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MOLECULAR CHARACTERIZATION OF AN EGYPTIAN ISOLATE OF TOMATO MOSAIC *TOBAMOVIRUS*

[32]

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ABSTRACT

Tomato plant is recorded to be one of the most economical vegetable crops in Egypt. This crop was found to be widely and severely infected with tomato mosaic *tobamovirus* (ToMV). An Egyptian isolate of ToMV was subjected to molecular characterization. The virus was purified from tobacco leaves using differential centrifugation.The electron microscopy of purified virus preparation showed the presence of rod-shaped virus-like particles with a model size close to 300 X 17 *nm*. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed a single coat protein band with molecular weight of about 17 KDa. ToMV coat protein gene (*cp*) was isolated and amplified using immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR) and its size length on agarose gel was found to be 479 bp. The nucleotide sequence of the viral genome was studied, as a genome composed of 6383 nucleotides was sequenced and its organization was addressed. Furthermore, the similarity between the ToMV-Egy genome and some overseas isolates was determined. Similarity between ToMV-Egy and five TMV isolates from USA, Australia, China, Japan and Russia was ranged from 79 to 99% and from 55 to 98% based on the nucleotide sequence and the deduced amino acids, respectively .

Key Words: ToMV, Purification, SDS-PAGE, Coat protein, IC-RT-PCR, Genome, Sequence

INTRODUCTION

Tomato (*Lycopersicon esculantum* L.) is one of the most economical vegetable crops worldwide which is subjected to different pathogens (**Broadbent, 1964; Brunt *et al* 1996**) .In Egypt, the cultivated area of such crop at the year 2003 was about 430207 feddan and gave a yield of about 6328725 tons (**Egyptian National Agricultural Library site: http://nile.enal.sci.eg/**). The virus diseases were found to be the most serious diseases as they reduce the yield as well as the quality of such crop (**El-Hammady *et al* 1983**).

Tobamoviruses were reported to be one of the most economically important virus group infecting tomato (**Van Regenmortel *et al* 2000**). **Brunt *et al***

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(1996) reported that tobacco mosaic *tobmovirus* (TMV) causes mosaic symptoms with financial loss ranged from 12 to 33 % of tomato yield.

The stability of tobamoviruses can be recognized as their longevity *in vitro* is several years and concentration in sap is often over 1 g/l (**Harrison *et al* 1971**) so virus yield can be high as 10 g/kg of fresh infected tissues (**Stace-Smith and Martin, 1993**). The virus particles are rigid helical rods with 300 nm in length and 17 nm in width (**Harrison *et al* 1971 and Sadik *et al* 2000**), having five percent ss-RNA with a molecular weight of 2×10^6 Da surrounded by coat protein subunits with a molecular weight of $17\text{-}18\times10^3$ Da (**Brunt *et al* 1996**).

The size of Tobamoviruses genome was found to be 6383-6395 nt and consisting of three genes coding for replicase, movment and coat viral proteins (**Ohno *et al* 1999 and Geolet *et al* 2002**).

Several ToMV strains have been placed into two groups depending on the location of the assembly origin within the last stretch of 1000 nucleotides.The assembly origin of group 1 (common strain) is located between 850 to 975 nt from the 3' end, and that of the group 2 (Cowpea strain and cucumber green mottle mosaic virus) is between 300 to 500 nt (**Fukuda *et al* 1980 and Meshi *et al* 1983**).

This investigation aimed to study some molecular characters of an Egyptian isolate of ToMV, therefore isolation, amplification and determination of ToMV-CP gene size using IC-RT-PCR were carried out. Sequencing the viral genome of this isolate and its relationship to some overseas TMV isolates were also undertaken.

MATERIAL AND METHODS

Virus source

In this study, an isolate of ToMV was obtained from Laboratory of Virology, Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Shubra El-Kheima, Egypt. This isolate was biologically confirmed by mechanical inoculation on some differential hosts, i.e. *Datura metel, Nicotiana glutinosa, N.tabacum cv.* White Burley, *Lycopersicon esculantum cv.*Duke and finaly maintained on *N. tabacum* cv. White Burley .

Virus purificartion

Two hundred grams of systematically ToMV-infected leaves from *N. tabacum* cv. White Burley were frozen in liquid nitrogen and ground into a fine powder. The virus was purified using differential ultracentrifugation as mentioned by **El-Ahdal *et al* (1984)**. The final pellets were resuspended in 1 ml of 0.05 M sodium phosphate buffer, pH 7.2 and kept at 4°C. The purified preparations were evaluated by electron microscopy as a negative staining technique described by **Milne and Lesemann (1984)** was used for determination of the virus particle morphology. The grids were examined with a Philips 400T transmission electron microscope, Electron Microscopic Unit, Specialized Hospital, Ain Shams University, Cairo, Egypt.

Purified preparation was also evaluated spectrophotometry and viral yield was calculated according to the equation given by **Noordam (1973)**.

