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Single Strand Conformational Polymorphism (SSCP) Strain Analysis and Partial Nucleotide Sequence of Maize Yellow Stripe Virus (MYSV) Segment 3 Genomic RNA

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The ambisense RNA3 of maize yellow stripe virus (MYSV) was detected in MYSV- infected maize tissues using a rapid, accurate and simple Immunocaptured reverse transcription polymerase chain reaction (IC/RT-PCR) method All MYSV infected maize tissues in this investigation were reverse transcribed and subsequently amplified *in vitro* from field plants. A newly designed specific non-degenerate primers based on the nucleotide sequence of the MYSV-RNA3 have been used. The primers F3: 5`-CTCAGCCATAGCCACATTACTCAGT-3` and R4: 5`-CTTTCAGGGTCAATTAGTTAGAG-3` amplified a DNA copy of approx. 720 base pairs. The IC/RT-PCR products were subsequently cloned in thimidinylated pGEM-T-easy vector for cDNA- DIG- labelling probe. The IC/RT-PCR technique is a powerful method for rapid detection of MYSV virus as well as it has a greater sensitivity and less laborious that it was unnecessary to extract the viral RNA from the particles for reverse transcription. The Single Strand Conformational polymorphism (SSCP) technique showed that each RT-PCR product for the given regions of MYSV with different symptoms represented a population structure with one predominant haplotype. To determine more accurately the population structure, we cloned RT-PCR product for only one MYSV infected sample. Seven clones were amplified by PCR and the corresponding PCR products were used for SSCP analysis. Almost one haplotype was clearly predominant that all clones displaced an identical SSCP pattern to that of the original RT-PCR product. Our MYSV isolate showed 97- 98 % identity with the published MYSV Maize yellow stripe virus segment 3 genomic RNA. This study proved that, MYSV-RNA3 of studied isolates that showed different symptoms (coarse stripe, fine stripe and chlorotic stunt) are the same of that one previously sequenced under Accession (No. AJ969414).

Keywords: hybridization, IC/RT-PCR, MYSV, sequencing, southern blot and SSCP.

The Tenuiviruses are most unusual type of viruses, they are pathogens of plants of the Gramineae family. Six viruses belong to this group as follow: Rice stripe virus (RStv), Maize stripe virus (MStv), Rice hoja blanca virus (RHBV), Rice grassy stunt virus (RGSV), Echinochloa hoja blanca virus (EHBV) and Urochloa hoja blanca (UHBV) (Gingery, 1988 and Falk and Tsai, 1998) as well as Maize yellow stripe virus (MYSV) was suggested as a tentative member of the Tenuivirus genus (Ammar *et al.*, 1984). Tenuiviruses are transmitted by delphacid planthoppers

but, MYSV is transmitted by a cicadellid leafhopper, *Cicadulina chinai* (Gingery, 1988 and Ammar *et al.*, 1989). Tenuiviruses are non-enveloped plant viruses, with possibly a negative ssRNA genome (Francki *et al.*, 1991).They contain four or five single stranded RNA segments , and the genetic information on RNA2, RNA3 and RNA4 is arranged in an ambisense coding fashion. For all members of this family, the 5` and 3` ends of the RNA segments are complementary, potentially forming a panhandle structure and this probably explains the circularity that can be observed in the individual RNPs in the electron microscope (Ramirez and Haenni, 1994; Falk, and Tsai, 1998). The 5` and 3` - terminal nucleotides are conserved between the different RNA segments of a given virus and also between the segments of viruses of the same genus. Interestingly, the eight conserved terminal nucleotides of the genome segments of Tenuiviruses are identical to the terminal sequence of those of phlebovirus (Ramireze and Haenni, 1994). It was therefore of importance to design a maize yellow stripe virus (MYSV) specific primers for using in RT-PCR specific amplification. In this study we performed IC/ RT-PCR, SSCP analysis and nucleotide sequence for samples showing difference in type and intensity of symptoms. The partial nucleotide sequence data have been submitted to the Genebank nucleotide sequence database and have been assigned the accession No. GQ289374.

