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MOST GRACIOUS, MOST MERCIFUL

J. King Saud Univ., Vol. 22, *Agric. Sci.* (1), pp. 1-7, Riyadh (2010/1431H.)

Biological, Serological and Molecular Identification of *Lettuce mosaic virus* from Field Infected Lettuce in Saudi Arabia

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(Received 21/6/1430H.; accepted for publication 24/10 /1430H.)

Keywords: DAS-ELISA, Host range, LMV, RT-PCR, Sequence.

Abstract. Mosaic symptoms were observed on field grown lettuce (*Lactuca sativa* Linn.) plants at Al-Hair area in Riyadh region. Double antibody sandwish-enzyme linked immunosorbent assay (DAS-ELISA) was used to detect the causal virus in the infected sap using antisera against some lettuce viruses. Polyclonal antibodies specific to *Lettuce mosaic virus* (LMV) reacted positively while no reaction was observed with any of the other tested viruses including *Alfalfa mosaic virus* (AMV), *Cucumber mosaic virus* (CMV), *Turnip mosaic virus* (TuMV) and *Tomato spotted wilt virus* (TSWV). As a result of inoculation with crude sap extracted from diseased lettuce leaves *L. sativa, G. globosa*, *Chenopodium amaranticolor* Cost and Reyn, and *C. quinoa* plants showed some virus-like symptoms while *D.* stramonium, *N. glutinosa* and *N. tabacum* showed no symptoms. Reverse transcription-Polymerase chain reaction assay (RT-PCR) was used for the detection and identification of the virus from nucleic acid extracts of infected lettuce plants using specific primer for the detection of the 3` end of the NIb gene together with the 5` end of the CP gene (region II). The viral DNA amplified product was approximately 346 bp as estimated by agarose gel electrophoresis. The 346 bp DNA fragment from LMV isolate was purified and sequenced. The comparative nucleotide sequence analysis showed 92.4 to 98.8% similarity with LMV isolates recovered from Brazil, France, China, Audran, AF199 and strain E. These results may suggest that the nucleotide sequence homology between the Saudi Arabian isolate, and the French, Chinese and Brazilian isolates are closely related. This is the first report of LMV on lettuce in Saudi Arabia.

Introduction

Lettuce is one of the most important and popular leafy vegetables that is mainly consumed as salad in the Kingdom of Saudi Arabia. It is grown in almost all agricultural regions both in the open field and in the greenhouses. Many viruses such as *Alfalfa mosaic virus* (AMV), *Broad bean wilt virus 1* (BBWV-1), *Beet western yellows virus* (BWYV), *Cauliflower mosaic virus* (CaMV), *Cucumber mosaic virus* (CMV), *Lettuce mosaic virus* (LMV), *Pea seed-borne mosaic virus* (PSbMV), *Turnip mosaic virus* (TuMV) and *Tomato spotted wilt virus* (TSWV) were reported to infect lettuce (Davis *et al.*, 1997, Moreno *et al*., 2005). *Lettuce mosaic virus* (LMV) is one of the viruses that seriously infect this crop and causes outbreaks (Raid *et al.*, 1996) and substantial yield losses (Patterson *et al.*, 1986). LMV belongs to the genus Potyvirus within the family *Potyviridae* (Barnett *et al*., 1995; Tomlinson, 1964). The genomic organization of LMV is typical of potyviruses, with a single positive-sense genomic RNA of 10.080 nucleotides (nt) encapsidated as flexuous rods. The viral genomic RNA has a virally encoded protein linked covalently at its 5´end, a poly-A tail at its 3´end, and contains a single open reading frame (ORF) which encodes a large polyprotein with 3255 amino acids (Revers *et al*., 1997b Shukla *et al*., 1994). LMV is transmitted by aphids in a non-circulative manner, and is seed-borne in lettuce (Zerbini *et al*., 1995; Dinant and Lot, 1992; Ryder, 1973; Tomlinson, 1970). LMV is one of the most important pathogens of lettuce which causes one of the major virus diseases of the lettuce crop world-wide (Tomlinson, 1970). Symptoms observed in infected plants included dwarfing, poor heading, mottling, and vein clearing (Dinant and Lot, 1992). LMV isolates show a large array of biological

