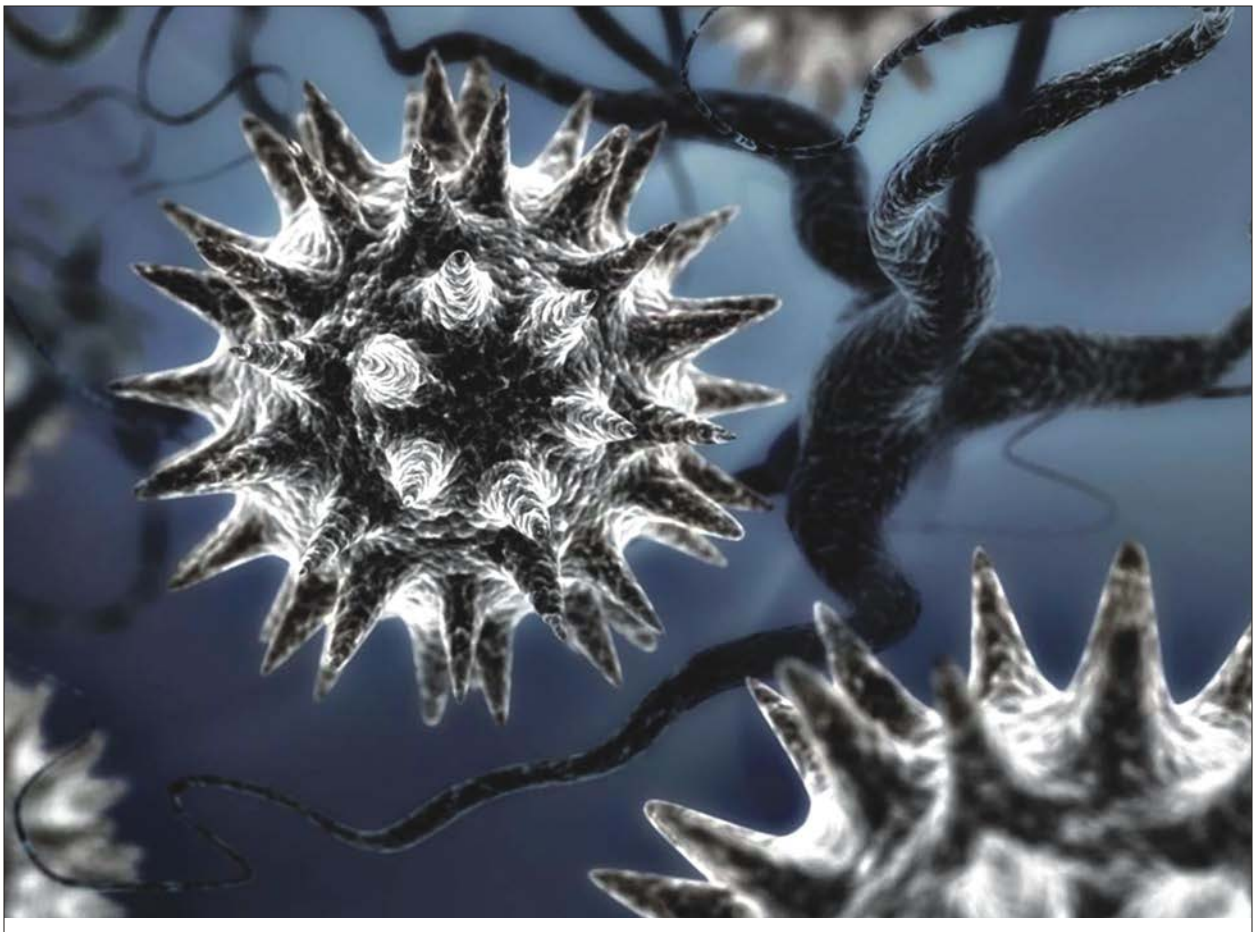

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Differentiation Study between Alfalfa Mosaic Virus and Red Clover Mottle Virus Affecting Broad Bean by Biological and Molecular Characterization

[1]Sabry Y.M. Mahmoud, [2]Abdel-Sabour G.A. Khaled and [3]Karel Petrzik
[1]Department of Agricultural Microbiology, Faculty of Agriculture,
Sohag University, Sohag (82786), Egypt
[2]Department of Genetics, Faculty of Agriculture,
Sohag University, Sohag (82786), Egypt
[3]Department of Plant Virology, Institute of Plant Molecular Biology, Biology Centre,
Academy of Sciences, Branisovska 31, Ceske Budejovice 370 05, Czech Republic

Abstract: This study aimed to identifying the causal virus (es) inducing wilting and necrotic symptoms on broad bean plants. Amplification of total RNA extracted from infected broad bean yielded 200-550 bp using *Comovirus*-specific primers. The amplified cDNAs was cloned, sequenced and analyzed. Nucleic acid sequence analysis of smallest and biggest bands revealed of 100 and 95% sequence identity with other Red Clover Mottle Virus (RCMV) and Alfalfa Mosaic Virus (AMV) isolates, respectively. Two viruses were isolated and biologically purified by single local lesion method on *Phaseolus vulgaris* plants. The obtained isolates were mechanically transmitted and has narrow host range included most plants belonging to Fabaceae and Solanaceae. The AMV was aphid transmitted in a non-persistent manner through *Myzus persicae* and *Aphis craccifora*. In contrast, RCMV don't transmit. Electron microscopy has revealed presence of AMV particles within the cytoplasm; also, non-aggregated particles packed side by side in the vacuoles of *Nicotiana tabaccum* var. White Burley cells was showed. On the other hand, spherical particles, about 30 nm in diameter were shown on partially purified RCMV preparations. Biological and molecular properties of the Egyptian isolates from broad bean established its identity as an AMV and RCMV, respectively.

