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DETECTION AND IDENTIFICATION OF AN EGYPTIAN ISOLATE OF CUCUMBER MOSAIC VIRUS (CMV) AFFECTING SUGARBEET USING NON-RADIOACTIVE RNA PROBE

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ABSTRACT:

Detection and identification of cucumber mosaic virus (CMV) RNA was carried out from sugar beet leaves by using reverse transcription and polymerase chain reaction (RT-PCR). The coat protein gene (RNA 3) was amplified selectively by using cucumovirus-specific coat protein gene primers. No DNA product of any length was produced when healthy leaves or tobacco mosaic virus RNA was used as templates. Dot-blot hybridization using CMV-CP/RNA probe also confirmed the presence of CMV genome in sugarbeet-infected tissues. The double-stranded PCR product (650 bp) was cloned into pGEM-T-Easy vector and the insert was partially sequenced. The multiple nucleotide sequence alignment of the CMV/CP-EG displayed 75 % homology with CMV subgroup II published nucleotide sequences of Fny-CMV, NT9-CMV, Q-CMV and Trk7-CMV strains.

INTRODUCTION:

Cucumber mosaic virus (CMV) is the species of the genus *Cucumovirus* within the Family *Bromoviridae* (Rybicki, 1995, Van Regenmortel *et al*., 2000). CMV is one of the most widespread plant viruses in the world. Cucumber mosaic virus (CMV) infected as many as 191 host species in 40 different families of vegetables, ornamentals and other plants. Important greenhouse crops affected by CMV include tomato (*Lycopersicon esculentum*), cucumber (*Cucurbits spp*.) and sweet pepper (*Capsicum annuum*). The primary vector is an aphid but several species of aphid infest the glasshouse (although tomatoes are not a preferred host of aphids) with the most common species being green peach aphid (*Myzus persicae*) and the cotton or melon aphid (*Aphis gossypii*). All aphids in the glasshouse are female and give birth to live young. The offspring can reproduce in 7 - 10 days and one female can produce 60 - 100 young over the course of a 20-day period. This makes them a very effective vector of CMV. Crop workers can mechanically transmit CMV from one plant to the next through sap carried on their hands and clothes. If workers are transmitting the disease, the pattern will be linear (i.e. up and down the rows). If aphids are responsible for the transmission, the pattern spread will be circular in nature. The genome of CMV consists of three capped plus sense single stranded RNAs (Peden and Symons, 1973). Of the tripartite CMV genome RNAs 1 and 2 are separately encapsidated and encode the viral polymerase subunits 1a and 2a, respectively. Proteins translated from RNAs 1 and 2 are associated with viral genome replication (Nitta *et al*., 1988). RNA 2 moreover encodes an additional protein, 2b, expressed from a subgenomic mRNA (Ding *et al*. 1994). This 2b protein is capable to suppress the host RNA silencing mechanism and thereby this protein is also involved in symptom severity (Brigneti *et al*. 1998). Also RNA 3 is bicistronic, coding for the viral movement protein (MP), and the viral coat protein (CP). Like the 2b protein, the CP is expressed via the formation of a subgenomic RNA molecule, denoted RNA 4, which is encapsidated in a single virus particle together

with RNA 3 (Palukaitis *et al.* 1992).

CMV has an extremely wide host range and many different strains have been identified (Douine *et al.* 1979; Kaper and Waterworth, 1981). Based on phylogenetic analysis of the CP ORF and rearrangements in the 5' non-translated region (NTR) of RNA 3, CMV strains can be divided into three subgroups: IA, IB, and II (Quemada *et al*. 1989, Anderson *et al*. 1995, Palukaitis and Zaitlin, 1997, and Roossinck *et al.* 1999).

The complete nucleotide sequences of the genomic RNAs of several CMV isolates have been reported and numerous isolates of CMV have been classified into two major subgroups - I and II - on the basis of biological and serological properties and nucleotide sequence homology (Quemada *et al.*, 1989, Palukaitis *et al*. 1992, Anderson *et al*. 1995). New sub-grouping of subgroup I into subgroups IA and IB, has been proposed recently on the basis of the nucleotide sequence of the 5' nontranslated region of RNA 3 (Palukaitis and Zaitlin, 1997; Roossinck *et al.* 1999). Subgroup I isolates of CMV are the most predominant and of major economic importance. Outbreaks of diseases incited by CMV infections have caused significant yield losses in many economically important crops (Tomlinson, 1987).

