CUCUMBER MOSAIC VIRUS SUBGROUP IA FREQUENTLY OCCURS IN THE NORTHWEST IRAN

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Summary. – To monitor genetic variation between Cucumber mosaic virus (CMV) isolates of northwest Iran, samples of cucurbitaceous plants expressing symptoms similar to those caused by CMV were collected. The samples were first screened by ELISA to detect CMV and to determine its subgroup. All detected CMV isolates appeared to be subgroup I (S-I). When total RNA from the samples was subjected to RT-PCR with a pair of primers corresponding to the CMV coat protein (CP) flanking regions, the expected ~870 bp DNA fragment was amplified at 18 samples of 34 tested. *Msp*I restriction analysis of 18 amplified products produced two DNA fragments with sizes about 530 and 330 bp corresponding to *Msp*I profile of CMV S-I. The amplification products of four representative samples were cloned and nucleotide sequences of 1–5 clones from each isolate were determined. The clones from each isolate were over 99% identical and also the isolates themselves were only up to 2% divergent. These isolates clustered in subgroup IA clade on a consensus phylogenetic tree and formed a distinct subclade suggesting that the isolates have originated from a common source.

Key words: Cucumber mosaic virus; diversity; restriction analysis; phylogenetic tree; sequencing

Introduction

Cucumber mosaic virus, the type species of the genus *Cucumovirus,* the family *Bromoviridae* has a wide host range of over 1000 species and infects a great variety of important crop plants. CMV is one of the most economically important plant viruses (Palukaitis *et al*., 1992; Rizos *et al*., 1992; Roosinck, 2001). In fields, the virus is mechanically transmissible by plant sap and is spread by more than 85 aphid species in a non-persistent manner (Edwardson and Christie, 1991).

The segmented genome of CMV comprises 3 single-stranded genomic and 2 subgenomic RNAs. RNA1 and RNA2 code for the components of a viral replicase. RNA3 codes for the 3a movement protein and 3b CP. However, the CP transcript (RNA4) is expressed subgenomically, if the internal promoter in the intergenic region of RNA3 is recognized (Choi *et al*., 1999). A 5 ORF, the 2b ORF, is also encoded on RNA2 and implicated in virus systemic spread (Ding *et al*., 1994).

Many CMV isolates have been described, which are placed in S-I and S-II on the basis of the serological relationship, peptide mapping of the CP, nucleic acid hybridization, and nucleotide sequence identity (Palukaitis *et al*., 1992). Further division of S-I into S-IA and S-IB has been proposed based on the sequences of the 5' non-translated region of RNA3 and the CP gene (Roossinck *et al*., 1999; Roossinck, 2002).

The aim of the study was to identify and characterize the CMV isolates occurring in the northwest region of Iran relating to the determination of their subgroup. A vast variety of cucurbits are grown in this region and the crops are vulnerable to CMV infections. In the presence of a wide host range, CMV may undergo a variation and the study of such changes is important for elucidation of the virus epidemiology.

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Abbreviations: CMV = Cucumber mosaic virus; CP = coat protein; DAS-ELISA = double-antibody sandwich ELISA; MAb(s) = monoclonal antibody(ies); ORF = open reading frame; PAb(s) = polyclonal antibody(ies); p.i. = post inoculation; TAS-ELISA = triple-antibody sandwich ELISA; S-I, II = subgroup I, II

Materials and Methods

Virus isolates and biological purification. Leaves from 143 cucurbitaceous plants including cucumber (*Cucumis sativus* L.), squash (*Cucurbita pepo*), and pepper (*Capsicum annum* L.) exhibiting symptoms characteristic for CMV (mosaic, mottling, stunting, fruit blistering, and leaf wrinkle) (Palukaitis *et al*., 1992) were collected from several locations in the northwest region of Iran. Portions of each sample were tested by ELISA, inoculation to the experimental host plants, and RT-PCR. The remainder of the sample was preserved on $CaCl_2$ for future reference. Saps of the infected samples, extracted in 0.1 mol/l phosphate buffer pH 7.2 were rubbed on carborundum-dusted young leaves of glasshouse-grown plants including *C. pepo, C. sativus, Chenopodium quinoa*, and *Nicotiana rustica*. Each local lesion, developed on *C. quinoa* was used to inoculate *C. pepo* to purify each isolate from a possible mixed infection.