Key words: Alfalfa mosaic virus, identification, DAS-ELISA, phylogenetic relationship, nucleotide sequences, red clover mottle virus, RT-PCR

INTRODUCTION

Faba bean (*Vicia faba* L.) is an economically important legume crop in many countries. In Egypt, it is considered the most popular food legume crop. Worldwide, this crop is known to be naturally infected by about 44 viruses (Cockbain, 1983), which cause considerable yield losses.

In Egypt, many viruses have been identified including AMV. However, until now no trails to study status of that virus or other unidentified viruses on faba bean have been done. Since, its first report by Weimer in 1931 (Jung *et al*., 2000), AMV has been found in most countries infecting many plants. The virus is easily transmitted by sap inoculation and by

Corresponding Author: Sabry Y.M. Mahmoud, Department of Agricultural Microbiology, Faculty of Agriculture, Sohag University, Sohag (82786), Egypt

several aphid species in a non-persistent manner to some of 430 plant species in 51 dicotyledonous families (Jung *et al*., 2000; Rivas *et al*., 2005; Gillaspie *et al*., 2006; Nair *et al*., 2009). It can cause extensive yield losses and economic damage (Van Regenmortel and Pinck, 1981). The AMV is one of the most biologically variable plant viruses and numerous natural variants having different pathogenicity (Hajimorad and Francki, 1991). The AMV is the type species of the genus *Alfamovirus* and belongs to the family Bromoviridae. It has a tripartite single-stranded genome. RNAs 1, 2, 3 and subgenomic RNA4 are separately encapsidated into bacilliform particles which are 18 nm wide and have lengths characteristic of the RNA encapsided (about 56, 43, 35 and 30 nm, respectively) (Sehnke and Johnson, 1994; Thole *et al*., 1998). The three genomic RNAs are not infective. Infection can start only in the presence of RNA4 or its translation product of Coat Protein (CP). Strains that have been studied in most detail are the Leiden and Madison isolates of strain 425 (425L and 425 M) (Hagedorn and Hanson, 1963), the Strasbourg isolate (S) (Walter *et al*., 1985), the 15/64 and VRU strains (Crill *et al*., 1970) and Korean isolates of strain KR (KR1 and KR2) (Jung *et al*., 2000). The complete nucleotide sequence of the AMV RNAs has been determined (Cornelissen *et al*., 1983a, b). RNA1 and RNA2 contain single open reading frame (ORF) encoding the viral replicas subunits (P1 and P2, respectively). RNA3 contains two ORFs encoding the movement protein and CP (Taschner *et al*., 1991). The CP is expressed from a fourth subgenomic RNA (sgRNA).

The RCMV is a member of the genus *Comovirus*, family Secoviridae, which represents nonenveloped plant viruses with icosahedral capsids and bipartite, single-stranded, positive-sense RNA genomes. As in other *comoviruses*, the two RNA molecules are encapsidated separately in isometric particles composed each of a large (L) and a small (S) coat proteins (Lomonossoff and Johnson, 1991). Two strains (O and S) of RCMV have been characterized (Lapchic *et al*., 1998; Lin *et al*., 2000) and the complete nucleotide sequence of the genome of strain S has been determined (Shanks *et al*., 1986). The RCMV has a mode of gene expression similar to *Cowpea mosaic virus* (CPMV), the type member of the group (Shanks *et al*., 1986).

During a survey in kafr El-Sheikh governorate, for virus infection in faba bean fields (2006-2008), viral symptoms such as severe chlorosis, necrosis, rolling, ring spots on leaves and generally plant stunting were observed. They were spread during the growing season causing considerable quantitative deleterious effect on plant yield. Therefore, the objective of this investigation was to isolate and identify the causal virus(es) by biological and molecular methods.

MATERIALS AND METHODS

This study was carried out in cooperation between Agricultural Microbiology Department, Faculty of Agriculture, Sohag University, Egypt and Plant Virology Department, Institute of Plant Molecular Biology, Academy of Sciences, Czech Republic in the framework of personal scientific cooperation since 2006.

Viral Source

The incidence of virus-like symptoms including chlorosis, necrosis, rolling, ring spots on leaves, stunting and plant death (Fig. 1) was about 60% in broad bean plants at Kafr El-Sheikh Governorate. The infection agent(s) were mechanically transmitted by macerating in 0.01 M phosphate buffer and (pH 7.0) to leaves of *Ph. vulgaris*, *V. faba, Chenopodium amaranticolor* and *C. quinoa* and *Nicotiana tabacum.* A preliminary study by DAS-ELISA

Fig. 1: Leaf rolling, stunting, necrosis and plant death showing on naturally infected faba bean

using polyclonal antibodies of Cucumber mosaic virus (CMV), Bean yellow mosaic virus (BYMV), Broad bean stain virus (BbSV) and Faba bean necrotic yellow vein virus (FBNYVV) was done.

Molecular Study

Extraction of RNA and RT-PCR Procedures

The RNA for reverse transcription and real time-PCR was isolated from about 0.1 g of infected leaves of faba bean sample with Nucleospin RNA plant kit (Macherey Nagel, Germany) according to the manufacturer's recommendations and eluted with 30 μL of water. Complementary DNA (cDNA) was synthesized from 7 μL of RNA with iScript synthesis kit (Bio-Rad) in 10 μL reaction volume. *Comovirus*-specific primers 5'-AANCCRGANGGDATNC-CRCAYTC-3' and 5'-GGATTGATACCTACCTGGCA-3' were used for amplification of 181 bp long segment of the RNA polymerase gene.

The 20 μL PCR reaction mixture contains 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM each of dNTPs, 1 U Dream Taq DNA polymerase (Fermentas, Lithuania), 2 μL of cDNA and 20 pmol of primers. The amplifications were performed in an amplification program of 35 cycles with 30 sec denaturation at 95°C, 1 min annealing at 45°C and 1 min synthesis at 72°C. All samples were analyzed on 1.5% agarose gel electrophoresis.