Materials and Methods

Virus source:

Incidence of MYSV was studied in maize fields in ten Governorates i.e. Lower Egypt (Qalyobia, Menofia, Sharkia, Gharbia, Ismailia and Kafr El-Sheikh) and Middle Egypt (Giza, Fayoum, Beni Suef and Minia) during 2004 and 2005 growing seasons. Summer (first week of July till end of August) and Nily (mid of September till end of October) were the periods of field inspection. Surveyed fields were selected randomly in each Governorate (Abdel-Kader, *et al.*, 2007).

Maize plants of different ages were estimated by visual examination for MYSV symptoms. Leaf samples showing typical symptom of MYSV and those gave positive reaction in ELISA were collected from Giza governorate and used for the following experiments.

One step IC/RT-PCR assay:

Thermo-resistant polypropylene PCR tubes were coated with 50 µl antiserum raised specifically against MYSV virus (1/8000 dilution, kindly provided by Dr. Amal Mahmoud) and incubated for overnight at 4°C. The tested sample was carried out as described for ELISA in a dilution of 1/10 by grinding 0.3gm of infected tissue in 3 ml sample extraction buffer, (4mM $NaHPO_4$, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, PVP, pH 7.4). Antigen trapping on the inner surface of the PCR tubes occurred overnight at 4°C. The tubes were then washed twice with PBS-T, and once with deionized water. Extra care was taken to avoid cross-contamination between wells. The one step RT- PCR reaction was carried out in 50µl total volume of amplification mixture to reach the final concentrations of: 10mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% (W/V) gelatin, 4mM $MgCl_2$, 200 µM dNTPs, 0.1 µM each

primer, the virus sense primer **F3:** 5`-CTCAGCCATAGCCACATTACTCAGT -3` and virus antisense primer **R4:** 5`-CTTTCAGGGTCAATTAGTTAGAG -3`, 2.5 units of the thermostable Taq DNA polymerase (Promega), 40 units ribonuclease inhibitor (Roach), 200 units of M-MLV reverse transcriptase (Promega) and incubating 45 min at 42°C. The amplification proceeded in the thermocycler (Uno) at 94°C for 5 min, and through 35 cycles of 94°C for 30 s and 49°C for 30s and 72°C for 2min, with a final step at 72°C for 10 min. Aliquot (5µl) of PCR amplified cDNA and 2µl of loading dye (0.02% bromophenol blue, 0.02% xylene cyanol, 20% sucrose) were directly analyzed by gel electrophoresis as described by Sambrook *et al.* (1989).

cDNA probe labeling:

cDNA probe was labeled with digoxigenin, using PCR- Dig labeling technique according to Roche, Boehringer Mannheim crop., Indianapolis, IN, USA, protocol. The cDNA-PCR product was used as a template for the polymerase chain reaction using PCR Dig Labeled dNTPs mixture. The PCR reaction was performed in 50 µl total volume containing 10mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% (W/V) gelatin, 4mM MgCl2, 200 µM dNTPs, 0.2 µM each primer, the virus sense (F3) and antisense primer (R4) and 2.5 units of the thermostable Taq DNA polymerase (Promega). The amplification was proceeded in the thermocycler as previously described. Southern blot hybridization was carried out according to Southern (1975).

Cloning and Nucleotide Sequencing:

Amplified cDNA- RNA3 core region fragment of the expected size (720bp) of MYSV was directly cloned into linearized and thymidylated PGEM-T-easy plasmid vector. The construct was transformed into *Escherichi coli* JM109 competent cells (Promega) according to Sambrook *et al.* (1989). Recombinant colonies were screened and tested for the presence of the MYSV - RNA3 core region cDNA fragment using the PCR technique followed by southern blot hybridization using non radioactive cDNA Dig labeled probe specific for MYSV RNA 3 core region (data not shown). Briefly, two clones (clone No. 4 and 5) carrying MYSV- DNA fragment (720 bp) which represents the core region of RNA3 (segment 3) were sequenced in one direction using the M13-21 forward primer. The sequence was carried out using ABI PRISM model 310 versions 3.4 semi-adaptive version 3.2 at Gene Link DNA sequencing service, New York, USA. Sequence analysis was performed using Bio Edit program version 5.0.6.

