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ORIGINAL ARTICLE

Molecular characterization of a *cucumber mosaic cucumovirus* isolated from lettuce in Egypt

Ali M. El-Borollosy [a,*], **Hoda M.A. Waziri** [b]

[a] *Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, P.O. Box 68, Hadayek Shubra 11241, Cairo, Egypt*

[b] *Department of Virus & Phytoplasma, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt*

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Abstract *Cucumber mosaic cucumovirus* (CMV) was isolated from lettuce plants (*Lactuca sativa*) showing virus like symptoms. Isolation was performed depending on specific polyclonal antibodies and *Chenopodium quinoa* as a local lesion host. Virus was purified from 200 gm of virus-infected *Nicotiana tabacum* cv. White Burley leaves giving $A_{260/280}$ ratio of 1.21 and a yield of 1.7 mg. Purified virus preparation was used for rabbit immunization to produce specific polyclonal antibodies. IgGs were purified and evaluated by indirect enzyme linked immunosorbant assay (I-ELISA) to determine the dilution end point which found to be 1:512. Electron micrographs showed spherical virus particles of about 30 nm in diameter. Virus coat protein (CP) molecular weight was determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), giving a single band of 25 kDa within resolving gel. Immunocapture-reverse transcriptase-polymerase chain reaction (IC-RT-PCR) was used for the amplification of CMV coat protein gene (*cp*), the appearance of 657 bp bands confirmed the expected size of such gene. Comparing virus *cp* gene sequence with the sequences of seven overseas isolates confirmed that the under study isolate was related to the CMV subgroup I.

Introduction

Lettuce (*Lactuca sativa*) belongs to the Compositae family is a very popular crop in Egypt used as a salad constituent and for its seed oil. It is an annual plant native to the Mediterranean area. Cultivation may have started as early as 4500 BC, perhaps initially for the edible oil extracted from its seeds. Salad lettuce was popular with the Ancient Greeks, Romans and Egyptians which was used as a fertility medicine. Cultivated lettuce was probably derived from the so called wild or prickley lettuce (*Lactuca sierriola*) which was loose and leafy. Firm heading forms became well developed in Europe by the 16th Century (Ryder et al., 2003).

Lettuce mosaic potyvirus (LMV), *Cucumber mosaic cucumovirus* (CMV) are the major viruses infecting lettuce worldwide causing serious loss of crop quantity and quality (Soleimani et al., 2011). Symptoms in lettuce caused by

* Corresponding author.
E-mail address: ali.elborollosy@yahoo.com (A.M. El-Borollosy).

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LMV or CMV consist of stunting, chlorosis, mosaic and improper heading of infected plants (Cock, 1968; Bruckart and Lorbeer, 1975).

CMV, genus: *Cucumovirus*, family: Bromoviridae, is one of the most widespread plant viruses with extensive host range infecting about 1000 species including cereals, fruits, vegetables and ornamentals (Roossinck, 1999). The virus is readily transmitted in a non-persistent manner by more than 75 species of aphids (Palukaitis et al., 1992). CMV is a multicomponent virus with a single stranded positive sense RNA. RNAs 1 and 2 are associated with viral genome replication while RNA 3 encodes for movement protein and coat protein. Numerous strains of CMV have been classified into two major subgroups (subgroups I and II) on the basis of serological properties and nucleotide sequence homology (Palukaitis et al., 1992; Madhubala et al., 2005; Oraby et al., 2008). The subgroup I has been further divided into two groups (IA and IB) by phylogenetic analysisRoossinck et al., 1999.

The present investigation aimed to isolate CMV from lettuce plants in Egypt, and identifying it depending on some molecular characteristics.

Materials and methods

Virus isolation

Fifty samples of lettuce (*L. sativa*) leaves showing virus-like symptoms were collected from open fields of Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Leaves showing mosaic mottling and malformation were collected in small plastic bags and kept at 4 °C. Isolation was performed serologically depending on I-ELISA according to Koenig (1981) using polyclonal antibodies specific for *Lettuce mosaic potyvirus* (LMV), *Cucumber mosaic cucumovirus* (CMV) and *Tobacco mosaic tobamovirus* (TMV) (Agdia Inc., USA). Biological isolation was done by virus mechanical inoculation in *Chenopodium quinoa* as a local lesion host. Virus was maintained on *Nicotiana tabacum* cv. White Burley under greenhouse conditions (28 °C ± 2).