Cloning and Sequences Analysis

Three fragments of about (200, 380 and 500 bp) were individually cloned to pre-cleaved blunt-ended vector plasmid pJET1.2/blunt (2974 bp, Fermentas) and sequenced using the BigDye sequencing terminator kit, version 3.1 (PE Biosystems, UK) then analyses on ABI PRISM 310 sequencer (PE Applied Biosystems, USA).

Multiple alignments of amino acid sequences were obtained using the default options of the AligX, Vector NTI Advance 9.1 program (Invitrogen). Search homologies with proteins from the BLAST basic local alignment search tool of National Center of Biotechnology Institute (NCBI) was done with the FASTA (Pearson and Lipman, 1988) and TBLASTN program 2.2.22+ (Altschul *et al*., 1990).

Phylogenetic Trees

Red Clover Mottle Virus

The amino acid sequence corresponding to 200 bp fragment was compared with most corresponding known other viruses belonging to *Comovirus* genus of Comoviridae family. Phylogenetic tree was constructed with deduced amino acid sequences of Red clover mottle

virus-Egyptian isolate (RCMV-Eg, GQ923687), Red clover mottle virus (RCMV, CAA46104), Squash mosaic virus (SqMV-R, BAB62139), Squash mosaic virus isolate CH 99/211 (SqMV-P1, ABZ89551), Squash mosaic virus of RdRp (SqMV-RdRp, CAG27555), Bean pod mottle virus (BPMV-R, ABP96717), Bean pod mottle virus (BPMV-P, AAD09629), Bean pod mottle virus of K-Hancock1 strain (BPMV-Ha, AAM73715), Bean pod mottle virus of K-Hopkins1 strain (BPMV-Ho, AAM73718), Bean pod mottle virus of subgroup I (BPMV-I, AAW69768), Bean pod mottle virus of subgroup II (BPMV-II, AAW69769), Radish mosaic virus isolate 1 (RaMV1, ACE06773), Radish mosaic virus (RaMV-P, BAF75830), Radish mosaic virus of California strain (RaMV-Ca, BAG84602), Radish mosaic virus (RaMV-R, AAY32935), Cowpea mosaic virus (CPMV-R, ABP96716), Cowpea mosaic virus (CPMV, CAA25029) and Andean potato mottle virus (APMV, AAA42422).

Alfalfa Mosaic Virus

The amino acid sequence corresponding to 550 bp fragment was compared with Alfalfa mosaic virus, the sole member of *Alfamovirus* genus and some other corresponding known viruses belonging to *Ilarvirus* genus of Bromoviridae family. Phylogenetic tree was constructed with predicted amino acid sequences of Alfalfa mosaic virus Egyptian isolate (AMV-Eg, GQ923686), Alfalfa mosaic virus of Leiden strain 425 (AMVTc-2, AAA46289), Prune dwarf virus (PDV, AAB39537), Fragaria chiloensis latent virus (FCILV, AAV68306), Humulus japonicus latent virus (HjLV, AAS86438), Prunus necrotic ringspot virus (PNRSV, AAK69474), American plum line pattern virus (APLPV, AAK15026) and Apple mosaic virus (ApMV, AAD55722).

The phylogenetic trees were constructed using the ClustalX2 Multiple Sequence Alignment Program (Thompson *et al*., 1997) with 1000 bootstrap replicates. Protein function analysis was performed using the fowling software (http://www.ebi.ac.uk/Tools/InterProScan/).

Viral Isolation

The viruses from naturally infected broad beans were mechanically transmitted by macerating in 0.01 M phosphate buffer (pH 7.0) to leaves of *Ph. vulgaris* and single local lesions were taken for biological purification. Two isolates from differed single local lesions were then propagated in *Nicotiana tabacum* var. White Burley or *Ph. vulgaris* and three cycles of purification were repeated. Purified virus isolates were confirmed by study on the basis of host range, symptomatology, mode of transmission and stability in the infectious sap. Also, viruses were insured by electron microscopy.

Biological Studies

Host Range and Symptomatology

Host range study was performed using the symptomatic leaf extracts of *N. tabacum* and *Ph. vulgaris*. Each leaf extract grounded in extraction buffer (0.01 M Na_2HPO_4, pH 7.2; 0.01 M sodium sulfite; 1 mM EDTA) and then inoculated onto *V. faba*, *Vigna radiata*, *V. unguiculata*, *Ph. vulgaris*, *Cicer arietinum*, *Lens esculenta*, *Lupinus termis*, *Pisum sativum*, *C. amaranticolor*, *C. quinoa*, *Beta vulgaris*, *Capsicum annuum*, *Datura metal*, *D. stramonium*, *Solanum nigrum*, *N. tabacum* var White Burley, *N. glutinosa*, *Sonchus oleraceus*, *Gomphrena globosa*, *Ocimum basilicum* and *Rumex acetosa*. Ten seedlings of each host plant were inoculated and each inoculation was repeated three times. All tested plants were kept in a greenhouse (20-25°C) and observed daily for symptoms development.

Insect Transmission

Two aphid species *Myzus persicae* and *Aphis craccifora* (provided from Plant protection department, Faculty of Agriculture, Sohag University) were used for transmission test. The aphids were raised on healthy cabbage (for *M. persicae*) or wheat plants (for *A. crassifora*) free of viruses. The aphids were placed onto *N. tabacum* or *Ph. vulgaris* systemically infected leaf pieces for acquisition feeding for 1 h in a Petri dish after 2 h fasting. Aphids were moved onto healthy three-leaf seedlings overnight (5 insects per plant) for inoculation feeding before being killed. A total of 10 plants were inoculated for each species of aphids. Mosaic symptoms on plants were observed 20 days post-inoculation. The virus-free insects were maintained on five healthy *N. tabacum* or *P. vulgaris* plants as control.

