Bioassay and Phylogeny of Five Iranian Isolates of *Cucumber mosaic virus* from Different Hosts Based on *CP* Gene Sequence

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Abstract

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Using double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), *Cucumber mosaic virus* (CMV) was detected in 31 out of 132 symptomatic leaf samples collected from different hosts of Urmia province of Iran, during 2011–2012. In biological assays, five different host isolates caused severe mosaic in *Nicotiana* species and *Capsicum annum* without significant difference in severity. Based on phylogenetic analysis of coat protein nucleotide and deduced amino acid sequence, two isolates were clustered into subgroup IA, while other three isolates were grouped into IB subgroup of CMV. All Urmian isolates shared a common *Msp*I, and no *Eco*RI and *Bsu*RI restriction sites. In contrast to S-IA isolates, the second *Msp*I site was found at 473–476 position of only S-IB isolates, which could be used to differentiate two S-IA and S-IB subgroups. Here, we report the first case of *Abutilon theophrasti* infection, as a new reservoir weed host for CMV in the world.

Keywords: CMV; coat protein; genetic variation; phylogeny; symptomology; subgroup I

Cucumber mosaic virus (CMV) is type species of the genus *Cucumovirus*, family *Bromoviridae* (King *et al.* 2012) with the widest host range among plant viruses (Berniak *et al.* 2010). CMV has been very successful in rapidly adapting to new hosts and new environments (Roossinck 2002) and causes great losses in vegetables, ornamentals, and fruits (Palukaitis & Garcia-Arenal 2003). The virus is mechanically transmissible by plant sap and spread by more than 80 aphid species in the non-persistent manner. The genome of CMV consists of three single-stranded positive-sense RNA species (Palukaitis & Garcia-Arenal 2003)

The CMV strain variation has been studied by serological assay (Wilson & Halliwell 1985), dsRNA analysis (Pares *et al.* 1992), PCR-RFLP (Rizos *et al.* 1992), and microarray methods (Deyong *et al.* 2005); thereby, it has been separated into two main subgroups, subgroup I (S-I) and subgroup II (S-II), on the basis of serological relationships, peptide mapping of the coat protein (CP), nucleic acid hybridisation, and nucleotide sequence identity (Palukaitis *et al.* 1992). Another division of subgroup I into IA and IB has been proposed based on sequence data, analysis of 5'-non-translated region of RNA3 of several strains, and phylogenetic analysis of CP (Roossinck *et al.* 1999; Roossinck 2002). The nucleotide sequence identity between CMV subgroup II and I strains ranges from 69% to 77%, while above 90% within subgroups (Palukaitis *et al.* 1992). Phylogenetic analysis of 15 CMV strains based on different gene sequences was not congruent and did not completely support the subgrouping indicated by the *CP* gene. This indicates that different RNAs may have independent evolutionary histories (Roossinck 2002).

Investigations on different tomato and cucurbit isolates of CMV in Iran revealed the existence of subgroups I and II with more prevalence of CMV-I, while the S-IA was more frequent than S-IB (Bashir *et al.* 2006, 2008; Nematollahi *et al.* 2012; Arafati *et al.* 2013). Although, this virus is endemic in the cultivated fields of different crops in the northwest of Iran, differentiation of field crop infecting isolates of CMV has not been carried out so far.

In this work, host range, symptom expression, and genetic diversity of CMV from different field crops and olericultures in Urmia vicinity, northwest of Iran, were analysed and compared with CMV isolates from other parts of the world based on nucleotide and deduced amino acid sequence of ORF CP.

MATERIAL AND METHODS

Sampling. Several surveys were conducted in the fields of beet, bean, tomato, olericultures, and different cucurbits in Urmia vicinity (west Azerbaijan, Iran) during 2011–2012 cultivation seasons (April to October). Totally, 132 leaf samples showing viral symptoms such as mosaic, mottling, and yellowing were collected from above mentioned crops and also the weeds growing in the same fields.

ELISA. All field-collected samples were analysed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark & Adams 1977) using CMV-specific polyclonal antibody (DSMZ, AS-0929) and their leaf extracts in ELISA extraction buffer (1 : 10). Mechanically inoculated symptomatic and mock-inoculated cucumber plants were used as positive and negative controls. The absorbance of each well was measured at 405 nm (OD_{405}) by ELISA-reader (Beckman AD 340S; Beckman Coulter, Fullerton, USA). Samples were considered positive only if the absorbance value was more than three times higher that of negative control.

