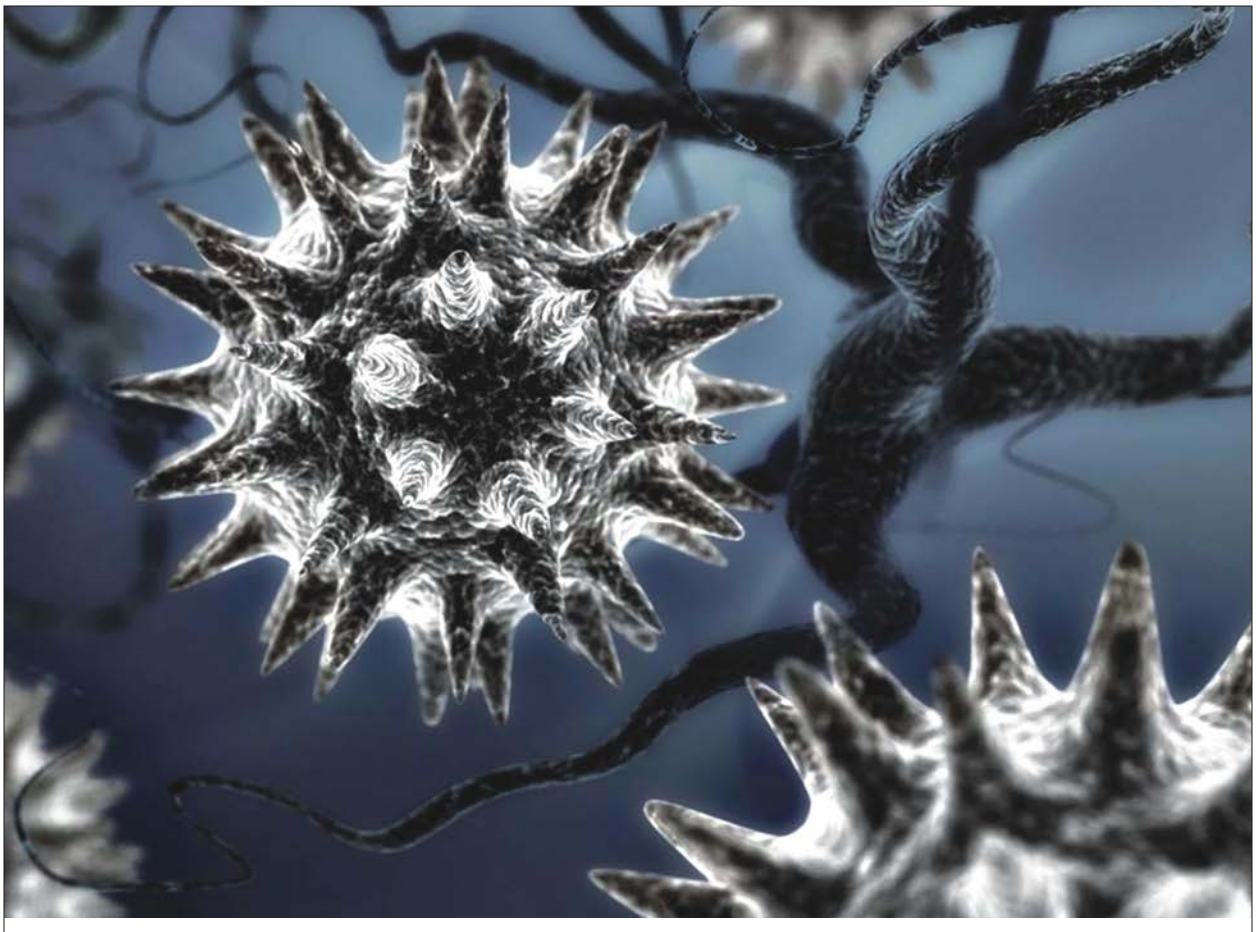

International Journal of Virology

ISSN 1816-4900

Academic Journals Inc.

www.academicjournals.com

International Journal of Virology 3 (3): 107-116, 2007
ISSN 1816-4900

Use of Helper Component Proteinase Gene to Identify a New Egyptian Isolate of Watermelon Mosaic Potyvirus

[1]T.Z. Salem, [1]S.M. El-Gamal and [1,2]A.S. Sadik
[1]Agricultural Genetic Engineering Research Institute (AGERI),
Agricultural Research Center (ARC), 9 Gamaa Street, Giza, 12619, Egypt
[2]Department of Agriculature Microbiology, Lab of Virology, Faculty of Agriculture,
Ain Shams University, P.O. Box 68, Hadayek Shobra, Cairo, 11241, Egypt

Abstract: In this study, an Egyptian isolate of potyvirus known to be zucchini yellow mosaic potyvirus (ZYMV) was isolated from leaves of the cultivar Giza 1 watermelon plants which cultivated in Agricultural Experimental Research Station, Sids, Beni Suef, Egypt. The double antibody sandwich (DAS)-enzyme-linked immunosorbent assay (ELISA) was carried out using polyclonal antibodies specific to ZYMV. The helper component proteinase gene (*Hc-pro*) of WMV was isolated by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers based on the DNA sequence of ZYMV virus, strain KR-PA. The blast searches identify the *Hc-pro* sequence as a part of Water Melon Mosaic Potyvirus (WMV). The sequence analysis showed that the isolated *Hc-pro* gene belongs to a new isolate of WMVs and its DNA sequence and deduced amino acid were compared with the *Hc-pro* of the French, China and Pakistan isolates. This study showed that the Egyptian isolate is closer to Pakistan isolate than French and China isolates, however, the phylogenetic tree showed that using one gene is not enough to relate these isolates to each other.