Assay of *in vitro* Stability

Dilution End Point (DEP), Thermal Inactivation Point (TIP) and longevity *in vitro* (LIV) of the virus isolates were determined according to Noordam (1973) by using *C. amaranticolor* and *C. quinoa* as indicator plants.

Electron Microscopy (EM)

Partial Purification of the Virus Isolates

First Isolate

According to Hajimorad and Francki (1991), Systemically infected *N. tabacum* leaves were homogenized in 2 volumes (w/v) of extraction buffer (0.2 M Na_2HPO_4; 0.3% 2-mercaptoethanol; 0.01 M EDTA, pH 7.6) and clarified by 0.5% cold mercaptoethanol; 0.01 M EDTA, pH 7.6) then clarified again by 5% cold chloroform and butanol (1: 1= v/v). After polyethylene-glycol (PEG, MW 6000, 8% w/v) precipitation, the sediments were resuspended in a buffer containing 0.02 M Na_2HPO_4, pH 7.6 and 1 mM EDTA followed by centrifugation through a 30% sucrose cushion for 2 h at 30.000 rpm at 4°C. The virus pellet was then resuspended in 0.5 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0). Virus concentration was estimated using an assumed extinction coefficient of 4.9 (Vloten-Doting and Jaspars, 1973).

Second Isolate

According to Shanks *et al*. (1986), systemically infected *Ph. vulgaris* leaves were grind in 0.1 M phosphate buffer, pH 8; containing 0.02 M 2-mercaptoethanol and 10% (w/v) sucrose. The extract was filtered through cheesecloth and clarified by low speed centrifugation. Supernatant was mixed with equal volume of chilled 1, 1, 2-trichloro- 1, 2, 2-trifluoro-ethane 9 freon (113) and emulsified briefly. Aqueous phase was polyethylene-glycol (PEG, MW 6000, 8% w/v) precipitated and then the sediment was treated as mentioned above.

Electron Microscopic Examination

Virus Particles

Electron microscopic examination of the partially purified suspension of tow isolates were negatively stained with 2% phosphotungstic acid (PTA). A small droplet of purified virus preparations was placed on a carbon-coated Formvar grid for 1 min and excess was removed. Another drop of 2% PTA, pH 6.0 was placed on the same grid for 1 min. Excess stain was removed by touching the edge of grid with a filter paper. After air drying the grids were examined by electron microscopy.

Ultrathin Sections

Fresh leaf samples collected from *N. tabacum* or *Ph. vulgaris* systemically infected plants were fixed in 3% glutaraldehyde, in 0.05 M phosphate buffer (pH 7), for 3 h. Samples were rinsed several times in 0.05 M phosphate buffer and then were post fixed with 1% OsO_4 in 0.05 M phosphate buffer for 2 h. Samples were rinsed several times with 0.05 phosphate buffer and taken to the laboratory. Samples were then dehydrated in a gradient acetone series and embedded in Spurr's medium (Spurr, 1969). Ultrathin sections (60 nm thick) were stained with uranyl acetate and lead citrate. Specimens were viewed with a Jeol-1010 transmission electron microscope at 100 kV (Unit of Electron Microscopy at Sohag University, Egypt).

RESULTS AND DISCUSSION

Preliminary biological indication and serological analysis by DAS-ELISA indicated probably presence of *Comovirus*-isolate. Hence, RT-PCR was directly used to ensure this hypothesis.

Molecular Study

RT-PCR and Nucleotide Sequences

RT-PCR assays performed with two *Comovirus*-specific primers for amplification of 181 bp long segment of the RNA polymerase gene. The results of RT-PCR showed three different bands (about 200, 380 and 550 bp) (Fig. 2). Once the sequences were determined, two fragments, 163 nucleotide (nt) and 569 nt were obtained from smallest and biggest bands, respectively. These sequences were disposed in the GenBank under accession numbers (GQ923686) and (GQ923687) for RCMV-Eg and AMV-Eg, respectively.

Analysis of Viral Sequences

From *in silico* comparison of alignment of the amino acid sequences corresponding to 163 nt fragments, with most other corresponding known viruses revealed identical percentages 70, 72, 75, 79, 81 and 100% for SqMV-R, (SqMV-RdRp and RaMV), (APMV and SqMV), CPMV, BPMV and RCMV isolates, respectively. The Phylogenetic tree showed that the Egyptian isolate clustered along with RCMV at 1000 bootstrap value (Fig. 3) and the same clustering of isolate was observed with the multiple alignment (Fig. 4).

The RCMV-Eg amplified sequence encodes a polypeptide of 44 amino acid (aa) with a predicted molecular weight of 5.567 kDa, identified as the RNA binding and RNA-directed

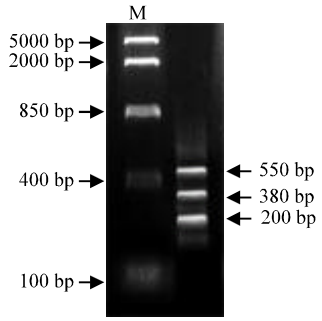

Fig. 2: RT-PCR products amplified from total RNA of infected leaves of faba bean sample

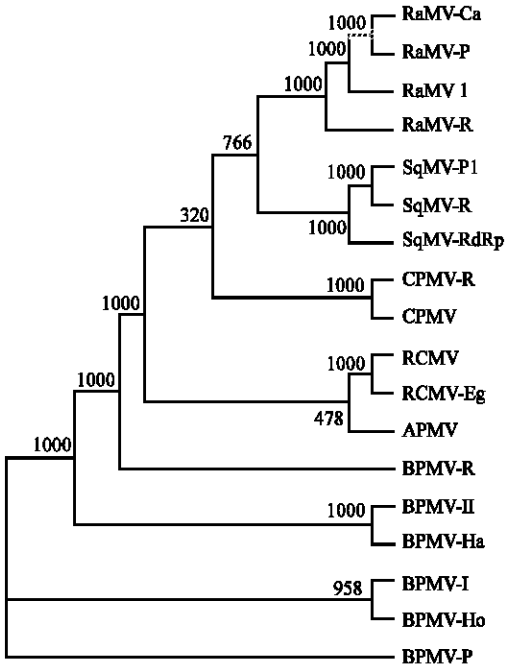

Fig. 3: Phylogenetic tree constructed with deduced amino acid sequences of RCMV-Eg with most *Comovirus* genus of Comoviridae family