Virus isolates, maintenance and inoculation of plants. Among CMV infected samples, five isolates from different hosts (Table 1) were chosen for biological and molecular investigation. Viral isolates were biologically purified by three serial single local lesion transfer on *Chenopodium quinoa* leaves, then propagated on *Cucurbita pepo* and *Nicotiana tabacum* plants by mechanical inoculation in 0.05M phosphate buffer, pH 7, containing 0.25% Na-DIECA (inoculation buffer). One month later, symptomatic plants were serologically tested to confirm their infection with CMV and systemically infected plants were used as the source of each CMV isolate for subsequent studies. Selected CMV isolates were mechanically inoculated on nine different test plants. Symptoms were recorded at 5–30 days post inoculation (dpi) and infection status of all inoculated plants were checked at 30 dpi by DAS-ELISA. Plant species used were *Chenopodium quinoa*, *C. amaranticolor,* capsicum (*Capsicum annum*), cowpea (*Vigna unguiculata*), broad bean (*Vicia faba*), zucchini (*C. pepo*), and tobacco (*N. tabacum, N. glutinosa*, and *N. rustica*).

Immunocapture RT-PCR (IC-RT-PCR). Coat protein gene of CMV isolates was amplified by immunocapture RT-PCR protocol (Nolasco *et al.* 1993), using F4 (forward) 5'-TTGAGTCGAGTCATG-GACAAATC-3'/F3 (reverse) 5'-AACACGGAATCA-GACTGGGAG-3' primer pair designed by Lin *et al.* (2004). Briefly, fresh leaf tissue of *N. tabacum* plant, infected with each CMV isolate, was macerated in PBST buffer containing 2% PVP and incubated overnight at 4°C in PCR vials which had been coated with CMV IgG before. cDNA synthesis was performed in 20 µl reaction mixtures containing 4 µl 5 × RT-buffer, 5 pmol CMV-F3 primer, 0.5mM dNTPs, 5mM DTT, 20 U RNAse inhibitor (Fermentas GmbH, St. Leon-Rot, Germany), and sterile distilled water. The mixtures were incubated at 72°C for 4 min, then immediately placed on ice and 100 U M-MuLV reverse transcriptase (Fermentas GmbH, St. Leon-Rot, Germany) was added to each reaction and followed by incubation at 42°C for 1 h in a thermocycler (Palm cycler, CG196; Corbett Research, Mortlake, Australia). PCR reactions were performed in 25 µl

Table 1. List of Urmian *Cucumber mosaic virus* isolates

Isolate	Accession No.	Original host	Geographical location
Ab	KJ173756	abutilon	Gharegoseile, Urmia, Iran
Ce27	KJ173754	celery	Sheykhsarmast, Urmia, Iran
Cu49	KJ173755	cucumber	Arablu, Urmia, Iran
To33	KJ173753	tomato	Sheykhsarmast, Urmia, Iran
Tob	KJ173757	tobacco	Institute of Tobacco Research, Urmia, Iran

Table 2. Accession number, original host and the location of CMV strains used for phylogenetic analysis

Strain	Accession No.	Original host	Subgroup	Country of origin[a]
CMV-22Huahine	FN554693	*Commelina diffusa*	IB	French Polynesia
CMV-ABI	L36525	gladiolus	IB	South Korea
CMV-Ajs4	JX025999.1	tomato	IB	Iran
CMV-B2	AB046951	*Musa sapientum*	IB	Indonesia
CMV-Bas3	JX025989.1	cucumber	IB	Iran
CMV-Fny	U20668	–	IA	USA (NY)
CMV-G10	AY541691	tomato	IB	Greece
CMV-G2	AY450854	tomato	IB	Greece
CMV-Gpp	AJ131623	gladiolus	IA	The Netherlands
CMV-KM	AB004780	melon	IA	[Japan]
CMV-M2	AB006813	–	II	[Japan]
CMV-Nd-Cs	AY429437	*Arachis hypogaea*	IB	The Netherlands
CMV-Ns	AJ511990	*Nicotiana glutinosa*	IA	Hungary
CMV-Ny	U22821	–	IA	[Australia]
CMV-PE	AF268597	passion flower	IB	China
CMV-Q	M21464	–	II	Australia
CMV-R	Y18138	*Rununculus* sp.	II	[France]
CMV-S337	AY871069	*Cucumis* sp.	IA	Iran
CMV-SD	AB008777	–	IB	[China]
CMV-SH12	AY871067	*Cucumis sativus*	II	Iran
CMV-Sn	U22822	–	II	[Australia]
CMV-SP103	U10923	*Spinacia oleracea*	II	USA (AR)
CMV-TN	AB176847	tomato	II	Japan
CMV-WL	D00463	–	II	USA (NY)

