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Research Papers

Characterization of potato and tobacco isolates of *Cucumber mosaic virus* from Syria and the first report on CMV satellite RNA from potato

Mohamad CHIKH ALI[1,2], Abdul Mohasen Said OMAR[3], Tetsuo MAOKA[4], Keiko T. NATSUAKI[5] and Tomohide NATSUAKI[1]

[1] Laboratory of Plant Pathology, Faculty of Agriculture, Utsunomiya University, Mine-machi 350, Utsunomiya 321-8505, Japan
[2] Department of PSES, University of Idaho, Moscow, ID83843-2339, USA
[3] Department of Crop Science, Faculty of Agriculture, Aleppo University, Aleppo, Syria
[4] National Agricultural Research Center for Hokkaido Region (NARCH), Sapporo, Japan
[5] Laboratory of Tropical Plant Protection, Department of International Agricultural Development, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya, Tokyo, 156-8502, Japan

Summary. *Cucumber mosaic virus* (CMV) has been reported from potato production areas in Europe, USA, Japan and more frequently in regions with warm climates such as Egypt, India, Saudi Arabia and Syria. As it is considered as an uncommon virus in potato, the characterization of potato isolates of CMV is far behind those from other hosts. In addition to potato, CMV is a common virus infecting many crops in Syria, but nothing is known about its molecular characteristics. The present study aimed to characterize Syrian CMV isolates collected from potato and neighboring tobacco fields. All potato isolates of CMV (total of four) co-infected potato plants with *Potato virus Y* (PVY) which is the most frequent potato virus in Syria. According to the sequence analyses of the coat protein (CP) coding region, three potato and three tobacco CMV isolates were found to be closely related regardless of the host species or geographic origin, and all belonged to the IA strain subgroup of CMV. A potato CMV isolate, PoCMV7-5, readily infected solanaceous plants in which it induced systemic infection, but was less infectious to other hosts including those of *Leguminosae* and *Cucurbitaceae*. When inoculated on potato plants, PoCMV7-5 alone or with various PVY strains was able to cause local but not systemic infection in all potato cultivars inoculated. PoCMV7-5 contained heterogeneous variants of satellite RNA which varied in length due to A or/and T deletion/insertion at approximate nucleotide position 225–240. This is the first report on CMV satellite RNA from potato.

Key words: IA strain subgroup, *Potato virus Y*, mixed infection, host adaptation, *Solanum tuberosum*.

Introduction

Cucumber mosaic virus (CMV, genus *Cucumovirus*, family *Bromoviridae*) is the type member of this genus. This virus has three positive-sense single stranded RNAs, namely RNA1, RNA2 and RNA3, which are encapsidated separately in CMV particles. The coat protein (CP) is expressed by a sub-genomic RNA designated as RNA4 (Palukaitis and Gracia-Arenal, 2003). CMV has a very broad host range and has caused epidemic diseases in many crops in the world (Palukaitis *et al*., 1992). According to serological relationships, peptide mapping of the CP and molecular analyses of genomic RNAs, CMV strains have been classified into two subgroups, subgroup I and subgroup II (Palukaitis *et al*., 1992; Palukaitis and Gracia-Arenal, 2003). Subgroup I have been further divided into IA and IB (Palukaitis and Gracia-Arenal, 2003).

CMV is a helper virus of a linear single stranded satellite RNA (satRNA) that depends on CMV for replication, encapsidation and transmission (Palukaitis and Gracia-Arenal, 2003). SatRNAs are usually 332–342 nucleotides (nt) long, even though

Corresponding author: M. Chikh Ali
Fax: +1 208 885 7760
E-mail: mhmdsaidsyr@hotmail.com

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satRNAs with 386–405 nt have been reported from Japan and Italy in association with the Asian CMV isolates (Palukaitis and Gracia-Arenal, 2003). The satRNA may not affect the pathogenesis of CMV, or can enhance the induction of necrosis or chlorosis or attenuate the symptoms on hosts, with the latter as the most common (Palukaitis *et al*. 1992; Simon *et al*., 2004). The symptom modification of CMV from satRNA depends on the virus strain, satRNA and host plant (Palukaitis and Gracia-Arenal, 2003).