Key words: *Hc-pro,* WMV, ZYMV, SMV, CMV, PRSV, RT-PCR, ELISA, DAS

INTRODUCTION

Plant viruses are a persistent threat toward production of both watermelon and other cucurbits in Egypt as well as in the World. There are several common viruses that can affect cucurbits, including zucchini mosaic potyvirus (ZYMV), watermelon mosaic potyvirus (WMV), papaya ringspot potyvirus (PRSV) (Verma, 1988) and cucumber mosaic cucumovirus (CMV). Depending on the type of viral infection, the infected plants show stunted, mottled, or crinkled light green colored leaves. Moreover, fruits may be irregular in shape, mottled or warty. In Fact the superinfection is a frequent phenomena of potyviruses which can be detected in infected cucurbit plants in particularly, watermelon for example by PRSV and WMV. In Egypt, ZYMV is known to be one of the most abundant potyviruses that infect cucurbits. However, the common methods of detecting the infected cucurbit plants were based on morphological and serological methods. Therefore, differentiation between ZYMV infection and WMV infection is not convenient using the conventional methods especially due to their morphological similarity although differentiation among WMVs was done using monoclonal antibodies (Desbiez *et al*., 2007). ZYMV was first reported in *Cucurbita pepo*, in Italy by Lisa *et al*. (1981). Afterwards the presence of the virus was reported in Algeria, Australia, Egypt, Germany, Israel, Japan, Jordan, Lebanon, Morocco, Spain, Taiwan, England, Turkey and USA (Davis, 1986; Brunt *et al*., 1996). WMV was not isolated or identified in Egypt and the infected

Corresponding Author: Tamer Zaki Salem, Department of Microbial Molecular Biology, AGERI, ARC, 9 Gamaa Street, Giza, 12619, Egypt Tel: (202) 5734421 Fax: (202) 573-1574

watermelons and cucurbits were known to be infected with ZYMV. WMV is probably distributed worldwide (Purcifull *et al*., 1984) mostly in temperate and Mediterranean regions and was first reported in *Citrullus lanatus* by Webb and Scott (1965). They reported that the WMV-infected watermelon showed yellowish (chlorosis), mosaic, mottled, deformed or/and stunted leaves and stems. WMV is transmitted by at least 29 species of Aphids, such as *Myzus persicae* or *Aphis craccivora* (Edwardson and Christie, 1986). WMV has been known to be transmitted by mechanical inoculation and to infect more than 170 plant species belonging to 27 families (Shukla *et al*., 1994). At the molecular level, WMV is closely related to SMV in most of its genome and could consider as a divergent strain of this virus, moreover, it also appears to share a recombination with Bean common mosaic virus (BCMV) in the P1 coding region (Desbiez and Lecoq, 2004). The first report of full-sequenced WMV was from France (WMV-Fr) (Desbiez and Lecoq, 2004) followed by China (WMV-CHN) (Wu *et al*., 2006) and Pakistan (WMV-Pk) (Ali *et al*., 2006).

Most of the genes that used to identify WMV were using the polyprotein (P1) gene or the Coat Protein (cp) gene. Helper component proteinase (*Hc-pro*) was not used before to relate WMVs to each other. *Hc-pro* known to fulfill many functions in viral life cycle (Urcuqui-Inchima *et al*., 2001) it was recognized as indispensable helper factor for virus host-to-host transmission by Aphid vectors (Thornbury *et al*., 1985). It has a protease activity to release itself from the precursor polyprotein (Oh and Carrington, 1989). Not only *Hc-pro* has a general enhancer function of infectivity and genome amplification but also cell-to-cell and systemic movement in plant (Kasschau *et al*., 1997). Recently it has been identified as a suppressor of Post Transcriptional Gene Silencing (PTGS) (Anandalakshmi *et al*., 1998; Brigneti *et al*., 1998; Kasschau and Carrington, 1998, Pruss *et al*., 2004). Due to new molecular methods have been emerged as a new trend to resist plant viruses such as using RNA Interference (RNAi), it is important to identify and know the genomic sequence of the viruses of interest. In this study we isolated the *Hc-pro* from a viral isolate used to be known in Egypt as ZYMV but post gene isolation and sequencing showed its similarity to WMVs. This step was very important in order to resist WMV using molecular techniques which would be impossible since it was known to be ZYMV. A comparative study between the WMV-Eg and WMV-Fr, WMV-CHN and WMV-Pk was performed to show the relationship of the Egyptian isolate to the other isolates.

