Partial biological and molecular characterization of a Cucumber mosaic virus isolate naturally infecting *Cucumis melo* in Iran

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Abstract Melon seedlings showing systemic chlorotic spots and mosaic symptoms were collected in central part of Iran, and a virus was isolated from diseased plants by mechanical inoculation. The virus systemically infected the most inoculated test plants by inducing mosaic symptoms, while, in the members of Fabaceae family and *Chenopodium quinoa* induced local lesions. Agar gel diffusion test using a polyclonal antiserum against a squash Cucumber mosaic virus (CMV) isolate showed the presence of CMV in the mechanically inoculated plants (designated CMV-Me). The virus was purified by polyethylene glycol precipitation and differential centrifugation. A polyclonal antiserum was produced against the virus that reacted specifically with virus antigen in ELISA and agar gel diffusion tests. The virus was molecularly characterized by PCR amplification of the full length of the coat protein gene using cucumovirus genus specific primer pair CPTALL-3/CPTALL-5 and sequence analysis of the resulting product. No RNA satellite was detected using the primer pair CMVsat3H/sat5T7P. Phylogenetic analysis based on the coat protein amino acid sequences showed that CMV-Me belongs to Subgroup IB. These results may be helpful in melon breeding programs, focusing on plant resistance to plant viruses including CMV.

Keywords *Cucumis melo* · CMV subgroups · Purification · Phylogenetic analysis

Cucumber mosaic virus (CMV) is the type member of the genus Cucumovirus (family *Bromoviridae*) with spherical particles containing three linear single-stranded positive sense RNA molecules denoted RNA-1, RNA-2, and RNA-3, which encode four viral proteins. The viral RNA segments are encapsidated by the viral coat protein (CP) that is expressed through a subgenomic RNA (RNA4) transcribed on RNA3 [6, 11].

CMV is one of the main important plant viruses causing severe economic losses on host plants around the world. It has a worldwide distribution and is distinct from other members of the family *Bromoviridae*, since CMV strains have a very wide host range, infecting of about 200 species in over 100 families, including fruit crops, vegetables and ornamentals, both monocots and dicots [12]. Furthermore, 80 species of aphid in 33 genera transmit CMV strains to a wide range of plant species in a nonpersistent manner. The wide host range, efficient aphid transmission and segmented genome of the virus make it easily occur in different ecological niches in nature and produce new strains. Therefore, researches on virus control strategies based on resistant hosts and breeding programs are very important and to aim this, virus detection by serological and molecular tests and gathering virus genetic information are important.

Recent classification of CMV isolates has been based on serological tests, nucleic acid hybridization and restriction fragment length polymorphism, and CMV isolates have been classified into two main subgroups denoted I and II. Subgroup I members have been further divided into IA and IB based on phylogenetic analyses, and appeared to be more prevalent in nature [18].

In Iran, CMV is one of the most widely distributed and economically important plant viruses and has been reported from many different host plants such as banana, sugar beet,

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pepper, violet, zinnia, cucumber, tomato, lettuce and cucurbits [1, 5, 7–9, 13, 16, 17]. We previously described CMV isolates on hoary cress from Shiraz and cucumber from Esfahan, Iran, which were belonged to II and IA subgroups, respectively [14]. This paper describes partial biological and molecular characterization of a local CMV strain obtained from melon in central part of Iran. Results showed that the virus is belonged to CMV IB subgroup. Virus purification, antiserum preparation and phylogenetic analysis are discussed.

