RESEARCH PAPER

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Unconventional P-35S sequence identified in genetically modified maize

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The Cauliflower Mosaic Virus 35S promoter sequence, CaMV P-35S, is one of several commonly used genetic targets to detect genetically modified maize and is found in most GMOs. In this research we report the finding of an alternative P-35S sequence and its incidence in GM maize marketed in Jordan. The primer pair normally used to amplify a 123 bp DNA fragment of the CaMV P-35S promoter in GMOs also amplified a previously undetected alternative sequence of CaMV P-35S in GM maize samples which we term V3. The amplified V3 sequence comprises 386 base pairs and was not found in the standard wild-type maize, MON810 and MON 863 GM maize. The identified GM maize samples carrying the V3 sequence were found free of CaMV when compared with CaMV infected brown mustard sample. The data of sequence alignment analysis of the V3 genetic element showed 90% similarity with the matching P-35S sequence of the cauliflower mosaic virus isolate CabbB-JI and 99% similarity with matching P-35S sequences found in several binary plant vectors, of which the binary vector locus JQ693018 is one example. The current study showed an increase of 44% in the incidence of the identified 386 bp sequence in GM maize sold in Jordan's markets during the period 2009 and 2012.

Introduction

The introduction of genetically modified organisms (GMOs) and their products has made a great contribution to the world's economic development[1,2] and has the potential to improve food functional properties,[3] enhancement of the tolerance to abiotic stress,[4] production of pathogen-resistant crops,[5] and plants for the promotion of human health.[6] Risk assessment tests have indicated that genetically modified products that carry authorized events are better characterized than other non-regulated plant derived foods. Although authorization of GM food is controlled by regulations, the ENGL ad hoc working group on unauthorized GMOs[7] has indicated that there are challenges facing detection of unauthorized GMOs (UGM). Therefore, risk assessment tests should show that GM crops are safe and authorized before release to the environment and for consumption.[8,9] Today, P-35S, T-*nos*, *bar*, *CP4-epsps*, and P35-*pat* are the five genetic elements which are recommended to detect GM products.[10-12] The 35S promoter, derived from the common plant virus, cauliflower mosaic virus (CaMV), is part of the transgenic construct in most GM crops being grown commercially, for example maize, soy, canola, and papaya.[10]

This study used PCR as the main specific molecular detection method for GMOs and DNA sequencing for identification and characterization of an alternative P-35S sequence in GM maize. The study also shows the incidence of both the typical 123 bp DNA sequence and the unconventional 386 bp sequence in the identified GM maize samples when using the typical primer set to detect the P-35S GM target.

Results

Detection and incidence of 386 bp sequence in GM maize products

The origin of the extracted DNA from the maize products was confirmed by the amplification of a 277 bp long maize specific piece of DNA as described (data not shown).[11,13,14] Detection of the CaMV 35S promoter of the CabbB-JI sequence in our samples was performed by using a specific primer set for CaMV P-35S (p35S-cf3, F and p35S-cr4, R)[13] to amplify a 123 bp fragment (**Fig. 1**). Amplification of the 123 bp DNA fragment was observed in 29 samples (72.5%), indicating that they were carriers of CaMV P-35S. From the 29 identified GM maize samples, 9 (31%) showed one DNA fragment of 123 bp that was

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Table 1. Number of maize samples (food or feed) carrying 123 bp and 386 bp DNA fragments which were amplified by specific primers CaMV P-35S (p35S-cf3, F and p35S-cr4, R). Forty maize samples were used for detection for GMOs

Type of sample	Number of samples	Number of GM samples	Samples carrying only 123 bp sequence	Samples carrying 123 bp and 386 bp sequences
Food	20	18	3	15
Feed	20	11	6	5
Total	40	29	9	20

Table 2. The 386 bp amplified DNA fragment (V3) detected in GM maize by CaMV P-35S specific primers (p35S-cf3, F and p35S-cr4, R) used for detection of the 123 bp fragment. The sequence was used in the BLAST as query sequence and MATLAB sequence alignment analysis

