Molecular Charactrization Of *Apple Mosaic Virus* (ApMV) Isolated From Strawberry Plants In EGYPT.

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Abstract— *Apple Mosaic Virus* (ApMV) isolated from symptoms and symptomless strawberry plants and identified with a specific antiserum using Double Antibody Sandwich ELISA (DAS-ELISA). Survey was conducted through three successive seasons during 2013-20l6 on commercial strawberry fields in four governorates in Egypt. The percentages of infection which recorded through seasons from the survey were 4, 2.1, 2.4% (Al-Dair-region) and 2.8, 5.5, 3.2% (Kafer Al-Sahapy-region) in El-Qalubia governorate. In El-Beheira governorate the percentage recorded 3.7, 2.5, 0.0% (Badr-region) and 1.3, 1.7, 3.7% (Al-Nobaria- region). El- Monofia (Quesna-region) recorded 1.9, 1.6, 2.3%. Ismailia (fayed-region) was recorded 2.1, 5, and 4.5% respectively. ApMV mechanically transmitted from infected strawberry plants onto 16 host species belonging to seven families. Mottling, local lesion, leaf deformation, systemic and dwarf plants were appeared two weeks' post inoculation on indicator host *Cucumus Sativus*. Reverse transcription polymerase chain reaction (RT-PCR) was used to amplify 262 bp fragment using specific primers for the viral coat protein gene, as a tool for molecular diagnosis. The amplified PCR fragments were cloned, sequenced and compared with other of those sequences available in GenBank. Results observed that, the Egyptian ApMV isolate was A+T, G+C (139, 123) identical content with Yunnan isolate. Obtained sequence comparison with the isolates available in the GenBank indicated that ApMV Egyptian isolate shared 98% identity with Yunnan isolate followed by 97% USA isolate, 95% UK isolate ,94% Turkey isolate, 92% Australia and Belarus isolates, and 89% with India isolate.

Index Terms— : Strawberry, ApMV, Host range, Survey, DAS-ELISA, RT-PCR, Sequence.

1 INTRODUCTION

Strawberry (*Fragaria x ananassa* Duch.) is one of the most economic vegetable crops in Egypt and considered the main cash crop for strawberry growers in some governorates. It is one of the most favorite and delicious fruits of which the demand has been increased in Egypt for local consumption and for exportation. Strawberry production is increasing annually, the world production exceeding 4 million tons, [20]. The total cultivated area of strawberry in the world (241109 Ha) and total world production of strawberries about (4516810) per ton. Egypt Occupies the fourth position of strawberries production after USA, Turkey and Spain. The total area of Strawberries orchards in Egypt are (13999.2) feddan with an approximate yearly production (242297) per ton according to the statistics of [5]. Strawberry can be infected by more than 30 viruses [12]. Many viruses form a major threat to the strawberry industry in Egypt, causing severe economic losses.

Apple mosaic virus (ApMV), a species of the genus *Ilarvirus*, family *Bromoviridae* [14], is found worldwide, ApMV has been reported to naturally infect a number of hosts in the *Rosaceae* family including *Rosa* spp [19 and 1]. It exists as a number of strains that cause a diversity of symptoms on many *Rosaceous* species i.e., rose [18]. ApMV has a wide host range, including woody and herbaceous plants. It is capable of infecting over 65 species in 19 families, by either experimental or natural routes [6,11 and 7] ApMV was isolated from leaves and flower parts. The virus can be transmitted with plant sap by mechanical inoculation. ApMV is a positive-sense single-stranded RNA [14].

Apple Mosaic Virus was detected, isolated and characterized on hosts plants and by DAS-ELISA, among molecular methods, RT-PCR has proved to be the most rapid, sensitive and reliable technique for detecting RNA, viruses in infected plants [10]. Thus, the use of PCR technology is an important step to optimize and speed up strawberry viruses' diagnosis.

The present work aimed to study several aspects of ApMV including disease distribution in some Egyptian Governorates, identification and characterization of the Egyptian isolate by host range, symptomatology, serologically, biologically and molecular studies. In addition to, sequence analysis to measure the similarities and differences between the Egyptian isolate and the isolates available in the Gen-Bank.