MATERIALS AND METHODS

Virus Isolation and Propagation

Leaf samples of watermelon plants cv. Giza 1 showing virus-like symptoms were collected from the open field at Agricultural Experimental Research Station, Sids, Beni Suef Governorate, Egypt. These samples were transferred to the lab in ice and kept at -20°C till used.

The infectious sap was prepared and then Eskandarani squash plants were mechanically inoculated in the presence of an abrasive (Carborandom, 600 mesh) as described by Allam *et al*. (2000). The inoculated plants plus the control were kept for symptoms developing under controlled greenhouse at biocontainment rooms for 4 weeks.

ELISA Detection

The Double Antibody Sandwich (DAS)-enzyme-linked immunosorbent assay (ELISA) was carried out using polyclonal antibodies specific to zucchini yellow mosaic potyvirus (ZYMV) raised at AGERI, ARC, Giza, Egypt as described by Clark and Adams (1977).

RT-PCR

Total RNA was extracted from infected Eskandarani squash plants by SV total RNA Isolation system (Promega) according to the manufacturer's instructions. RT-PCR was performed according to

the manufacturer's manual of one step RT-PCR kit (QIAGEN) using Forward primer (ZvHpF) 5' ATG TCG TCG CAA CCG GAA GTT CAG TTC TTC and Reverse primer (ZvHpR) 5' TTA CCA ACT CTG TAA TGC TTC ATC TCG C designed according to the nucleotide sequence of HC-pro of ZYMV strain KR-PA (AY278998, Kwon *et al*., 2005). The reaction mixture (50 μL) was containing: 400 μM dNTPs, 0.6 μM of each primer and 1.1 μg total RNA template. The thermal cycler program was as the following: 50°C for 45 min; 95°C for 15 min for one cycle followed by 40 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 2.5 min and 72°C for 10 min. The PCR product was purified from the gel by the gel extraction kit (Qiagen) and cloned into pGEM-T Easy vector system I (Promega) and transformed into *Escherichia coli JM109* strain.

Sequencing Analysis

Three different clones carrying Hc-pro were completely sequenced in the genomic facility in AGERI, ARC, Egypt. Each clone was sequenced in both directions by using M13F and M13R universal primers using the Big Dye terminator reaction mix (Applied Biosystems). The sequences were run on a 3700 ABI automated sequencer. Sequencher 4.1 (Gene Codes Corporation) was used to assemble the contiguous sequences. BLAST searches (Altschul *et al*., 1990) were performed on the resulted sequence to verify authenticity before any phylogenetic analyses were undertaken. The DNA and deduced amino acid (aa) sequences were also compared with the other Hc-pro from closely related isolates of the same virus using GenDoc program.

Phylogenetic Analyses

The DNA sequence of the Hc-pro isolated from the WMV-Eg strain was aligned using Lasergene Megaline program (DNAStar, Madison, WI) against the three Hc-pro sequences from the completely sequenced WMVs (France strain AY437609, Chinese strain DQ399708 and Pakistani strain AB218280). The sequences used for alignment were trimmed to the size of the Hc-pro gene without the primer sequences used for PCR. The alignments were performed on the DNA and amino acid basis. The ancestral relationships among the four WMV's are presented as a phylogenetic tree created by the lasergene megaline program.

RESULTS AND DISCUSSION

In this investigation, an Egyptian isolate of WMV, used to be known as ZYMV in Egypt, was obtained. This virus was isolated from watermelon leaves naturally exhibited virus-like symptoms and propagated by mechanical inoculation of the infectious sap obtained from infected squash plants Eskandarani cultivar.

DAS-ELISA was used as a confirmatory test using polyclonal antibodies specific to ZYMV which serologically related to WMV. ELISA showed that no positive reaction was found in the healthy sample that used as a control compared to the infected plants which showed values ranged from 0.853 to 0.918 at A_{405} nm (Table 1). The results indicate that the virus under investigation is either ZYMV or related to it.

Table 1: DAS-ELISA detection of the Egyptian isolate of WMV-Eg used to be known as ZYMV in squash plants mechanically inoculated with the infectious sap extracted from virus-infected watermelon samples

	ELISA detection					
	Dilutions					
	1:10		1:15		1:20	
Samples	ELISA value	Result	ELISA value	Result	ELISA value	Result
Infected	0.871	+	0.918	+	0.853	+
Negative control (Healthy)	0.293	-	0.358	-	0.261	-