Sequence	Sequences of amplified fragment (5′ → 3′)	Length of sequence
V3	TCCACGTCTT TCAAGCAAGT GGATTGATGT GATGGTCCGA TTGAGACTTT TCAACAAAGG GTAATATCCG GAAACCTCCT CGGATTCCAT TGCCCAGCTA TCTGTCACTT TATTGTGAAG ATAGTGGAAA AGGAAGGTGG CTCCTACAAA TGCCATCATT GCGATAAAGG AAAGGCCATC GTTGAAGATG CCTCTGCCGA CAGTGGTCCC AAAGATGGAC CCCCACCCAC GAGGAGCATC GTGGAAAAAG AAGACGTTCC AACCACGTCT TCAAAGCAAG TGGATTGATG TGATATCTCC ACTGACGTAA GGGATGACGC ACAATCCCAC TATCCTCGCA AGACCCTTCC TCTATATAAG GAAGTTCATT TCATTTGGAG AGGAAG	386 bp

amplified by the CaMV P-35S promoter specific primers. The remaining 20 GM maize samples (69%) unexpectedly contained a second DNA fragment of 386 bp termed V3 which was also amplified in addition to the 123 bp DNA fragment (**Table 1**). The 386 bp DNA fragment was neither found in standard wild type maize (ERM-BF413a) nor in standard GM maize MON810 (**Fig. 2A**). Further analysis of 16 GM maize samples carrying V3 fragment showed that five contained the genetic event MON863 as detected by specific primers MON863-F and MON863-R, which amplified the DNA fragment by 84 bp (**Fig. 2B**).[11,13] In addition, nested PCR experiments showed that seven of the identified GM maize samples contained the hsp70 exon1-intron1 region of maize MON810.[11,113, 14] Further tests were performed to exclude possible contamination of the tested GM maize samples with CaMV. The tested samples were found free of CaMV when compared with a CaMV infected brown mustard sample (**Fig. 2C**). The analysis was based on the amplification of 89 bp DNA fragments by CaMV-F and CaMV-R primers that were not detected in the identified GM maize but were identified in the standard CaMV infected brown mustard sample.[15]

Sequence analysis of 386 DNA fragment

The sequence of the V3 fragment comprises 386 bp (**Table 2**). Our sequence alignment results showed that the P-35S sequences of MON810[16] and MON863[17] gave low comparability with the V3 sequence. The analysis showed that the 233 bp MON810 P-35S sequence contained 204 bases that matched the V3 sequence; this represents 52.8% of the V3 sequence. Similarly, the V3 sequence showed 49% similarity with the MON863 P-35S sequence. Sequence comparison of V3 with the cauliflower mosaic virus genome composed of 8024 bp, accession V00141/J02048 (http://www.ncbi.nlm.nih.gov/nuccore/V00141), showed 24 differences, 20 base pair substitutions, and four deletions, and the matching V00141 sequence is located between positions 7048 and 7435 (**Fig. 3**). The 4883 bp sequence, derived from the cauliflower mosaic virus isolate CabbB-JI sequence[17] that included the P-35S3 region between base positions 396 and 1779, was found suitable for sequence alignment analysis for V3 sequence. The matching sequence was located between positions 1340 and 1727 along CabbB-JI P-35S region (**Fig. 4**). Deletion of four bases and 34 base pair substitutions of transitions or transversion types were identified in V3 sequence as compared with CabbB-JI P-35S promoter sequence. This means there is 90% similarity between V3 sequence and P-35S matching sequence. Further searches in the GenBank of NCBI were carried out to look for matching sequences. The V3 sequence was used as query sequence and was searched in the BLAST database to produce an ordered list of matching sequences. The Blast search of the V3 query sequence performed in April 2013 returned 210 Blast Hits (sequences) representing the best matching subset of sequences based on the Pairwise Local Alignment along with V3 sequence generated in this study. The first 12 sequences showed similar Max scores equal 691 and E-value equals zero; the others showed lower scores and values. It is worth mentioning that the lower the E-value, or the closer it is to zero, the more "significant" the match is (BLAST-FAQs). The results presented in **Figure 5** illustrated alignment analysis of the V3 sequence (lower sequence) with the JQ693018 (upper sequence), representative of the best 12 matching sequences. Apparently, we can find a few mismatches along the V3 sequence at positions: 1, 11, 12, and 336 in comparison with the JQ693018 matching sequence and the similarity was 99%.