Virus purification

Leaves of *N. tabacum* cv. White Burley (200 gm) showing severe symptoms were harvested 2 weeks post-inoculation, kept at −20 °C overnight and used for virus purification. Virus particles were purified using a modified protocol of Lot et al. (1972) as described by El-Afifi et al. (2007). The virus yield was determined spectrophotometrically by considering A_{260} 5.0 = 1 mg/ml (Sarma et al., 2001). Preparations were negatively stained with 2% phosphotungastic acid (PTA) according to Griffin (1990) and examined with a Philips EM400T transmission electron microscope, Specialized Hospital, Ain Shams University, Cairo, Egypt.

Antiserum production

Polyclonal antiserum was produced in a New Zealand white rabbit by injecting purified virus preparation depending on the method described by Madhubala et al. (2005) (1 mg of virus with Freund's incomplete adjuvant, 1:1) intramuscularly six times at 10 days interval. The animal was bled 15 days after the last injection and the antiserum was collected.

Immunoglobulins G (IgGs) purification was carried out as mentioned by Clark and Adams (1977). Final dialyzed proteins were loaded on 10 ml of diethylaminoethyl (DEAE) cellulose column (Whatman Inc., USA), eluted using half strength phosphate buffer saline (PBS) and collected in microtube. Absorption of fractions was measured at 280 nm and concentration of IgGs was adjusted to 1 mg/ml (OD_{280} = 1.46) using half strength PBS, then stored at 4 °C. IgGs dilution end points were determined using I-ELISA against clarified infectious tobacco sap and healthy tobacco sap was used as a control.

SDS–PAGE of virus coat protein

The molecular weight of virus CP subunits 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).

IC-RT-PCR for CMV cp gene amplification

The IC-RT-PCR was performed using infected tobacco leaf collected 15 days post-CMV inoculation. Immunocapturing and cDNA synthesis was carried out as described by Minafera and Hadidi (1994). The following primers (Invitrogen Corp., USA) were used for the amplification of CMV *cp* gene were designed depending on CMV *cp* gene sequences collected from PubMed (GeneBank) web site (http://www.ncbi.nlm.nih.gov):

5′ATGGACAAATCTGAATCAAC3′ (Sense).
5′TCAAACTGGGAGCACCCCAG3′ (Antisense).

PCR was performed as described by Ghosh et al. (2002) using PerkinElmer Cetus Thermal Cycler PE 9700 (PerkinElmer Inc., USA). Five microlitre from resulting cDNA were transferred to tube containing 45 µl PCR reaction mixture. PCR program was 94 ° initial melting for 3 min followed by 35 cycles of 94 °/1 min, 55 °/1 min, 72 °/2 min and 72 °/10 min final extension.

For PCR product analysis, 1.5% agarose gel was used and electrophoresis was carried out in Sub-Cell DNA apparatus (Bio-Rad® Lab., USA) at 80 V. The amplified gene band was visualized on an UV Transilluminator and photographed by Gel Documentation System (AlphaImager® TM1220, Documentation and Analysis system, Canada).

Purification of PCR product

DNA fragments representing viral *cp* gene were purified from agarose gel using the gel slicing and melting method described by Wieslander (1979). The final pellets were washed with 1 ml of 70% ethanol, then left to dry in air and resuspended in 30 µl TE buffer and stored at −20 °.

CMV cp gene cloning, sequencing and sequence analyses

The *cp* genes were ligated into pGEM®-T vector plasmids, which were used for *Escherichia coli* JM109 competent cells

transformation according to Promega's pGEM®-T Easy Cloning kit manual's instructions. Sequencing was carried out at Gene Analysis Unit (VACSERA, Agouza, Cairo, Egypt) using ABI Prism® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA). Sequencing products were resolved in an automated sequencer model 310 (Applied Biosystems, USA).

DNA sequences of CMV *cp* genes from different worldwide isolates were collected from GeneBank (http://www.ncbi.nlm.nih.gov) as demonstrated in Table 1. These sequences were compared with the nucleotide sequence of the understudy CMV lettuce isolate to draw homology tree and determine similarity degrees. Molecular analysis was carried out using the DNAMAN Software, version 7.0 (Lynnon Corp., Canada).

Results

Virus isolation

CMV was isolated from naturally infected lettuce plants (showing mosaic and malformation) (Fig. 1). Collected samples gave only positive I-ELISA results with CMV specific antiserum and produced chlorotic local lesions on *C. quinoa*. Three cycles of local lesions isolation were performed and the last produced lesions were inoculated on *N. tabacum* cv. White Burley plants.