variability, with variations in the severity of symptoms induced, seed transmissibility, and ability to overcome the three resistance genes described in lettuce cultivars (Pink *et al*., 1992a, b). The complete nucleotide sequences of the genomes of two LMV isolates, LMV-0 (EMBL #X97704) and LMV-E (#X97705), have been determined (Revers *et al*., 1997b). Two of these genes, *mo11* and *mo12*, are recessive and are believed to be either closely linked or allelic (Dinant and Lot, 1992; Lot and Deogratias, 1991; Pink *et al*., 1992b). The control of lettuce mosaic relies on prophylactic measures such as the elimination of contaminated commercial seed lots (Tomlinson, 1962) and on genetic resistance (Dinant and Lot, 1992). The mo1 alleles also provide control of seed transmission because, even in the tolerance cases, LMV accumulates in the mother plants containing mo11 or mo12, but does not access the embryo (Dinant and Lot, 1992; Pink *et al*., 1992b; Bos *et al*., 1994). The present study aims at the identification of a disease agent associated with mosaic in lettuce plants in Riyadh region, Saudi Arabia using biological, serological and molecular techniques.

Material and Methods

Source of virus isolate and detection

Twenty leaf samples were collected from naturally infected lettuce plants showing mosaic symptoms in Al-Hair area, Riyadh region. The detection of lettuce viruses in these samples was carried out serologically using DAS-ELISA procedure. ELISA kit's for five lettuce viruses were purchased from Agdia (Agdia Inc., 30380 Country Road, Elkhart, Indiana 46514 USA). Briefly, ELISA procedures were applied in the same way explained by the manufacturing company, which were not remarkably different from those indicated in the original procedure by Clark and Adams (1977). Each of the four micro titer plates was coated with antibodies against AMV, CMV, TuMV, TSWV and LMV after being diluted with the coating buffer. Subsequent to incubation and washing, aliquots of 100 ul of each sample (which was extracted in the extraction buffer) were added in two wells of each plate. 100 ul of the proper dilutions of the relevant antibody-alkaline phosphatase conjugate were dispensed in the wells of each plate subsequent to washing plates from samples sap. P-nitrophenyl phosphate solution was then added in the wells of each plate after washing from the conjugate solution. The plates were incubated for 1 hour. The reaction was then stopped using 3M NaOH, and the absorbance values were measured at 405 nm by minireader II, Dynatech product.

The leaf tissues of a representative isolate were homogenized in a prechilled mortar and pestle with 0.01 M phosphate buffer, pH 7.2, containing 0.1% sodium sulphite (Na_2SO_3) using an extraction ratio 1:4 (w/v). Inoculum was used to inoculate *Lactuca sativa* Linn., *Chenopodium amaranticolor* Cost and Reyn, *C. quinoa* L., *Gomphrena globosa* L., *N. tabaccum, N. glutinosa,* and *D. stamonium* plants previously dusted with carborundum (600 mesh) using a pad of cheesecloth or the index finger. These plants known to react characteristically with LMV (Christie *et al*., 1968; Nelson and Mckittric, 1969; Tomlinson, 1970; Fegla *et al*., 1990) included *Chenopodium quinoa L., C. amaranticolor* Costa and Ryen, *Gomphrena globosa* L., and *L. sativa*. Plants were maintained in the greenhouse and observed for symptom development.

RT-PCR amplification and nucleotide sequencing of LMV

Extracts and RNA purification from 30 mg of infected and healthy leaf samples were prepared using SV–Total RNA Isolation System according to the manufacturer's protocol (Promega, CA, USA). RT-PCR was done with one step RT-PCR using the QIAGEN One Step RT-PCR Kit. The used oligonucleotide primers designed according to Revers *et al*. (1997a) were used to prime the amplification of the 3` end of the NIb gene together with the 5` end of the CP gene (region II) (Fig. 1, courtesy of Revers *et al*., 1997a). The sequencing primers were P3: 5`- ATT CGA AAA TTY AAR TGG TG-3` position 8825 to 8848 and P4: 5`- GCG TTB ATG TCG TCG TCY TT-3 position 9152 -9171 (R=A and G; Y=C and T; B=C, G and T). The reaction was set up according to the manufacturer's recommendations. RT-PCR reaction mixture was amplified using the following cycling parameters: hold at 50°C for 30 minutes (RT step), hold at 95°C for 15 minutes (hot start to PCR), then subjected to one cycle of amplification: 92°C for 20 s, 42°C for 20 s and 72°C for 40 s, 35 cycles of amplification: 92°C for 20 s, 56°C for 20 s and 72°C for 40 s, and a final incubation at 72°C for 7 min. For electrophoresis analysis, aliquots of 10 ul each PCR amplified DNA products were mixed with gel loading buffer. Separation was done on a 1% agarose gel in 1xTBE buffer (1x = 89 mM Tris, 89 mM borate, and 2 mM EDTA, pH 8.3). DNA was stained with ethidium bromide added to the gel at a concentration of 0.5 ug/ml. DNA was visualized on a UV transilluminator and photographed using DNA