Discussion

Our studies started in early 2009 to survey GM maize and to monitor GM elements in food and feed products in Jordan, and since then, the main target has been to use CaMV P-35S to identify the GM maize. The results obtained in 2009 showed that 18% of tested food and feed maize samples were carrying 123 bp DNA fragments,[14] and 25% of detected GM maize samples were carrying an additional DNA fragment of 386 bp (unpublished results). However, further work reported in this study which was carried on during 2011–2012 showed that the incidence of genetically modified maize in Jordan increased significantly. 72.5% of tested maize samples were GMOs, and 69% of identified GM maize products were carriers of second DNA fragments

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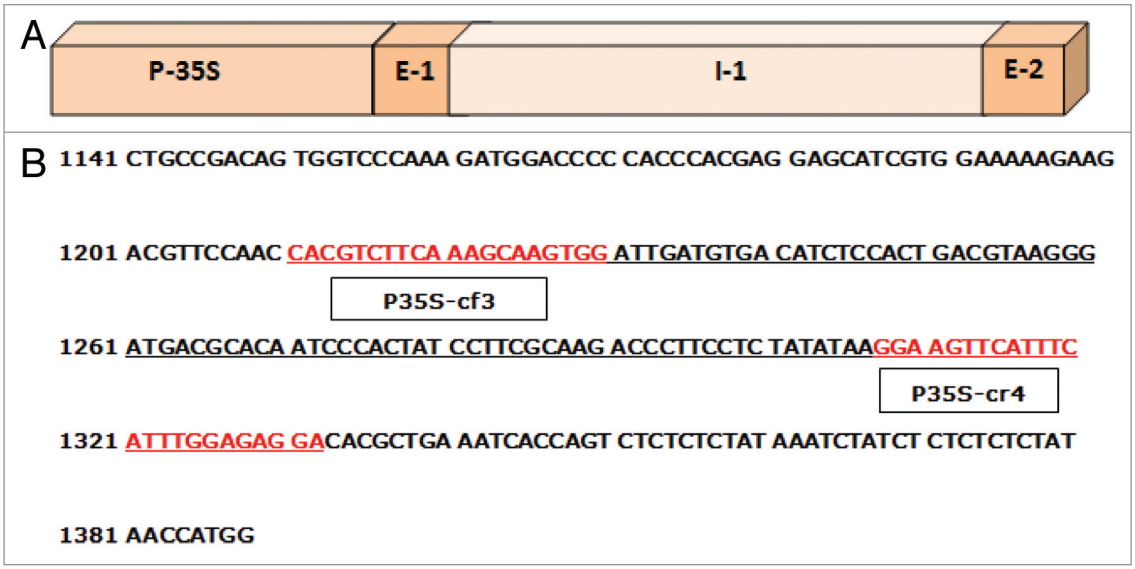

Figure 1. (**A**) Schematic representation of part of the MON810 cassette including P-35S, *hsp70* intron (I-1), and two exons (E-1 and E-2). (**B**) The origin of the 123 bp fragment (underlined); the sequence is PCR amplified by specific primers P-35S-cf3 and P-35S-cr4 shown in red.