Melon plants with systemic chlorotic spots, leaf deformation and mosaic symptoms were collected in a local garden in Abarkouh (Yazd province), Iran. Symptomatic tissues were extracted in 0.1 M potassium phosphate buffer (1:10; w:v), pH 7, and mechanically inoculated to the test plants previously dusted with carburandum powder. Eight to ten test plants from each species were inoculated. The presence of CMV in symptomatic samples and inoculated test plants was verified using a polyclonal antiserum raised against a squash CMV isolate by agar gel diffusion. The isolated virus designated as CMV-Me was biologically purified and propagated by serial single lesion transferring on zucchini (*Cucurbita pepo*) and maintained on *Nicotiana glutinosa* and *Nicotiana tabacum* var. Turkish during the course of this study. The plants were maintained in a greenhouse and monitored for developing virus symptoms up to 3 weeks post inoculation. Symptomless plants were assessed by agar gel diffusion or back inoculation.

A simple procedure was set up for purification of CMV-Me. Systemically infected leaves of zucchini plants were harvested 10–15 days after infection and extracted in a warning blender in three volumes of 0.5 M sodium citrate buffer, containing 5 mM EDTA, pH 6.5 and the resulting juice was filtered through a double layer of gauze. Clarification of the extract was carried out by using a chloroform step (final concentration of 20 %) followed by stirring at 4 °C for 20 min and centrifugation at 10,000 rpm for 20 min. The virus was precipitated after addition of polyethylene glycol (PEG) 6000 and NaCl to the final concentrations of 10 % and 0.5 M, respectively, and centrifugation at 10,000 rpm for 20 min. The pellet was resuspended in appropriate volume of 0.05 M of extraction buffer, left stand for 30 min and centrifuged at 10,000 rpm for 10 min. Further virus purification was achieved by subjecting the resulting preparation to another cycle of PEG precipitation and the final preparation was dissolved in 0.05 M borate buffer containing 5 mM EDTA, pH 9 and stored at –20 °C. All centrifugation steps were carried out at 4 °C using a Sorvall SS-34 rotor.

A polyclonal antiserum was prepared against the virus after five injections of the purified virus preparation (0.5 mg/ml) mixed with Freund's complete adjuvant (v:v) into a rabbit. Bleeding was carried out 10 days after the last

injection and antiserum was isolated and stored at –20 °C. IgG was precipitated by saturated ammonium sulfate solution and isolated using chromatography on DEAE-cellulose column as described [3].

Total RNA was extracted from systemically infected leaf tissue of zucchini plants by using DENAzist total RNA extraction kit (DENAzist Asia, Iran) as described by manufacturer's instructions. The integrity and quality of the total RNA was checked in 1 % agarose gel and quantified by NanoDrop ND-1000 spectrophotometer. For cDNA synthesis, about 200 ng total RNA along with 1 µl of 10 pM of CMV-CP genus specific primer CPTALL-3 [4], was denatured at 65 °C for 10 min, immediately cooled on ice followed by addition of 2 µl of 10 mM of dNTP mix (Cinnagene, Iran) and five unit of M-MLV reverse transcriptase (Fermentas, Lithuania) as described by manufacturer's instructions. PCR amplification was carried out using 2 µl of the resulting cDNA in a total volume of 20 µl consisting of 18 µl of sterile distilled water, 1 µl of 10 pM each of the primers CPTALL-3/CPTALL-5 [4], 0.75 µl of 50 mM MgCl₂, 0.25 µl (0.3 unit) of *Taq* DNA polymerase (Cinnagene), 2 µl of 10 × PCR buffer and 1 µl of 10 mM of dNTP mix. PCR was performed in a Techne thermal cycler with initial denaturing at 94 °C for 3 min and then 35 cycles of denaturing at 94 °C for 1 min, annealing at 45 °C for 1 min and extension at 72 °C for 1 min with a final extension step at 72 °C for 10 min. PCR product was analyzed in 1 % agarose gel staining with ethidium bromide.

The amplified PCR product was purified using AccuPrep PCR purification kit (Bioneer, South Korea) according to manufacturer's protocol and used for sequencing by Macrogen (South Korea). The sequence data were compiled, analyzed and used for a search in the GenBank database using BLAST program. The deduced coat protein amino acid sequences of 22 to 37 CMV isolates belonging to different subgroups and geographical locations were retrieved from the GenBank database and compared with that of CMV-Me using MEGA5 software. A maximum likelihood phylogenetic tree was constructed based on 100 replicates bootstrapping the aligned sequences to resolve the taxonomic position of the virus.