[a]The country of origin is indicated where known; in the cases where the country of origin is not indicated, the country where the sequence data was obtained is given in brackets

reaction mixtures containing 5 μl cDNA, 2.5 μl of 10× PCR-buffer, 4mM MgCl$_2$, 2 pmol of each F3 and F4 primer, 0.2mM dNTPs, and 1.5 U SmarTaq DNA polymerase (Sinaclon, Tehran, Iran). The cycling parameters were initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 10 minutes. Amplified fragments of CMV isolates in expected size were extracted from agarose gel by GF-1 nucleic acid extraction kit (Vivantis Technologies, Subang Jaya, Malaysia), then they were directly sent for sequencing in both directions to Bioneer Inc. (Daejeon, South Korea) using the respective PCR primer pair.

Sequence analysis. The nucleotide sequences of *CP* gene of all Urmian isolates were compared with the counterpart sequences of 23 CMV isolates (Table 2) available in GenBank. Multiple sequence alignment of nucleotide and deduced amino acid sequences was carried out using optimal alignment methods of the software packages DNAMAN v. 7 (Lynnon Biosoft, Quebec, Canada) and MEGA v. 5 (Center for Evolutionary Medicine and Informatics, Tempe, USA) (Tamura *et al.* 2011), respectively. Phylogenetic relationships were inferred using MEGA v. 5 based on the neighbour joining method (Saitou & Nei 1987) with a 100 replicate bootstrap search. *Peanut stunt cucumovirus* strain ER (PSV-ER) was defined as out-group species.

RESULTS

CMV isolates and symptomology. Thirty one out of 132 symptomatic leaf samples collected from different crop fields and weed plants, including *C. sativus* (10), tomato (8), pepper (6), *N. tabacum* (2), *C. pepo* (2), and one of each bean, celery and *Abutilon theophrasti* were positive for CMV by DAS-ELISA. Five isolates
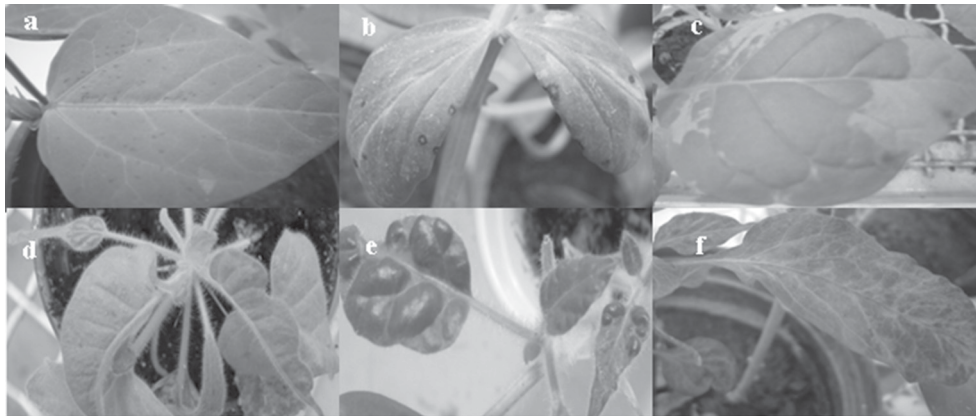
Figure 1. Disease symptoms caused by Urmian CMV-SI isolates on host plants.Local lesion on *V. unguiculata* (a) and *V. faba* (b) at 7 dpi; severe mosaic on *N. tabacum*, *N. glutinosa*, *N. rustica* and *C. annum* (c–f) at 21 dpi

of CMV from different hosts did not show reliable difference in the kind and severity of symptoms induced on inoculated test plants. *C. quinoa* and *C. amaranticolor* showed chlorotic local lesions at 5–6 dpi. Viral isolates caused small and large necrotic local lesions on inoculated leaves of *V. unguiculata* and *V. faba*, respectively, at 7 dpi. All isolates caused severe mosaic often with blisters and leaf distortion on *N. tabacum, N. glutinosa, N. rustica,* and *C. annum* at 21 dpi (Figure 1)*.* Infection of all symptomatic inoculated plants was confirmed by DAS-ELISA in proper time after symptom development.