2 MATERIALS AND METHODS

SOURCE OF SAMPLES AND FILED INSPECTION:

Plant samples showing symptoms and symptomless were collected from four governorates (El-Qalubia, El- Beheira, El-Monofia and El-Ismailia) during the spring and early summer of three successive seasons (2013/2014, 2014/2015 and 2015/2016). 3009 samples were collected from different localities of strawberry cv. (Festival, Camarosa, Florida, montakhab, Fortuna, winter star and Rosa Linda) plants carrying different external symptoms like that caused by viruses, and tested using ELISA.

ENZYME-LINKED IMMUNE SORBENT ASSAY (DAS ELISA)

The samples were tested serologically using DAS-ELISA technique demonstrated by [4] using ApMV polyclonal antibodies, according to the manufacturer's instructions. Positive and negative control were used, absorbance was measured at 405 nm using ELISA reader (BioTeK-ELX808).

ISOLATION OF APMV

Samples of infected plants which gave positive results with ELISA were used for isolating this targeted virus under greenhouse conditions using specific indicator host plant for ApMV.

MECHANICAL TRANSMISSION AND HOST RANG

Strawberry plants which give positive reaction with ELISA were used as a source of mechanical infection to the indicator host rang (Strawberry, *Chenopodium* and *Cucumber*). Sixteen species and varieties belonging to families (*Rosaceae*, *Solanaceae*, *Cucurbitaceae*, *Chenopodiaceae*, *Leguminosae*, *Lamiaceae* and *Compositae*) were mechanical inoculated using ApMV and maintained under greenhouse conditions for 20 days. Plants showed symptoms and symptomless were checked by back inoculation to the indicator hosts, results was confirmed by DAS-ELISA.

MOLECULAR DETECTION

Total RNA Extraction and Reverse transcription (RT-PCR)

Total RNA was extracted from leaves and flowers of infected strawberry plants using plant total RNA Mini kit (RBC) as described in the manual instruction. Reverse transcription polymerase chain Reaction was carried out on RNA preparations with Reverse Verso™ one-Step RT-PCR Reddy Mix Kit (Thermo scientific). RT-PCR was performed in 25 µl total volume containing 5.5 µl of nuclease - free water, 4 µl (1ng) of total RNA, 12.5 µl of one step PCR master mix, 0.5 µl (10µM) specific forward and reverse primers (Table 1), 0.5 µl Verso enzyme mix and 1.5 µl RT-Enhancer. Synthesis of cDNA was performed at 50°C for 15 min, followed by denaturation at 95°C for 2 min. Amplification was carried out for 35 cycles under the following conditions: denaturation at 95°C for 20 sec, annealing at 62°C 30 sec. extension at 72°C for 30 sec, followed by a final extension for 5 min at 72°C. Amplified products were detected by agarose gel electrophoresis.

ELECTROPHORESIS ANALYSIS

The PCR product were electrophoresed in 1% agarose gel in -0.5x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 120 volt. 100bp DNA molecular weight markers (ABgene, UK) for 1 hour and stained with ethidium bromide (0.5 µl/ml) [15]. The fragments were photographed using UV lamp in gel-documentation (Bio Rad, Gel Doc XR system 170-8170).

TABLE: (1) PRIMER SEQUENCES AND THE EXPECTED SIZE OF THE AMPLIFIED COAT PROTEIN GENE OF APMV CDNA.

Virus	Primer sequence	Expected size	Reference
ApMVf	5'-ATCCGAGTGAACAGTCTATCCTCTAA-3'	262bp	Menzel *et al.*, 2002
ApMVr	5'-GTAACTCACTCGTTATCACGTACAA-3'		

CLONING OF PCR AMPLIFIED FRAGMENTS OF APMV

The amplified 262 bp of ApMV cDNA product was directly cloned into pCR™ TOPO vector using TA™ Cloning system (Invitrogen, Carlsbad, CA).