Outbreaks of diseases incited by CMV infections have caused significant yield losses in many economically important crops (Tomlinson, 1987). Unfortunately, for most crops, suitable sources of natural resistance to CMV have not become available for breeding (Watterson, 1993). Hence, to date, most crops can only be protected from CMV infections by taking phytosanitary measures, by early detection and by using virus-free starting material.

The current study reports the molecular techniques that can be used in early detection of CMV using non-radioactive RNA probe and comparing the partial nucleotide sequences of the CP-encoding RNAs 3 of CMV Egyptian isolate infecting sugarbeet plants with that of some published strains.

MATERIALS AND METHODS:

1. Source of virus isolate: Samples of sugar beet plants showing mosaic yellow and stunting symptoms were collected from Nobaria Governorate during season 2003. Some experiments were carried out in the greenhouse to isolate and identify the causal agent.

2. Isolation of the virus isolate: Samples of sugar beet leaves suspected to be infected with the virus were ground in a sterilized mortar and pestle in 0.05 M potassium phosphate buffer, pH 7.2 and then pressed through a double layer of cheesecloth. Expressed sap was used in mechanical inoculation as described by Noordam (1973).

Young leaves of healthy sugar beet plants and other host's inoculated seedlings and control were kept in an insect-proof greenhouse.

The single local lesions technique described by Kuhn (1964) was used for biological purification of the virus. Single local lesion developed on *Ch. amaranticolor* L. as local lesion host plant was used to inoculate sugar beet.

3. Virus identification: Identification of the isolated virus was based on studying the host range and symptomatology, mode of transmission and serological reaction.

3.1.Host rang and symptomatology: Ten plant species and varieties belonging to four families were mechanically inoculated with infectious sap expressed from sugar beet plants, the respective source mentioned above then seedling of the same species and age were inoculated with water and used as a control. Four weeks later, symptomless plant was checked for virus infection by back inoculation to *Ch. amaranticolor* and by using DAS – ELISA technique.

3.2. Insect transmission: Colonies of *Myzus persicae* sulm, *Aphis fabae* scop and *Aphis craccivora* koch were obtained from the stock

culture of plant virus research section, Plant Pathology Research Institute. Insects were reared on cabbage seedlings under insect proof cages virus–free aphids starved for one hour, were allowed to feed on virus–infected sugar beet leaves (*Beta vulgaris* cv. H. Poly) for acquisition feeding periods of 5 – 10 minutes. Five aphids/plant were used for inoculation feeding period of 24 hrs. The procedure was used for the control, except that virus free aphids were used. The aphids killed by spraying with Malathion (0.15%). Symptoms and percentage of transmission were recorded for one month.

4 – Serological reaction: ELISA Kit was supplied by SANOFI, Saint, Animals, Paris France. DAS – ELISA technique and tissue blot immunoassay (TBIA) techniques were used for virus detection as described by Clark and Adams (1977) and Lin *et al*. (1990) respectively.

5-Isolation of CMV/ dsRNA: CMV double stranded RNA (dsRNA) was isolated from infected tissues, as described by Jordan and Dodds (1985). All steps of isolation were performed at room temperature. 0.1 gm sugar beet tissues were transferred to a plastic centrifuge tube and 1 volume of phenol pH 4.0 (water-saturated phenol equilibrated with 50 mM acetate buffer pH 4.0) was added, and the tissue was homogenized completely. The tube was tightly capped and vigorously, shaken for 3 minutes. The phenol and aqueous phases were separated by centrifugation and the aqueous phase was re-extracted with 8 volumes of chloroform-isoamyl alcohol 4:11 v/v. After centrifugation the upper phase was transferred to a new tube and 0.75 volume of isopropanol was added, and nucleic acids were precipitated from the mixture by three cycles of freezing and thawing. The precipitated RNA was collected by centrifugation; the pellet was washed with 60% ethanol, dried and dissolved in TE buffer 10mM Tris, 1mM EDTA pH 8.0). The dsRNA fractions were analyzed by agarose gel electrophoresis, and the gels were stained with ethidium bromide.

