminator. Two pairs of oligonucleotide primers (Alpha DNA - Canada) were used to detect each of *Zein* gene, CaMV *P35S* promoter and *NOS* terminator (Table 1). The primers were diluted to final concentration of 10 pmol/µl with sterile distilled deionzed water.The PCR thermal profile differed in response to the used primer (Table 2-a, b and c).

Table 1: Primer details and description of their sequence.

Primer	*Sequence*	*Amplicon Size bp*	*Gene specificity*	*Reference*
ZE03	**5'**- AGT GCG ACC CAT ATT CCA G-**3'**	**277**	*Zein*	[13]
ZE04	**5'**-GAC ATT GTG GCA TCA TCA TTT-**3'**			
P35S 1-5'	**5'**-ATT GAT GTG ATA TCT CCA CTG ACG T-**3'**	**101**	*P35S Promoter*	[13]
P35S 2-3'	**5'**-CCT CTC CAA ATG AAA TGA ACT TCC T-**3'**			
NOS118-F	**5'**-GCA TGA CGT TAT TTATGA GAT GGG-**3'**	**118**	*NOS Terminator*	[14]
NOS118-R	**5'**-GAC ACC GCG CGC GATAAT TTA TCC-**3'**			

Table 2-a: PCR thermal profile used with ZE03 and ZE04 primers.

Steps	*Temperature ^{o}C*	*Time (min:sec)*	*No. of cycles*
Initial Denaturtion	95^{o}	4:30	1 cycle
Denaturtion	96^{o}	1:45	40 cycle
Annealing	60^{o}	2:00	
Extension	72^{o}	2:00	
Final Extension	72^{o}	4:50	1 cycle

Table 2-b: PCR thermal profile used with P35S 1-5' and P35S 2-3' primers.

Steps	*Temperature ^{o}C*	*Time (min:sec)*	*No. of cycles*
Initial Denaturtion	95^{o}	10:00	1 cycle
Denaturtion	95^{o}	1:00	45 cycle
Annealing	55^{o}	2:00	
Extension	72^{o}	2:00	
Final Extension	72^{o}	7:00	1 cycle

Table 2-c: PCR thermal profile used with NOS118-F and NOS118-R primers.

Steps	*Temperature ^{o}C*	*Time (min:sec)*	*No. of cycles*
Initial Denaturtion	95^{o}	10:00	1 cycle
Denaturtion	95^{o}	00:25	50 cycle
Annealing	62^{o}	00:30	
Extension	72^{o}	00:45	
Final Extension	72^{o}	7:00	1 cycle

Template DNA and with no primer represent the negative control. The PCR products were electrophoresed in 2.5% agarose gel with 1x TBE buffer (54g of Tris-Base, 27.5g of Boric acid and 20ml of 0.5M EDTA (pH=8.0). Gel was visualized and documented with aid of gel documentation system (Consort - Belgium). Amplification products were separated and

discriminated by using capillary electrophoresis (CE) by an automatic analyzer, which allows the separation and the identification of the amplified products and determine accurately their molecular weight.

The *P35S* promoter and *NOS* terminator amplicons were excised from the agarose gels and purified by QIAquick gel extraction kit (Qiagen - Germany) according the suppliers instructions. Purified products were sequenced in Saint Joseph University (Beirut – Lebanon) by using Genetic Analyzer (ABI-3130). PCR products were purified after cycle sequencing with aid of MinElute PCR Purification Kit (Qiagen - Germany). The BLAST (Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/)) and ChromasPro (1.6 version) softwares were used to analyze the sequence and to conduct the alignment of the sequences with the transformation vector.

Results and Discussion

The validity of DNA samples for amplification assay was confirmed since all these samples showed positive results for *Zein* gene (Fig. 1) with molecular size of 277 bp.