SSCP analysis:

SSCP analysis was performed as previously described by (Amin, 1999; Sabek *et al.*, 1999 and Rubio *et al.*, 1996). 2 µl of the RT-PCR or PCR product was added to 9 µl of denaturing solution (95% formamide, 20mM EDTA, PH8.0, 0.1% bromophenol blue) and boiled for 5 min followed by chilling on ice for 5 min. Samples were resolved by electrophoresis in 8% polyacrylamide gels at 200 volts at 4°C for 3 hr. SSCP products were visualized using silver staining (Teresa *et al.*, 1998).

Results and Discussion

The naturally infected maize plants showing Leaf stripping, yellowing. Stunting and stem epinasty symptoms (Fig.1) were collected. The virus was transmitted by *Cicadulina chinai* vector in the greenhouse. Fig. (2) illustrate the symptoms on Maize caused by MYSV in greenhouse (fine stripe, coarse stripe and chlorotic stunt).

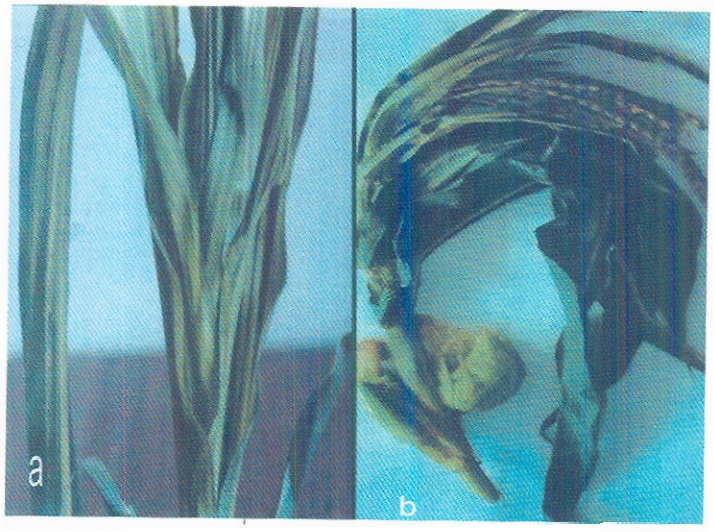

Fig. 1. Natural-MYSV infection: Symptoms in the fields on corn. (a) Leaf stripping, yellowing. (b) Stunting and stem epinasty.

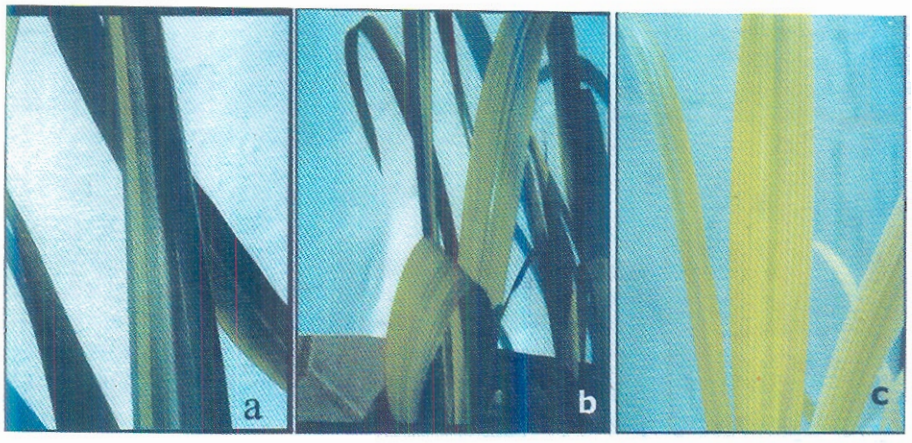

Fig. 2. Symptoms on Maize caused by vector-transmitted MYSV in the greenhouse. a: fine stripe, b: coarse stripe, c: chlorotic stunt.