Virus purification

UV absorption data show that $A_{260/280}$ ratio for purified virus preparation was 1.21, while yield was 1.7 mg virus/200 g infected leaves. Electron micrographs show spherical virus particles of about 30 nm in diameter (Fig. 2).

Antiserum production

Specific antiserum was raised against the isolated CMV, IgGs were purified and antiserum titer was determined using I-ELISA. Data in Table 2 show that antiserum reacted up to dilution of 1/512 with clarified infectious tobacco sap, while negative results were obtained with the healthy sap.

Molecular weight of CMV CP

Results in Fig. 3 showed that the SDS–PAGE analysis of the purified CMV preparation indicating the CMV CP. The protein appeared as one band with a molecular weight of about 25 kDa.

Table 1 Source of CMV coat protein gene sequences used for comparisons.

Country of origin	Accession number	CMV subgroup
China I	EU926956	I
Egypt	JQ013954	I
India	EU600216	II
Japan	AB070622	I
Korea	AB109908	II
Syria	AB448691	I
USA	GU362669	I

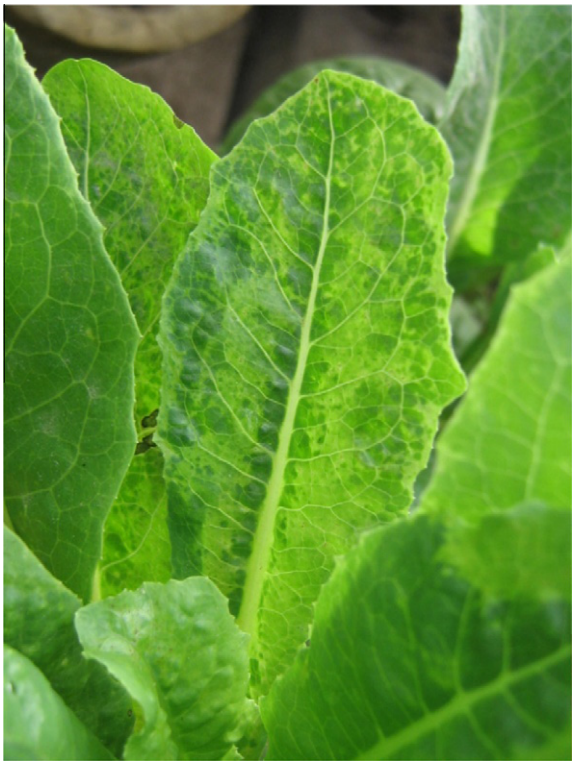

Fig. 1 Lettuce plant infected with CMV showing mosaic, mottling and malformation.

IC-RT-PCR for CMV cp gene amplification

The agarose gel analysis of the PCR product indicated a single band with size length of 657 bp which is the expected size for the CMV *cp* gene Fig. 4.

CMV cp gene sequencing and sequence analyses

The nucleotide sequence of CMV *cp* proved that it is consists of 657 base, started with ATG and ended with TTG, as start

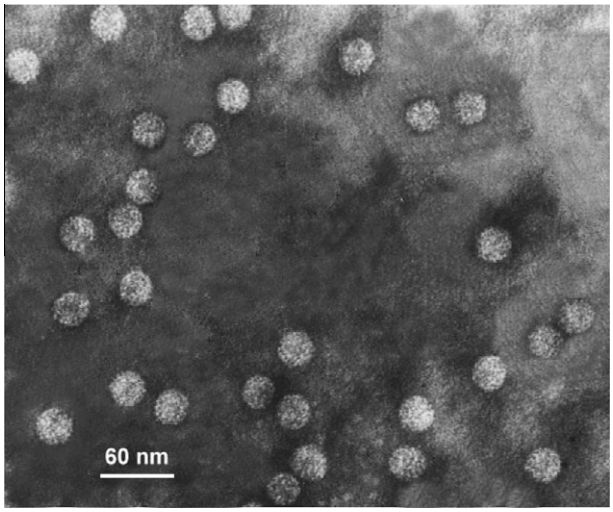

Fig. 2 Electron micrograph of negatively stained purified CMV preparation.

Table 2 CMV IgGs evaluation against infected and healthy tobacco sap.