SEQUENCE AND ANALYSIS

ApMV isolated from cloning using mini-preparation were sequenced in Macrogen Inc. (Seoul, Korea). Similarities and differences were analyzed between ApMV Egyptian isolate and the isolates available on the Gen-Bank using DNA-MAN software.

3 RESULTS AND DISCUSSION

Source of samples and filed inspection

A survey of *Apple Mosaic Virus* (ApMV) disease was conducted in some Egyptian governorates during the growing seasons of 2013/2014, 2014/2015 and 2015/2016. The strawberry plants were randomly taken from different regions following an imaginary line from the fields' outer border to the center. Collected samples were tested by ELISA during the three seasons for the presence of viral infections. External symptoms were observed on some strawberry samples suspected to be virus infection such as mottling, yellowing, leaf curling, leaf deformation, cup shape, vein banding and stunting, in as observed in Fig (1).

Fig. (1): Naturally infected strawberry showing different viral like external symptoms. (A) mottling, (B) yellowing, (C) vein banding, (D) ring spot in flower, (E) cup shape and leaf crinkle, (F) leaf deformation

ApMV isolates were detected in all surveyed governorates, varied from region to region and from season to season. The percentages of infection which recorded from the survey were 4, 2.1, 2.4% (Al-Dair-region) and 2.8, 5.5, 3.2% (Kafer Al-Sahapy-region) in El-Qalubia governorate. In El-Beheira governorate the percentage was 3.7, 2.5, 0.0 % (Badr-region) and 1.3, 1.7, 3.7% (Al-Nobaria- region). El- Monofia (Quesna-region) recorded 1.9, 1.6, 2.3%. Ismailia (fayed-region) was 2.1, 5, and 4.5% respectively.

Table (2), and Fig. (2). In general, from a total of 1070 strawberry samples collected during the first season 2.7% were ApMV positive, the second season samples 974 and the percentage of was 3.0%, while from a total of 965 samples collected during the third season, 2.8% of samples were ApMV positive.

TABLE (2): SURVEY OF THE NATURAL OCCURRENCE OF APMV ON STRAWBERRY PLANTS GROWING AT DIFFERENT LOCATIONS IN EGYPT DURING THREE SUCCESSIVE SEASONS 2013- 2016.

Governorate	Percentage of ApMV infection using DAS ELISA								
	2013/2014			2014/2015			2015/2016		
	tested	infected	%infection	tested	infected	%infection	tested	infected	%infection
EL-Qalubia	380	13	3.4%	350	13	3.7%	360	10	2.8%
El-Beheira	340	9	2.6%	380	8	2.1%	275	5	1.8%
EL- Monofia	160	3	1.8%	124	2	1.6%	130	3	2.3%
Ismailia	190	4	2.1%	120	6	5%	200	9	4.5%
Total	1070	29	2.7%	974	29	3%	965	27	2.8%

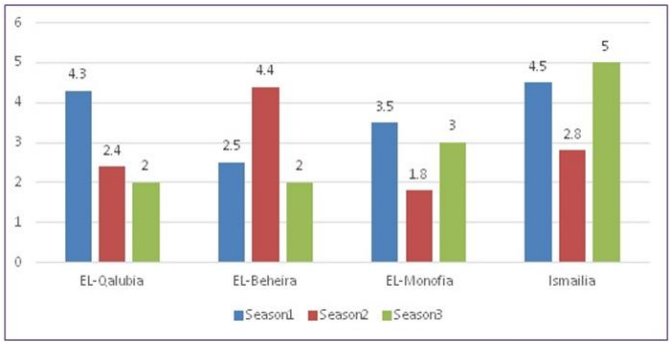

Fig (2). Histogram showing incidence of ApMV virus in strawberry plants collected from different locations in Egypt during 2013-2016.

Data obtained from the survey in the main strawberry production areas showed that the virus was recorded in all surveyed Governorates. From a total of 3009 samples collected during the three seasons, 2.83% of samples were ApMV positive. The highest infection rates were recorded in Qalubia, Ismailia, Monofia and Beheira respectively, the lowest infection rate was recorded in El- Beheira and EL-Monofia respectively. Same results were confirmed with [9] who identified the ApMV for the first time in Egypt. ApMV is distributed worldwide wherever its hosts are present [8].