For all of Tenuiviruses, the 5` and 3` ends of the RNA segments are complementary, potentially forming a panhandle structure and they need a long time of denaturation in presence of high denatured substance. The 5` and the 3` terminal nucleotides are conserved between the different RNA segments of a given virus and also between the segments of viruses of the same genus (Ramirez and Haenni, 1994). The previous studies reported that tenuivirus-specific primer, a 23-nt primer 5' CCCGGGCGGCCGCACACAAAGTC 3' designed by de Miranda *et al.* (1994), was used to amplify the extracted RNAs (from infected plant) by RT-PCR. Also, authors used methylmercuric hydroxide (CH3HgOH) in the previous method used by Mahmoud (2001) which is not available for us in the present work. It was therefore of importance to design a newly specific non-degenerate primers for maize yellow stripe virus (MYSV) based on the nucleotide sequence of the ambisense MYSV-RNA3 for using in RT-PCR specific amplification. Also, IC/RT-PCR assay was used to overcome this problem.

In this study, newly designed specific primers (F3 and R4) based on the nucleotide sequence of the MYSV- RNA3 have been used in Immunocaptured / Reverse Transcriptional-Polymerase Chain Reaction (IC/RT-PCR) assay under more realistic conditions for the first time and successfully amplify a DNA copy of approximately 720 base pairs. Extraction of total RNA from samples was unnecessary because bands were observed whereas the reverse transcriptase and PCR reaction mixtures had been applied directly to the particles in a single closed – tube, this result is similar to that reported (Nolasco *et al.*, 1993 and Mehta *et al.*, 1997). The IC/RT-PCR products amplified from MYSV infected maize plant using the specific primers showed an intense DNA fragment at the expected size regardless of the amount of immunoglobulin used to coat the Eppendorf tubes.

Detection of Maize Yellow Stripe Virus (MYSV) by IC/RT-PCR was performed from five MYSV infected samples (from Sorghum and Maize plants as two different hosts) showing different symptoms and reacted positively with ELISA using MYSV antiserum. The 5 tested MYSV infected samples, each produced a DNA fragment of the same size after IC/RT-PCR regardless the visual field symptoms All amplification products gave the expected size [approximately 720 nucleotides (nts)], as shown in Fig. (3 A). Detection of Maize Yellow Stripe Virus (MYSV) by IC/RT-PCR was performed from three MYSV infected sugarcane plants showing ELISA negative. No amplified product was observed from three MYSV infected sugarcane plants in compare with the PCR product from infected three maize plants with different symptoms (data not shown)

The main advantages of IC/RT-PCR of the system are the high sensitivity obtained, the simplicity, the low risk of contamination and the easy establishment of adequate conditions. The method has been successfully applied to the detection of MYSV using the specific antiserum. It is clear that the IC/RT-PCR assay has a greater sensitivity and useful in overcoming many difficulties encountered with serological methods, such as low antigen titer, availability of antibodies, and cross-reactivity of antibodies with heterologous antigens.