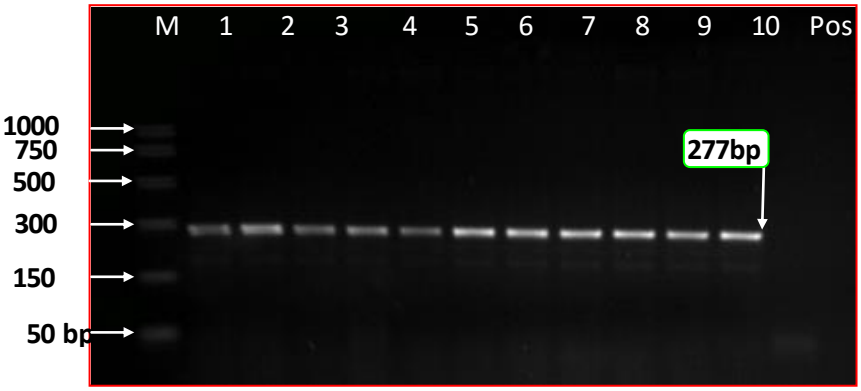


Figure 1: PCR amplified products of 10 maize DNA samples using *Zein* primer.
M= Molecular marker 1kb (Bench top PCR marker); Neg= Negative Control; Pos=Positive Control.

PCR analysis detected a total of 10 DNA samples out of 72 as genetically modified maize, nine of these were positive for *P35S* promoter (Fig.3) and *Nos* 3' terminator (Fig.4). The tenth sample was infected by insect, therefore it was neglected. Verification of PCR ampliconse revealed that all the detected GM maize were of MON810 type (Fig.2) engineered with (*cryIAb*) gene under the control of the cauliflower mosaic virus *P35S* promoter and *NOS-118* terminator.

The expected amplification ampliconse resulted from using each pairs of the specific primers were 101 bp and 118 bp in size confirming the presence of *P35S* and *Nos* 3', respectively.

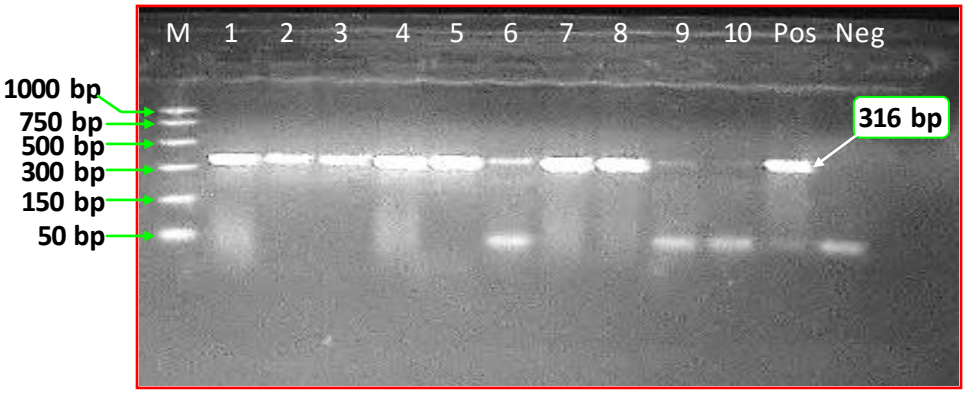


Figure 2: PCR amplified products of maize DNA samples using *ZE03* and *ZE04* primers. M= Molecular marker 1kb (Bench top PCR marker); Neg= Negative Control; Pos=Positive Control.

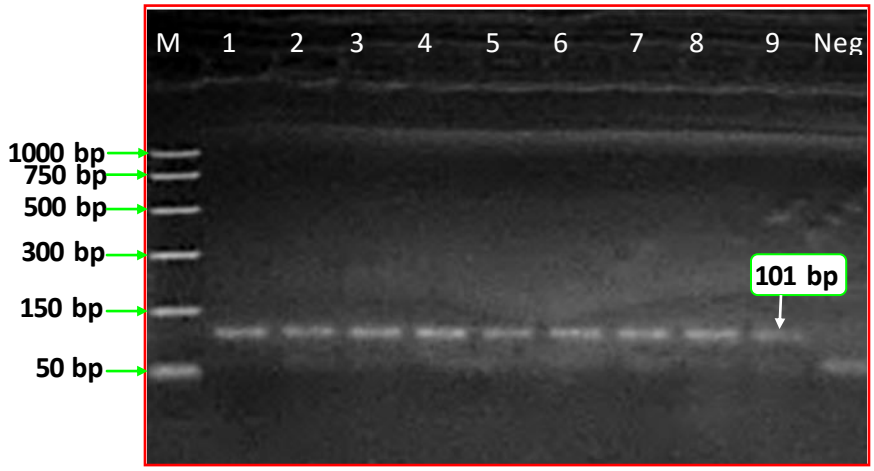


Figure 3: PCR amplified products of maize DNA samples using *P35S 1-5'* and *P35S 2-3'* primers. M= Molecular marker 1kb (Bench top PCR marker); Neg= Negative Control.