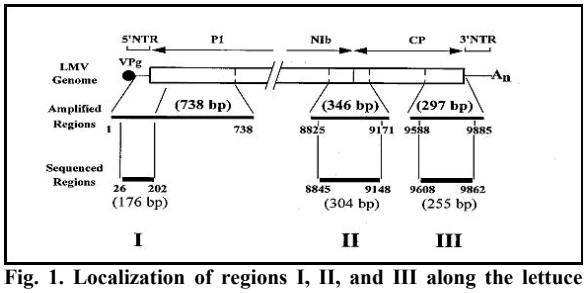

Fig. 1. Localization of regions I, II, and III along the lettuce mosaic virus (LMV) genome. The size in base pairs of the amplified (middle line) or sequenced (bottom line) regions are given in parentheses. The positions of the fragments along the LMV genome are given. The LMV genomic regions in which the studied fragments are included are labeled at the top 5-NTR (5-nontranslated region), P1, NIb, coat protein (CP), and 3-NTR (3-nontranslated region).

documentation gel analysis. 50 bp PCR marker (Promega) was used to determine the size of RT-PCR amplified cDNA products.

An amplified DNA fragment of expected size (346 bp) of the 3` end of the NIb gene together with the 5` end of the CP gene (region II) were electrophoresed in 1% TAE buffer (40 mM Tris, 40 mM acetate, and 1 mM EDTA). Bands of the expected size were excised and recovered with a Wizard PCR clean up kit (Promega). The nucleotide sequence of this LMV isolated gene was carried out in one direction with the specific complementary primer at King Faisal Specialist Hospital & Research Center, Biological and Medical Research Department, Riyadh, Kingdom of Saudi Arabia using AB3730xl DNA Analyzer, from Applied Biosystem-HITACHI. Sequence analyses were performed and the homolog tree analyses were done using DNAMAN trial version 5.2.10 program (Lynnon BioSoft., Quebec, Canada, www.lynnon.com). The following GenBank accession numbers for different LMV isolates were used in the comparison: AF418578, AF525082, AF525083, AJ278854, AJ297630, AJ306288, AJ488153, AY372280, AY167756, AY156088, AY12908, EF423619, Z78225, Z78224, Z78223, X97705, X97704, and X65652.

Results and Discussion

Virus symptoms and detection

Under field conditions, naturally infected lettuce plants showed typical mosaic symptoms accompanied with irregular growth of leaves and occasionally veinal necrosis and bronzing (Fig. 2A). Infected plant failed to form heads. To detect and identify the causal virus (es) associated with those symptoms developed on lettuce plants grown under field conditions, several samples of symptomatic plants were collected from Al-Hair fields, Riyadh region, extracted and serologically tested using DAS-ELISA. LMV antiserum reacted positively with the tested extracts while no reaction was detected with any of the other antisera tested against AMV, CMV, TuMV or TSWV. ELISA is considered positive when the absorbance of the tested sample is at least double the absorbance of the control.

Mechanically inoculated lettuce plants developed symptoms similar to those found on naturally infected ones. Reaction on inoculated diagnostic host leaves indicated the probability of LMV presence. The virus induced systemic yellow mottling, mosaic and leaf distortion symptoms on inoculated lettuce plants (Fig. 2C). The virus induced necrotic local lesions, usually with reddish margins on inoculated leaves of *C. amaranticolor* 10-13 days post inoculation, followed by systemic yellow veinal flecks 5-7 days later (Fig. 2B). Local lesions but without reddish margins appeared on inoculated leaves of *C. quinoa*, 10-13 days post inoculation (DPI) followed by conspicuous systemic vein symptoms with twisting and stunting of apical leaves. *G. globosa* reacted 8-10 days post inoculation with chlorotic lesions which later became necrotic on inoculated leaves (Fig. 2D). No symptoms were observed on inoculated *N. glutinosa*, *Datura stramonium* and *N. tabacum*. Lack of symptoms on these three plant species and the symptoms expressed on the field-infected and the inoculated plants in this investigation were similar to those reported for LMV earlier (Brunt *et al.*, 1996).