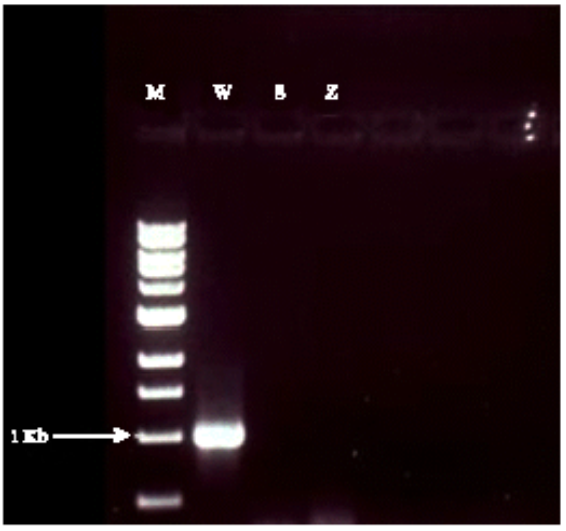

Fig. 1: RT-PCR analysis to confirm the WMV authenticity. M is 1 Kb ladder (Fermentas), W refers to the usage of WMV specific primers, S refers to the usage of SMV specific primers and Z refers to the usage of ZYMV specific primers

In Egypt, the virus under investigation was identified as ZYMV since it usually gives positive results with DAS-ELISA against ZYMV antibodies and since Watermelon is one of the variable hosts of ZYMV. During different study to isolate Hc-pro from ZYMV, RT-PCR was used to isolate the Hc-pro using primers designed based on the ZYMV-sequence alignment from the Basic Local Alignment Search Tool (BLAST). The isolated ~ 1.3 Kb was cloned in the pGEM-Teasy vector and sequenced. Results showed that the isolated Hc-pro is similar to Hc-pro of WMVs.

In addition to perform RT-PCR and sequencing three times, another PCR experiment was performed to eliminate the possibility of WMV superinfection with other viruses such as SMV or ZYMV. The primers used were designed to distinguish the WMV, SMV and ZYMV from each other by targeting unique DNA sequence in each virus. All forward primers were designed based on the 5' UTR of the coat protein DNA sequences and the reverse primers were designed based on a unique region in the 3' UTR of each of the viral genomes. The results showed expected band (~ 1kb) with the WMV-based primers and no band with the SMV, or ZYMV-based primers (Fig. 1).

From the previous results, the virus under investigation can identify as a new Egyptian isolate of WMV (WMV-Eg). Due to the lack of information provided for the WMV Hc-pro sequence, the alignment was performed using the Hc-pro sequences from the complete genome of WMVs which were available recently in 2004 for WMV-Fr (AY437609) isolated in France and 2006 for WMV-CHN (DQ399708) and WMV-Pk (AB218280) isolated from China and Pakistan, respectively (Desbiez and Lecoq, 2004; Ali *et al*., 2006; Wu *et al*., 2006). For the sake of accuracy, the DNA sequence homologous to ZYMV primers was excluded from the DNA alignment because their designs were based on the ZYMV sequence. The DNA alignment of Hc-pro of WMV-Eg, WMV-Fr, WMV-CHN and WMV-Pk showed 195 nucleotide differences out of 1318 bp, 145 of them were variable amongst all WMVs while 50 of them were exclusive to WMV-Eg alone (Fig. 2). The nucleotide differences in Hc-pro of WMV-Eg are the highest amongst all