6-RT-PCR: Approximately 5µg of dsRNA extracted from both CMV-infected *sugar beet* plants and from tobacco plants infected with TMV were used as templates for cDNA synthesis. First strand cDNA synthesis was initiated with primer **(**5'-CCCCGGATCCTG GTGGCCTT-3'), complementary to the conserved ultimate 3' terminal 10 nucleotides of all CMV RNA 3. Second strand cDNA synthesis was primed with degenerated primer CMVCP-2 (5'-CCCCGGATCCACATCAYA GTTTTRAGRTTCAATTC-3'),corresponding to nucleotide 1102 to 1126 of RNA 3, just upstream of the RNA 4 sub-genomic promoter. The RT-PCR reaction was carried out using Retro-tools reverse transcriptase (Biotools) and subsequent PCR amplification was performed in the same tube by the same enzyme (Single tube assay). The cDNA was amplified for 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. The RT-PCR fragments were analyzed on 1% agarose and then gene cleaned from the agarose gel prior to cloning into pGEM-T-Easy vector (Promega, MA, Wisconsin).

7-DNA cloning and sequencing: CMV/CP PCR products of the expected size were extracted from agarose gel using (GFX DNA Extraction kit, Amersham Pharmacia) and eluted in nuclease free water. Purified DNA was ligated to the pGEM-T vector (Promega) and trans-formed to *E. col*i. The recombinant plasmid was denoted as pHEA11. The cloned sequence of CMV/CP EG-isolate was compared with that of subgroup I and subgroup II CMV strains, whose sequences were available in the literatures and in Gen-Bank, i.e. Fny-CMV (Owen *et al.* 1990, D10538) and NT9-CMV (Hsu *et a*l. 1995, D28780) for subgroup I CMV, and Q-CMV (Davies and Symons, 1988, J02059) and Trk7-CMV (Salanki *et al.* 1994, L15336).

8-Preparation of non-radioactive RNA probe: CMV/ RNA probe for transcripts was synthesized *in vitro* with T7 RNA polymerase (Roche Diagnostics) using pGEM-T- Easy plasmid DNA (pHEA11) that encoded the CMV/CP gene. The Riboprobe kit from Boehringer, Mannheim was used to produce a RNA probe as following: Purified template (25 ng/ µl), (1µg DIG-UTP), 2µl RNase inhibitor (20U/µl), 2µl RNA polymerase (20 U/µl), ddH_2O up to 18µl. The mixture as incubated at 37^{o}C for 2 hours and (2µl) of DNase I-RNase free was added and incubated at 37 ^{o}C for 15 minutes. 2 µl of EDTA pH 8.0 were added and the reaction was precipitated by adding 2.5µl of 4 M LiCl, 75µl prechilled 100% ethanol, and left at -70 ^{o}C for 1/2 hour. The riboprobe was spinned at 13000 x g for 15 minutes in the cold, washed with 500 µl of ice cold 70% ethanol. The pellet was dry briefly and dissolved in 100 µl of water and 0.2 µg of probe/ml of hybridization buffer was used. After incubation in a blocking buffer that contained 100 mM maleic acid (pH 7.5), 150 mM NaCl, and 2% blocking reagent (Roche Diagnostics), the samples were incubated overnight in blocking buffer supplemented with 1000-fold diluted DIG-specific anti-bodies conjugated with alkaline phosphatase. Color was developed by incubation in alkaline phosphatase [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 0.3% Tween-20] supplemented with NBT-BCIP as substrate.

9. Dot-blot hybridization: DIG-labeled RNA probe developed from the amplified coat protein sequence of cDNA clone (pHEA11) was used in dot-blot hybridization assays to assess specificity. For dot- blot analysis, extracted saps from CMV sugar beet infected tissues and tobacco plants were spotted onto nitrocellulose membrane and cross linked under UV cross linker between 2,500 and 10,000 µJoule/cm^2 for 3min. Hybridization was carried out as described by Webster and Barker (1998) using a DIG-labeled RNA probe and Goat-anti-DIG-AP conjugate in a dilution of 1:2500.