ELISA. Leaves from collected samples were ground 1:10 (weight/volume) in extraction buffer and subjected to double-antibody sandwich (DAS)-ELISA (Clark and Adams, 1977) with anti-CMV polyclonal antibody (PAb) (DSMZ, Germany). Then, the ELISA-positive samples were further subjected to the indirect triple-antibody sandwich (TAS)-ELISA (Khetarpal and Kumar, 1995) with CMV subgroup-specific monoclonal antibodies (MAbs) (DSMZ, Germany) to determine the subgroup affiliation of each isolate. Absorbance values of ELISA plate wells were measured by Anthos 2002 ELISA Plate Reader (Austria). The threshold was set as twice the mean absorbance value of the healthy control plants.

RNA extraction. Total RNA from the leaves of infected samples was extracted as described elsewhere (Rowhani *et al*., 1993) with minor modifications. Leaf tissue (100 mg) from each sample was homogenized in 1 ml extraction buffer [91 mmol/l K_2HPO_4, 30 mmol/l KH_2PO_4, 292 mmol/l sucrose, 0.22 mmol/l bovine serum albumin, fraction V, 0.8 mmol/l polyvinyl pyrrolidone 25, 30 mmol/l ascorbic acid, pH 7.6] and transferred into a 1.5 ml Eppendorf tube and subjected to centrifugation at 1050 x g for 4 mins in a Heraeus Megafuge 1.0R rotor 3041 (Germany). The aqueous phase was decanted into another tube and centrifuged at 16,800 x g for 20 mins. The resultant pellet was suspended in 200 µl buffer (10 mmol/l EDTA, pH 8, 50 mmol/l Tris, 0.1% of 2-mercaptoethanol) and after adding of 25 µl of 10% SDS incubated at 60°C for 20 mins. Then, 80 µl of 5 mol/l potassium acetate was added and incubated on ice for 30 mins. After centrifugation at 16,800 x g for 15 mins, the supernatant was collected and transferred into a fresh tube followed by adding of 0.1 volume of 3 mol/l sodium acetate and an equal volume of cold isopropanol before keeping at -20°C for 1 hr. The tubes were centrifuged at 16,800 x g for 20 mins and the resulting pellet was washed with 70% ethanol. After drying, the pellet was suspended in 40 µl RNase-free sterile distilled water and stored at -20°C.

RT-PCR and cloning. RT-PCR was performed on the every ELISA-positive and some ELISA-negative samples. RT was performed by the use of the CMV CP 5' primer (5'-GCTTCTCCGC GAG-3'). All the reagents used in the assay were from Fermentas and the assay was done according to the manufacturer's instruction. CMV CP 5' and 3' (5'-GCCGTAAGCTGGATGGAC-3') primers corresponded to nt 1149–1161 and nt 1998–2015 of CMV-Q RNA3, respectively (Rizos *et al*., 1992). PCR was performed by the use of *Taq* DNA polymerase and a thermal profile with an initial denaturing at 94°C for 2 mins, then 35 cycles each consisting of 94°C for 30 secs, annealing at 50°C for 30 secs, polymerization at 72°C for 1 min, and a final polymerization at 72°C for 5 mins. PCR products were run on 1.2% agarose in 0.5 X TBE buffer stained with 0.5 µg/ml ethidium bromide, visualized, and photographed by a gel documentation apparatus. Restriction analysis of the PCR products was done with *Msp*I. Then, the fragments amplified from the isolates D, L7, CB, and TA2 were ligated into plasmid pTZ57R/T (Fermentas) and cloned in *Escherichia coli* strain TG1 as described elsewhere (Bashir and Hajizadeh, 2007). Clones of isolate D, L7, CB, and TA2 were subjected to dideoxy terminator cycle sequencing in Macrogen Inc. (Seoul, South Korea) using the universal M13F and M13R primers.