MECHANICAL TRANSMISSION AND HOST RANG OF APMV

The obtained results from mechanical inoculation by rubbing finger in one-direction on cotyledon or primary 2-6 leaves of hosts using sap inoculations for ApMV indicated that, symptoms on infected plants differ according to the plant species. From 16 tested host plants, ApMV infected 16 hosts. Mosaic and vein bending on *Fragaria ananassa*, mosaic on *Ch. amaranticolor*, Chlorotic Local lesions on *Ch. quinoa*, Systemic yellow mosaic on *Phaseolus vulgaris*, vein clearing and chlorosis on *Vicia faba*, Chlorotic local lesions, systemic and stunting on *Cucumis sativus*, Chlorosis on *Datura metal*, leaf malformation on *Solanum melongena*, Leaf crinkling on *Lycopersicon esculentum*, Systemic infection on *Nicotiana glutinosa*, Chlorotic and mottle on *Nicotiana benthemina*, Leaf malformation on *Capsicum annuum*. Table (3) and Fig (3).

TABLE (3): HOST RANGE AND SYMPTOMS RECORDED AFTER MECHANICAL INOCULATION OF APMV AND TESTED BY ELISA.

No.	Family	Hosts	Symptoms infectivity	Incubation period (days)	ELISA test
1	*Rosaceae*	*Fragaria ananassa*	Mosaic and vein bending	20 days	+
2	*Chenopodiaceae*	*Ch. amaranticolor*	Mosaic	12 days	+
		Ch. quinoa	Chlorotic Local lesions	10 days	+
3	*Leguminosae*	*Phaseolus vulgaris*	Systemic yellow mosaic	14 days	+
		Vicia faba	Vein clearing and chlorosis	20 days	+
4	*Cucurbitaceae*	*Cucumis sativus*	Chlorotic local lesions, systemic and stunting	15 days	+
5	*Solanaceae*	*Datura metal*	Chlorosis	14 days	+
		Datura stramonium	No symptoms	15 days	-
		Solanum melongena	Leaf malformation	20 days	+
		lycopersicon esculentum	Leaf crinkling	14 days	+
		Nicotiana glutinosa	Systemic infection	14 days	+
		Nicotiana occidentalis	No symptoms	15 days	-
		Nicotiana benthemina	Chlorotic and mottle	15 days	+
		Petunia hybrid	Leaf mosaic	15 days	+
		Capsicum annuum	Leaf malformation	15 days	+
6	*Compositae*	*Zinnia elegans*	No symptoms	20 days	-

Fig. (3): Symptoms of APMV on herbaceous indicator plants, (A)Mosaic and vein bending *Fragaria ananassa*,(B) Chlorotic Local lesions on *Ch. quinoa*, (C) Mosaic on *Ch.amaranticolor*, (D) Systemic infection on *Nicotiana glutinosa*,(E) Leaf malformation on *Solanum melongena*, (F) Leaf crinkling on *lycopersicon esculentum*, (G) Leaf malformation on *Capsicum annuu*m, (H) Chlorosis on *Datura metal*, (I) Chlorotic and mottle, tip malformed on *Nicotiana benthemina*, (J) Chlorotic local lesions, systemic and stunting on *Cucumis sativus*, (K) Vein clearing and chlorosis on *Vicia faba*, (L) Systemic yellow mosaic on *Phaseolus vulgaris*.

The obtained data reviled that ApMV Egyptian isolate had limited host range these results agreed with [2] who reported that ApMV can infect about 20 species, usually symptomlessly. These results agreed with [8] who reported that ApMV can be transmitted, although with difficulty, by mechanical inoculation of sap from woody plants to several herbaceous species. Same results were confirmed by [19].

MOLECULAR DETECTION

Total RNA Extraction and Reverse transcription (RT-PCR)

Samples of infected plants which gave positive results with DAS-ELISA were used for total RNA isolated using one step RT PCR amplification. Electrophoresis analysis of RT-PCR product showed a single amplified fragment at 262 bp Fig. (4). the size of PCR product was expected 262 bp similar results confirmed by [13].