IC-RT-PCR, sequence comparison, and phylogenetic analysis*.* IC-RT-PCR of systemically infected plants with five Urmian CMV isolates resulted in amplification of a 678 bp long DNA fragment, as expected (Figure 2). No products were amplified by IC-RT-PCR from sap extracts of healthy plants.
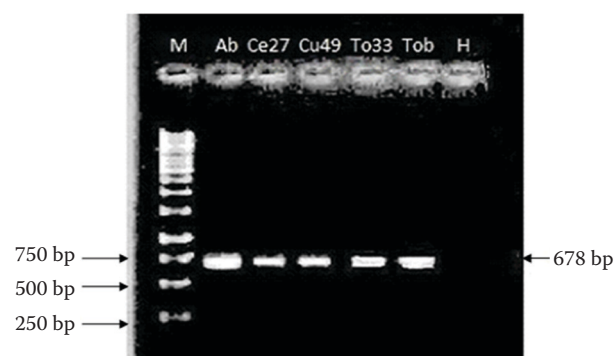

Figure 2. Electrophoretic pattern of amplified coat protein cDNA fragment of Urmian CMV isolates by IC-RT-PCR using F4/F3 primer pair

Lanes 1–5: CMV isolates Ab, Ce27, Cu49, To33, Tob; lane 6: healthy tobacco plant (H); M: Gene Ruler 1Kb DNA Ladder (Fermentas, GmbH, St. Leon-Rot, Germany)

The amplicons comprised the complete *CP* gene in fragment of 657 bp (including start and stop codons). Following nucleotide sequence alignment, our Urmian isolates showed over 93% nucleotide sequence identity with each other. Among five isolates, Cu49 and Tob were completely identical and showed 100% nucleotide sequence identity with S-I isolates (Fny, Ny), and 98.9% with Iranian S337 isolate. On the other hand, two identical Ce27, To33 isolates in nucleotide sequence had their highest identities (99.1%) with Ab isolate, whereas all three isolates had 98.3–98.7% identity with two S-I isolates G2 and G10. In contrast, less than 80% sequence identity was observed between five Urmian isolates and compared S-II isolates (Table 3). Sequence comparison of amplified CMV coat protein gene from five Urmian CMV isolates revealed base changes at positions 39, 57, 534, 597, and 615 in Tob isolate, but without change in the corresponding coded amino acid sequences.

Nucleotide sequence analysis of *CP* gene revealed that all Urmian isolates share a common CCGG *Msp*I (*Hpa*II) restriction site at 445–448 position, without any *Eco*RI and *Bsu*RI restriction sites, as a characteristic pattern of CMV S-I. Surprisingly, in contrast to S-IA isolates, the second *Msp*I site was found at 473–476 position of only S-IB isolates To33, Ce27, and Ab, like it was present in the same position of other members of S-IB clade (Figure 4). The second *Msp*I site could be considered as a useful character to differentiate the S-IA and S-IB subgroups.

In phylogenetic analysis of *CP* gene, two neighbour joining trees based on nucleotide and deduced amino acid sequence alignment were similar in topology and showed three major clades representing subgroups S-IA, S-IB, and S-II (Figure 3). Subgroups S-IA and