Raising polyclonal antibodies specific to the ToMV isolate

Antiserum against the ToMV was prepared by injecting two adult New Zealand white rabbits (2-4 kg) with purified viral preparation according to the method described by **Makkouk and Gumpf (1976)** .The injections were at one week interval, first dosage (3.5 mg) was taken intramuscularly with complete adjuvant, the second was injected intravenously (2.5 mg). The third and the fourth injections (3.5 mg each) were taken intramuscularly with incomplete adjuvant. The normal serum was obtained before starting the injection schedule by bleeding the rabbits. The clarified serum, was pipetted, placed in Eppendorf tubes and stored at -20°C.

Isolation and purification of immunoglobulins G (IgG)

IgGs were isolated from the antiserum according to **Steinbuch and Audran (1969).** One ml of virus antiserum was added to 2 ml of 0.06 M sodium acetate buffer (pH 4.8) and dialyzed three times against the same buffer; 0.082 ml Caprylic acid was added with stirring and left for 30 min at 28 °C. Mixture was centrifuged at 8000 rpm for 10 min; supernatant was dialyzed two times against 0.05 phosphate buffer (pH 7.2) for 4 h. The IgGs were precipitated using saturated ammonium sulfate and collected by centrifugation at 8000 rpm for 10 min. Pellets were resuspended in 1ml distilled water. Concentration of IgG was adjusted to 1 mg/ml (1.4 optical density (OD) at 280 nm) and stored at -20 °C.

Antiserum titer

Indirect ELISA (I-ELISA) **(Koenig and Paul, 1982)** was used to determine the specificity as well as the dilution end point (DEP) of the purified antiserum. Twelve double fold dilutions of the antiserum were use against clarified ToMV tobacco infectious sap. As a control, the normal serum (dilusion 1/2) and healthy sap were used.

SDS-PAGE of ToMV coat protein

The molecular weight of coat protein subunits of ToMV was determined by SDS-PAGE using 4% stacking gel on a 12% resolving gel and the buffer system as described by **Laemmli (1970)** and **Shukla and Ward (1988).**

Molecular studies

1- IC-RT-PCR

IC-RT-PCR was carried out according to **Weidemann and Maiss (1996)** with some modifications for the isolation and amplification of ToMV-CP gene. Purified virus particles were captured in ToMV-IgG coated wells of an ELISA plate. The viral RNA was released by adding 25 µl of Tris buffer [10 mM Tris-HCL (pH 8.0) containing 1 % Triton X-100] to each well and ELISA plates was kept at 65 °C for 5 min. The cDNA was created using the coat protein gene reverse primer and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV Reverse Transcriptase). The following primers were used for the amplification of CP gene:
5'ATGTCTTACTCAATCACTTC3'(forward) and 5'ATTTAAGATGCAGGTG

CAGA 3′ (reverse) (purchased from Invitrogen Corp., USA). A 50 µl PCR reaction mix contained the primers (1µM final concentration each), Taq DNA polymerase (1.0 U), 200 µM of each dNTPs, 1X PCR reaction buffer . Reaction was overlaid with 50 µl mineral oil. PCR conditions were 94°C initial melting for 3 min followed by 35 cycles of 94 °C /1 min, 55°C /1 min and 72°C /2 min, with 72 °C /10 min final extension.

The PCR product was analyzed on 1 % agarose and the size length of the CP gene was determined using Gel-Pro Analyzer software (Media Cybernetics, USA).

2. Sequence of the ToMV genome

The nucleotide sequence of the full length of ToMV isolate genome was determined using Sanger's dideoxy method (**Sanger *et al* 1977**). The method based on the fact that a nucleotide with a dideoxyribose sugar (missing OH group on 3' carbon) terminates polymerization of DNA. A primer complementry to one spot of the viral cDNA was annealed. The annealed templates were separated into 4 tubes each having all 4 dNTPs and only one of the dideoxy (ddNTP). DNA polymerase was used for the synthesis of new strands in the presence of 35S-ATP. The products in each tube (different radiolabeled population of nucleotide fragments) were analyzed on polyacrylamide gel using gel documentation system. ToMV full genome sequencing was done at Molecular Virology Group-Biotechnology, Group-Department of Plant Biology, The Royal Veterinary and Agricultural University (KVL), Copenhagen, Denmark.

Sequence analysis

DNA sequences related to ToMV genome of different isolates were collected from GeneBank (**http://www.ncbi.nlm.nih.gov**). These isolates are from: Australia [NC_002692, **Lee *et al* 2002** (Personal communication)], China [AS395129, **Shao *et al* 2001** (Personal communication)], Japan (X02144, **Ohno *et al* 1999**), Russia [Z92909, **Belenovich *et al* 1997** (Personal communication)] and USA (NC_001367, **Geolet *et al* 2002**). These sequences were aligned with the nucleotide sequence of the Egyptian ToMV (ToMV-Egy) using the DNASIS software (**Hitachi Software Engineering Co., Ltd.**). DNA sequence was translated to the deduced amino acids and aligned using DNASIS software. Similarity between isolates was carried out using GeneDoc software (**http://www.psc.edu/biomed/genedoc/**) and phylogenic trees were drawn using MEGA2 software (**http://www.megasoftware.net/**)

RESULTS & DISCUSSION

The ToMV isolate causes severe mosaic symptoms on tomato plants was propagated on *N. tabacum cv* .White Burley after it was biologically confirmed and found to give necrotic local lesions on *D. metel* and *N. glutinosa* plants, severe mosaic and mosaic accompanied with vein banding on tomato and tobacco, respectively. These results were also reported by **Brunt *et al* (1996)**.