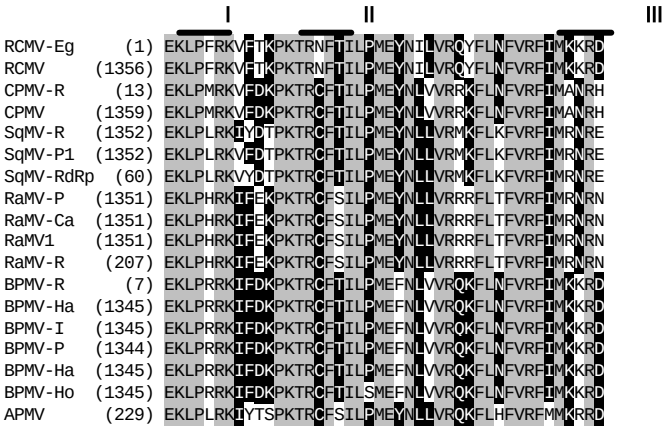

Fig. 4: Alignment of the amino acid sequences of RCMV-Eg with most *Comovirus* genus of Comoviridae family viruses. Regions with identical sequences are highlighted in gray. Amino acid residues that are conserved sequences in the majority of the amino acid sequences of proteins of viruses are highlighted in black

RNA polymerase (RdRp-1) due to the presence of conserved stretches common to the corresponding protein of *Comoviruses*, three longer amino acid stretches [I:(EKLP), II:(KPKTR) and III:(FVRFIM)] were identical in all figured sequences (Fig. 4).

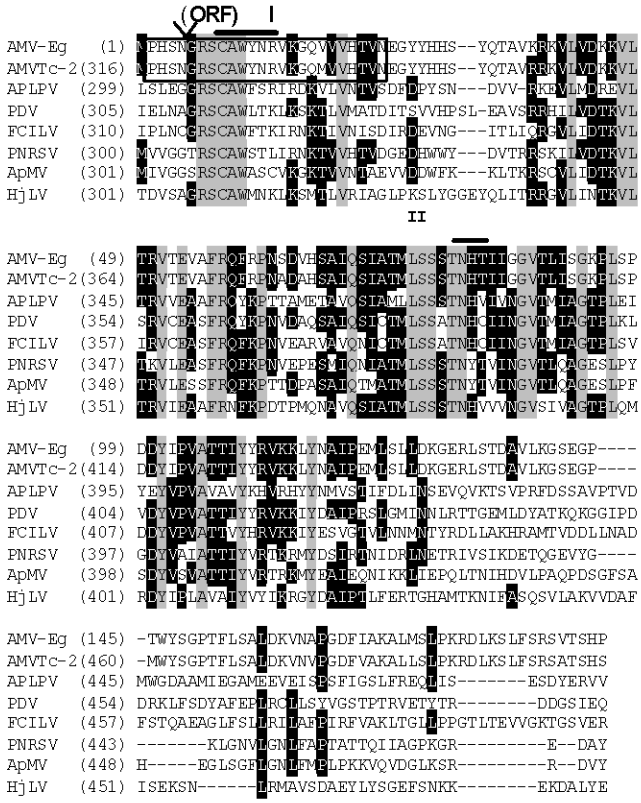

Fig. 5: Alignment of the amino acid sequences of AMV-Eg with AMV Tc-2 of *Alfamovirus* genus and some viruses of the *Ilarvirus* genus. Regions with identical sequences are highlighted in gray. Amino acid residues that are conserved sequences in the majority of the amino acid sequences of proteins of viruses are highlighted in black

On the other hand, computer-assisted analysis of the assembled amino acid sequences corresponding to 569 nt fragments with AMV Tc-2 and some other corresponding known viruses revealed identical percentages of 38, 39, 47, 52 and 95% for APLPV, (FCILV and ApMV), (HjLV and PNRSV), PDV and AMV Tc-2 isolates, respectively (Fig. 5). The phylogenetic tree relationships deduced from multiple alignments of Egyptian isolate with others showed two major clades that clustered at 404 bootstrap values (Fig. 5). The first clade contained AMV-Eg that clustered along with AMV Tc-2 of the same Genus at 1000 bootstrap value, whereas the second clade comprised the other *ilarvirus*. This is consistent with the clustering based on the sequence alignments (Fig. 6).

The obtained sequence of AMV-E g (569 nt in size) contains a short Single Open Reading Frame (ORF) that initiates with a start codon (AUG) at nt position 1-3 from 5' end and terminates with an amber stop codon (UAG) at nt position 59-61. The result of multiple alignments of AMV-E g with others showed that this short ORF is conserved between AMV-Eg and AMV Tc-2 isolates (Fig. 5).

The AMV-E g amplified sequence encodes a polypeptide of 190 aa with a predicted molecular weight of 21.049 kDa, identified as the RNA binding and mRNA methyltransferase

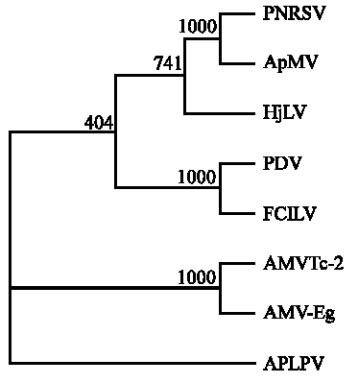

Fig. 6: Phylogenetic tree constructed with deduced amino acid sequences of AMV-Eg with AMV Tc-2 of *Alfamovirus* genus and some viruses of the *Ilarvirus* genus

due to the presence of conserved stretches common to the corresponding protein of the Bromoviridae family. The multiple alignments of AMV-Eg with others showed identical amino acid stretches, two amino acid blocks [I: (RSCAW) and II: (LSS)] were identical in all deduced amino acid sequences (Fig. 5).