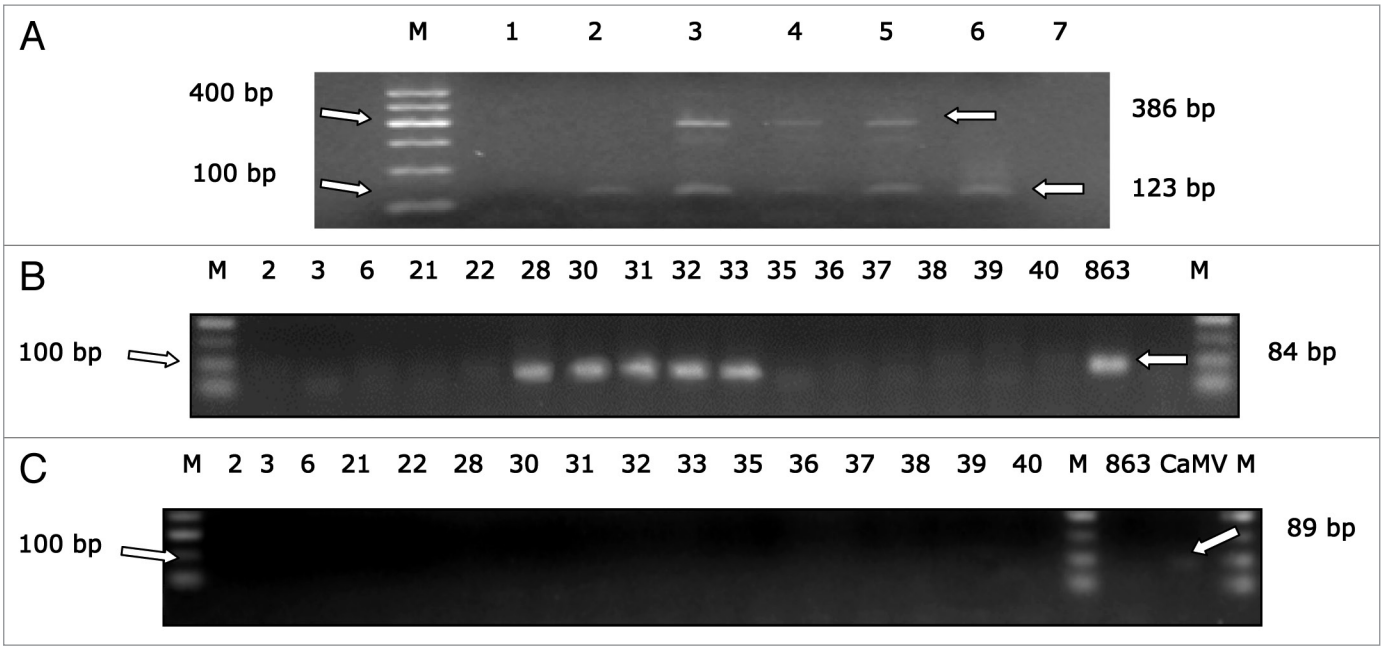

Figure 2. (**A**) Detection of amplified standard 123 bp fragment and V3 sequence of 386 bp, sequences detected by CaMV P-35S (p35S-cf3, F and p35S-cr4, R) in maize products obtained from Jordan's market. M, refers to the 100 bp DNA standard ladder; 1, standard wild type maize (ERM-BF413a); 2, standard GM maize (MON810); 3–7, tested maize products. (**B**) Numbers 2–40 indicate sample numbers, 863 is the reference sample MON863 maize, M refers to the 50 bp DNA standard ladder. (**C**) Numbers 2–40 indicate sample numbers, CaMV indicates mustard sample weakly infected with the cauliflower mosaic virus, M refers to the 50 bp DNA standard ladder.

of 386 bp sequence. This means an increase of 54.5% and 44% in the incidence of GM maize and the newly identified 386 bp sequence. MON810 and MON863 events were identified in the current study, and the V3 element was found in GM maize carrying both genetic events. According to published results and our BLAST search, the two genetic events, MON810 and MON863, had P-35S sequences[16,17] which showed low comparability with V3 sequence. But 90% similarity was observed between V3 sequence and CabbB-JI P-35S.[18] Our recent search in GenBank revealed another sequence of CaMV "V00141.1" that showed 24 mismatches with V3.