Host plant's reactions to CMV-Me after mechanical inoculation are summarized in Table 1. CMV-Me caused local infection in plants from *Fabaceae*. Disease symptoms were highly progressed on plants from *Cucurbitaceae*, leading to subsequent death of the plants. Laboratory infected melon plants exhibited symptoms similar to naturally infected plants including systemic chlorotic spots, mosaic, leaf deformation and death of the plants (Fig. 1a).

Use of chloroform and differential centrifugation in purification of the virus led to removal of the most of green

Table 1 Experimental host range of Cucumber mosaic virus melon strain (CMV-Me) after mechanical inoculation

Test plants	Symptoms on the leaves
<i>Chenopodium quinoa</i>	NLL
<i>Cucumis melo</i>	CLL, CS, M, LD, RS, SCS, DE
<i>C. sativus</i>	CLL, CS, Mo, M, LD, RS, SCS, DE
<i>Cucurbita pepo</i>	CLL, CS, M, LD, RS, SCS, DE
<i>Phaseolus aueus</i>	NLL
<i>P. vulgaris</i>	NLL
<i>Vicia faba</i>	NLL
<i>Vigna unguiculata</i>	NLL
<i>Capsicum annuum</i>	CS
<i>Nicotiana glutinosa</i>	D, LD, M, SS
<i>N. rustica</i>	D, LD, M, SS
<i>N. tabacum</i> var. Turkish	CS, M, LD

CLL chlorotic local lesions, CS chlorotic spots, D dwarfing, DE death, LD leaf deformation, M mosaic, Mo mottling, NLL necrotic local lesions, RS ring spot, SCS systemic chlorotic spots, SS shoe string

pigments in the final preparation. A yield of 2 mg virus per 100 g of infected tissue was estimated for the purified preparation. The prepared CMV-Me antiserum could specifically detect the virus in crude extract of infected plants in agar gel diffusion, after absorption (Fig. 1b). In indirect ELISA, 1/1500 dilution of isolated IgG (with concentration of 1 mg/ml) gave the best result when used

with 1/10 dilution of the crude extract of CMV-infected plant and 1/3000 dilution of goat anti-rabbit IgG.

After RT-PCR of the total RNA extract from CMV-Me infected zucchini (Fig. 1c), the expected ca. 900 bp fragment was amplified (Fig. 1d). No satellite was detected to be associated with the virus using the primer pair CMVsat3H/sat5T7P [15]. Sequence analysis showed that the sequenced fragment (Accession No. KT626606) is 861 bp in length and contains the full length of CP gene of CMV-Me. It is 657 nts long, beginning with an AUG start codon and terminating with an UGA stop codon, coding for a deduced 218 aa with an estimated MW of 23.9 kDa and an isoelectric pH of 10.4. Sequence analysis for amino acid composition also showed that Arginine is the major (13.3 %) and Cysteine is the minor (0.43 %) component of the CP of CMV-Me. It has a *Hind*III restriction enzyme site starting at nucleotide 304 from the 5' end.

The sequence of the fragment was used for searching in the GenBank database using BLAST program and results showed that the CP amino acid sequence of CMV-Me has the most similarity with those of CMV subgroup I-B members. It had 99 % amino acid sequence identity with Bas3 isolate (AGG16139) of squash and Ker.mel.2 isolate (AFZ62495) of melon from Iran, CMV-G2 (AAR23529) and G10 (AAS48545) tobacco isolates from Greece, Vir56 isolate (AAY19285) of yellow oleander from Italy and JK isolate (ABM46612) of lemmongrass from India. Due to the high similarity of the virus with the above mentioned isolates, only the coat protein amino acid sequence of the isolate Bas3 was included in phylogenetic analysis. This analysis confirmed the above result and revealed that CMV-Me formed a subclade with isolates SD, Bas3, Ajs4; TR15, CNO3, P3613, BQ6, Calicut, CMVJMBr; Gerbera Silves; Lucknow, all members of subgroup I-B that used for comparison in this study (Fig. 2, arrow).