Table 3. Percentage of nucleotide sequence identities among *CP* gene of five Urmian CMV isolates and 24 other isolates

| CMV isolates	Ab	Ce27	Cu49	To33	Tob	Fny	G2	G10	22Huahin	ABI	Ajs4	B2	Bas3	GPP	Km	M2	Nd-Cs	Ns	Ny	PE	Q	R	S337	SD	SH12	Sn	SP103	Tn	WL
Ab	100																												
Ce27	98.9	100																											
Cu49	93.8	94.2	100																										
To33	98.9	94.2	94.2	100																									
Tob	93.8	94.2	100	94.2	100																								
Fny	93.8	94.2	100	94.2	100	100																							
G2	98.5	98.8	93.6	98.8	93.6	93.6	100																						
G10	98.6	98.9	93.8	98.9	93.8	93.8	99.8	100																					
22Huahin	97.4	97.5	93.2	97.5	93.2	93.2	97.7	97.8	100																				
ABI	93.2	93.3	94.2	93.3	94.2	94.2	93.3	93.5	93.9	100																			
Ajs4	98.3	98.5	94.2	98.5	94.2	94.2	98.2	98.3	97.5	94.1	100																		
B2	96.2	96.0	93.0	96.0	93.0	93.0	96.0	96.0	96.6	93.6	96.0	100																	
Bas3	98.5	98.9	94.1	98.9	94.1	94.1	98.6	98.8	97.7	93.8	98.8	96.5	100																
GPP	93.0	92.8	96.5	92.8	96.5	96.5	92.8	93.0	92.8	93.6	93.6	91.9	93.0	100															
Km	93.0	93.2	97.1	93.2	97.1	97.1	93.2	93.3	93.2	93.8	93.9	92.5	93.3	98.2	100														
M2	77.1	77.1	77.2	77.1	77.2	77.2	77.2	77.4	77.5	77.7	77.8	78.1	77.1	77.5	78.1	100													
Nd-Cs	93.0	93.4	93.7	93.4	93.7	93.7	93.4	93.6	94.3	96.9	94.3	92.8	93.9	93.3	93.4	77.8	100												
Ns	94.1	94.2	99.4	94.2	99.4	99.4	93.6	93.8	93.2	93.8	94.2	93.8	94.1	96.2	96.5	77.2	93.4	100											
Ny	93.8	94.2	100	94.2	100	100	93.6	93.8	93.2	94.2	94.2	93.0	94.1	96.5	97.1	77.2	93.7	99.4	100										
PE	93.5	93.6	94.7	93.6	94.7	94.7	93.3	93.5	93.5	93.3	94.5	93.3	94.4	93.6	93.9	76.0	92.8	94.1	94.7	100									
Q	77.2	77.2	77.4	77.2	77.4	77.4	77.4	77.5	77.6	78.0	78.0	78.1	77.2	77.7	78.3	99.4	78.1	77.4	77.4	76.1	100								
R	76.8	76.8	77.4	76.8	77.4	77.4	76.9	77.1	77.2	77.7	77.5	78.0	76.8	77.2	78.3	99.1	78.0	77.2	77.4	75.8	98.8	100							
S337	93.2	93.6	99.2	93.6	99.2	99.2	93.3	93.5	92.9	93.8	93.6	92.7	93.5	96.3	97.0	76.9	93.3	98.6	99.2	94.1	77.1	77.1	100						
SD	93.9	94.1	94.4	94.1	94.4	94.4	94.2	94.4	94.9	98.6	94.5	94.4	94.5	93.8	94.5	78.0	96.8	93.9	94.4	93.5	78.3	78.0	94.2	100					
SH12	78.0	77.8	78.8	77.8	78.8	78.8	78.0	78.2	78.9	80.5	78.8	79.4	78.0	79.4	80.1	99.6	80.9	78.8	78.8	77.3	99.2	98.7	78.4	80.5	100				
Sn	76.6	76.6	77.1	76.6	77.1	77.1	76.8	76.9	77.0	77.5	77.4	78.0	76.6	77.1	78.0	99.1	77.5	76.8	77.1	75.8	98.8	98.6	76.8	77.8	98.5	100			
SP103	76.8	76.8	76.8	76.8	76.8	76.8	76.9	77.1	76.9	77.1	77.5	77.7	76.8	76.8	77.4	98.3	77.2	76.8	76.8	75.2	98.6	97.7	76.5	77.4	97.7	97.7	100		
Tn	76.9	76.6	76.8	76.6	76.8	76.8	76.9	77.1	77.3	77.4	77.4	77.7	76.6	77.1	77.7	99.5	77.5	76.8	76.8	75.5	99.2	98.9	76.5	78.0	99.2	98.9	98.2	100	
WL	76.8	76.8	76.9	76.8	76.9	76.9	76.9	77.1	77.2	77.5	77.5	77.8	76.8	77.2	77.8	99.4	78.0	76.9	76.9	75.7	99.1	99.1	76.6	77.8	99.2	98.8	98.0	99.2	100