RT-PCR amplification and nucleotide sequencing of LMV

LMV was detected in naturally-infected lettuce plant tissues by RT-PCR. RT-PCR amplification of viral RNA was carried out on the total RNA isolated from naturally-infected and uninfected plant leaves using specific primers (P3 and P4) for LMV successfully amplified 346 bp fragments from LMV-infected plants but not from uninfected ones. The PCR parameters described in this investigation successfully amplified this gene of the Saudi Arabian isolate of LMV.

RT-PCR was performed on total RNA extracted from 30 mg tissues of plants infected with LMV and of healthy plants using the SV total RNA Isolation System Kit (Promega). The RNA was reverse transcripted using reverse transcriptase. The reverse transcription reaction was primed with the

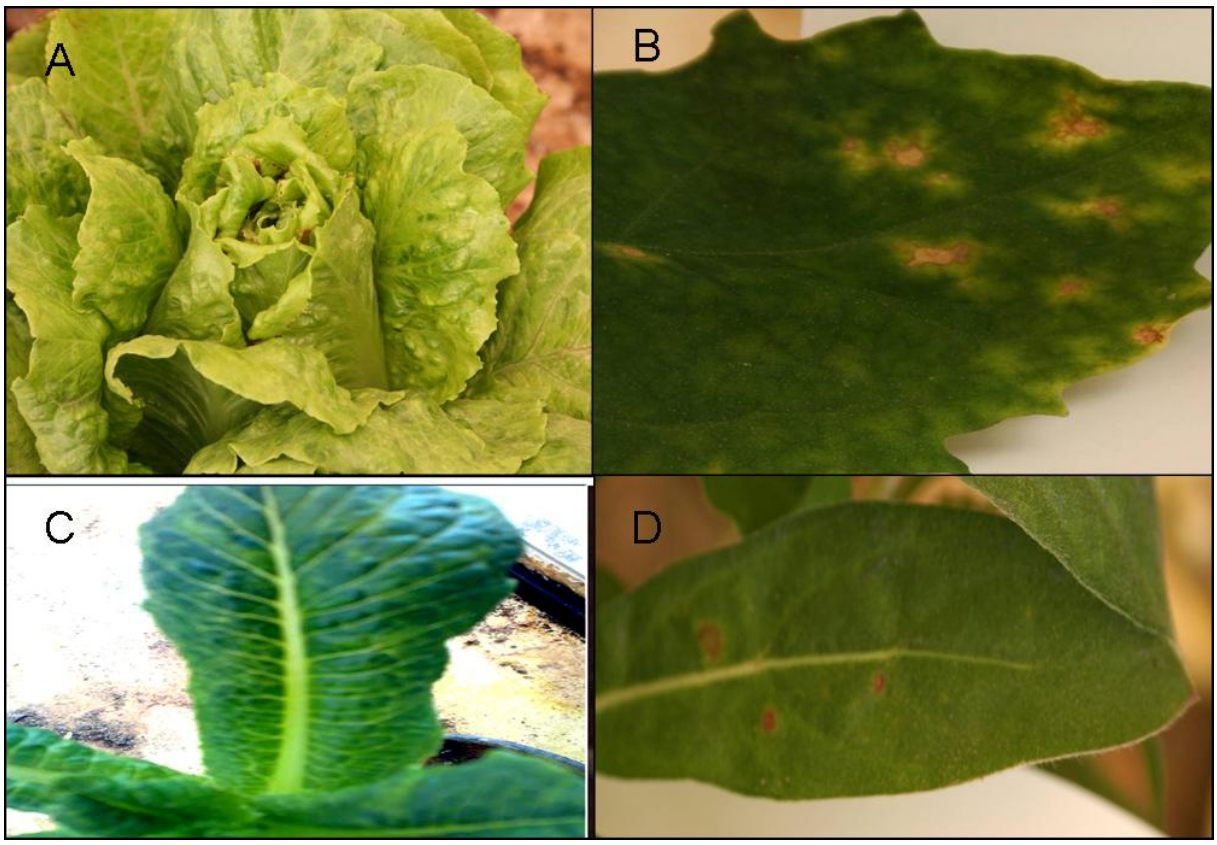

Fig. 2. Symptoms of LMV on infected host plants: (A) mosaic symptoms on naturally infected lettuce. (B) necrotic local lesions on a leaf of *Chenopodium amaranticolor*, (C) mosaic symptoms on a mechanically inoculated lettuce plant, and (D) necrotic local lesions on inoculated *Gomphreana globosa* leaf.