RESULTS AND DISCUSSION

Isolation and Identification: The naturally infected sugar beet showing yellow, severe mosaic and stunting were collected from Nobaria Governorates. After the successive single local lesion transfers in *Ch. amaranticolor*, and gave positive reaction with antiserum to CMV in ELISA, the resulting virus isolate was propagated in sugar beet and *N. rustica* which developed the same symptoms as those seen in naturally infected plants and the obtained symptoms were similar to those illustrated by El–Kady *et al*. (1985) and Soliman (2003).

Host range and symptomatology: Reaction of ten plants species and varieties belonging to four families to virus infection is shown in Table 1.

Table 1: Host range and reaction of different hosts to CMV isolated from *Beta vulgaris*.

Family	Species	Cultivars	Symptoms	SA
Chenopodiaceae	*Beta vulgaris L.cvs.*	H – poly	Ym	+
		Beta poly	Ym	+
		Chenopodium amaranticolor Coste & reyn	NLL	+
		Chenopodium quniou Wild	NLL	+
Amrantha ceae		*Gomphrena globosa L.*	NLL	+
Solana ceae	*Nicotiana tabacum. cv.*	White Burley	M	+
	N.rustca L.		SM	+
	N.glutinosa L.		M	+
Leguminosae	*Vicia fabae* L. cv.	Gimd 3	M	+

The obtained isolate of CMV reacted

positively with ten plants. Symptoms produced on the artificially inoculated plants by virus isolate was systemic symptoms produced on the tested *Beta vulgaris* plants *cv* H–Poly and Beta poly which gave sever reaction (yellow mosaic) with the virus on the other hand it induced necrotic local lesions on the inoculated leaves of *Ch. amararticolor, c. quinoa* and *Gomphrena globosa* these results were in agreement with those reported by EL–kady *et al*. (1985) Amin, (1989) and Soliman (2003). Other host reactions mentioned in the result developed systemic symptoms as a result of CMV infection such as: *Nicotiana tabacum* cv white Burley, *N. glutinosa, N. rustica and vicia fabae L.* In general, the obtained results concerning the host range were in agreement with the finding of others (EL-kady *et al.* 1985, Espin *et al.* 1994 and Saker, 2004)

Table 2: Transmission of CMV by three aphid species.

Aphids	No. of infected plant	% infection
M.persicae	6/10	60
Aphis craccivora	4/10	40
Aphis faba	0/10	0

Insect transmission: The current isolate of CMV was found to be transmitted mechanically and by two aphid species namely *Myzus persicae* and *Aphis craccivora*. Results in table 2 indicated that *M. persicae* was most effective vector in transmitting the virus in the non–persistent manner under the green house conditions than the *Aphis craccivora.*

Ym: yellow mosaic, NLL: necrotic local lesions, M: mosaic, SM: stunting mosaic.

On the other hand, the *Aphis faba* was failed to transmit CMV. Percentage of transmission was ranged between (40-60%) respectively. Similar results were obtained by (EL-kady *et al.* 1985, Espin *et al.* 1994 and Saker, 2004).

Serological reaction: The routine diagnosis of CMV is carried out mainly by serological methods, which are sufficiently sensitive to detect CMV infection in sugar beet as reported by (Maat, 1980). ELISA and TBIA were successfully used in identifying the isolated virus as (CMV). Positive reaction obtained with CMV specific antiserum by DAS–ELISA proved that the virus under study is CMV. The use of the ELISA technique greatly facilitated the identification of viruses and provided more accurate and consistent results as did symptomatology and host range. However, serological methods cannot distinguish CMV isolates that contain additional sequences outside the CP ORF. This might be one of the reasons that CMV isolates containing additional sequences in the 3' NTR have been reported previously, neither in sugar beet nor in other crops. Consequently, recombination events may be much more common among plant-infecting viruses, but are simply not detected due to the common use of serological methods rather than RNA-based detection systems.

Double-stranded RNA (dsRNA) analysis: dsRNA analysis (fig. 1) resulted in a banding profile typical of that seen with members of the cucumovirus family of plant viruses as reported by Fisher *et al*., (1997) and Nameth and Steininger (1997). Plants positive for cucumovurus-like dsRNA were tested with a direct antibody sandwich enzyme-linked immunosorbent assay (ELISA). ELISA results confirmed the presence of CMV in all symptomatic plants tested. No evidence of dsRNA or CMV was found in any of the asymptomatic plants tested.