The sequences of the amplified region (841 nt) was aligned with the corresponding region of the previously reported CMV strains/isolates (Table 1) and Peanut stunt virus, strain ER as the outgroup species (Naidu *et al*., 1993) by the use of GeneDoc (Nicholas and Nicholas, 1997). For construction of a phylogenetic tree, the CP flanking sequences were also removed and the analysis was based on the CP region (654 nt). Consensus parsimonious trees were inferred by using Phylip Package 3.6 (Felsenstein, 2004). First, the alignment file was submitted to 100-replicate bootstrap by Seqboot. Then, parsimonious trees were generated by the use of Dnapars with the multiple data analysis option. Finally, a consensus tree with branches over 50% bootstrap support was chosen by the use of Consensus program and viewed by Treeview (Page, 1996).

The sequences reported in this paper have been submitted to GenBank and assigned the accession numbers DQ002876 to DQ002885.

Results

ELISA and glasshouse inoculation

The DAS-ELISA detected CMV in 34 out of the 143 tested cucurbits. All 34 positive samples gave reaction with CMV S-I specific MAb in TAS-ELISA. None of the tested samples reacted with the CMV S-II MAb (Table 2).

Mosaic, leaf curling, and stunting developed on the inoculated squash plants 4–5 days post inoculation (p.i.). The cucumber plants expressed mild mosaic at 4–5 days p.i. Yellow local lesions on the leaves of *C. quinoa* appeared 7–10 days p.i. Mosaic, stunting, and asymmetric leaves occurred on *N. rustica* 14–20 days p.i. Subsequently, TAS-ELISA with the S-I MAb performed on samples from the symptomatic plants resulted in the positive reaction (data not shown).

PCR, cloning, and sequencing

Electrophoresis of the PCR products revealed that among tested 28 ELISA-positive samples, the expected ~870 bp fragment was amplified in 18 samples (Table 2). Digestion of the PCR products with *Msp*I yielded two fragments with sizes about 530 and 330 bp (data not shown).

Table 1. Abbreviations, accession numbers, and origin of CMV strains/isolates

Abbreviation	Acc. No.	Strain/isolate	Original host[a]	Country of origin[b]
Kor	L36251	CMV strain Korea		South Korea
Km	AB004780	CMV-Km	Melon	[Japan]
N	D28486	CMV strain N		Japan
HL	AB049568	CMV-Lily	Lily	Japan
O	D00385	CMV strain O		Japan
Y	D12499	CMV strain Y		Japan
D8	AB004781	CMV-D8	Japanese radish	Japan
Is	U43888	CMV strain banana	*Musca acuminata*	[Israel]
M	D10539	CMV strain M		UK
Hawaii	U31219	CMV strain Hawaii	*Musca* sp.	Hawaii
Fny	U20668	CMV strain Fny		USA (New York)
C7-2	D42079	CMV strain C7-2		Japan
As	AF013291	CMV strain As		[South Korea]
K	AF127977	CMV strain K		China
SD	AB008777	CMV-SD		[China]
NT9	D28778	CMV strain NT9		Taiwan
IA	AB042294	CMV-IA		Indonesia
G2	AY450854	CMV strain G2	Tobacco	Greece
Sa	AB109909	CMV strain Sa	*Stellaria aquatica*	South Korea
Q	M21464	CMV strain Q		Australia
Kin	Z12818	CMV isolate Kin		UK
Wem	U61285	Wemmershoek CMV		South Africa
Trk7	L15336	CMV strain Trk7		Hungary
TN	AB176847	CMV strain TN	Tomato	Japan
WL	D00463	CMV strain WL		USA (New York)
M2	AB006813	CMV-M2		[Japan]
B23	AY871071	CMV -B23	Cucumber	Iran
B13	AY871070	CMV-B13	Cucumber	Iran
S337	AY871069	CMV-S337	*Cucumis* sp.	Iran
SH17	AY871068	CMV-SH17	Cucumber	Iran

[a]Only for the isolate, whose host is cited in the accession. [b]Country shown in square brackets –the respective strain or isolate was studied without referring to the virus origin.

Confirmation of insertion of the PCR products in the cloning plasmid was done by double digestion with *Eco*RI/*Bam*HI or *Eco*RI/*Hind*III that released the expected fragment.