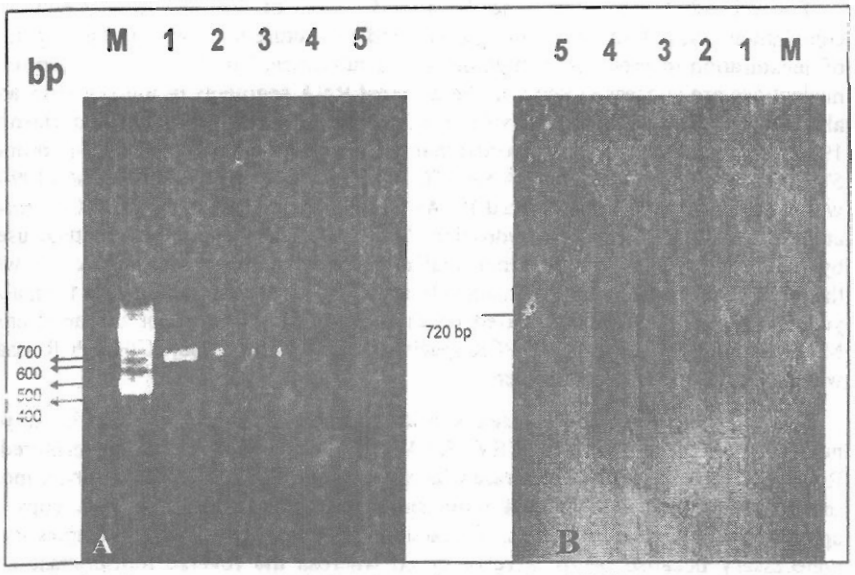

Fig. 3. A: 1% agarose gel electrophoresis analysis of IC/RT-PCR amplification products from MYSV infected samples showing different symptoms. M: 100bp DNA ladder (Promega), lane1: sample showing coarse stripe on Sorghum., Lane 2: coarse stripe with yellowing, lane 3: fine stripe, lane 4: coarse stripe on maize, lane 5: chlorotic stunt on maize B: Southern blot using the cDNA dig labeled probe.

For more confirmation and accurate detection of MYSV in tested samples, the MYSV cDNA was used for creation of Dig labeled- cDNA probe, specific for MYSV-RNA3 core region, for Southern blot hybridization. The MYSV cDNA probe was clearly hybridized with the PCR products that amplified using F3 and R4 primers from MYSV –infected plants and showed sharp signals having the same intensity (Fig. 3 B).

The introduction of digoxigenin (Dig)-labeled DNA and RNA probes (Màs *et al.*, 1993; Dietzgen *et al.*, 1994 and Harper and Creamer, 1995) has eased the need for RNA purification and for radioactive probes, which have short life and present safety hazards. The dig-labeled probes combined with colorimetric visualization were capable of detecting different types of viruses with a high degree of specificity. However, our results revealed that the Dig-probe with nylon membrane was sensitive; in addition, the results were obtained faster and the probe was used without safety concern and could be stored without loss of activity, this came in agreement with Lion and Haas (1990) and Narvàez *et al.* (2000).

Southern blot hybridization assay showed a high sensitivity than agarose gel electrophoresis analysis as reported by Hadidi and Yang (1990). The MYSV-cDNA specific probe, Southern blot hybridization were used to establish the authenticity and specificity of the RT-PCR products.

Several studies used different assays to explain the differentiation of MYSV symptoms (fine, coarse stripe and chlorotic stunt) whether because the presence of different strains or due to symptoms development (Ammar *et al.*, 1989; Ammar *et al*, 1990a; Ammar *et al.*,1990b; Mahmoud, 1996; Aboul-Ata *et al.*, 1996 and Mahmoud, 2001). Single stranded confirmation polymorphism (SSCP) analysis was used to confirm strain recognition. Only one MYSV genomic region of five samples showing difference in type (coarse stripe, fine stripe and chlorotic stunt) and intense (severe and mild) of the symptoms was used for SSCP. All SSCP patterns showed the same SSCP profile patterns indicating that the RT-PCR products for the given region represented a population structure with one predominant haplotype (Fig. 4). To determine more accurately the population structure we cloned RT-PCR product for only one sample showing two different symptoms. Seven clones were amplified by PCR and the corresponding PCR were used for SSCP analysis. Almost one haplotype was clearly predominant and displaced an identical SSCP pattern to that of the original RT-PCR product (data not shown). Haplotype frequency determined by SSCP analysis was used to estimate the genetic diversity showed that there is no diversity between either inter-isolate or intra-isolate for the only examined region but may be they could show a difference if we used SSCP for another different regions in all the genomic RNAs. This result demonstrated that the different symptoms of MYSV (fine-stripe, coarse-stripe and chlorotic stunt) which, usually are produced on different leaves of the same plant, could be represent different stages of MYSV infection in maize plants rather than different pathogens or isolates which is in agreement with the previous studies.