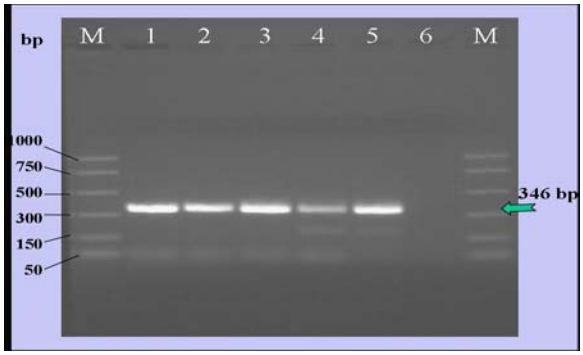

Fig. 3. An approximately 346-bp DNA fragment was amplified from LMV-infected lettuce (as indexed by DAS-ELISA) by RT-PCR with an LMV primer pair (lane 1, 2, 3, 4 and 5). No DNA fragment was amplified from uninfected plants as ensured by (DAS-ELISA) (lane 6). DNA 50 bp PCR molecular marker (lane M).

complementary primer specific for LMV. The resulting complementary DNA (cDNA) was amplified by PCR after adding the complementary and homologous primers. Electrophoresis analysis of RT-PCR product revealed a single amplified fragment of 346 bp. Obtained data in Fig. 3 illustrate the agarose gel electrophoresis of RT-PCR amplified LMV cDNA from infected lettuce plants. Viral targets are detected by molecular procedures with higher sensitivity and reliability than other methods (Olmos *et al*., 2005). Consequently, the prospect of detecting plant viruses by nucleic acid amplification has increased, especially for viruses occurring in very low concentrations in plants or vectors. Previous studies have shown that different nucleic acid amplification techniques are available to detect stylet borne viruses with different sensitivity ranges and also, that a number of proteins can be used for the capture phase, thus obviating the need for virus specific antibodies (Olmos *et al*., 1996).

The DNA fragment amplified from LMV-infected lettuce was purified, and its nucleotide sequence was determined. The gene sequence of the Saudi Arabian isolate of LMV obtained from Al-Hair area was found to be composed of 346 nucleotides in length. A multiple alignment was done along with the previously obtained sequences by GenBank sequence data. As shown in Fig. 4, the comparative sequence analysis of the nucleotides sequence revealed that the

sequence of this DNA fragment was 99.7% identical to the gene sequence of LMV isolates (strain="0") (#X97704, #X65652) and #AF525082 recovered from lettuce from France and Brazil respectively. 99.2% similarity with (#AF525083) and 98.8% similarity with LMV isolate "SaoPaulo" (#AF418578), (#AY372280), (#AY156088) and (#AY167756) and 96.5% similarity with LMV isolate (#AY129086) recovered from lettuce from Brazil. 98.6 % similarity with (#AJ297630) and (#AJ306288) Yuhang isolate, 94.5% similarity with Beijing isolate (#EF423619) and 94.2% similarity with HZ isolate (#AJ488153) isolated from lettuce from China. 93.1% similarity with isolate 13 (#Z78223), 92.7% similarity with isolate 1 (#Z78225) and Audran isolate (#Z78224), 92.4% similarity with isolate "AF199" (#AJ278854) and strain E (#X97705).

Sequence-based clustering's of LMV isolates were consistent when three different algorithmic approaches (neighbor joining, maximum likelihood, and parsimony) were used, and did not reveal new branches in the dendrograms beyond the three major clusters previously identified (LMV-Yar, LMV-Greek, and LMV-RoW). No new member of the Yar cluster was identified, and this branch still contains a single, molecularly divergent isolate, LMV-Yar (Pink *et al*., 1992a, b; Revers *et al*., 1997a). Seven additional isolates from Greece were placed into the Greek cluster. However, some isolates from lettuce, ornamentals or weeds harvested in 1999 and 2000 in Greece, either from Athens or Crete, were placed in the RoW cluster. LMV-RoW was previously named LMV-WE-C based upon the Western European and Californian origin of the isolates known at that time (Revers *et al*., 1999) but it was renamed because it now includes isolates from all parts of the world represented in this analysis (West Africa, North Africa, North America, South America, East Asia, Australia, and Europe).