Sequences of 5 clones from isolate D2, 3 clones from each of the isolates CB and TA2, and 1 clone from isolate L7 were determined. It was revealed that an expected 841 bp fragment (excluding the primer sites) was amplified from the isolates, which covered the complete CP gene (654 nt) plus 79 and 105 nt of 5' and 3' flanking regions, respectively. It was also revealed that 2 clones of the isolate D2 (D2-I1C1 and D2-I1C2) and TA2 (TA2-I1 and TA2-I3) were identical. Accordingly, sequences of 10 clones belonging to 4 isolates were submitted to GenBank and assigned the accessions.

Sequence and phylogenetic analysis

Alignment of these sequences with previously reported CMV isolates/strains showed that the CMV clones from Iran belonged to S-IA with 95–97% sequence identities to the S-IA strains Km, Fny, O, Kor, and Y (Table 1). The identities between the Iran isolates and the S-IB strains (C7-2, SD) were 93–94%. In comparison to the CMV S-II strains Kin and Q, identities of only 76–78% existed between the Iran isolates and the S-II. Furthermore, there were 98–99% identities between the isolates reported from Iran in the current study.

In the phylogenetic tree, clones from the isolates D2, TA2, CB, and L7 fell out in CMV S-IA subclade being more closely related to the strains O, Y, D8, Is, M, Hawaii, and Fny (Fig. 1). In addition, the S-IA members formed two clusters, one of which comprised the Iran isolates and the Korean strain (Kor) and the other encompassed the remaining S-IA strains.

Discussion

Determination of the subgroup affiliation of CMV isolates is important for elucidation of the virus epidemiology (Yu *et al*., 2005). Also, detection of CMV isolates and assessment of their genetic diversity are effective steps in the virus control, especially through genetic engineering (Lin *et al*., 2003).

In the present research, serological techniques were primarily applied to find out the infecting virus, because these tools are

Table 2. Characteristics of CMV isolates from Iran

Isolate	Geographical location	Original host	Serotype by ELISA	RT-PCR	Subgroup[a]	Phylogenetic position[b]
2A	Urmia	Cucumber	I	+	I	
K1	Urmia	Cucumber	I	+	I	
K7	Urmia	Cucumber	I	–	N/A	
F	Urmia	Peper	I	–	N/A	
CB	Basmenj	Squash	I	+[c]	I	IA
CB-a	Basmenj	Squash	I	+	I	
H3-3	Shabestar	Squash	I	N/T	N/A	
H4-4	Shabestar	Squash	I	+	I	
H2-2	Shabestar	Squash	I	+	I	
H5-5	Shabestar	Squash	I	+	I	
R2	Shabestar	Squash	I	N/T	N/A	
D2	Shabestar	Squash	I	+[c]	I	IA
D1	Shabestar	Squash	I	+	I	
D3	Shabestar	Squash	I	+	I	
BG3	Shabestar	Squash	I	N/T	I	
L9	Shabestar	Squash	I	–	N/A	
L10	Shabestar	Squash	I	N/T	I	
L1M2	Shabestar	Cucumber	I	–	N/A	
T6M1	Shabestar	Cucumber	I	–	N/A	
T6M2	Shabestar	Cucumber	I	–	N/A	
TA1	Tabriz	Squash	I	+	I	
TA2	Tabriz	Squash	I	+[c]	I	IA
TA3	Tabriz	Squash	I	+	I	
G3	Shabestar	Squash	I	+	I	
H3	Shabestar	Squash	I	+	I	
G4	Shabestar	Squash	I	–	N/A	
H2	Shabestar	Squash	I	–	N/A	
H1	Shabestar	Squash	I	–	N/A	
H4Z	Shabestar	Squash	I	N/T	I	
DB	Shabestar	Squash	I	N/T	I	
BS3	Bonab	Cucumber	I	+	I	
L7	Bonab	Squash	I	+[c]	I	IA
BH9	Bonab	Cucumber	I	–	N/A	
BH3	Bonab	Cucumber	I	+	I	

[a]Determined by *Msp*I digestion of the PCR products. [b]Based on CP sequences. [c]Cloned and sequenced PCR products. N/T = not tested; N/A = not applicable.