(**A**)

(**B**)
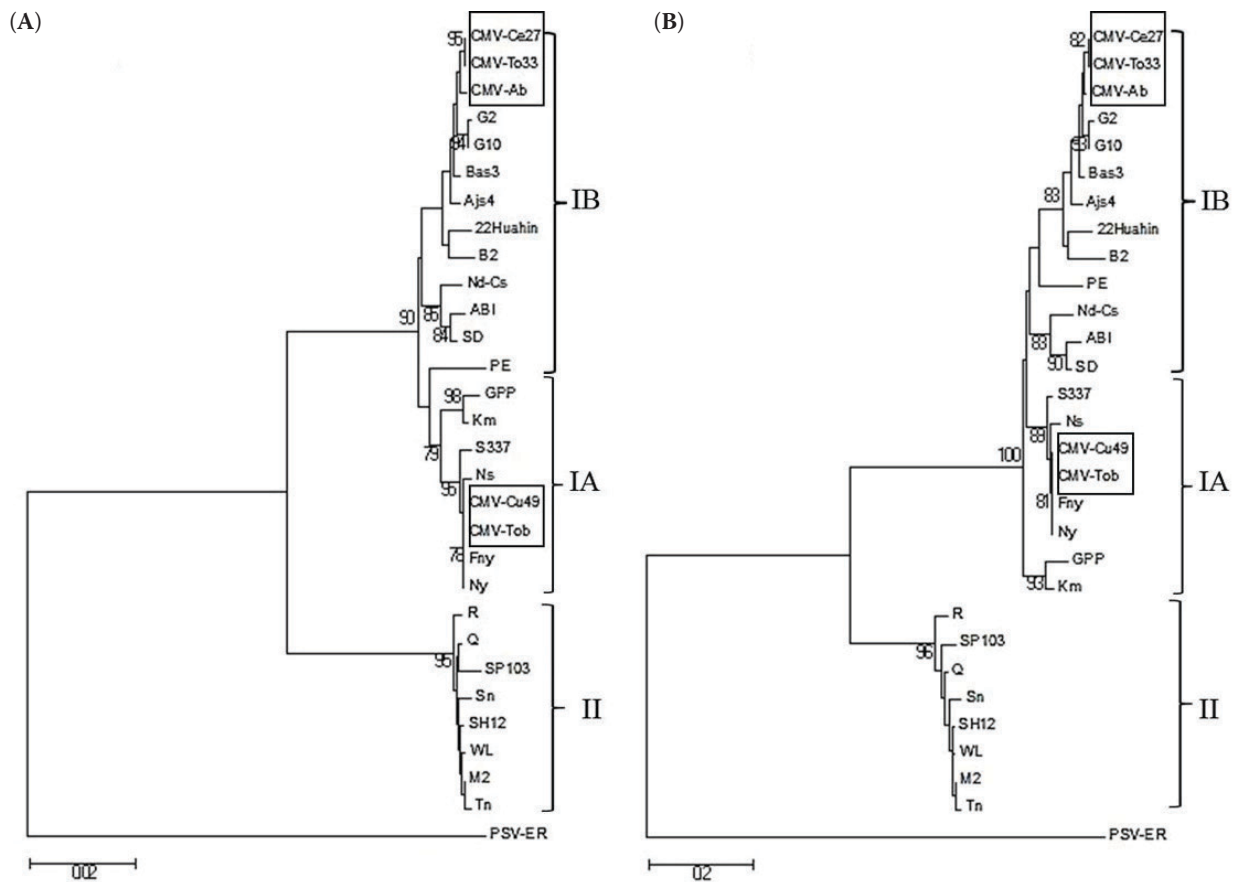
Figure 3. Phylogenetic trees of 29 CMV isolates based on their CP gene nucleotide (A) and deduced amino acid sequences (B)

Trees were reconstructed using neighbor joining method in Mega 5 with 100 bootstrap replicates; isolates belonging to different subgroups (IA, IB, and II) are labeled; bootstrap values (> 70%) are shown above the branches; all branches with < 70% bootstrap value were collapsed; urmian CMV isolates are in boxes.