Purification of ToMV isolate and its evaluation

A virus yield of about 4.9 mg/ml (about 86 mg per 100 gm infected *N. ta-*

bacum cv. White Burley leaves) was obtained with a good purity, A_{260} = 1.541 and $A_{280/260}$ of about 1.37 . The yield of tobamoviruses, which are very stable and present in large quantities in infected plants, was about 100 mg of virus per 100 g infected tissue (**Stace-Smith and Martin, 1993**). The purified virus preparation was negatively stained with 2% uranyl acetate and results in Figure (1) showed the presence of rod-shaped particles measuring 300 nm in length and 17 nm in width which found to be in full agreement with **Brunt *et al* (1996)**. Results showed that no virus particles were obtained from the healthy tobacco plants.

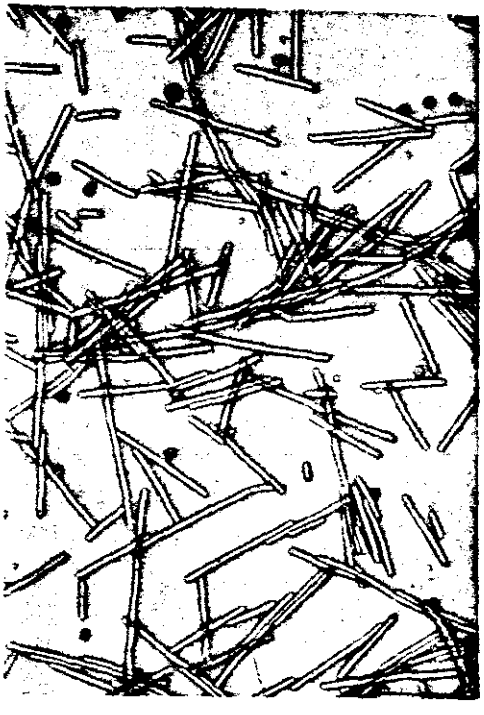


Figure 1. Electron microscopy of purified virus preparation stained with 2% uranyl acetate (Mag. X60,000).

Production of antiserum

For the ToMV isolate, a specific antiserum was produced, IgGs were purified and its titer was determined using I-ELISA. Data in Table (1) show that antiserum reacted up to dilution of 1/2048 with clarified infectious sap and no positive results were obtained with the healthy sap among the I-ELISA technique. This result agrees with that found by **El-Ahdal *et al* (1996)**.

Table 1. Determination of antiserum titer using I-ELISA.

Dilution	ELISA value at A_{405} nm	Result
Undiluted	2.173	+
1/2	2.001	+
1/4	1.446	+
1/8	1.351	+
1/16	1.319	+
1/32	0.953	+
1/64	0.705	+
1/128	0.661	+
1/256	0.630	+
1/512	0.495	+
1/1024	0.403	+
1/2048	0.195	+
1/4096	0.060	-
Control*	0.087	-

*=Clarified infected sap with normal serum (1/2). +=Positive. -=Negative.

Molecular weight of ToMV-CP

Results in Figure (2) show that the SDS-PAGE analysis of the purified ToMV preparation indicated that ToMV-CP appeared as one band with a molecular weight of about 17 KDa . This result was agreed with that found by **Gabrial and Lister (1973); Parent *et al* (1985) and Matthews and Dodds (1998)** who reported that MW of TMV-CP was 19, 16 and 17.5 KDa, respectively.

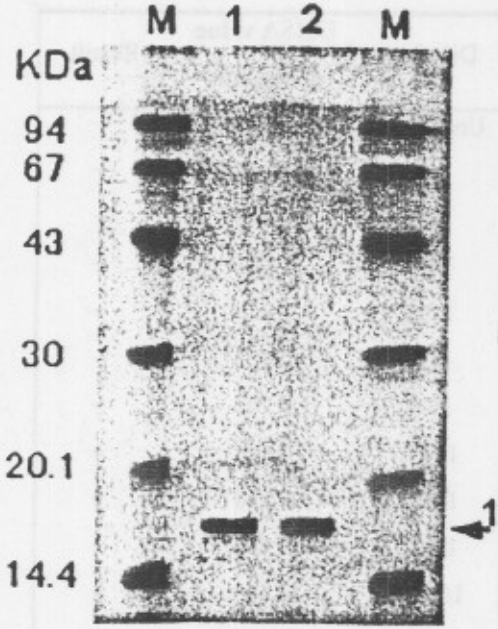


Figure 2. 12% SDS-PAGE of purified virus preparations (Lanes 1 and 2). M: Protein marker (Promega, USA).