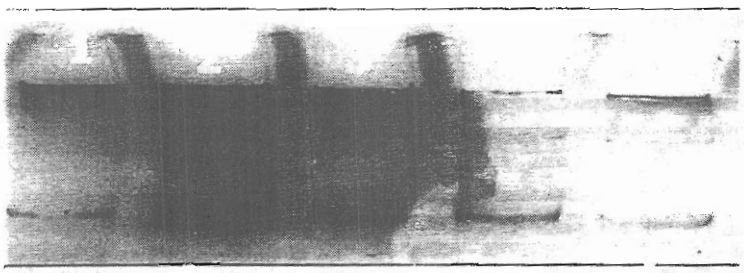

Fig. 4. Single strand conformational polymorphism (SSCP) analysis of RT-PCR product from five infected maize plants showing different MYSV symptoms lanel: sample showing coarse stripe on Sorghum. Lane 2: coarse stripe with yellowing on maize, lane 3: fine stripe on maize, lane 4: coarse stripe on maize, lane 5: chlorotic stunt on maize.

In the present study, cloning and sequencing of MYSV-RNA3 was used to determine and characterize viral RNA. The 720 bp amplified product of the isolate MYSV was legated into the pGEM-T-easy vector and cloned. Only two clones of MYSV isolate was sequenced. The partial nucleotide sequence of RNA3 (segment 3) of Maize yellow stripe virus (MYSV) was determined and revealing 720

nucleotides as expected as shown in Fig. (5) with deduced 240 amino acids. Comparison of the MYSV –RNA3 core region with those of another tenuivirus isolates revealed no significant similarity with most tenuivirus isolates ((Rice stunt (RSV), maize stripe virus (MSV), and rice hoga blanka virus (RHBV)) (Katutani *et al.*, 1990 & 1991 and Huiet *et al.*, 1991). The nucleotide sequence alignment Fig.(5) of Our MYSV isolate (MYSV clone 4 and 5) showed 97- 98 % identity with the nucleotide sequence of MYSV Maize yellow stripe virus segment 3, partial sequence, genomic RNA with accession No. AJ969414 (Ammar *et al.*, 2007).