These results might suggest that V3 sequence is an alternative form of P-35S sequence. Further, this study showed 99% similarity between the 386 bp sequence and the matching sequences found in several binary plant vectors, of which binary vector locus JQ693018 is one example.[19,20] Only four base pair changes

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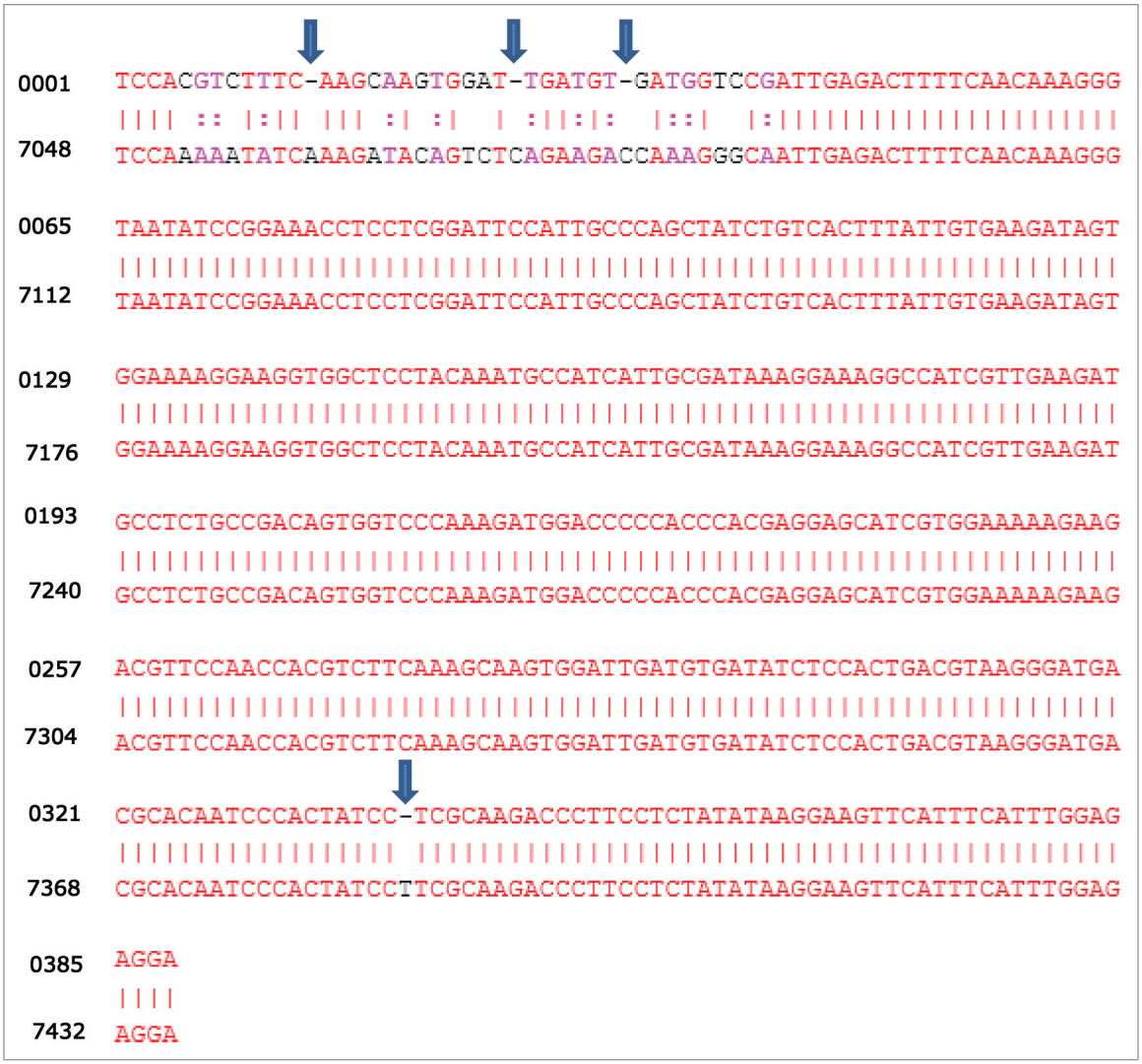