Of the CMV isolates molecularly characterized from Iran so far, only CMV-Ld [14] is belonged to II subgroup and the most of the remaining have been isolated from squash and belonged to IA subgroup. Subgroup IB members seems to be less prevalent than IA and occur on tomato in Iran [2, 10]. Although the sequence data of another melon infecting CMV isolate is available in GenBank, it has been no report on the properties of the virus in Iran, yet, and this work present data for the first time on properties of a subgroup IB member of CMV naturally infecting melon in Iran.

As plants from *Fabaceae* reacted locally to CMV-Me, they may be regarded as candidates for searching the resistance genes against the virus. In comparison with CMV-Ld, a member of serogroup II that induced typical line pattern on test plants and systemically infected faba bean [14], this symptom was not observed on any of the test plants inoculated with CMV-Me, and faba bean showed only local symptom in response to the isolate. This

Fig. 1 **a** Disease symptoms induced in melon after mechanical inoculation of CMV-Me. **b** Immunodiffusion reaction of CMV-Me polyclonal antiserum (As) with healthy (H) and CMV-Me infected (I) zucchini crude extract. **c** Total RNA extracted from CMV-Me infected zucchini and **d** the resulting RT-PCR product (lane 1) using cucumovirus genus specific primer pair CPTALL-3/CPTALL-5, after electrophoresis in 1 % agarose gel and staining with ethidium bromide. M represents DNA ladder

Fig. 2 A maximum likelihood phylogenetic tree constructed after multiple sequence alignment of deduced coat protein gene amino acid sequences of the selected CMV strains including CMV-Me (arrow). Numbers at the nodes represent branches with more than 50 % bootstrap scores. Virus isolates and GenBank Accession Numbers are: AFD98309, ISPaVe05; BAJ79035, PoCMV9-11; ALE59664, LOR-A; CAH17693, RT52; CAH17692, RT88; AY871069, S337; AY871070, B13; AY871068, SH17; CAB77390, Mf; ABW81209, CB; EF620777, CMV-Cu; AB008777, SD; JX025977, Bas3; KT626606, CMV-Me; AGG16155, Ajs4; AJ810264, TR15; AJ810261, CNO3; AJ810266, p3613; AGW51600, BQ6; ABU62578, Calicut; BAR13149, CMVJMBR; AFV99522, Gerbera Silves; ABM46606, Lucknow; CAH17689, RT68; AJ810253, RT67; BAC76759, PaFM; ABN03949, Tsh; AF198103, Ld; DQ018292, LY; AF063610, S; AB006813, M2; L15336, Trk7; BAA00358, WL; CAE51065, AL; AAY42628, P26; AAY42630, D-1; AF127976, LS. *Peanut stunt virus* (PSV, AY775057) was used as an out-group

may be related to different host range determinants in the two virus isolates.

The reaction of faba bean plants following virus inoculation, could be classified as immune (no lesion development), semi-sensitive (a few lesions were produced on inoculated leaves) and sensitive (more than 150 lesions per each inoculated leaf), when inoculated with a definitive virus inoculum dilution (1/10), indicating the genetic variation, and the presence of horizontal resistance genes against the virus in faba bean plants.

Since the purification method presented in this study, does not require high speed and sucrose density gradient centrifugation steps, it offers a simple and an efficient, time and cost saving procedure for virus purification.

The occurrence of virus isolates belonged to all three CMV subgroups in Iran is epidemiologically important. It provides multiple opportunities for genomic reassortment, rearrangement, and development of recombinant virus strains that can overcome the currently available resistance genes.