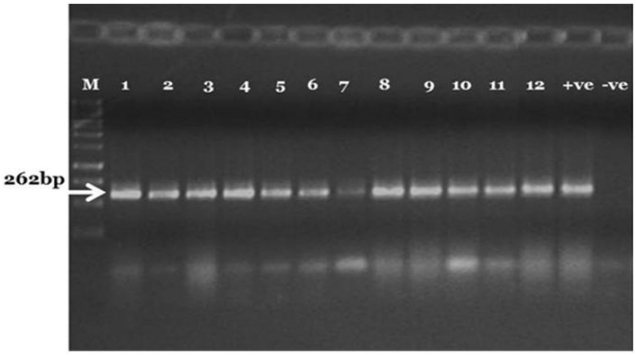

Fig. (4): 1% Agarose gel electrophoresis analysis of RT-PCR showing the amplified fragment of (ApMV) from isolated from strawberry plants. (M) 100bp DNA molecular weight marker (ABgene, UK). Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 strawberry samples collected from different locations in Egypt and tested positive for ApMV, +ve Positive control, –ve Negative control.

The obtained results agree with [19,12 and 20] who found that ApMV was detected in strawberry by using reverse transcription polymerase chain reaction (RT-PCR).

NUCLEOTIDE SEQUENCE ANALYSIS

Molecular characterization of ApMV genome has been done. Fresh leaf tissues of F. ananassa were applied for extraction and PCR amplification. The amplified PCR fragments were used for cloning, sequencing and comparing the sequencing with other of those sequencing available in Gen-Bank. The DNA sequence was performed using the PCR product 262 bp of the coat protein gene obtained when mers specific to virus under study were used and cloned. Nucleotide sequence analysis of several clones of amplified ApMV cDNA obtained from RNA extracts of infected leaves from different geographical locations were determined. The leotide sequence of ApMV Egyptian isolate showed in Fig. (5). the sequence was aligned by using DNAMAN program with isolates for ApMV available in the Gen-Bank.

Fig (5): Nucleotide sequence of cloned DNA of 262 bp from the coat protein gene of ApMV isolated from strawberry plants in Egypt.

Comparison between bases composition of coat protein gene sequence for ApMV Egyptian isolates with isolates of ApMV available in the GenBank was done to determine A+T, G+C content as shown in Table (4), the Egyptian ApMV isolate was A+T, G+C identical content with ApMV Yunnan isolate and recorded 139, 123 respectively. The results showed that the Egyptian isolate nucleotides arranged with the highest number of Adenine 71 (27.09%) and Guanine (G) 79 (30.15%), then Thymine (T) 68 (25.95%) and Cytosine (C) 44 (16.79%) respectively. Table. (4): Showed the comparison between bases composition of coat protein gene sequence for ApMV Egyptian isolate and seven ApMV isolates available in Gen-Bank.

TABLE. (4). COMPARISON BETWEEN BASES COMPOSITION OF COAT PROTEIN GENE SEQUENCE FOR APMV EGYPTIAN ISOLATE AND SEVEN APMV ISOLATES AVAILABLE IN GENBANK.

ApMV isolated	Total Base pair	Bases											
		A		C		G		T		A+T		C+G	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Egypt	262	71	27.1	44	16.8	79	30.2	68	26	139	53.1	123	46.9
Yunnan	262	71	27.1	44	16.8	79	30.2	68	26	139	53.1	123	46.9
USA	262	70	26.7	44	16.8	77	29.4	71	27.1	141	53.8	121	46.2
UK	262	74	28.2	42	16	79	30.2	67	25.6	141	53.8	121	46.2
Turkey	262	73	27.9	45	17.2	79	30.2	65	24.8	138	52.7	124	47.3
Australia	262	73	27.9	48	18.3	74	28.2	67	25.6	140	53.4	122	46.6
Belarus	262	76	29	46	17.6	74	28.2	66	25.2	142	54.2	120	45.8
India	262	74	28.2	50	19.1	68	26	70	26.7	144	55	118	45