Figure 3. Local alignment using "swalign" of MATLAB for the matching sequences of V3 (upper sequence) and part of cauliflower mosaic virus genome V00141/J02048 (lower sequence). Arrows show the four deletions in V3 sequence, see the text for details.

at positions 1, 11, 12, and 336 were observed along the analyzed region of JQ693018. The mismatches at positions 1, 11, and 12 along V3 sequence were reproducible and were observed in sequencing replicates of V3 fragments which were obtained from several GM maize samples.

Therefore, we suggest that the V3 sequence is an alternative sequence of P-35S, and it is closer to matching the sequence of binary locus JQ693018. At present knowledge there is no evidence about the possible origin of the V3 sequence of 386 bp and how it was introduced in the identified GM maize, taking into account that tested standard MON810, MON863, and blank maize samples did not contain 386 bp sequences. Another argument in this context that should be considered is the close similarity between the V3 sequence and the matching sequence in the binary vector locus JQ693018. These results might give further insight for future theoretical and experimental work to investigate possible molecular mechanisms, which gave rise to the 386 bp sequence. Further efforts, sequencing work, and bioinformatics analysis are required to investigate and explain how that V3 sequence was formed and introduced into the commercial GM maize marketed in Jordan. In conclusion, we showed in this study an alternative form of P-35S in GM maize. The current state of knowledge gives positive proof about the safety of commercially-available GM crops for human consumption, and it is fact that GM crops are the answer for global food and feed demands. Nevertheless, there are concerns about the problems to detect disseminated UGM,[22,23] and this gives the need for thorough monitoring of the GM events.

Materials and Methods

Maize food and feed products

Maize samples (500–1000 g per sample) used for food or feed were obtained during the period from April 2011 and February 2012 from the Department of Animal Wealth Laboratories, Ministry of Agriculture, Food and Drug Testing Administration,