CMV is an uncommon virus in potato and has occasionally been reported in potato crops in some parts of Europe (Chrzanowska *et al*., 2004), in regions with warm climates such as Egypt and India (Jeffries, 1998), central California (Somerville *et al*., 1987), Saudi Arabia (Al-Shahwan *et al*., 1997) and Japan (Matsunami *et al*., 1972; Kano *et al*., 1985; Sato *et al*., 2001). The rare incidence of CMV in potatoes is due to the high resistance of potato cultivars to this virus (Celebi-Toprak *et al*., 2003; Chrzanowska *et al*., 2004). This resistance restricts CMV replication and movement within inoculated leaves and prevents systemic infection by this virus (Chrzanowska *et al*., 2004). At high temperatures, however, CMV infection occurs systemically in most of the resistant cultivars (Celebi-Toprak *et al*., 2003) which might explain the frequent incidence of CMV from potato in regions with warm climates.

In Syria, CMV is the second main virus in cucurbits after *Zucchini yellow mosaic virus* (ZYMV; Haj Kassem *et al*., 2005) and is a common virus in legumes (Makkouk and Attar, 2003). In potato, CMV has recently been detected using serological means (Haj Kassem *et al*., 2006). Though it is a common virus in Syria, nothing is known about the molecular characteristics of Syrian CMV isolates and to which subgroup they belong. The objective of the present study was to the characterize four potato (Chikh Ali *et al*., 2008) and three tobacco CMV isolates collected in Syria, and characterize CMV satRNAs from potato.

Materials and methods

Virus isolates

During the autumn season of 2006, four CMV isolates were collected from two potato fields in Aleppo and Hama provinces, Syria. In addition three CMV isolates were collected from a tobacco field next to a potato field in Hama province.

Biological tests

A potato CMV isolate, namely PoCMV7-5, which was originally found as a mixed infection with PVY, was maintained in *Nicotiana tabacum* cv. Samsun. *Datura stramonium* was used to separate this isolate from the PVY infection. To study biological characteristics, a range of indicator plants were inoculated with PoCMV7-5, at least two plants each. Inoculated plants were allowed to grow in an incubator at 23°C with a photoperiod of 16 h.

To investigate its ability to infect potato, potato cultivars Maris Bard, Desiree, King Edward and Nishiyutaka were inoculated with PoCMV7-5 alone or along with various PVY strains including PVY^{O}, PVY^{NTN} and $PVY^{N}W$. Potato plants were grown in an incubator at 24 or 28°C and a photoperiod of 16 h or in a greenhouse under the natural condition of Hokkaido region, Japan during May and June (2008).

Serological tests

Compound direct ELISA using a CMV kit (Agdia, Elkhart, IN, USA) and a PVY monoclonal antibody which detects all PVY isolates (4C3; Agdia, Elkhart, IN, USA) were used to test inoculated plants according to the manufacturer's instructions.

Molecular tests

RNA extraction

Total RNA was extracted from dried (20 mg) potato or tobacco leaves using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Resultant pellets were dissolved in 125 μL of RNase-free water.

Nucleic acid sequencing

To determine the CP gene of CMV isolates, CP coding region was amplified using IC-RT-PCR according to the method described by Choi *et al.* (1999). PCR products were cloned and sequenced according to Chikh Ali *et al.* (2008).

Detection and sequencing of satRNA

To detect and determine the sequence of CMV satRNA, the forward primer AK1 (5′-GTTTTGTTTGTTGGAGAATTGCG-3′) and the reverse primer BK1 (5′-GGGTCCTGTAGAGGAATGT-3′) which amplify a segment of 391bp for the 55-1 clone of CMV

satRNA (accession No. AB072502) were used. Positive control was a tobacco cv. White Burley leaf infected with the clone 55-1 along with the helper CMV, whereas the negative control was non inoculated tobacco. Nucleotide sequencing was carried out as described above. A total of 14 clones were sequenced.

Sequence analysis

Sequence analysis was conducted using the DNASIS software (Hitachi Software Engineering Co., Japan). The CP homology was searched using the BLAST program provided by the NCBI (http://www.ncbi.nlm.nih.gov/). For multiple alignment, CLUSTAL X ver. 1.81 (Thompson *et al*. 1997) was used with default parameters. Phylogeny inference was conducted using the maximum parsimony (MP) method by PAUP* version 4.0 beta (Swofford, 1998) using the HSearch option and 100 bootstrap resamplings. The phylogenetic tree was constructed based on the nucleotide sequences of the CP gene of the Syrian CMV isolates along with other CMV isolates available in DDBJ/EMBL/GenBank databases. The *Peanut stunt virus* (PSV; U15730) CP gene was used as an outgroup. Isolate names and DDBJ/EMBL/GenBank database accession numbers are shown in Figure 2.