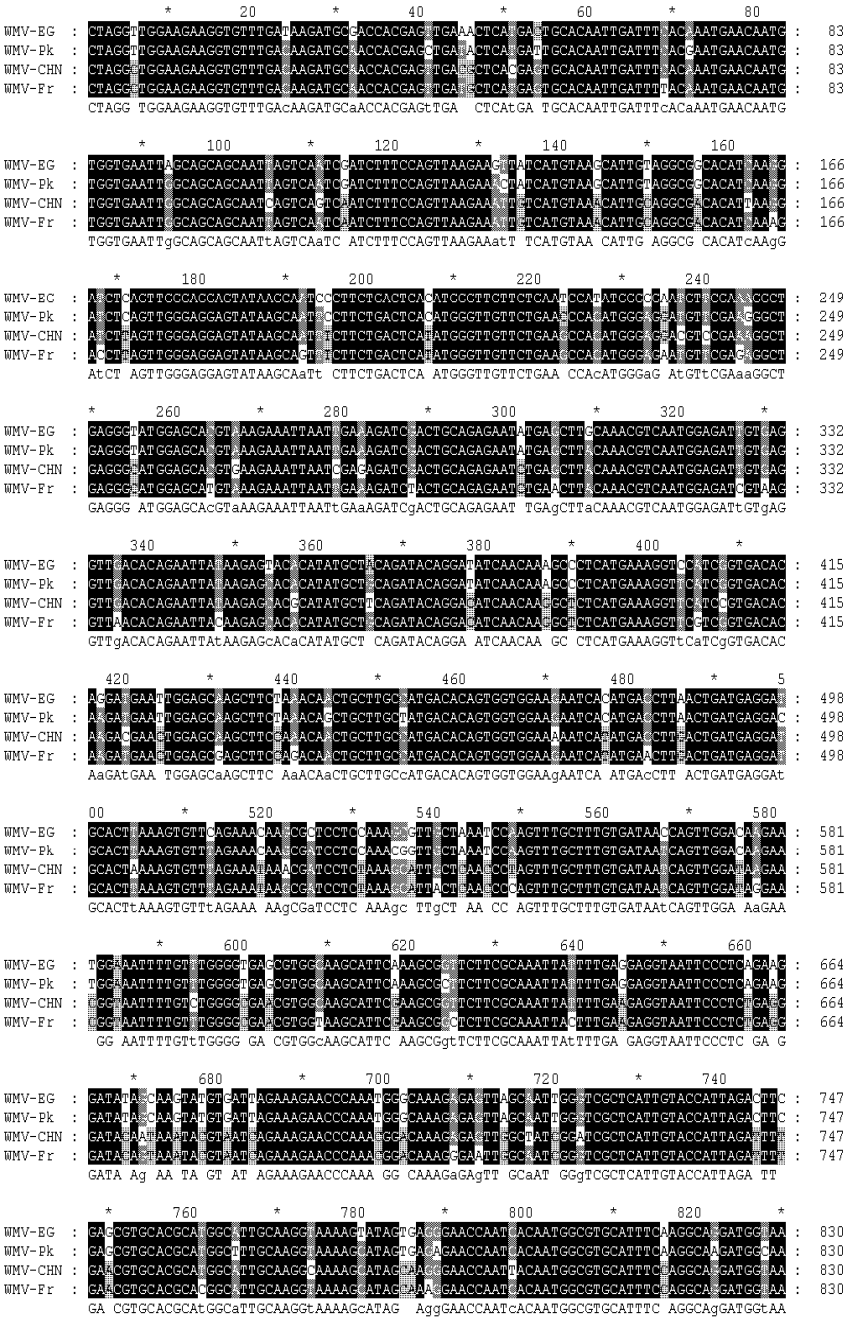

Fig. 2: Continued

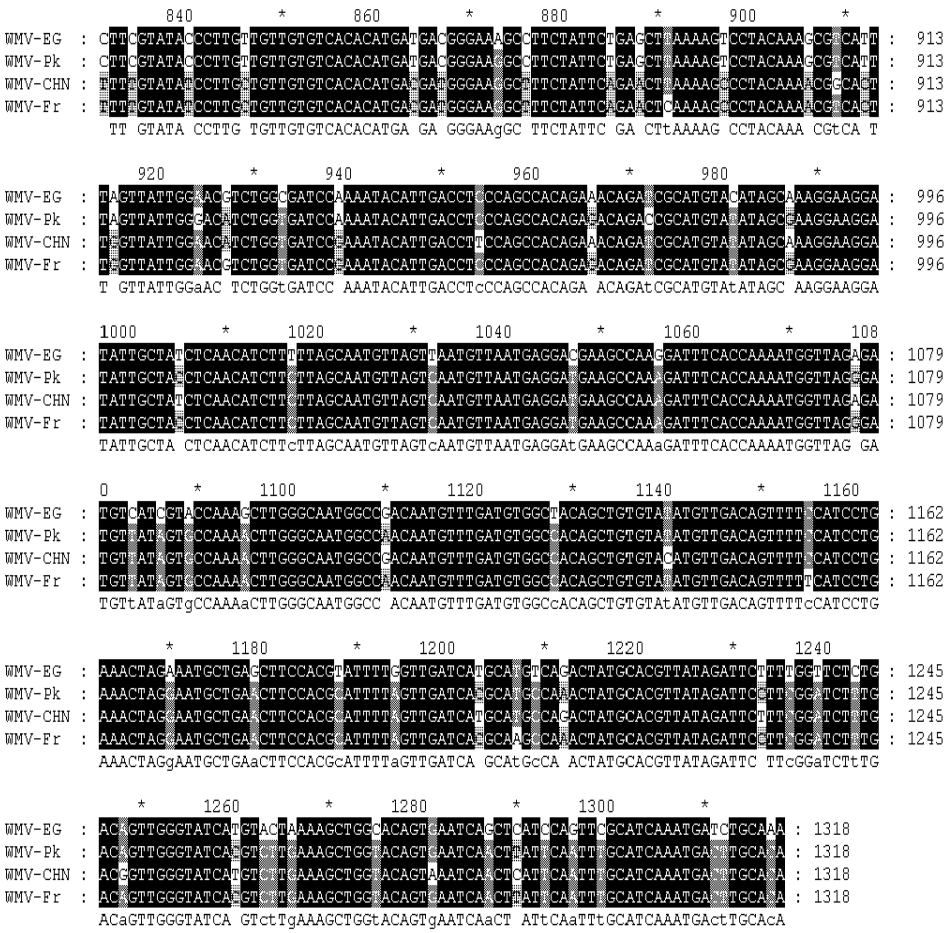

Fig. 2: Nucleotides alignment of Hc-pro among different WMVs isolated from Egypt, France, China and Pakistan. Hc-pro of WMV-Eg showed the highest exclusive variable nucleotides (50 nt) comparing to other WMVs which showed 30, 29 and 18 nt differences in WMV-Fr, WMV-CHN and WMV-Pk, respectively