Biological Study

At the end of the molecular study, the authors were sure to have a mixed infection of two viruses (RCMV and AMV), but they need to separate them and confirm it via biological definition and EM.

Host Range Studies

Characteristic development symptoms of AMV and RCMV isolates observed on Fabaceae, Solanaceae and on other indicator plants are summarized in Table 1, Fig. 7a-i and 8a-f. In general, more sever symptoms were induced by AMV than that of RCMV. So that, clear differences in symptoms between two isolates were obtained. Symptoms induced by AMV isolate were greatly influenced by environmental conditions. RCMV induced only necrotic local symptoms on *C. amaranticolor* and *Lupinus termis,* also induced necrotic ring spots on *S. nigrum.* In contrast, RCMV isolate induced necrotic ring lesions and local lesions in inoculated *V. faba* and *Ph. vulgaris* leaves followed by stem necrosis and sever mosaic, respectively, but induced mild mosaic directly on *Pisum sativum* without local infection. On the other hand, necrotic local lesions followed by a mild mottle, stem necrosis and plant death were noticed on *V. faba* after AMV inoculation. Also, local infections induced on *C. amaranticolor* and *Phaseolus vulgaris* turned to mosaic and chlorotic flecking as systemic infections, respectively. Mild mosaic was observed on *N. tabacum* var. White Burley, bright yellow mosaic induced on *Ocimum basilicum* and *Capsicum annuum* var. California but vein clearing and mosaic symptoms were induced on *Sonchus oleraceus* after inoculation with AMV isolate. The same isolate induced yellowing, shoot tip necrosis and stunting in *Cicer arietinum, Lens esculenta* and *Pisum sativum*, whereas induced chlorotic flecking and mottling on *V. radiate*. This isolate induced necrotic local lesions only on *Rumex acetosa V. unguiculata, Lupinus termis, C. quinoa* and *G. globosa.*

Insect Transmission

Three and five out 10 *N. tabacum* seedlings developed mosaic symptom 12 days after AMV transmission inoculation by each of the two aphid species *Myzus persica* and *Aphis*

Table 1: Differentiation between RCMV and AMV isolates via host range and symptoms after mechanical inoculation

	Symptoms induced by					
	RCMV isolate on			AMV isolate on		
Host plants	Inoculated leaves	Uninoculated leaves	Incubation period (day)	Inoculated leaves	Uninoculated leaves	Incubation period (day)
Phaseolus vulgaris	NLL	SM	5	NLL	CF	3-5
Vicia faba	NRL	SN	4	NLL	MM, SN, D	5
Pisum sativum	-	MM	8	-	Y, STN, S	12
Vigna radiata	-	-	-	-	CF, Mot	10
Vigna unguiculata	-	-	-	NLL	-	7
Cicer arietinum	-	-	-	-	Y, STN, S	12
Lens esculenta	-	-	-	-	Y, STN, S	12
Lupinus termis	BNLL	-	7	NLL	-	3-5
Chenopodium quinoa	-	-	-	NLL	-	7
C. amaranticolor	CLL	-	6	CLL	M	6
Beta vulgaris	-	-	-	-	-	-
Capsicum annum	-	-	-	-	BYM	18
Datura metel	-	-	-	-	-	-
D. stramonium	-	-	-	-	-	-
Solanum nigrum	NRS	-	-	-	-	-
Nicotiana tabacum var. White Burley	-	-	-	-	MM	12
N. glutinosa	-	-	-	-	-	-
Sonchus oleraceus	-	-	-	-	VC, M	12
Gomphrena globosa	-	-	-	NLL	-	8
Ocimum basilicum	-	-	-	-	BYM	20
Rumex acetosa	-	-	-	NLL	-	8

NLL: Necrotic local lesions, NRL: Necrotic ring lesions, BNLL: Black necrotic local lesions, CLL: Chlorotic local lesions, NRS: Necrotic ring spots, MM: Mild mosaic, SM: Severe mosaic, CF: Chlorotic flecking, SN: Systemic necrosis, D: Death, Y: Yellow; STN: Shoot tip necrosis, S: Stunting, BYM: Bright yellow mosaic, VC: Vein clearing, Mot, Mottling, -: No symptoms

cracciforae, respectively. On the other hand, no symptoms were detected on *Ph. vulgaris* plants after RCMV transmission inoculation by aphid species. Results confirming the transmissibility of AMV by these two aphid species, in contrast was noticed when RCMV isolate was used.

Stability *in vitro*

The infectivity test of AMV inoculum after various treatments showed that the Eg isolate had a TIP of 64°C and DEP of 10^{-3} and its infectivity was retained *in vitro* for up to 3 days. These values were 70°C, 10^{-6} and 14 days, respectively when RCMV isolate was tested.

AMV and RCMV Characteristics

The purified viruses had an absorption spectrum typical of viral nucleoprotein. The $A_{260/280}$ values for AMV and RCMV were 1.6 and 1.5, respectively. The yield of the viruses was approximately 2 and 3 mg per 100 g of fresh leaves. Purified preparations appeared to be not free from contamination as evidenced by EM observations. Bacilliform virus-like particles not showed on AMV-Eg preparations from *N. tabacum*. EM observation of RCMV-Eg infected *Ph. vulgaris* leaves revealed spherical particles in the sap, about 30 nm in diameter (Fig. 9).