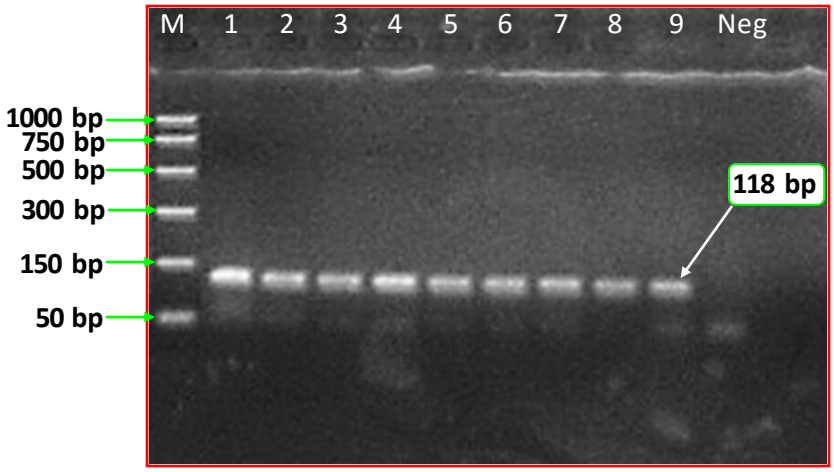


Figure 4: PCR amplified products of maize DNA samples using NOS118-F and NOS118-R primers. M= Molecular marker 1kb (Bench top PCR marker); Neg= Negative Control.

DNA sequencing was used to get unerring verification to the amplified products and locate the possible mutations in the sequence of *P35S* promoter and *NOS* terminator on the level of single nucleotide. Among the 10 detected GM samples three were extracted and purified by using QIAquick gel extraction kit and prepared for the DNA sequencing.

The BLAST (Basic Local Alignment Search Tool) software (http://www.ncbi.nlm.nih.gov/) was used to analyze the sequence. Alignment results indicated complete similarity between the constructed sequence of *P35S* and the reference sequence of CaMV genome in both forward and reverse strands (Fig. 5).

The nearly full identity between the constructed sequences of *P35S* compared with its respective sequence (Fig. 5) on the NCBI database (http://www.ncbi) indicated the its highly conservativeness. This result may minimize the concerns about the stability of such elements and assure that no large scale mutations will occur in their related nucleotide sequence. However, some have their own concerns beyond the element sequence as the new genes inserted into the plant's DNA appear to be merged with

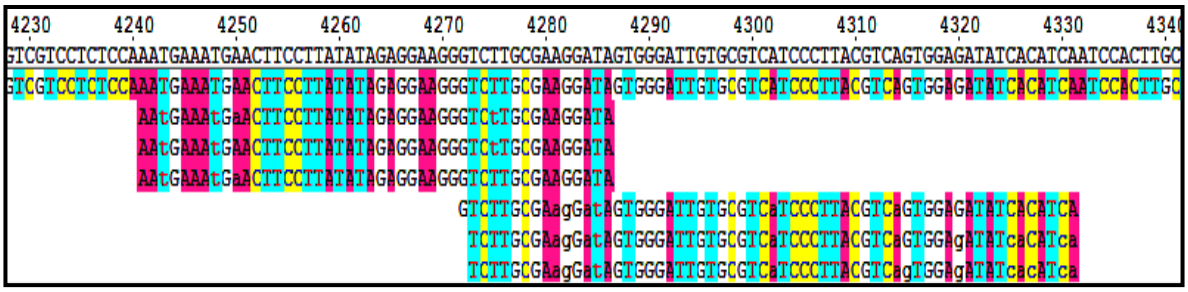
products from the plant's own genes, creating an irregular occurrence. The genes inserted into MON810 produce a variant of mRNA (the intermediary between DNA and proteins) which is likely to produce a protein different to the one tested by the producer because it is fused with the protein from the plant's own genes. Therefore, it is not known what toxin MON810 actually produces [15].