S-IB fell into two completely separate clades. Three out of five our isolates (Ab, Ce27, To33) were clustered in S-IB, whereas other two isolates (Cu49, Tob) were grouped in S-IA.

DISCUSSION

Differentiation of CMV isolates based on symptom severity on tobacco and capsicum has previously been reported by several researchers (Zhang *et al.* 1994). All five isolates used in this study developed severe mosaic, blistering, and leaf distortion in three *Nicotiana* species and capsicum, like S-I induced symptoms. In contrast to previous reports concerning more severe symptoms induced by S-IB than those of S-IA (Hellwald *et al.* 2000), our five isolates caused symptoms with similar severity on inoculated *Nicotiana* species and capsicum, regardless of their categorisation to subgroups IB or IA.

Differentiation and subgrouping of CMV isolates is important in epidemiology of this virus. Serological differentiation, PCR followed by restriction analysis (Rizos *et al.* 1992), and IC-RT-PCR (Yu *et al.* 2005) methods have been used for subgrouping of CMV isolates. The latter method is more applicable because of avoiding the extraction of viral or plant total RNA and can be easily performed in a single tube (Yu *et al.* 2005). In this research, we used IC-RT-PCR method for detecting and differentiation of Urmian CMV isolates resulting in amplification of *CP* gene cDNA. Analysis of nucleotide sequence data of amplified fragments from five CMV isolates confirmed efficiency of IC-RT-PCR technique to identify the isolates of CMV.

In both inferred phylogenies, based either on the nucleotide or deduced amino acid sequence data, Tob and Cu49, with 100% identity to CMV-Fny and CMV-NY, were clustered into CMV S-IA clade. Further three isolates (To33, Ce27, and Ab) were clustered together

Figure 4. Multiple alignment of CP nucleotide sequence (437- 517 nts) of five Urmian (in bold) and 24 selected isolates belonging to different subgroups (IA, IB and II)

MspI (CCGG) and BsuRI (GGCC) restriction sites are in bold and shown in solid rectangle; CMV isolates are abbreviated as in Tables 1 and 2

into S-IB clade and tomato isolates G2 and G10 from Greece were the closest ones in the tree. Of interest it is also that, among our five isolates, Ab (originated from weed *A. theophrasti*) showed the highest nucleotide sequence variation in comparison with other isolates. Based on our results, there was no correlation between *CP* gene sequence variation and geographical origin or original host plant, as described before (Hayakawa *et al.* 1989; Lin *et al.* 2004). To our knowledge, this is the first report of CMV infection of *A. theophrasti* in the world. Weed hosts function as a reservoir for the virus and serve as primary source of inoculum for the development of disease epidemics.

West Azerbaijan, with subtropical climate, is one of the most important provinces for Iran agriculture. To determine the prevalence of the CMV subgroups in different crops, areas and years, continuous studies on more samples are required. In this study, while none of the five Urmian isolates with different host origins belonged to subgroup II, it did not mean the absence of S-II isolates in this area of Iran. Our results were in accordance with findings of previous studies, showing the occurrence of both S-I and S-II on cucurbits with greater prevalence of S-I isolates in the northwest of Iran (Bashir *et al.* 2006, 2008),

which might be because of its broad host range in the world (Hayakawa *et al.* 1989). It seems that S-I members are the predominant strains in the tropics and subtropics, whereas the S-II strains predominate in temperate zones (Haase *et al.* 1989).

References

Arafati N., Farzadfar S., Pourrahim R. (2013): Characterization of coat protein gene of *Cucumber mosaic virus* isolates in Iran. Iranian Journal of Biotechnology, 11: 109–114.

Bashir N.S., Kalhor M.R., Zarghani S.N. (2006): Detection, differentiation and phylogenetic analysis of *Cucumber mosaic virus* isolates from cucurbits in the Northwest region of Iran. Virus Genes, 32: 277–288.

Bashir N.S, Nematollahi S., Torabi E. (2008): *Cucumber mosaic virus* subgroup IA frequently occurs in the Northwest Iran. Acta Virologica, 52: 237–242.

Berniak H., Kamińska M., Malinowski T. (2010): *Cucumber mosaic virus* group IA and II are represented among isolates from naturally infected lilies. European Journal of Plant Pathology, 127: 305–309.