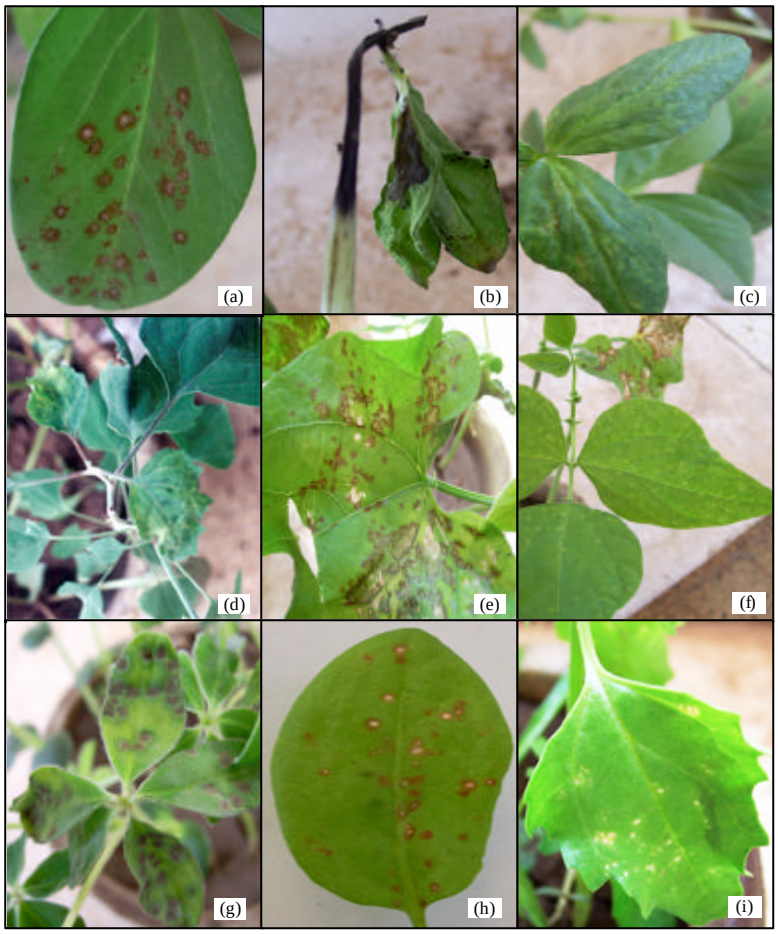

Fig. 7: Symptoms on test plants inoculated with Egyptian isolate of AMV from broad bean. a) necrotic local lesions on inoculated broad bean, b) systemic necrosis and death on broad bean, c) mild mosaic on broad bean, d) mosaic on *C. amaranticolor*, e) necrotic local lesions on bean, f) chlorotic flecking on uninoculated bean leaves, g) black necrotic local lesions on *Lupinus termis* leaves, h) necrotic local lesions on *Rumex acetosa* leaves and i) necrotic local lesions on *C. quinoa* leaves

Ultrathin Sections and Relation with Cells

Amorphous inclusion bodies have been seen as a raft containing particles packed side by side occur in the vacuoles of *N. tabacum*-AMV infected cells (Fig. 10a). On the other hand, inclusion bodies were not shown on *Ph. vulgaris*-RCMV infected cells, but many small vacuoles were distributed within these cells (Fig. 10b).

In the present study, the AMV and RCMV characteristics were studied using infected plant materials and compared the strain identity using sequence similarities of the *Comovirus*-specific primers. Leaves with chlorosis, necrosis, rolling, ring spots and plant stunting were collected from different broad bean growing areas in Kafr El-Sheikh Governorate and tested by DAS-ELISA against most viruses infect broad bean (data not shown). Due to negative ELISA reactions and their characteristic symptoms the samples

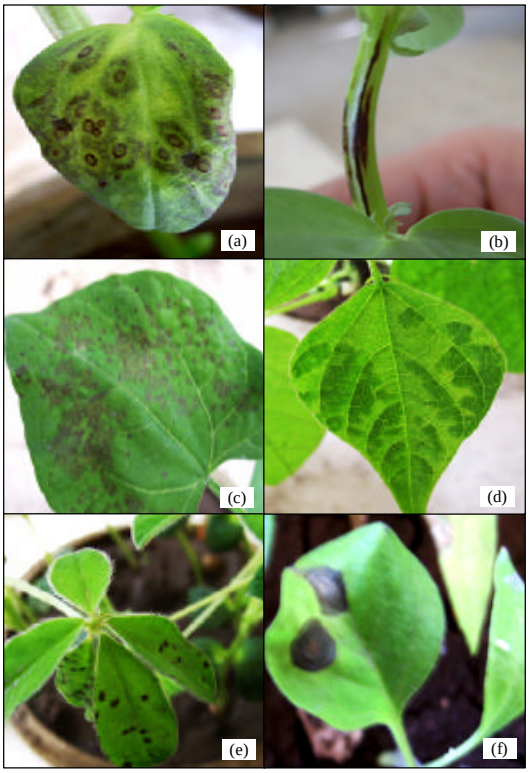

Fig. 8: Symptoms on test plants inoculated with Egyptian isolate of RCMV from broad bean. (a) necrotic ring spots on inoculated broad bean, (b) systemic necrosis on broad bean, (c) necrotic local lesions on bean, (d) severe mosaic on uninoculated bean leaves, (e) necrotic local lesions on *Lupinus termis* leaves and (f) necrotic ring spots on *S. nigrum* leaves

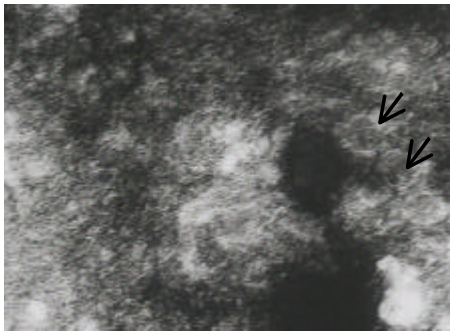

Fig. 9: Electron micrograph of partial purified particles of RCMV-Eg isolate. Bar indicates approximately 30 nm

were submitted to RT-PCR by using *Comovirus*-specific primers. Altogether, these results indicated that native field broad bean is infected by mixed infection with AMV and RCMV. Last evidence derived from only a partial nucleotide sequences, shown that comparison of