other WMVs, followed by WMV-Fr, WMV-CHN and WMV-Pk which showed 30, 29, 18 exclusive nucleotide differences, respectively. On the amino acid level, the alignment showed 21 amino acid (aa) differences amongst all 4 Hc-pro of WMVs, 13 a.a. of them were variable among all 4 Hc-pro WMVs while 8 were exclusive to WMV-Eg alone. The Hc-pro of WMV-Eg has the most exclusive a.a. differences followed by WMV-Fr by 7 differences, however on the contrary to the DNA results, WMV-Pk showed more a.a. differences (4) than WMV-CHN that has only one a.a. exclusive difference (Fig. 3). Although the DNA alignment showed nucleotide differences along the whole DNA sequence, the a.a. differences were restricted to the first two third of the a.a. sequence and the last third portion remained similar with only two a.a. differences. This indicates that the 3' portion is the most conservative domain of the Hc-pro protein. Hc-pro known to be consists of three regions: An N-terminal region essential in aphid transmission, central region has several functions such as RNA silencing suppression and a C-terminal region has proteinase activity (Plisson *et al*., 2003).

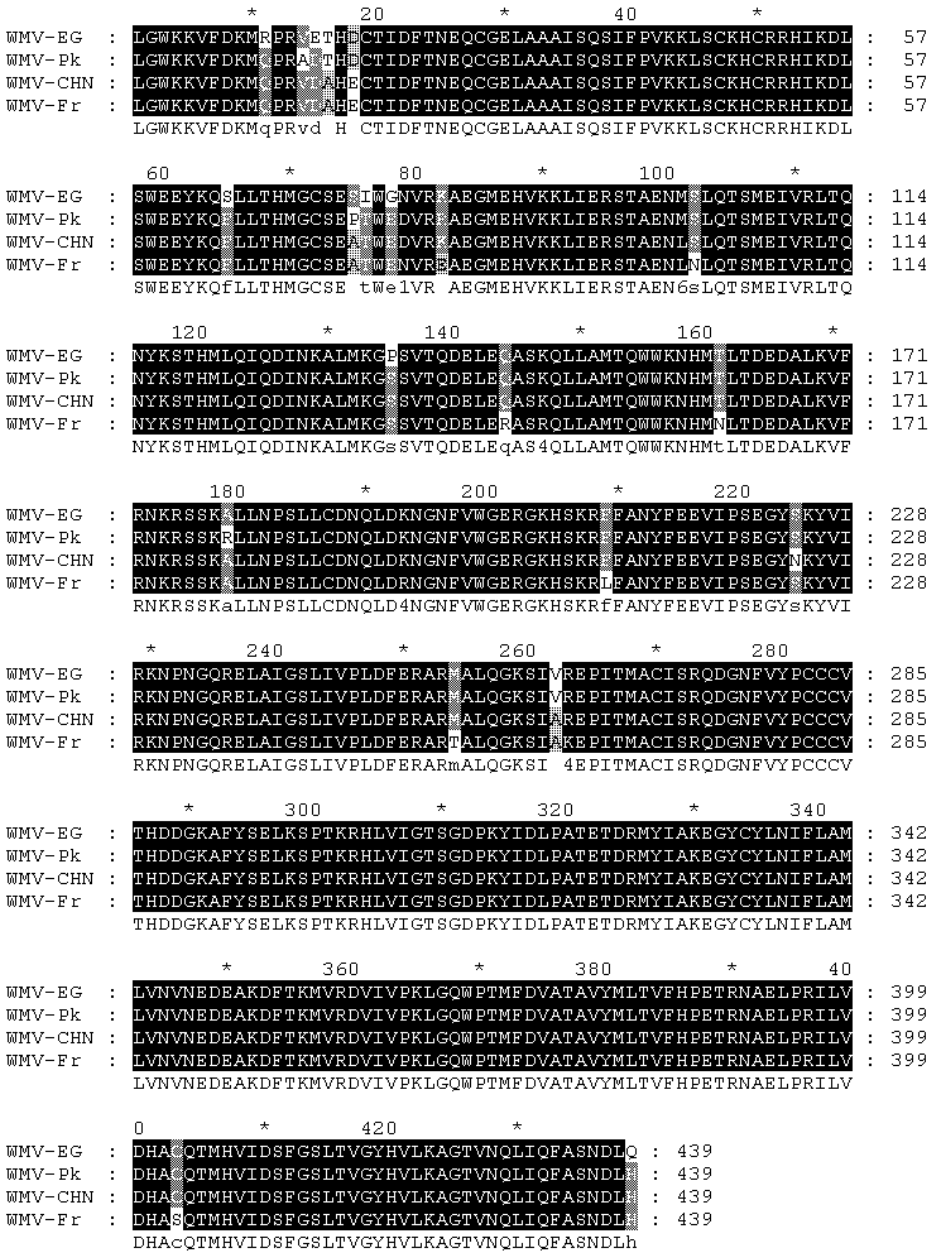

Fig. 3: Amino acids alignment of Hc-pro. Hc-pro of the Egyptian isolate (WMV-Eg) showed 8 exclusive a.a. which were the highest a.a. differences comparing to other WMVs