The detection of LMV and the infection of lettuce with this virus was the first documentation in Saudi Arabia. The spread of this virus is probably due to sowing virus-infected seeds and the occurrence of heavy population of its insect vectors in this region. The minor differences observed in the nucleotide sequence homology between the Saudi Arabian, French, Chinese and Brazilian isolates may suggest that all these isolates are closely related to each other. The results obtained from this study will help manage this disease in this crop.

Finally, being transmitted with seeds, aphids, and through mechanical means (Tomlinson, 1970; Brunt *et al*., 1996), the infection induced by this virus renders the crop unmarketable and the yield loss was reported to reach 100%. The effect of this virus on lettuce and probably on the other crops in Saudi Arabia will be threatening in presence of alternate hosts and weeds which serve as reservoirs for the virus, unless careful measures are implemented to use virus-free seeds and to control the reservoir plants and the aphid vectors of this virus.

Acknowledgements. The cooperation and technical assistance of Dr. Ahmed Ali Al-Qahtani at the Biological and Medical Research Department, King Faisal Specialist Hospital & Research Center, Riyadh, Saudi Arabia, is greatly appreciated.

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تعريف فيروس موزاييك الخس بالطرق البيولوجية والسيرولوجية والجزيئية من نباتات خس مصابة حقلياً في المملكة العربية السعودية

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(قدم للنشر في ١٤٣٠/٦/٢١هـ؛ وقبل للنشر في ٢٤ /١٠/١٤٣٠هـ)

الكلمات المفتاحية: فيروس موزاييك الخس، المدى العائلي، اختبار الإليزا، تفاعل البلمرة المتسلسل العكسي النسخ، التتابع النيوكليوتيدي

ملخص البحث. شوهدت أعراض موزاييك على نباتات خس (*Lactuca sativa* linn.) في حقول الحاير بمنطقة الرياض. تم استخدام اختبار الإليزا للكشف عن وجود المسبب الفيروسي المتسبب في حدوث هذه الأعراض وذلك باستخدام أمصال لعدة فيروسات يشك في تسببها في تكشف هذه الأعراض. تفاعلت الأجسام المضادة الخاصة بفيروس موزاييك الخس إيجابياً في اختبار الإليزا، بينما لم تشاهد أي تفاعلات مصلية للفيروسات الأخرى التي تصيب الخس والتي شملت فيروس موزاييك البرسيم (AMV)، وفيروس موزاييك الخيار (CMV)، وفيروس موزاييك اللفت (TuMV)، وفيروس تبقع وذبول الطماطم (TSWV). تكشفت أعراض على نباتات الخس *L. Sativa*، والمخلدة *Gomphrena globosa*، ونوعان من الزريح *Chenopodium amaranticolor* Coste & Reyn، و *C. quinoa* التي أعديت ميكانيكياً بعصارة من أوراق الخس التي شوهدت عليها الأعراض. لم تظهر أعراض على نوعي التبغ *Nicotiana glutinosa* و *N. tabaccum* عند إعدائهما بعصارة خام من نفس أوراق نبات الخس المصاب بالفيروس. استخدم اختبار تفاعل البلمرة المتسلسل العكسي النسخ (RT-PCR) للكشف عن الفيروس وتعريفه من مستخلص الحمض النووي الريبوزي لنباتات خس مصابة وذلك باستخدام بادئ متخصص للكشف عن المنطقة "٢" التي تشتمل على بداية الطرف ٥ لجين الغلاف البروتيني بالإضافة إلى الطرف ٣ لجين الأجسام المحتواة النووية من النوع B (Nib). قدر الحمض النووي (DNA) الناتج من تفاعل الـ RT-PCR المضاعف بما يعادل ٣٤٦ زوجاً من القواعد النيوكليوتيدية وذلك باستخدام الفصل الكهربائي على الأجاروز. تم تنقية قطعة DNA لعزلة فيروس موزاييك الخس التي حجمها ٣٤٦ كما تم تحديد التتابع النيوكليوتيدي لها في هذه المنطقة. أوضحت مقارنة تحليل التتابع النيوكليوتيدي أن هناك تماثلاً بنسبة ٩٢.٤ إلى ٩٨.٨٪ بين عزلة فيروس موزاييك الخس قيد الدراسة والعزلات التي سجلت في البرازيل وفرنسا والصين وأدران وسلالة AF199 وسلالة E. أوضحت نتائج التتابع النيوكليوتيدي أن التماثل بين عزلة السعودية والعزلات المذكورة أعلاه كان متقارباً جداً. يعتبر هذا أول تسجيل لفيروس موزاييك الخس في المملكة العربية السعودية.