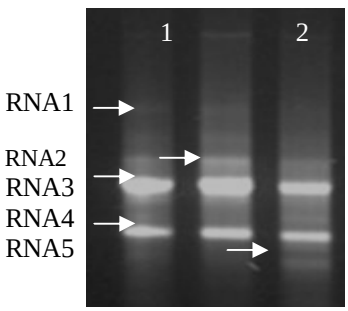

Fig. 1: 1.8% agarose gel showing the dsRNAs profile extracted from sugar beet plants infected with CMV (lanes 1 and 2), from *Nicotiana benthamian*a infected with (TMV) (Lane 3).

RT-PCR amplification of CMV/CP-EG strain: RT-PCR can be used to rapidly and sensitively detect plant viruses. Indeed, the RT-PCR method has proven to be more sensitive than ELISA and dot-blot hybridization (Hsu *et al*., 1995). Also CMV can be effectively detected with different sets of degenerate primers (Hsu *et al*. 1995, Singh *et al*. 1995, and Choi, *et al.* 1999). The set of primers used in this study amplified the CP ORF and flanking regions of CMV isolates from sugar beet crops successfully as shown in Figure 2.

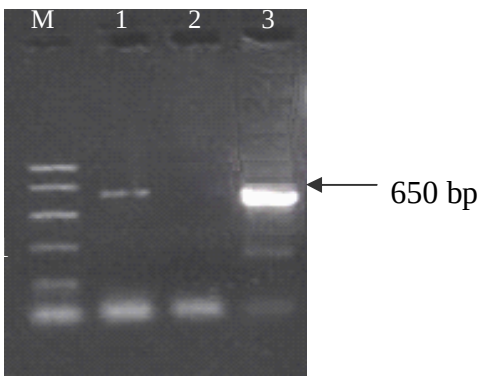

Fig. 2: Amplified DNA fragments of the CMV-EG isolate obtained by RT-PCR. Primers CMVCP-1 and CMVCP-5 were used to perform RT-PCR on ds RNA isolated from sugar beet plants (Lanes 1 and 3) infected with CMV. Lane 2: tobacco leaves infected with TMV (-ve control). M: PCR DNA size marker (Promega) is used.

Detection of CMV using non-radioactive RNA probe: DIG-labeled RNA probe developed from the amplified coat protein sequence of cDNA clone (pHEA11) was used in dot-blot hybridization assays to assess specificity. This probe specifically hybridized with crude extracts from sugar beet and tobacco, which infected with CMV-Egyptian strain. None of the RNAs of tobacco mosaic virus cross-reacted to the probe (Fig. 3). A non-radioactive RNA probe, derived from the RNA 3 of CMV-EG, was proven to be highly specific to detect CMV in infected beet tissues as shown in Fig. (3). In combination with the degenerated primer set and specific probe developed in this study, the detection of other strains of CMV can be achieved. Moreover, the probe can be efficiently used for differentiating isolates of CMV in infected tissues when applied in northern blot hybridizations. To date, the occurrence of RNA recombination has been established in several members of the Bromoviridae family, *Brome mosaic virus* (BMV) (Bujarski and Kaesberg, 1986) and *Cowpea chlorotic mosaic virus* (CCMV) (Allison *et al.* 1990).

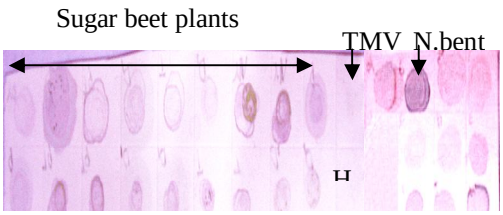

Fig. 3: The DIG-labeled RNA probe derived from the CMV cloned sequence of Egyptian isolate reacts with crude sap preparation extracted from sugar beet plants and reacts with crude extracts from *N. benthamian*a. TMV: crude extracts from tobacco mosaic virus (TMV) shows no reaction. H: healthy control.

Comparison of nucleotide and deduced amino acid sequences: The partial nucleotide sequences of the 3'-halves of RNA 3 of the CMV/CP Egyptian strain was compared with those of some strains in subgroup II of CMV, i.e. Fny (D10538), NT9 (D28780), Q (J02059) and Trk7 (L15336). The homologies at the nucleotide level are about 75 % as observed in (Fig. 4a & b).