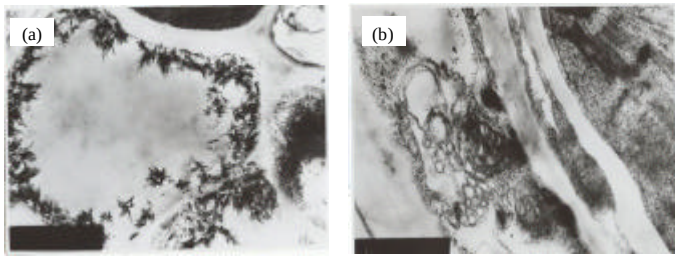

Fig. 10: Electron micrograph of thin section of (a) *N. tabacum* and (b) *P. vulgaris* AMV and RCMV-infected leaf cells, respectively. Amorphous inclusion bodies seen as a raft containing particles packed side by side in the vacuoles of AMV-infected tobacco cells (a). Small vacuoles were distributed within *P. vulgaris* cells infected with RCMV (b)

some gene sequence give good picture of relationship with the genus *Alfamovirus* or *Comovirus* as do comparisons of longer sequences (Pietersen *et al*., 1985; Neeleman *et al*., 1991). Previously, it has been difficult to classify AMV or RCMV strains, but now they can be differentiated more accurately based upon molecular properties (Kudela and Gallo, 1995; Lin *et al*., 2000). The PCR primers for *Comoviruses* were selected to amplification RNA-directed RNA polymerase (RdRp-1) and RNA binding and mRNA methyltransferase. RNA-directed RNA polymerase is an essential protein encoded in the genomes of all RNA containing viruses with no DNA stage (Koonin *et al*., 1989; Zanotto *et al*., 1996). Universal oligonucleotide primers complementary to conserved shard by all known members of a virus group have been shown to enable the identification of a new member of the different virus groups, *geminiviruses* (Rybicki and Hughes, 1990) and *potyviruses* (Langeveld *et al*., 1991). The obtained sequence of AMV-Eg (569 nt in size) contains a single short ORF that initiates with a start codon (AUG) at nt position 1-3 from 5' -end and terminates with an amber stop codon (UAG) at nt position 59-61. The position and sequence of RCMV-Eg corresponds well with supposed length of the amplification product of primers used, but the amplification of AMV-Eg was accidental, probably due to misspriming of the second primer. In AMV, RNA 2 contains several ORFs of 100 to 150 nt (Brederode *et al*., 1980). An ORF contains 663 nt concluding with a UGA stop coding in AMV RNA-3 (Barker *et al*., 1983). In AMV-RNAs 1 and 3 of strain 425, the 5' -proximal AUG codon is closely followed by a termination codon, whereas, in AMV-RNAs 2 and 4, the first AUG codon from terminal 5' -end is the beginning of long ORF (Koper-Zwarthoff *et al*., 1980; Cornelissen *et al*., 1983b). Both isolates supported amplification of the expected fragments, which was also found in AMV and RCMV strains. The amplified DNA fragments were subjected to sequence analysis. Results showed that the level of amino acid sequence identity in the RNA binding and mRNA methyltransferase and RdRp-1 compared with the nucleotide sequence identity of isolated AMV and RCMV strains were 95 and 100% with AMV-Tc2 and RCMV, respectively. Present results are agreement with findings of Jung *et al*. (2000) and Lapchic *et al*. (1998) for AMV and RCMV, respectively and El-Araby *et al*. 2009 when studied some potato viruses.

Isolation and host range studies showed that broad bean plants infected by two viruses, one of the *Alfamovirus* and the other of the *Comovirus*, this result agreement with previously reported on several crops (Paliwal, 1982; Valkonen *et al*., 1992; Lapchic *et al*., 1998). The symptoms on some test plants including *Rumex acetosa* and *S. nigrum* inoculated with AMV-Eg and RCMV-Eg, respectively showed some differences as compared to that of

previously described AMV isolated in Korea (Kudela and Gallo, 1995) and RCMV isolated in Sweden (Abdelmoeti, 1979). In general, AMV-Eg and RCMV-Eg could be clearly differentiated by host reactions in some indicator plants such as *C. amaranticolor, L. termis, C. arietinum* and *Ph. vulgaris*. AMV appears to be common in legume crops such as *Lablab niger, V. faba* and *Medicago sativa* in Sudan and Tanzania (Nour and Nour, 1962; Kaiser and Robertson, 1976). The combination of seed-infected plants and spreading by aphids may be resulted high levels of infection by AMV in broad bean fields in Egypt. Bacilliform virus particles of AMV-Eg not showed. This is in agreement with the previous report showing that structural integrity of AMV particles is not stable (Hajimorad and Francki, 1991). Keeping the virions structurally stable is extremely important because the low yield of particles often necessitates the bulking of virus preparations that may involve combining purified materials over several weeks to months (Pietersen *et al*., 1985; Valkonen *et al*., 1992). In general loss of infectivity of AMV started at a relatively low temperature and extended over a range of 20°C or more. The actual inactivation point depends on the concentration of the virus. Although, El-Attar *et al*. (1971) mentioned the detection of AMV from broad bean; this is the first report to fully describe the isolation and characterization of AMV and RCMV particles associated with broad bean in Egypt.

CONCLUSIONS

The AMV and RCMV were isolated from mixed infection of *V. faba* plants. Symptoms induced on different hosts are reported. The isolates described in this work showed symptoms, transmission, virus particles and RNA structure analogue to those of strains AMV Tc-2 and RCMV. Further details of molecular characteristics such as full-length genomic RNA sequences and other genes (like coat protein) will be required in order to reveal more knowledge's about the molecular characterization of these strains and relationship with other known AMV and RCMV strains.

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