cost-effective and well-established in our laboratory. For routine detection of CMV isolates, ELISA methods based on using PAbs or MAbs have been used widely. However, MAbs are more reliable for determining of the subgroup affiliation of isolates (Palukaitis and Zaitilin, 1997). Here, DAS-ELISA using a CMV-specific PAb gave positive reactions with 34 tested samples. The remaining samples were also collected from diseased plants, but they did not contain CMV as determined by ELISA. Some of these negative samples were subjected to RT-PCR and did not give any amplification of CMV fragments. These results suggested that other virus(es) might be involved in the infections of cucurbitaceous plants.

The inoculation tests provided further evidence for the presence of CMV in the examined isolates, because the inoculated plants developed symptoms resembling those incited by CMV and produced positive reactions in the ELISA (Palukaitis *et al*., 1992; Zitter *et al*., 1996).

None of the infected samples gave positive reaction with the CMV S-II specific MAb in TAS-ELISA. However, all samples reacted with CMV S-I MAb and it was concluded that all positive isolates belonged to the S-I subgroup. Prevalence of the S-I strains has been reported previously and more than 80% of the variants in the CMV population belonged to the S-I (Gallitelli, 2000).

Out of 34 ELISA-positive samples only 28 samples were subjected to the RT-PCR assay, because no fresh leaf material of the remaining six samples was left. The assay resulted in the amplification of approximately 870 bp fragments in 18 samples from 28 tested. Failure of the amplification in remaining 10 ELISA-positive samples could be due to the suboptimal PCR condition, particularly the heterogeneity of the primers, because they were designed according to the unusual strain, CMV-Q (Rizos *et al*., 1992). The other reason could be attributed to a low