IC-RT-PCR

The agarose gel analysis of the IC-RT-PCR product indicated a single band with size length of 479 bp which is the expected size for the ToMV- CP gene (Figure 3). Such result was in harmony with that found by **Choi and Park (1986) and Geolet *et al* (2002).**

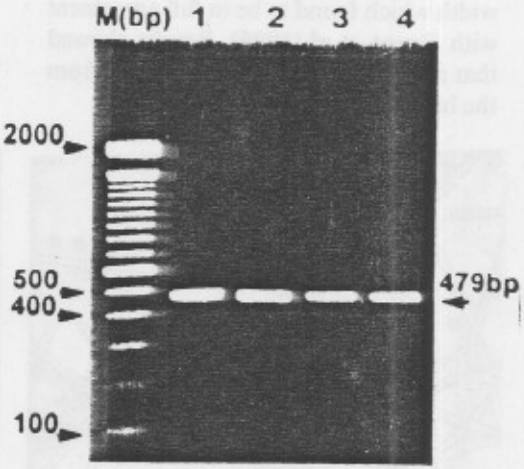


Figure 3. IC-RT-PCR for the amplification of ToMV- CP gene . Lane M : DNA Ladder (Promega, USA), lanes 1, 2, 3&4: ToMV-CP gene.

Sequence of TMV genome

The nucleotide sequence of the full genome of the applied ToMV isolate was studied. Results in Figure (4) showed that the genome is consisted of 6383 nucleotides, represents three genes as shown in Figure (5). The replicase gene (*rep* gene)

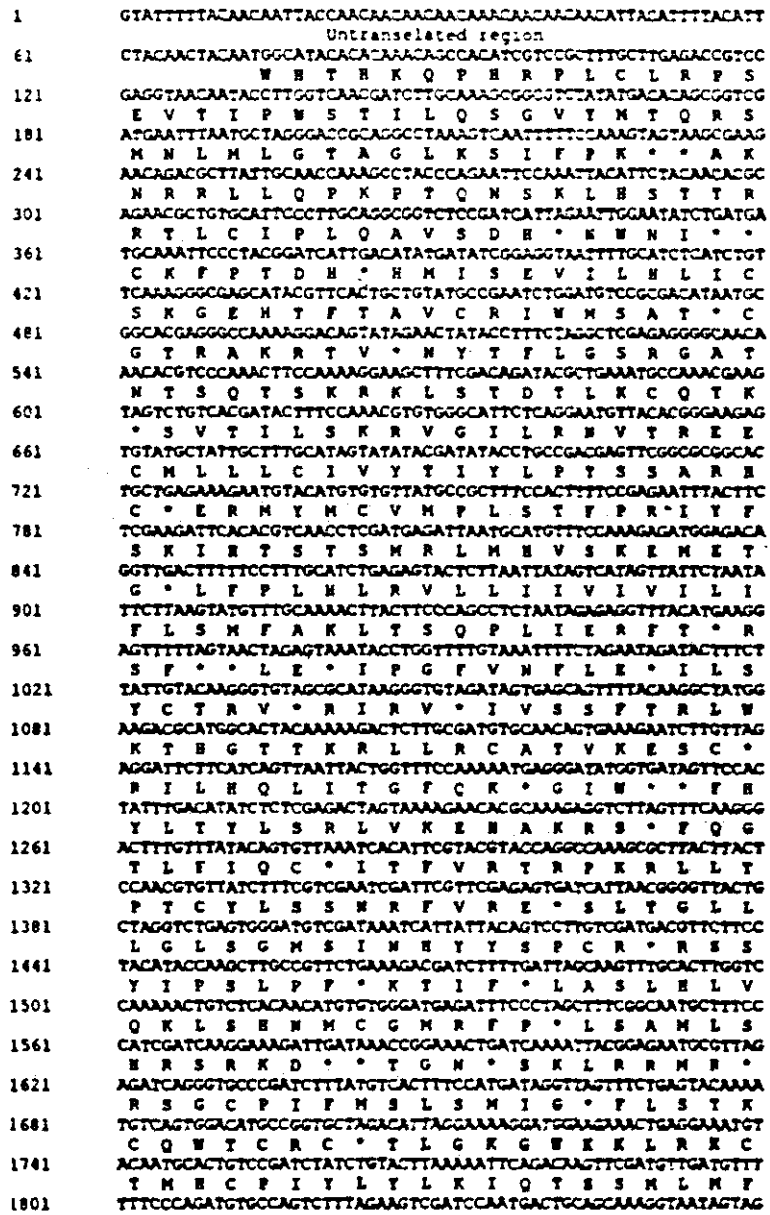
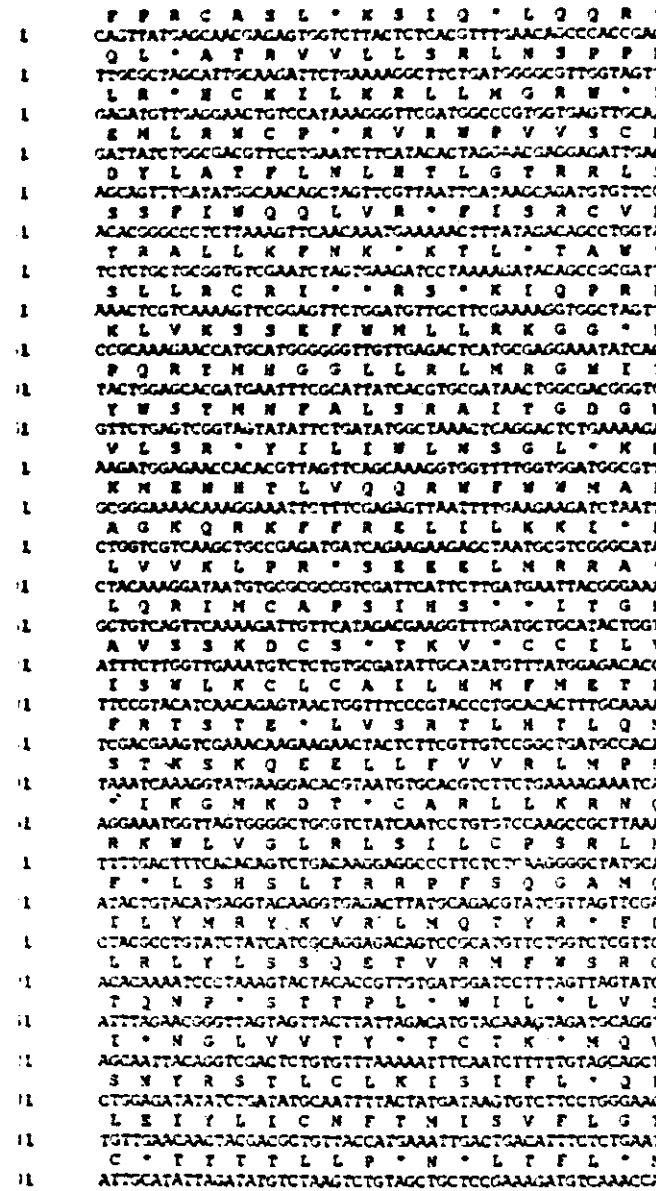