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CMV-EG  CNNGCTGACATCATAGTTTTGAGATTCAATTCCTTTTGC-TCCCTGTTGGGCCC—CCTT
D10538  TAAGCCTACATCATAGTTTTGAGGTTCAATTCCTCTTAC-TCCCTGTTGAGCCC--CTT-
D28780  TAAACCCACAACATATCTTTGAGGTTCAATTCCTCTCGT-TCCCTGTTGGGCCC—CTTT
J02059  TAAGTACACATCACAGTTTTAAGGTTCAATTCAATTTGCATCCCTGTTAGGCAAGGCCTT
L15336  TAAGTCCACATCACAGTTTTAAGGTTCAATTCCTTTTGC-TCCCTGTTGGGCCC--CCTT
                *** ** *  *** ** ********   *    ********  **    * *

CMV-EG  ACTTTCTCATGGATGCTTCTCCGCGAGTTAGCGTT--TAGTTGTTCACCTGA------
D10538  ACTTTCTCATGGATGCTTCTCCGCGAGATTGCGTTATTGTCTACTGACTATATAGAGAGT
D28780  ACTTTTTCATGGATGCTTCTCCACGAGATTGCGTT-TCGTCTACTTATCCTA---AGAGT
J02059  ACTTTCTCATGGATGCTTCTCCGCGAGTTAGCGTT--TAGTTGTTCACCTGA------
L15336  ACTTTCTCATGGATGCTTCTCCGCGAGATAGCGTT--TAGTTGTTTACCTGA------
        ***** ***************** **** * *****      *  * *    *

CMV-EG  GTC-GTGTTGTGTTTTG--------TTTTCGCGTCTTAGTGTGCCTATGGACAAATCTGG
D10538  GTTTGTGCTGTGTTTTCTCTTTTGTGTCGTAGAATTGAGTCGAGTCATGGACAAATCTGA
D28780  ATT-GTGTTGTGTTTTCTCTTT-GTGTAGTAGAATTGAGTCGAGTCATGGACAAATCTGA
J02059  GTC-GTGT-TTTCTTTG---------TTTTGCGTCTCAGTGTGCCTATGGACAAATCTGG
L15336  GTC-GTGTGTTTCTTTGTCTTTGTATTTTCGCGTCTTAGTGTACCTATGGACAAATCTGG
         *  ***   *  ***          *         * ***      **************

CMV-EG  TCTCCCAATGCTAGTAGAACCTCCCGGCGTCGTCGCCCGCGTAGAGGTTCTCGGTCCGC
D10538  ATCAACCAGTGCTGGTCGTAACCGTCG---ACGTCGTCCGCGTCGTGGTTCCCGCTCCGC
D28780  ATCAACCAGTGCCGGTCGTAATCGTCG---ACGTCGTCCGCGTCGTGGTTCCCGCTCCGC
J02059  CTCTCCCAATGCTAGTAGAACCTCCGG---TCGTCGCCCGCGTAGAGGTTCTCGGTCCGC
L15336  ATCTCCCAATGCTAGTAGAACCTCCCGGCGTCGTCGCCCGCGTAGAGGTTCTCGGTCCGC
         **  *** ***  ** * *       *    ***** ****** * ***** ** *****

CMV-EG  T---TCTGGTGCGGATGCAGGGTTGCGTGCTTTGACTCAGCAGATGCTGAGACTCAATAA
D10538  CCCCTCCTCCGCGGATGCTAACTTTAGAGTCTTGTCGCAGCAGCTTTCGCGACTTAATAA
D28780  TCCCTCCTCCGCGGATGCTAACTTTAGAGTCTTGTCGCAGCAGCTTTCGCGACTTAACAA
J02059  T---TCTGGTGCGGATGCAGGGTTGCGTGCTTTGACTCAGCAGATGCTGAGACTCAATAA
L15336  T---TCTGGTGCGGATGCAGGGTTGCGTGCTTTGACTCAGCAGATGCTGAAACTCAATAA
            **    ********    **  * *  *** * ****** *   *  *** ** **