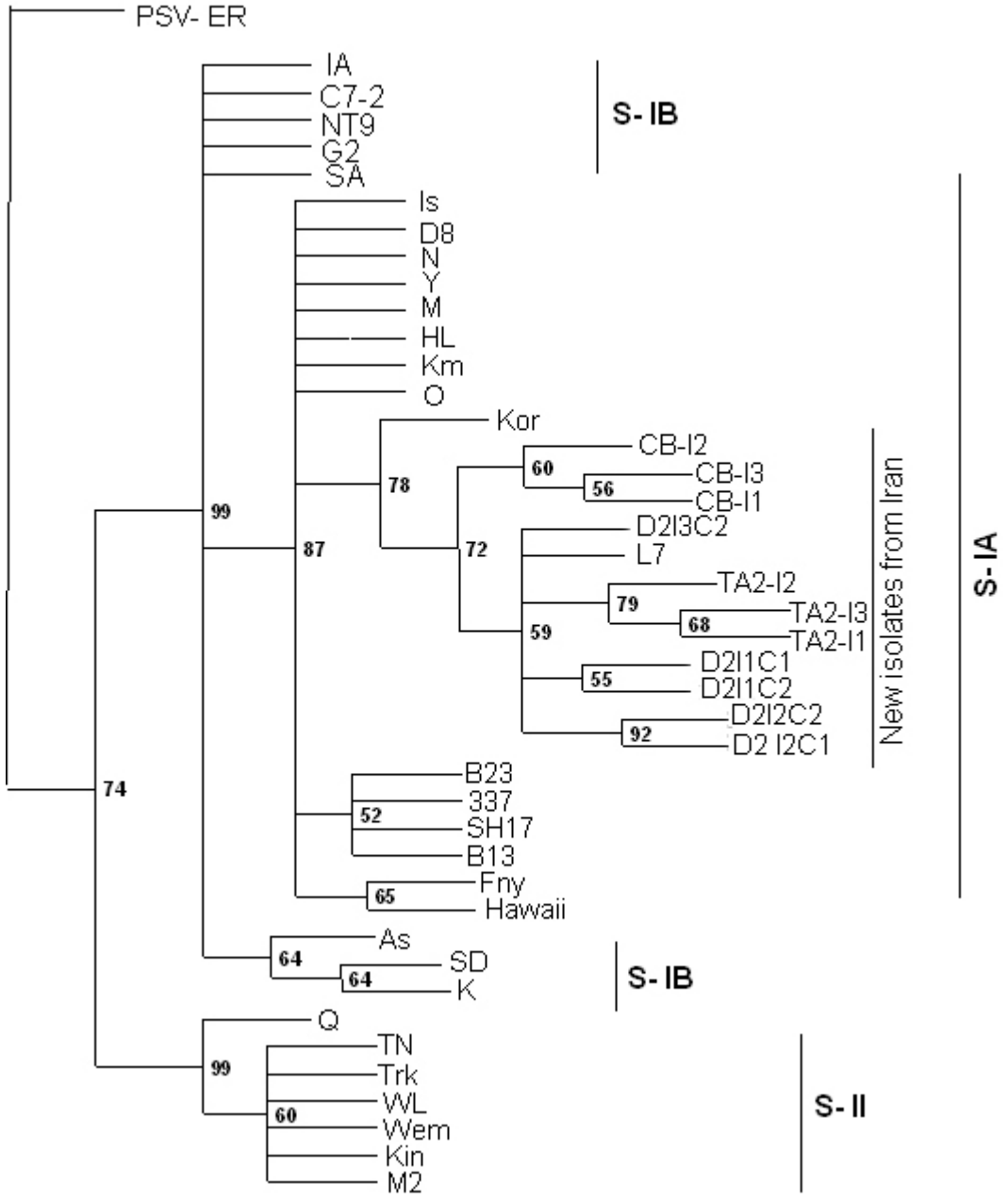

Fig. 1

Phylogenetic tree of CMV isolates based on CP cDNA sequences

Branches with less than 50% bootstrap support (shown above the branch lines) were collapsed. Isolates b23, 337, SH-17, and b13 were previously determined as isolates from Iran. The new Iranian isolates are indicated with a vertical line. D2-I1C1, D2-I1C2, D2-I2C1, D2-I2C2 and D2-I3C2 are clones of the isolate D2; CB-I1, CB-I2 and CB-I3 of CB; TA2-I1, TA2-I2 and TA2-I3 are of TA2; L7 is a clone of the isolate L7.

quality of the RNA preparations, because the collected samples were kept at 4°C until processing that resulted in discoloration. Therefore, it was suggested that cucurbit samples should be subjected to RNA extraction immediately after the sampling.

Restriction analysis of the CMV CP cDNA isolates with *Msp*I is an established technique in differentiation of the virus isolates (Rizos *et al*., 1992; Singh *et al*., 1995). This method is helpful for classification of the isolates into groups according to their restriction profiles and at the same time

this method minimizes the number of PCR product species being submitted to the sequencing. CMV isolates from Iran produced two *Msp*I fragments of approximately 530 and 330 bp in size providing another proof that these isolates were CMV S-I. This analysis also showed that none of the infected samples contained mixed population of the virus restrictotypes, because no combined profile was obtained with any of the samples. This result may suggest that the isolates have originated from a common infection source such as from the seeds.

In the phylogenetic parsimony trees, the CMV isolates from Iran were placed with the S-IA members. It has been earlier proposed that the S-I isolates should be split by placing the Asian CMV strains in the S-IB subgroup and the remaining S-I members in the S-IA subgroup (Palukaitis and Zaitilin, 1997). However, other studies have shown that the Italian strain Ifn and some US isolates belonged also to the S-IB.

Phylogenetic analysis of CMV on the basis of the CP sequences is suggestive of a radial evolving behavior (Roossinck *et al*., 1999). This virus has gone through a narrow bottleneck and from that point it has spread worldwide. The first radiation that occurred with the S-II strains was a worldwide evolving event. These strains, after going through a second radiation, have given rise to the S-IB strains. This second radiation was initially thought to be a rather limited phenomenon, because until then, all the reported S-IB strains were coming from Asia. A third radiation event of S-IB strains that resulted in an emergence of S-1A strains was also global. The Iran isolates are prevalently S-IA members which implies that they have emerged recently, although it is impossible to put any timeline on such an evolutionary event.

The immediate outcome of this study involves an improvement in the detection of CMV local isolates and also better designing of the primers for RT-PCR according to these isolates.

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