Figure 4. DNA sequence and deduced amino acids of the full genome of ToMV-Egy isolate.



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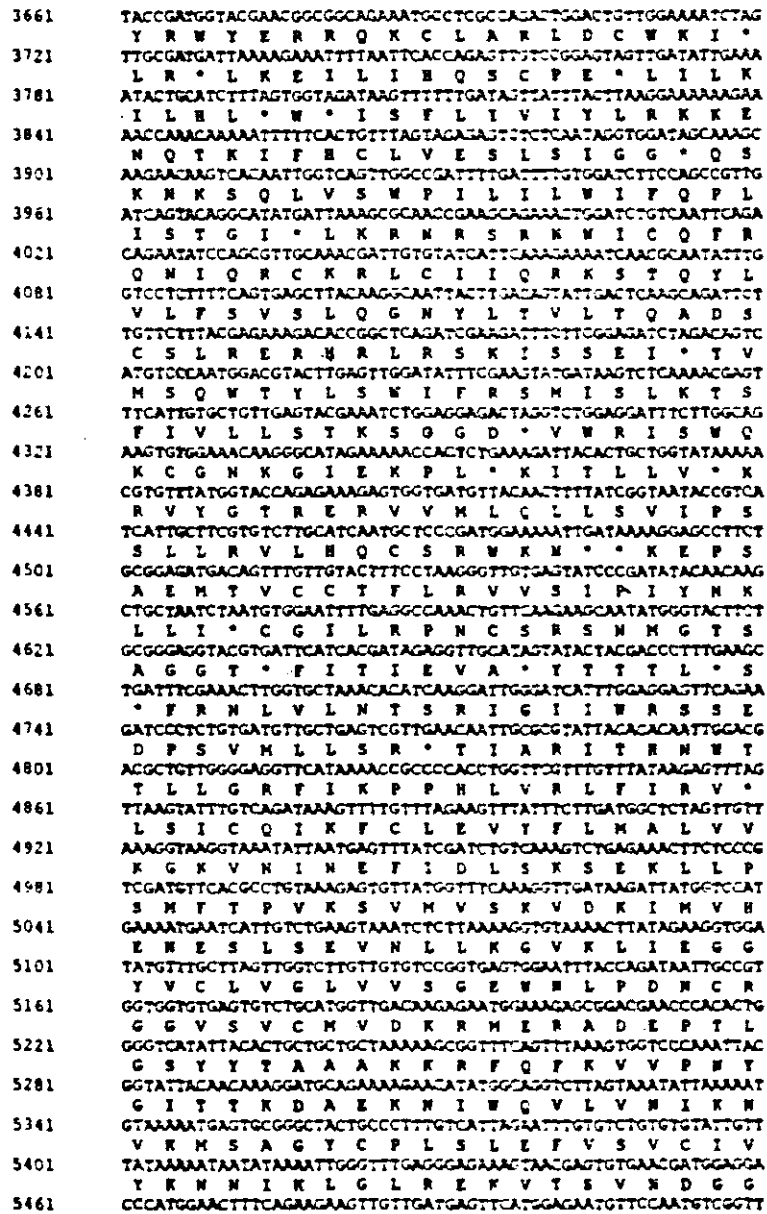