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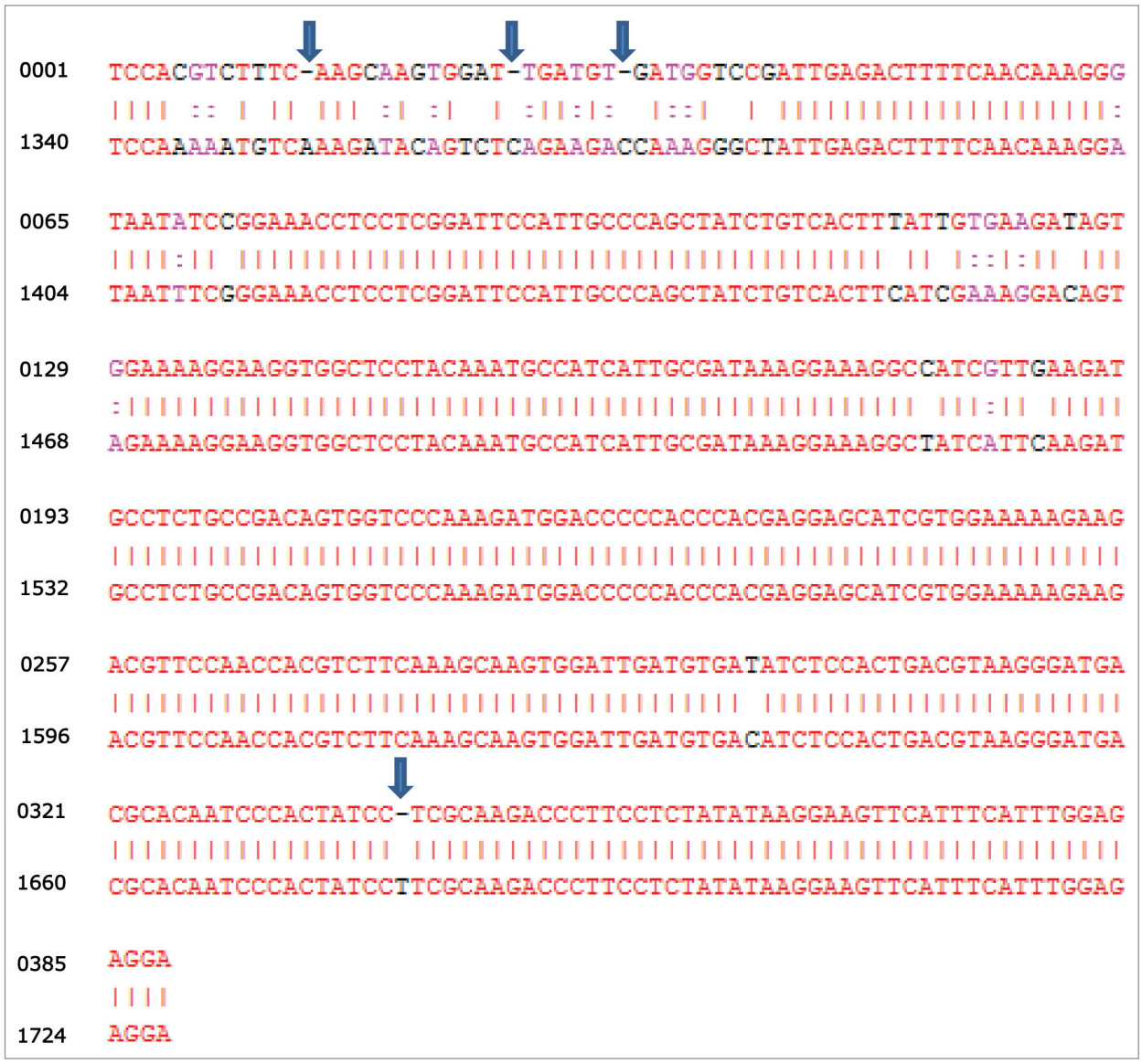

Figure 4. Local alignment using "swalign" of MATLAB for the matching sequences of V3 (upper sequence) and CabbB-JI P-35S region (lower sequence). Arrows show the four deletions in V3 sequence, see the text for details.

Royal Scientific Society Testing Laboratories, and local markets in Amman, Jordan. Standard blank maize (ERM-BF413a) and standard genetically modified maize samples (MON810 and MON863) were purchased from the European Commission, DG JRC, IRMM. Mustard plant infected with CaMV was a kind gift of Biolytix AG.

Genomic DNA extraction and DNA amplifications

Genomic DNA was extracted from 100 mg of homogenous ground samples by using NucleoSpin® Plant II kit from MACHEREY-NAGEL (MN) Switzerland. The extracted DNA was kept at –20 °C until further use. Primers used and the PCR amplifications conditions were described by Van den Eede et al.[11] and Querci et al.[13] The primers were synthesized at Alpha DNA and at Microsynth AG. Each PCR reaction mixture (50 µl final volume) contained: 5 µL of 10 × PCR Buffer, 5 µL of 25 mM $MgCl_2$, 0.25 µL of Taq DNA polymerase from the TopTaq TM PCR kit (Qiagen), 2.5 µL of 16 mM dNTPs (Promega), 1.25 µL of a 20 µM solution of each primer, 32.75 µL nuclease-free water, and 2 µL of extracted DNA (10.7–48.6 ng μL^{-1}). The amplifications were performed in the Applied Biosystem Thermocycler 9902. The amplification products of control and maize samples in parallel with DNA marker ladder of 50 bp and 100 bp (Qiagen) were separated on a 1.5% agarose gel, run with 3 V cm^{-1}, and visualized under UV light after staining with ethidium bromide for molecular size determinations of the DNA fragments.[21]