Results of *NOS* terminator sequence may support these concerns, as two mismatches with single nucleotide number 2747 both on the forward strand (Fig. 5). Also, another two mismatches occurred within nucleotide number 2802 in two samples both on the forward strand, while the reverse strand showed complete match. Such changes with single nucleotide might have resulted from the mismatches of *Taq* polymerase during PCR amplification to produce amplicons, especially when there are different results showed by forward and reverse primers, rather than the permanent changes themselves because changes of nucleotide location happens on single stranded sequence only [13].

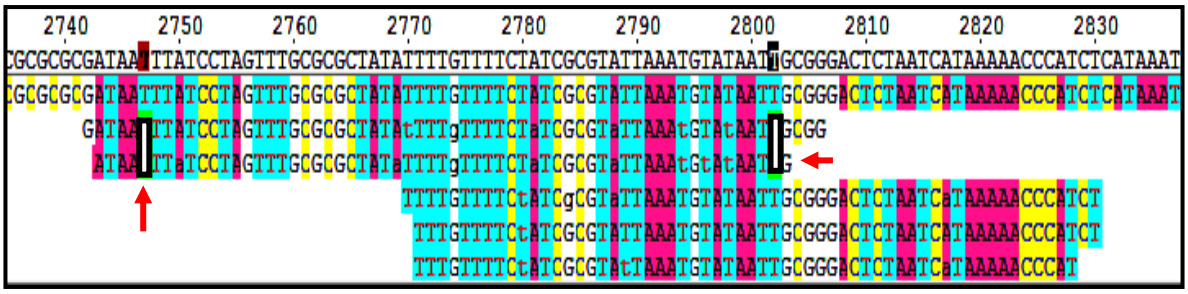
The maintenance of maize biological diversity and food biosafety require restricted importing and labeling system to monitor the GM maize seed status in Iraq. Also, different type of primers designed to target the wide spectrum of genetic events invading the biological system, accompanied with depending new tools for detecting GMO like RT-PCR will be necessary for detecting and quantifying recombinant DNA efficiently.