Figure 4. Continue

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                P  M  E  L  S  E  E  V  V  D  E  F  M  E  N  V  P  M  S  V
5521  AGACTCGCAAAGTTTCGAACCAAATCCTCAAAAAGAGGTCCGAAAAATAATAATAATTTA
                R  L  A  K  F  R  T  K  S  S  K  R  G  P  K  N  N  N  N  L
5581  GGTAAGGGGCGTTCAGGCGGAAGGCCTAAACCAAAAAGTTTTGATGAAGTTGGAAAAGAG
                G  K  G  R  S  G  G  R  P  K  P  K  S  F  D  E  V  G  K  E
5641  TTTGATAATTTGATTGAAGATGAAGCCGAGACGTCGGTCGCGGATTCTGATTCGTATTAA
                F  D  N  L  I  E  D  E  A  E  T  S  V  A  D  S  D  S  Y  *
5701  ATATGTCTTACTCAATCACTTCTCCATCGCAATTTGTGTTTTTGTCATCTGTATGGGCTG
                I  C  L  T  Q  S  L  L  H  R  N  L  C  F  C  H  L  Y  G  L
5761  ACCCTATAGAATTGTTAAACGTTTGTACAAATTCGTTAGGTAACCAGTTTCAAACACAGC
                T  L  *  N  C  *  T  F  V  Q  I  R  *  V  T  S  F  K  H  S
5821  AAGCAAGAACTACTGTTCAACAGCAGTTCAGCGAGGTGTGGAAACCTTTCCCTCAGAGCA
                K  Q  E  L  L  F  N  S  S  S  A  R  C  G  N  L  S  L  R  A
5881  CCGTCAGATTTCCTGGCGATGTTTATAAGGTGTACAGGTACAATGCAGTTTTAGATCCTC
                P  S  D  F  L  A  M  F  I  R  C  T  G  T  M  Q  F  *  I  L
5941  TAATTACTGCGTTGCTGGGGTCTTTTGATACTAGGAATAGAATAATCGAAGTAGAAAACC
                *  L  L  R  C  W  G  L  L  I  L  G  I  E  *  S  K  *  K  T
6001  AGCAGAATCCGACAACAGCTGAAACGTTAGATGCTACCCGCAGGGTAGACGACGCTACGG
                S  R  I  R  Q  Q  L  K  R  *  M  L  P  A  G  *  R  T  L  R
6061  TTGCAATTCGATCTGCTATAAATAATTTAGTTAATGAACTAGTAAGAGGTACTGGACTAT
                L  Q  F  D  L  L  *  I  I  *  L  N  N  *  *  E  V  L  D  Y
6121  ACAATCAAAATACTTTTGAAAGTATGTCTGGGTTGGTCTGGACCTCTGCACCTGCATCTT
                T  I  K  I  L  L  K  V  C  L  G  W  S  G  P  L  H  L  H  L
6181  AAATGCATAGGTGCTGAAATATAAAGTTTGTGTTTCTAAAACACACGTGGTACGTACGAT
                K                    Untranslated region
6241  AACGTACAGTGTTTTTCCCTCCACTTAAATCGAAGGGTAGTGTCTTGGAGCGCGCGGAGT
                                     Untranslated region
6301  AAACATATATGGTTCATATATGTCCGTAGGCACGTAAAAAAGCGAGGGATTCGAATTCCC
                                     Untranslated region
6361  CCGGAACCCCCGGTTGGGGCCCA
                                     Untranslated region
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Figure 4. Continue

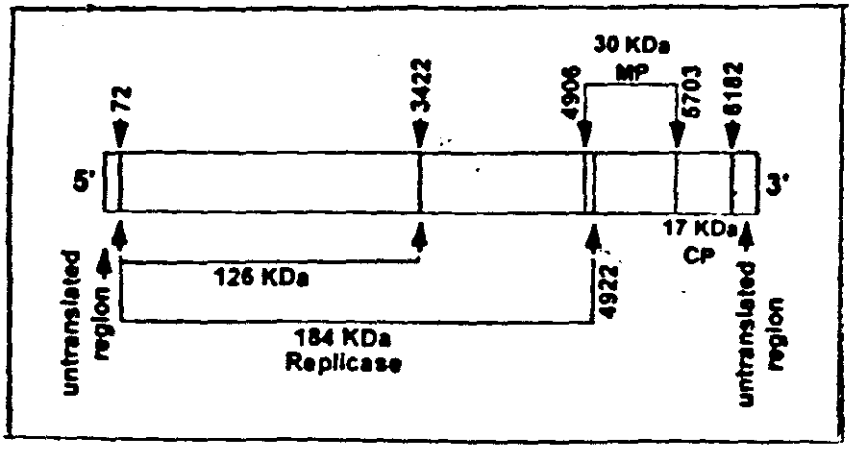

Figure 5. Organization of ToMV-Egy genome.