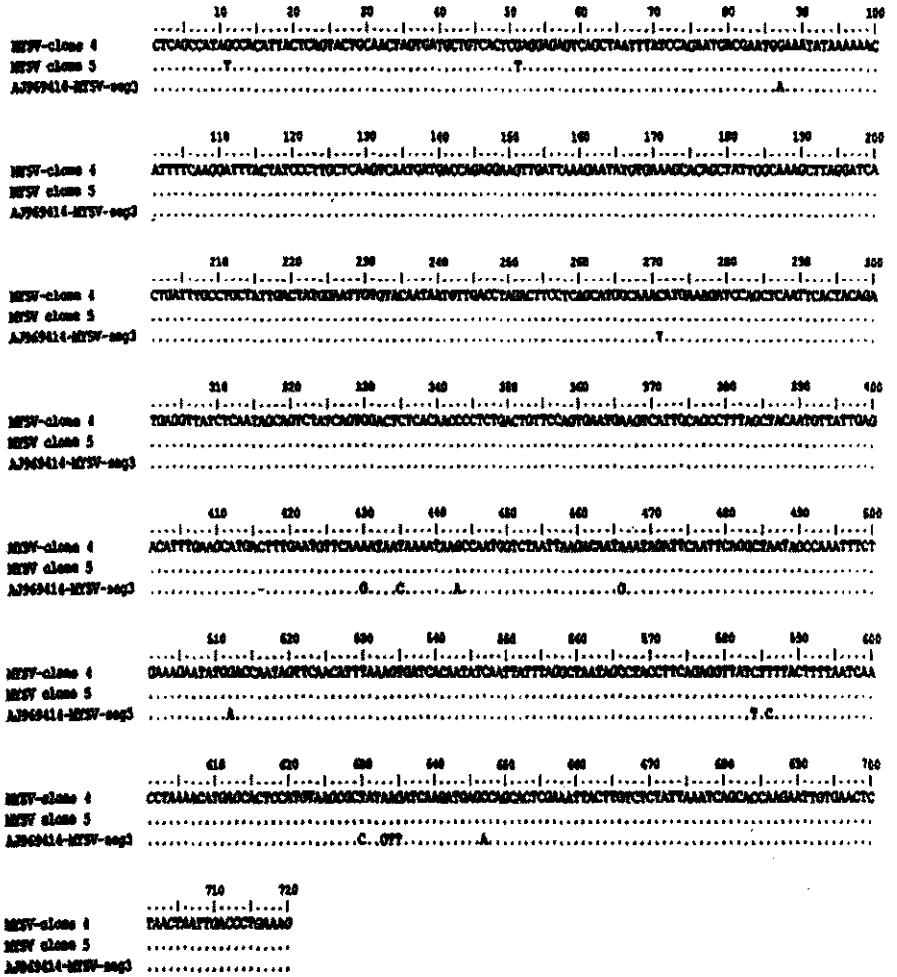

Fig. 5. Nucleotide sequence alignment of cloned MYSV- RNA3 -core region (clone 4 & 5) with another MYSV published sequence.

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تحليل الأنماط المتحورة للخيط المفرد للحامض النووى (SSCP) والتتابع النيكليوتيدى الجزئى من (RNA3) لفيروس التخطط الأصفر فى الذره (MYSV)

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لقد تم بنجاح الكشف عن الحامض النووى(ambisense RNA3) لفيروس التخطط الأصفر فى الذره MYSV فى الأنسجه المصابه بالفيروس بأستخدام تقنيه تفاعل البلمرة المتسلسل والتى تعتمد على ادمصاص الأجسام المضادة المتخصصه و التى أثبتت سرعتها و دقتها فى الكشف عن الفيروس. تصميم بادئات حديثه متخصصة اعتمادا على التتابع البيكليوتيدى المنشور مسبقا وقد أنتجت هذه التقنيه حزمة من الحمض النووى المتخصصة لهذا الفيروس حجمها حوالى ٧٢٠ قاعدة نيوكلوتيدية بأستخدام البادئ:
F3: 5`-CTCAGCCATAGCCACATTACTCAGT-3`و R4: 5`-CTTTCAGGGTCAATTAGTTAGAG-3`

تم عمل فصل واكثار الناتج من اختبارانزيم البلمرهPCR ، باستخدام اختبار الكلونة عن طريق استخدام الناقل pGEM-T. تم عمل مجس متخصص لهذا الفيروس يساعد فى عملية الكشف عن الفيروس . تم عمل تحليل الأنماط المتحورة للخيط المفرد للحامض النووى (SSCP) لمعرفة هل هناك اختلاف بين الاعراض المختلفة المميزة لمرض التخطط الاصفر فى الذرة وبين التركيب الوراثى لها، لما يتمتع به هذا الاختبار من قدرة عالية على التفريق بين العزلات المختلفة هذا بالاضافة الى سهولة الاجراء ،ولقد اظهرت النتائج انه لا يوجد اختلاف بينها وانما يرجع هذا الأختلاف فى الاعراض الى تطور المرض. تم عمل تحليل لتتابع قواعد الحمض النووى الناتج من تفاعل انزيم البلمرة المتسلسل ولقد اظهرت النتائج ان هناك تشابه كبير بين هذه العزلة والعزلة المنشوره فى بنك الجينات لهذا الفيروس بنسبة ٩٧-٩٨ %.