Clark M.F., Adams A.N. (1977): Characteristics of micro-plate method of enzyme linked immunosorbent assay

for the detection of plant viruses. Journal of General Virology, 34: 475–483.

Deyong Z., Willingmann P., Heinze C., Adam G., Pfunder M., Fery B., Fery J.E. (2005): Differentiation of *Cucumber mosaic virus* isolates by hybridization to oligonucleotides in a microarray format. Journal of Virological Methods, 123: 101–108.

Haase A., Ritcher J., Rabenstein F. (1989): Monoclonal antibodies for detection and serotyping of Cucumber mosaic virus. Journal of Phytopathology, 127: 129–136.

Hayakawa T., Mizukami M., Nakajima M., Suzuki M. (1989): Complete nucleotide sequence of RNA3 from Cucumber mosaic virus (CMV) strain O: comparative study of nucleotide sequences and amino acid sequences among CMV strains O, Q, D and Y. Journal of General Virology, 70: 499–504.

Hellwald K.H., Glenewinkle D., Hauber S., Pfanzenk Z. (2000): Increased symptom severity in pepper plants after co-inoculation with two *Cucumber mosaic virus* subgroup I strains in comparison to single inoculations of the corresponding wildtype strains. Journal of Plant Disease and Protection, 107: 368–375.

King A.M.Q., Adams M.J., Carstens E.B., Lefkowitz E.J. (2012): Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses. San Diego, Elsevier Academic Press.

Lin H.X., Rubio L., Smythe A.B., Falk B.W. (2004): Molecular population genetics of *Cucumber mosaic virus* in California: evidence for founder effects and reassortment. Journal of Virology, 78: 6666–6675.

Nematollahi S., Bashir N.S., Rakhshandehroo F., Zamanizadeh H.R. (2012): Phylogenetic analysis of new isolates of *Cucumber mosaic virus* from Iran on the basis of different genomic regions. Plant Pathology Journal, 28: 381–389.

Nolasco G., de Blas C., Torres V., Ponz F. (1993): A method combining immunocapture and PCR amplification in a microtiter plate for the routine diagnosis of plant viruses and subviral pathogens. Journal of Virological Methods, 45: 201–218.

Palukaitis P, Garcia-Arenal F. (2003): *Cucumber mosaic virus*. Description of Plant Viruses. Availabe at http://www.dpvweb.net/dpv/showdpv.php?dpvno=400

Palukaitis P., Roossinck M.J., Dietzgen R.G., Francki R.I.B. (1992): Cucumber mosaic virus. Advances in Virus Research, 41: 281–348.

Pares R.D., Gillings M.R., Gunn L.V. (1992): Differentiation of biologically distinct Cucumber mosaic virus isolates by PAGE of double-stranded RNA. Intervirology, 34: 23–29.

Rizos H., Gunn L.V., Pares R.D., Gillings R.M. (1992): Differentiation of Cucumber mosaic virus isolates using the polymerase chain reaction. Journal of General Virology, 73: 2099–2103.

Roossinck M.J. (2002): Evolutionary history of *Cucumber mosaic virus* deduced by phylogenetic analysis. Journal of Virology, 76: 3382–3387.

Roossinck M.J., Zhang L., Hellwald K.H. (1999): Rearrangements in the 5' nontranslated region and phylogenetic analyses of Cucumber mosaic virus RNA 3 indicate radial evolution of three subgroups. Journal of Virology, 73: 6752–6758.

Saitou N., Nei M. (1987): The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4: 406–425.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., Kumar S. (2011): MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731–2739.

Wilson A.D., Halliwell R.S. (1985): Characterization and field studies of a Cucumber mosaic virus isolate from spinach in the Winter Garden area of Texas. Plant Disease, 69: 751–754.

Yu C., Wu J., Zhou X. (2005): Detection and subgrouping of *Cucumber mosaic virus* isolates by TAS-ELISA and immunocapture RT-PCR. Journal of Virological Methods, 123: 155–161.

Zhang L., Hanada K., Palukaitis P. (1994): Mapping local and systemic symptom determinants of Cucumber mosaic cucumovirus in tobacco. Journal of General Virology, 75: 3185–3191.

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