starts from 72 to 4922. The movement protein gene (*mp* gene) starts from 4906 to 5700. The coat protein gene (*cp* gene) starts from 5703 to 6182. These genes started with ATG and ended with TAA, as start and stop codons, respectively, as shown in Table (2). The size length of *rep,mp* and *cp* genes were 4851 , 795 and 479 and encoded three proteins with molecular weights of about 184 , 30 and 17 KDa , respectively . Data of **Sadik *et al* (2000)** indicated that MW of TMV-RNA was about 6400 b, i.e., 2 X 10^6 Da .**Choi and Park (1986)** reported that the MW of TMV-RNA was 2 X 10^6 , 2.03 X 10^6 Da and 2.1 X 10^6, respectively.

The nucleotide sequence of the ToMV-full genome in this study and the deduced amino acids were compared with five overseas TMV isolates from Australia **[Lee *et al* 2002** (Personal communication)] ,USA **(Geolet *et al* 2002),** Japan **(Ohno *et al* 1999),** China **[Shao *et al* 2001** (Personal communication)] and Russia **[Belenovich *et al* 1997** (Personal comunication)]. Similarity ranged from 79 to 99% and from 55 to 97% between the ToMV-Egy isolates and those of overseas were obtained based on DNA sequence (Table 3) and deduced amino acids (Table 4), respectively.

The 5-terminus of the ToMV genomic RNA is capped with 7-methyl guanosine which is necessary for virus infectivity , this result agreed with **(Knapp and Lewandowski, 2001).** Both 126 and 183 KDa proteins are translated directly from the genomic RNA and are required for efficient replication **(Ishikawa *et al* 1991 and Lewandowski & Dawson,**

Table 2. Comparison between the organization of ToMV-Egy genome and five TMV isolates from different countries

Isolates	Genome size (base)	*rep* gene		*mp* gene		*cp* gene	
		Start-Stop	PMW (KDa)	Start-Sop	PMW (KDa)	Start-Stop	PMW (KDa)
Australian	6383	72-4922	184	4906-5700	30	5703-6182	17.5
		ATG-TAA		ATG-TAA		ATG-TAA	
Chinese	6395	69-4919	183	4903-5709	30	5712-6191	17.6
		ATG-TAA		ATG-TAA		ATG-TGA	
Egyptian	6383	72-4922	184	4906-5700	30	5703-6182	17.0
		ATG-TAA		ATG-TAA		ATG-TAA	
Japanese	6384	72-4922	180	4906-5700	30	5703-6182	17.0
		ATG-TAA		ATG-TAA		ATG-TAA	
Russian	6383	72-4922	184	4906-5700	30	5703-6182	17.7
		ATG-TAA		ATG-TAA		ATG-TAA	
American	6395	69-4919	183	4903-5709	30	5712-6191	17.0
		ATG-TAA		ATG-TAA		ATG-TGA	

PMW = Protein molecular weight.

Table 3. Similarity (%) between the DNA sequences of full genome of five TMV isolates compared to ToMV-Egy isolate.

Isolates	Australian	Chinese	Egyptian	Japanese	Russian	American
Australian	*					
Chinese	79	*				
Egyptian	99	79	*			
Japanese	99	79	98	*		
Russian	99	79	98	99	*	
American	79	98	79	79	79	*

Table 4. Similarity (%) between the amino acids sequence of ToMV-Egy and five TMV overseas isolates.

Isolates	Australian	Chinese	Egyptian	Japanese	Russian	American
Australian	*					
Chinese	55	*				
Egyptian	97	54	*			
Japanese	98	54	97	*		
Russian	98	54	97	97	*	
American	54	95	55	55	55	*

2000). The 30 kDa MP and the 17 kDa CP are expressed from individual 3'-co-terminal subgenomic mRNAs (**Knapp and Lewandowski, 2001**) .The MP is required for both cell-to-cell and long-distance movement,and is expressed early in the infection process (**Watanabe *et al* 1984 and Lehto *et al* 1990**). CP is required for long distance movement in many hosts and is expressed later in infection, reaching maximal accumulation 24-72 h post-infection (**Siegel *et al* 1978**). The 3' untranslated region (NTR) can be folded into a series of pseudoknots (**Knapp and Lewandowski, 2001**) and a terminal tRNA-like structure that will specifically aminoacylate with histidine. The 5'-NTR is a strong translational enhancer (**Gallie and Walbot, 1992**). Both the 5'- and 3'-NTRs contain cis-acting elements required for replication (**Takamatsu *et al* 1991; Buck, 1999 and Chandrika *et al* 2000**).