References

1. Ahoonmanesh A, Alavi V, Mosahebi Mohammadi gh. Concurrent presence of *Cucumber mosaic virus* in certain tomato growing areas of Iran. Iran. J Plant Pathol. 1997;33:111–25.
2. Arafati N, Farzadfar Sh, Pourrahim R. Characterization of coat protein gene of *Cucumber mosaic virus* isolates in Iran. Iran. J Biotech. 2013;11:109–14.
3. Ball E, Hampton R, De Boer S, Schaad N. Polyclonal antibodies. In: Hampton R, Ball E, De Boer S, editors. Serological methods for detection and identification of viral and bacterial plant pathogens. USA: APS Press; 1990. p. 33–54.
4. Choi SK, Choi JK, Park WM, Ryu KH. RT-PCR detection and identification of three species of cucumovirus with a genus-specific single pair of primers. J Virol Methods. 1999;83:67–73.
5. Farhangi SH, Mosahebi G, Habibi MK, Okhovvat SM. Occurrence, distribution and relative incidence of mosaic viruses infecting field grown squash in Tehran province, Iran. Commun Agric Appl Biol Sci. 2004;69:507–12.
6. Gallitelli D. The ecology of *Cucumber mosaic virus* and sustainable agriculture. Virus Res. 2000;71:9–21.
7. Ghotbi T, Bananej K. First report of *Cucumber mosaic virus* in banana from Iran. Plant Dis. 2005;89:914.
8. Golnaraghi A, Shahraeen N, Pourrahiam R, Farzadfar Sh, Ghasemi A. Occurrence and relative incidence of viruses infecting soybeans in Iran. Plant Dis. 2004;88:1069–74.
9. Izadpanah K. An annotated list of virus and virus-like diseases of plants in Fars. Farsi, English index: College of Agriculture, Shiraz university; 1983.
10. Nematollahi S, Sokhandan-Bashir N, Rakhshandehroo F, Zamanizadeh R. Phylogenetic analysis of new isolates *Cucumber mosaic virus* from Iran on the basis of different genomic regions. The Plant Pathol J. 2012;28:381–9.
11. Palukaitis P, Roossinck MJ, Dietzgen RG, Francki RIB. Cucumber mosaic virus. Adv Virus Res. 1992;41:281–348.
12. Pinto ZV, Rezende JAM, Yuki VA, Piedade SMS. Ability of *Aphis gossypii* and *Myzus persicae* to transmit *Cucumber mosaic virus* in single and mixed infection with two potyviruses to Zucchini Squash. Summa Phytopathol. 2008;34:183–5.
13. Rahimian H, Izadpanah K. Identity and prevalence of mosaic inducing cucurbit viruses in Shiraz, Iran. Phytopathol Z. 1978;92:305–12.
14. Rasoulpour R, Izadpanah K. Properties and taxonomic position of hoary cress strain of cucumber mosaic virus. J Plant Pathol. 2008;90:97–102.
15. Sato H, Hase S, Sugiyama M, Karasawa A, Suzuki T, Takahashi H, Ehara Y. A novel satellite RNA of *Cucumber mosaic virus* induces unique line-pattern mosaic symptoms in tobacco. J Phytopathol. 2000;148:47–51.
16. Sokhandan-Bashir N, Kalhor M, Zarghani S. Detection, differentiation and phylogenetic analysis of *Cucumber mosaic virus* isolates from cucurbits in the northwest region of Iran. Virus Genes. 2006;32:277–88.
17. Soleimani P, Mossahebi GH, Koohi-Habibi M, Zad J, Hosseini-Farhangi S. Occurrence and distribution of lettuce mosaic disease in Tehran province of Iran. Commun Agric Appl Biol Sci. 2004;69:513–7.
18. Yu C, Wu J, Zhou X. Detection and subgrouping of *Cucumber mosaic virus* isolates by TAS-ELISA and immunocapture RT-PCR. J Virol Methods. 2004;123:155–61.