The phylogenetic tree of Hc-pro on the basis of DNA and amino acids of the 4 WMVs showed that the WMV-Eg is close to the WMV-Pk, while the WMV-Fr is close to the WMV-CHN (Fig. 4 a and b). In spite of that when the three WMVs (WMV-Pk, WMV-CHN and WMV-Fr) were align together they showed confusing results. The alignment between the three WMVs included the Hc-pro, coat protein, P1 and the whole genome DNA on the basis of DNA and deduced a.a. sequences. WMV-Pk was distant from the other 2 WMVs (WMV-CHN and WMV-Fr) when Hc-pro

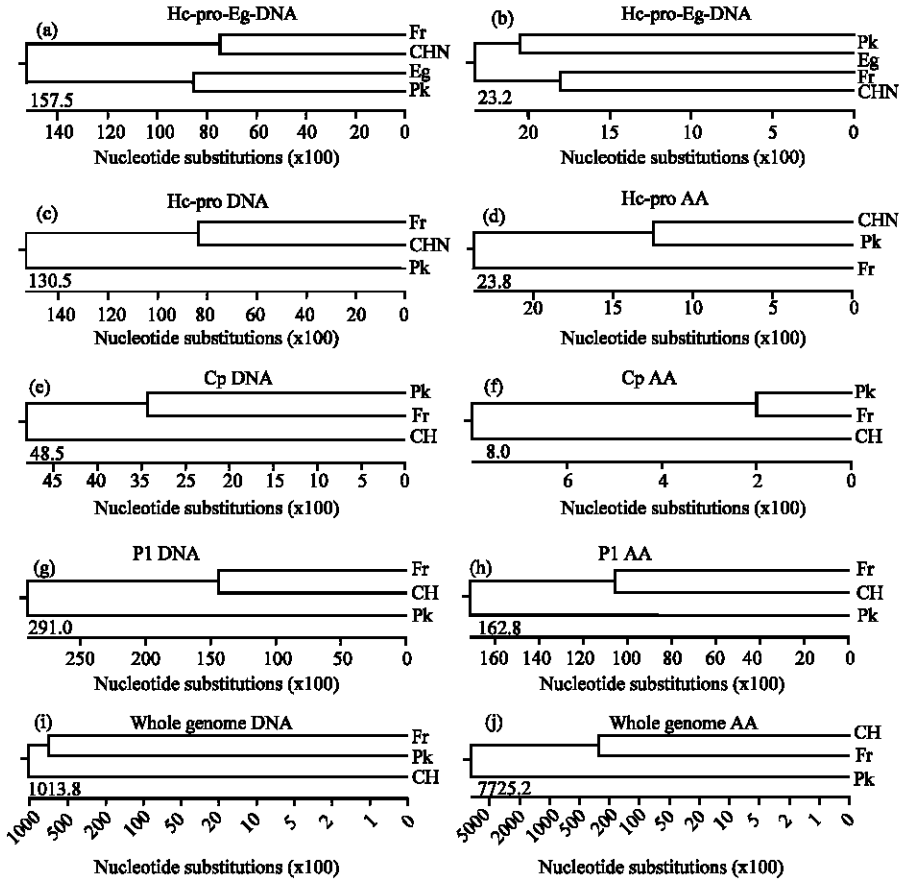

Fig. 4: Phylogenetic tree of Hc-pro of the Egyptian, French, Chinese and Pakistani WMV isolates based on DNA sequence (a) and deduced amino acids (b). A comparison between the French, Chinese and Pakistani WMV isolates were made on the DNA level for Hc-pro (c), CP (e), P1 (g) and the whole genome (I). While, the same was performed on the deduced a.a. for Hc-pro (d), CP (f), P1 (h) and the whole genome (j). The scale under the tree indicates the distance between sequences in substitution event units

(Fig. 4c and d) and P1 (Fig. 4g and h) were used for alignments on the basis of both DNA and a.a. levels and also when whole genome was used on the basis of deduced a.a. only (Fig. 4j). On the other hand, WMV-Pk was grouped with WMV-Fr and separated from WMV-CHN on the basis of coat protein sequence (Fig. 4e, f) alignments of both DNA and a.a. and on the basis of whole genome sequence alignments of DNA only (Fig. 4i). From the previous results we can include that the isolate under investigation is a new Egyptian isolate of WMV (WMV-Eg) and it seems closely related to the Pakistani isolate than the other 2 viruses, however, more sequences need to be done to confirm that. Also we can conclude that the relation between these viruses can not be confirmed by using one gene. Hence, the CP which is the most common gene used to relate viruses in this group is not legitimate since it showed the most controversy results amongst the other genes.

ACKNOWLEDGMENTS

We express our appreciation to Reda El-Sayed Salem from Dr. Salama's Lab in AGERI, ARC, GIZA, Egypt for his help with ELISA test.