The phylogenetic analysis of the present nucleotide (Figure 6) and deduced amino acids (Figure 7) showed two main

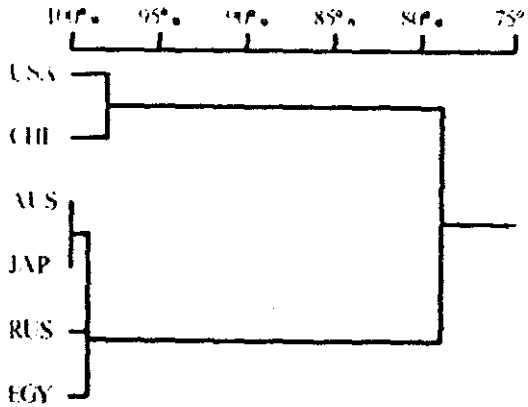

Figure 6. Phylogenetic analysis of different TMV isolates. A tree based on the DNA sequences of full genome of five TMV isolates compared to ToMV-Egy isolate.

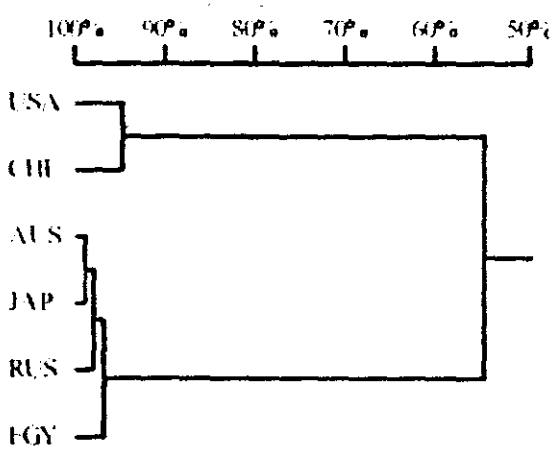

Figure 7. Phylogenetic analysis of different TMV isolates. A tree based on deduced amino acids of full genome of five TMV isolates compared to ToMV-Egy isolate.

groups. The first group included isolates from Australia, Japan and Russia. The second group included isolates from China and USA. The ToMV-Egy isolate was found to be too close to the first group with similarities 99 (Figure 6) and 97 % (Figure 7) on the basis of DNA sequencing and deduced amino acids, respectively. On the other hand, the ToMV-Egy isolate was too far to be an isolate in group two. As its identities with the second group as shown in Figure (6) and Figure (7) were 79 and 54 %, respectively.

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الخصائص الجزيئية لعزلة مصرية من فيروس موزيك الطماطم

[٣٢]

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تعتبر الطماطم من أهم محاصيل الخضر الأقتصاديه في جمهورية مصر العربية. يصاب هذا المحصول بأكثر من فيروس و التي من اكثرها انتشارا فيروس موزيك الطماطم. لذلك استهدف هذا البحث إلى دراسة الخصائص الجزيئية لعزلة مصرية من هذا الفيروس .لهذا فقد تم عمل تنقية لهذه العزلة بواسطة الطرد المركزي المتناوب السرعات.

وقد أوضح الفحص بالميكروسكوب الإليكتروني النافذ للتحضيرات المنقاة وجود جزيئات عصوية الشكل صلبة بأبعاد ٣٠٠×١٧ نانوميتر. وتم تقدير الوزن الجزيئي للغطاء البروتيني بواسطة SDS-PAGE وثبت وجود شريط واحد single band بوزن حوالي ١٧ كيلودالتون. تم عزل ومضاعفة جين الغلاف البروتيني للفيروس باستخدام تفاعل البلمرة المتسلسل والنسخ العكسي بعد ربط الجزيئات الفيروسية بالأجسام المضادة المتخصصة IC-RT-PCR . و تم اختبار ناتج التفاعل خلال الأجاروز جل و الذي اظهر وجود شريط واحد single band بحجم طولي حوالي ٤٧٩ زوج من النيوكليوتيدات. وقد قدر التتابع النيوكليوتيدي لجينوم الفيروس بأكمله والذي ثبت أنه يتكون من ٦٣٨٣ نيكليوتيده. ثم تم تحديد الجينات الثلاثة وهى جين التضاعف وجين الغطاء البروتيني وجين الحركة المكونة لجينوم الفيروس. وقد تم تقدير درجة التشابه بين العزلة المصرية وبعض العزلات الدولية من الولايات المتحدة الأمريكية، استراليا، الصين، اليابان وروسيا ووجد أن التشابه يتراوح بين ٧٩-٩٩٪ على مستوى التتابع النيكليوتيدي و٥٥-٩٨٪ على مستوى تتابع الأحماض الأمينيه المتوقع.

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