Sequencing and analysis of the of the 390 bp DNA sequence

The large DNA fragment of 386 bp was extracted and purified from agarose gel by using the PCR clean-up Gel extraction according to the manufacturer's protocol (NucleoSpin® Extract ll Kit purchased from MACHEREY-NAGEL). The extracted DNA was kept at –70 °C till further use. Frozen samples of

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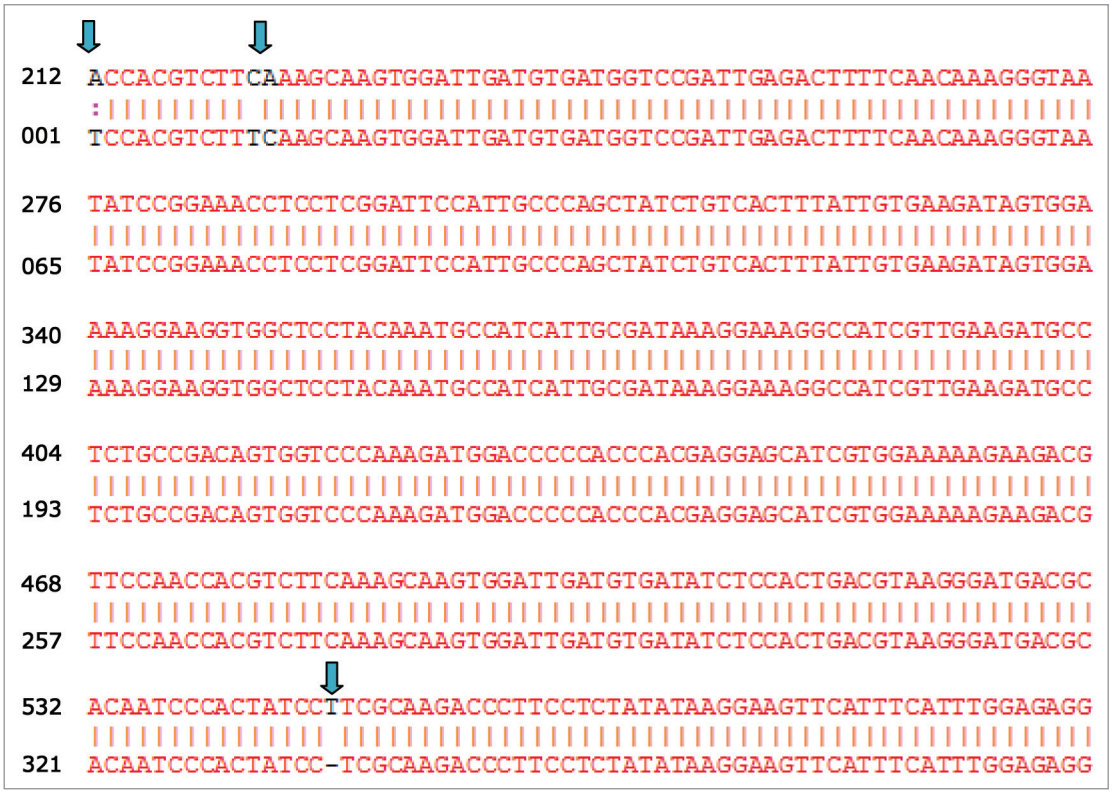

Figure 5. Results of local alignment using "swalign" of MATLAB for the matching sequence of JQ693018 (upper sequence) when compared with V3 sequence (lower sequence). Arrows show changes in the V3 sequence, three base pair substitutions and one deletion.

extracted DNA were then sent to Synergene Biotech GmbH (Schlieren) for DNA sequencing.

The Basic Local Alignment Search Tool (BLAST) of the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was searched for the matching sequences of 386 bp sequence. Further, MATLAB Bioinformatics toolbox 2012 was used to analyze DNA sequences of loci obtained in the BLAST search for the closest DNA sequences identified in BLAST database which are more likely matching 386 bp sequence.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgments

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