REFERENCES

Ali, A., T. Natsuaki and S. Okuda, 2006. The complete nucleotide sequence of a Pakistani Isolate of Watermelon mosaic virus provides further insights into the taxonomic status in the Bean common mosaic virus subgroup. Virus Genes, 32: 307-311.

Allam, E.K., I. Sohair, El-Afifi and A.S. Sadik, 2000. Inclusion bodies as a rapid mean for detection of some plant viruses. Proceedings 9th Congress of the Egyptian Phytopathology Soc., Giza, May 8-10, 2000, pp: 117-141.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman, 1990. Basic local alignment search tool. J. Mol. Biol., 215: 403-410.

Anandalakshmi, R., G.J. Pruss, X. Ge, R. Marathe, A.C. Mallory, T.H. Smith and V.B. Vance, 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA., 95: 13079-13084

Brunt, A.A., K. Craptree, M. Dallywitz, A. Gibs and L. Watson, 1996. Viruses of plants description and lists from the vide database. University Press, Cambridge, UK.

Brigneti, G., O. Voinnet, W.X. Li, L.H. Ji, S.W. Ding and D.C. Baulcombe, 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. Embo. J., 17: 6739-6746.

Clark, M.F. and N.E. Adams, 1977. Characterization of the microtiter plate method of enzyme-linked immunoassay (ELISA), for the detection of plant viruses. J. Gen. Virol., 37: 475-483.

Davis, R.F., 1986. Partial characterization of zucchini yellow mosaic virus isolated from squash in Turkey. Plant Dis., 70: 735-738.

Desbiez, C. and H. Lecoq, 2004. The nucleotide sequence of Watermelon Mosaic Virus (WMV, Potyvirus) reveals interspecific recombination between two related potyviruses in the 5' part of the genome. Arch. Virol., 149: 1619-1632.

Desbiez, C., C. Costa, C. Wipf-Scheibel, M. Girard and H. Lecoq, 2007. Serological and molecular variability of watermelon mosaic virus (genus Potyvirus). Arch. Virol., 152: 775-781.

Edwardson, J.R. and R.G. Christie, 1986. Viruses infecting forage legumes. Fla Agric. Exp. Stn Monog. No. 14, p: 454.

Kasschau, K.D., S. Cronin and J.C. Carrington, 1997. Genome amplification and long-distance movement functions associated with the central domain of tobacco etch potyvirus helper component-proteinase. Virology, 228: 251-262.

Kasschau, K.D. and J.C. Carrington, 1998. A counter defensive strategy of plant viruses: Suppression of posttranscriptional gene silencing. Cell, 95: 461-470.

Kwon, S.W., M.S. Kim, H.S. Choi and K.H. Kim, 2005. Biological characteristics and nucleotide sequences of three Korean isolates of Zucchini yellow mosaic virus. J. Gen. Plant Pathol., 71: 80-85.

Lisa, V., G. Boccardo, G. D'Agostion, G. Dellavalle and M. D'Aquilio, 1981. Characterization of a potyvirus that causes zucchini yellow mosaic. Phytopathology, 71: 667-672.

Oh, C.S. and J.C. Carrington, 1989. Identification of essential residues in potyvirus proteinase HC-Pro by site-directed mutagenesis. Virology, 173: 692-699.

Plisson, C., M. Drucker, S. Blanc, S. German-Retana, O. Le Gall, D. Thomas and P. Bron, 2003. Structural characterization of Hc-pro, a plant virus multifunctional protein. J. Biol. Chem., 278: 23753-23761.

Pruss, G.J., C.B. Lawrence, T. Bass, Q.Q. Li, L.H. Bowman and V. Vance, 2004. The potyviral suppressor of RNA silencing confers enhanced resistance to multiple pathogens. Virology, 320: 107-120.

Purcifull, D., E. Hiebert and J.R. Edwardson, 1984. Watermelon Mosaic Virus 2. CMI/AAB Descr. Pl. Viruses No. 293, pp: 7.

Shukla, D.D., C.W. Ward and A.A. Brunt, 1994. Genome Structure, Variation and Function. In: The Potyviridae. CAB International, Cambridge, pp: 74-112.

Thornbury, D.W., G.M. Hellman, R.E. Rhoads and T.P. Pirone, 1985. Purification and characterization of potyvirus helper component. Virology, 144: 260-267.

Urcuqui-Inchima, S., A.L. Haenni and F. Bernardi, 2001. Potyvirus proteins: A wealth of functions. Virus Res., 74: 157-175.

Verma, A., 1988. In: The Plant Viruses The Filamentous Plant Viruses. Milne, R.G. (Ed.), Plenum Press, Vol. 4. New York, pp: 371.

Webb, R.E. and H.A. Scott, 1965. Isolation and identification of Watermelon Mosaic Virus 1 and 2. Phytopathology, 55: 895.

Wu, Y.F., J.X. Zhang, R. Wang, Z.P. Luo, X.M. Wang and D. Yang, 2006. Accession No. DQ39970.