CMV-EG  ACCCTCGCCATTGGTCGTCCCACTCTTAACCACCCAACCTTCGTGGGTAGTGAAAGCTG
D10538  GACGTTAGCAGCTGGTCGTCCAACTATTAACCACCCAACCTTTGTAGGGAGTGAACGCTG
D28780  GACGTTAGCAGCTGGTCGTCCAACTATTAACCACCCAACCTTTGTGGGTAGTGAACGCTG
J02059  AACCCTCGCCATTGGTCGTCCCACTCTTAACCACC-AACCTTCGTGGGTAGTGAAAGCTG
L15336  AACCCTCGCCATTGGTCGTCCCACTCTTAACCACCCAACCTTCGCGGGTAGTGAAAGCTG
         **  * **   ********* *** ********* ****** *  ** ****** ****

CMV-EG  TAAACCCGG-TTACACTTTCACATCTATTACCCTGAAACCGCCTGAAATTGAGAAAGGTT
D10538  TAGACCTGG-GTACACGTTCACATCTATTACCCTAAAGCCACCAAAAATAGACCGTGGGT
D28780  TAAACCTGG-GTACACGTTCACATCTATTACCCTGAAGCCGCCGAAAATAGACCGCGGGT
J02059  TAAACCCGGGTTACACTTTCACATCTATTACCCTGAAACCGCCTGAAATTGAGAAAGGTT
L15336  TAAACCCGG-TTACACTTTCACATCTATTACCCTGAAACCGCCTGAAATTGAGAAAGGTT
        ** *** **  ***** ***************** ** ** **  **** **    ** *

CMV-EG  ATATTTTGGTAGAAGGTTGTCTTTGCCAGATTCAGTCACGGACTATGATAAGAAGCTTG
D10538  CTTATTACGGTAAAAGGTTGTTACTACCTGATTCAGTCACGGAATATGATAAGAAGCTTG
D28780  CTTATTATGGTAAAAGGTTGTTGCTACCTGATTCAGTCACGGAGTTCGATAAGAAGCTTG
J02059  CGTGTTTTGGTAGAAGGTTGTCTTTGCCAGATTCAGTCACGGACTATGATAAGAAGCTTG
L15336  CATATTTTGGTAGAAGGTTGTCTTTGCCAGATTCAGTCACGGACTATGATAAGAAGCAAG
        * * **  **** ********   * ** ************** *  **********  *

CMV-EG  GTTCGCGCATTCAAATCAGGATTAATCCTTTGCCGAAATTTGATTCTACCGTGTGGGTTA
D10538  TTTCGCGCATTCAAATTCGAGTTAATCCTTTGCCGAAATTTGATTCTACCGTGTGGGTGA
D28780  TTTCGCGCATTCAAATTCGAGTTAATCCTTTGCCGAAATTTGATTCTACCGTGTGGGTGA
J02059  TTTCGCGCATTCAAATCAGGATTAATCCTTTGCCGAAATTTGATTCTACCGTGTGGGTTA
L15336  TTTCGCGCATTCAAATCAGGATTAATCCTTTGCCGAAATTTGATTCTACCGTGTGGGTTA
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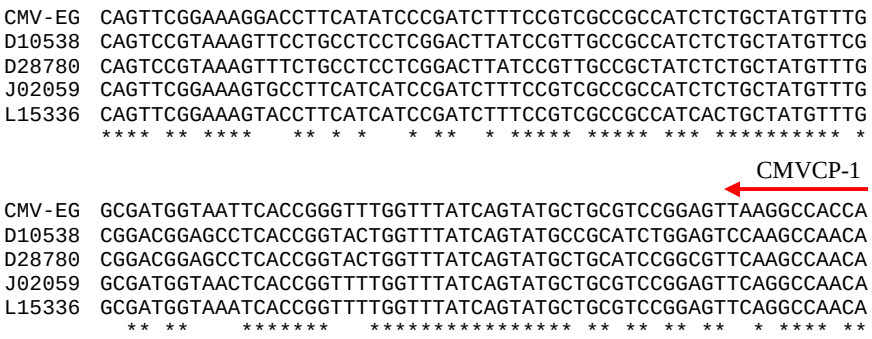

Fig. 4a: Multiple nucleotide sequence alignment of the coat protein gene of four CMV isolates with CMV-EG characterized in this study. Conservative sequences are marked by asterisk at the bottom of the alignment. Dashes denote gaps in the sequence. The primer sequences (CMVCP-1 and CMVCP-2) are denoted by red arrows.

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