References

1. Daniell, H. 2007. Transgene containment by maternal inheritance: Effective or elusive? *PNAS, 104 (17) : 6879–6880*.
2. Matsuoka, T., H. Kuribara, K. Takubo, H. Akiyama, H. Muira, Y. Goda, Y. Kusakabe, K. Isshiki, M. Toyoda and A. Hino. 2002. Detection recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J. of Agric. and Food Chem., 50: 2100-2109.*
3. Sahoo, L., J.J. Schmidt, J.F. Pedersen, D.J. Lee and J.L. Lindquist. 2010. Growth and fitness components of wild x cultivated *Sorghum bicolor* (Poaceae) hybrids in Nebraska. *Amer. J. of Bot., 97(10):1610-1617.*
4. Alfalahi, A.O. and F.W. Abdulqahar. 2010. Biota gene flow and genome contamination. *The Iraqi J. Agric. Sci. 41(2):126-149.*
5. Rimachi, L.F., J.A. Delgado, Y.A. Villasante and R. Ortiz. 2011. Detecting adventitious transgenic events in a maize center of diversity. *Electronic J. of Biotech., 14(4):1-12.*
6. Huang, H-Y. and T-M. Pan. 2004. Detection of genetically modified maize MON810 and NK603 by multiplex and Real-time polymerase chain reaction methods. *J. Agric. Food Chem., 52: 3264-3268.*
7. Ahmed, F. 2002. Detection of genetically modified organisms in foods. *Trend in Biotech., 20(5): 215-223.*
8. Holst-Jensen, A., S.B. Ronning, A. Lovseth and K.G. Berdal. 2003. PCR technology for screening and quantification of genetically modified organisms (GMOs). *Anal. Bioanal. Chem., 375: 985-993.*
9. Gurel, F., E. Arican, N. Gozukirmizi and S. Ari. 2011. Recent molecular tools for detecting transgenic events in genetically modified (GM) crop products. *Sci. Res. and Essays, 6(24): 5091-5099.*
10. Shrestha, H.K., K-K. Hwu and M-C. Chang. 2010. Detection of genetically modified maize (*Zea mays* L.) in seed samples from Nepal. *Afric. J. of Biotech., 9(34): 5581-5589.*
11. Hernandez, M., D. Rodriguez-Lazaro, D. Zhang, T. Esteve, M. Pla and S. Prat. 2005. Interlaboratory transfer of a PCR multiplex method for simultaneous detection of four genetically modified maize lines: Bt11, MON810, T25 and GA21. *J. Agric. Food Chem., 53(9):3333-3337.*
12. Shrestha, H.K., K.K. Hwu, S.J. Wang, L.F. Liu and M.C. Chang. 2008. Simultaneous detection of eight genetically modified maize lines using a combination of event- and construct-specific multiplex-PCR technique. *J. Agric. Food Chem., 56(19): 8962-8968*
13. Nguyen, C.T., R. Son, A.R. Raha and O.M. Lai. 2008. Detection of genetically modified organisms (GMOs) using molecular techniques in food and feed samples from Malaysia and Vietnam. *Int. Food Res. J., 15(2): 155-166.*
14. Lipp, M., A. Bluth, F. Eyquem, L. Kruse, H. Schimmel, G. Eede and E. Anklam. 2001. Validation of a method based on polymerase chain reaction for the detection of genetically modified organisms in various processed foodstuffs. *European Food Res. and Tech., 212: 497-504.*
15. Rosati, A., P. Bogani, , A. Santarlasci and M. Buiatti. 2008. Characterisation of 3′ transgene insertion site and derived mRNAs in MON810 YieldGard



A



B

Figure 5: The constructed sequences of *P35* S promoter (A) and *NOS118* terminator (B) compared with their respective sequence in the NCBI database.

استخدام تحديد تتابع DNA في الكشف عن وتشخيص الذرة الصفراء (Zea mays L.) المحورة وراثياً في العراق

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الخلاصة

استخدم تفاعل إنزيم البوليميريز المتسلسل (PCR) لتشخيص الذرة الصفراء المحورة وراثيا. أشارت النتائج إلى ان 10 من مجموع 72 عينة شملتها الدراسة كانت محورة وراثيا. تبين لدى التحقق من نواتج تفاعل PCR أن جميع عينات الذرة الصفراء المحورة وراثيا هي من نوع MON810 حورت وراثيا باستخدام جين *cryIAb* الخاضع لسيطرة محفز فايروس موزائيك القرنابيط *P35S* والناهي *NOS*. أكدت نتائج تحديد تتابع نكلوتايدات DNA لثلاث من العينات العشر المشخصة على أنها محورة وراثياً وجود كلا من المحفز *P35S* والناهي *NOS* هذا من جهة، ومن جهة أخرى أشارت نتائج المصفوفة الناتجة من المقارنة بين تتابع نكلوتايدات كلاً من المحفز والناهي للعينات المدروسة مع تتابعاتهما الموجودة في بيانات NCBI إلى أن هناك تطابقا كاملا في عينات المحفز، في حين وجدت حالة عدم تطابق في موقعين في تتابع الناهي كلاهما على الشريط الأمامي، وبينما صححت إحداهما من خلال تتابع الشريط المعاكس، يمكن أن تكون حالة عدم التطابق الأخرى ناتجة عن خطأ في عملية الاستنساخ بواسطة إنزيم البوليميريز. أثبتت هذه النتائج محافظة عالية للعناصر الوراثية المشخصة على تتابعاتها، وغياب تام للطفرة في القواعد المفردة. أوضحت نتائج التشخيص دخول الذرة الصفراء المحورة وراثياً غير المرخصة إلى القطاع الزراعي الوطني بدون إذن السلطات المختصة مما يزيد من خطر انتشار الذرة المحورة وراثيا في جميع أنحاء البلد مما يستدعي التحرك السريع لإرساء نظام رقابي وتشريعي صارم.