(A): Adenine (C): Cytosine (G): Guanine (T): Thymine

Nucleotide sequence analysis for 262bp amplified fragment from the coat protein gene of the ApMV genome showed similarity ranged from 98%, 97%, 95%, 94%, 92% and 89% when it compared with the other ApMV sequences available in the Gen-Bank. ApMV Egyptian isolate shared 98% identity with Yunnan isolate followed by 97% USA isolate, 95% UK isolate ,94% Turkey isolate, 92% Australia and Belarus isolates, and 89% with India isolate. Fig.(6). Showed phylogenetic tree were

constructed for the different sequences comparison of ApMV Egyption isolate with the available sequences in the GenBank. Fig.(7). Observed the amino acid translation for ApMV by using DNAMAN software.

The obtained results is agreement with [3] who mentioned that nucleotide sequence similarity in the CP gene of ApMV isolates from hazelnut were 90.3-99.9 and 85.2-90.8 % when compared to corresponding sequences of the ApMV strains available in Gen-Bank. [16] stated that the complete nucleotide sequences of Apple mosaic virus RNA 1 and 2 have been characterized. [17] Comparison of the sequences of three amplicons with nine complete available sequences of ApMV coat protein (CP) (from apple) at amino acid level revealed a maximum of 96% homology to a Korean isolate of ApMV.

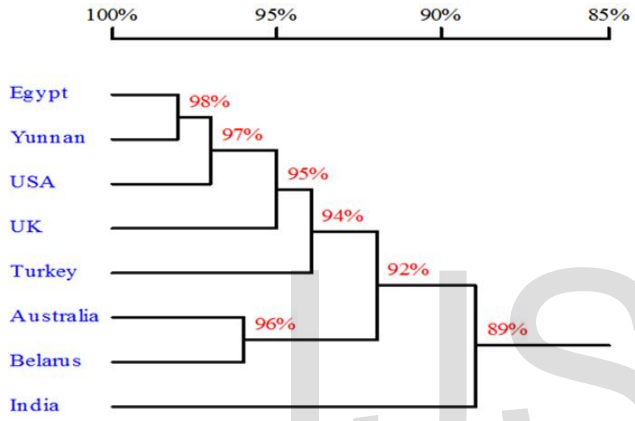

Fig (6). Phylogenetic tree constructed from multiple sequence alignment of coat protein gene of the Egyptian ApMV isolate with corresponding sequence of the seven ApMV isolates available in the GenBank.

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1       ATCCGAGTGAACAGTCTGTCCTCGAATGGATGGATTGGTTTGGTTGAGGATTACGATGAA
1        I  R  V  N  S  L  S  S  N  G  W  I  G  L  V  E  D  Y  D  E

61      AGTAATCCGAAAGGTCCGAATCCGATGGACCGATAGGGTTTCAAGAAAGACCAACCGAGA
21       S  N  P  K  G  P  N  P  M  D  R  *  G  F  K  K  D  Q  P  R

121     GGTTGGCAATGGGAAGCCGCTCCGAACACAACTTATGATGACTTCGTGAGGAAGTTTAGG
41       G  W  Q  W  E  A  A  P  N  T  T  Y  D  D  F  V  R  K  F  R

181     TTGGTTCTGGAGTTTAAGACGAATTTCGCCGCTGGTGCGAAGGTCTTTATGAGCGATTTG
61       L  V  L  E  F  K  T  N  F  A  A  G  A  K  V  F  M  S  D  L

241     TACGTGATAACGAGTGAGTTAC
81       Y  V  I  T  S  E  L
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Fig (7): Nucleotide sequences and predicted amino acid sequence of the cloned cDNA of the ApMV coat protein gene.

In conclusion, the present data confirmed that ApMV should be considered an important pathogen of strawberry, even if its presence in the surveyed regions is limited to well defined areas. Moreover, the establishment of a reliable, sensitive and quick molecular diagnostic method, such as the one-step RT–PCR will allow detection and identification of ApMV in the early stages of strawberry cultivation. Thus, ApMV control will be simpler and easier than previously and knowledge of the health status of strawberry for commercial and agricultural purposes will be improved.

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