Dilution	ELISA value at A_{405} nm	Result
Undiluted	0.922	+
1/2	0.895	+
1/4	0.801	+
1/8	0.753	+
1/16	0.677	+
1/32	0.611	+
1/64	0.564	+
1/128	0.505	+
1/256	0.489	+
1/512	0.397	+
1/1024	0.200	–
Control[a]	0.097	–

+ = Positive, – = negative.
[a] Clarified healthy sap with antiserum (dilution 1/2).

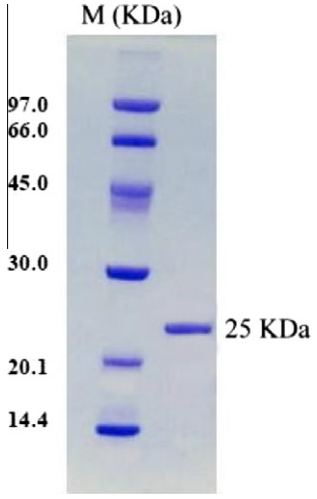

Fig. 3 SDS–PAGE of purified CMV preparation. M: Protein marker (Promega, USA).

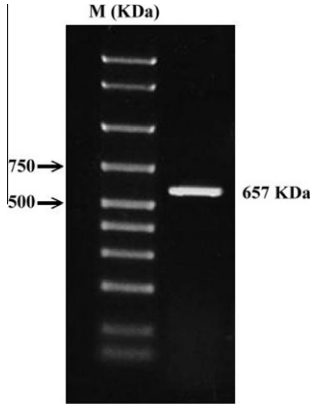

Fig. 4 IC-RT-PCR for the amplification of CMV *cp* gene. Lane M: DNA Ladder (Promega, USA).

and stop codons, respectively. Sequence was aligned with seven overseas isolates, the alignment was converted to homology tree (Fig. 5). The lettuce CMV isolate of this study proved 93% similarity with the virus isolates belonging to serotype

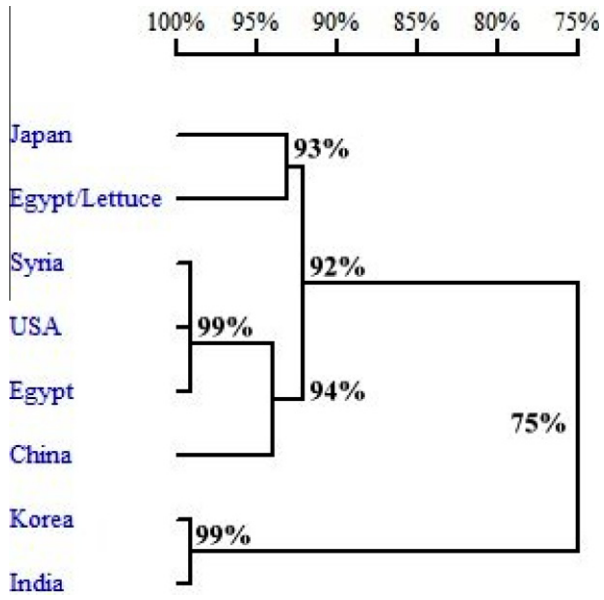

Fig. 5 Homology tree and similarity degrees between the isolated lettuce CMV and seven overseas isolates.

or subgroup I, while 75% was found with the isolates related to subgroup II.

Discussion

During the present investigation CMV was isolated from lettuce plants showing mosaic, mottling and malformation. The virus was confirmed by specific antiserum and indirect ELISA. Electron micrographs of the purified preparation revealed spherical particles with 30 nm in diameter. Results were in harmony with Madhubala et al. (2005), El-Afifi et al. (2007) and Oraby et al. (2008).

The virus was molecularly characterized, and with the aid of SDS–PAGE found to has a CP with molecular weight of 25 kDa. Also, IC-RT-PCR was used for virus *cp* gene amplification, which produced band with a size of about 657 bp, results were similar to those obtained by Madhubala et al. (2005) and Oraby et al. (2008) who worked on the characterization of CMV from Vanilla and sugar beet, respectively.

The present isolate gave 93% similarity with the Japanese isolate (belonging to subgroup I) and 92% with other isolates relates to subgroup I. On the other hand, 75% of similarity was obtained with the Indian and Korean isolates of subgroup II. Madhubala et al. (2005), Verma et al. (2006) and (Soleimani et al., 2011) characterized CMV from Vanilla, Geraniums (*Pelargonium* spp.) and lettuce plants, respectively, depending on molecular characteristics. Generally they isolated, characterized, cloned and sequenced *cp* gene, also aligned the nucleotide and amino acid sequences with other worldwide CMV isolates belonging to different subgroups.

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