Results

Biological tests

In *D. stramonium* inoculated with sap from potato sample 7-5 co-infected with CMV and PVY, local necrotic lesions appeared 2 days after inoculation. No systemic symptoms were developed up to 1 month after inoculation. CMV was detected only in inoculated leaves by ELISA, whereas PVY was not detected either in inoculated or upper leaves. CMV but not PVY (as confirmed by ELISA) was recovered from the inoculated leaves of *D. stramonium* and back inoculated on *N. tabacum* cv. White Burley (Table 1). The recovered isolate was designated as PoCMV7-5. The reaction of 18 indicator plants to PoCMV7-5 is shown in Table 2.

On potato cultivars Maris Bard, Desiree, King Edward and Nishiyutaka, local necrosis of various types followed by collapse of inoculated leaves (Figure 1) was observed, and no systemic symptoms were observed on potato plants. CMV was detected in inoculated but not in upper leaves by ELISA and RT-PCR in both single and mixed infections with various PVY strains including the original $PVY^{N}W$ isolate (separated from PoCMV7-5 using *D. stramonium*), PVY^{O} or PVY^{NTN} under various incubation temperature used.

Serological tests

All inoculated plants were tested by ELISA for the presence of CMV infection. ELISA results are summarized in Table 2.

Molecular tests

Nucleic acid sequencing

In the IC-RT-PCR, six CMV isolates including PoCMV7-5, produced bands of approximately 940 bp. The amplified sequences included the full CP

Table 1. Potato and tobacco samples infected with CMV.

Sample No.	Host plant/cultivar	Location	Symptom	ELISA (CMV)[a]	ELISA (PVY)[a]
6-5	Potato/Marfona	Hama	Mosaic	+	+
7-5	Potato/unknown	Hama	Mosaic	+	+
7-7	Potato/unknown	Hama	Mosaic and stunting	+	+
9-11	Potato/Agria	Aleppo	Yellowing and stunting	+	+
5-1	Tobacco/unknown	Hama	Mosaic	+	–
5-2	Tobacco/unknown	Hama	Mosaic	+	+
5-3	Tobacco/unknown	Hama	Mosaic	+	+

[a] + and – indicate positive and negative reactions, respectively.

Table 2. The host range of a potato CMV isolate, PoCMV7-5 collected from Syria

Family	Host plant	Symptoms [a]
Solanaceae	Potato cv. Maris Bard	N(+) / Sl(–)
	Potato cv. Nishiyutaka	N(+) / Sl(–)
	Nicotiana debneyi	nr(nt) / Mo(+)
	N. glutinosa	nr(nt) / M, N(+)
	N. benthamiana	nr(nt) / M, De(+)
	N. tabacum cv. Samsun	nr(nt) / M, N(+)
	N. tabacum cv. Xanthi	N(nt) / Mo, N(+)
	N. occidentalis	nr(nt) / De, N(+)
	Datura stramonium	Ll(+) / Sl(–)
Chenopodiaceae	*Chenopodium amaranticolor*	Ll(+) / Sl(–)
Leguminosae	*Vigna unguiculata*	Ll (+) / Sl(–)
	Pisum sativum cv. Snack	Sl(+) / Sl(–)
	P. sativum cv. Azumino 30nich Kinusaya	Sl(+) / Sl(–)
	Phaseolus vulgaris cv. Kentucky 101	N(+) / Sl(–)
Cucurbitaceae	*Cucurbita pepo* cv. Diner	Ll(+) / Sl(–)
	C. pepo cv. Hayato	Sl(–) / Sl(–)
	Cucumis sativus cv. Su-Yo	Sl(–) / Sl(–)
	C. sativus cv. Hanjiro-Fushinari	Sl(–) / Sl(–)
	C. melo cv. Ginsen	Sl(–) / Sl(–)
	Cirtullus lanatus cv. Kabuki	Sl(–) / Sl(–)

[a] nt, not tested; nr, not recorded; M, mosaic; Mo, mottle; N, necrosis; De, deformation; Ll, local lesion; Sl, asymptomatic; Inoculated leaves / Upper leaves; +, Positive by ELISA; –, negative by ELISA.

coding region as confirmed by sequence analysis. The DDBJ/EMBL/GenBank databases accession numbers are shown in Figure 2. The CP gene of Syrian CMV isolates comprised of 654 nt encoding for 218 amino acids, had identical amino acid sequences and shared nucleotide similarity of 99.2–99.7% (Table 3). The CP of Syrian CMV isolates shared 100% amino acid homology with CMV strain Fny and 99.39–99.97% with CMV strains from France and Spain. In the phylogenetic tree of the CP gene, Syrian CMV isolates fell into the sub-group IA together with the Fny strain (USA; accession No. D10538; Figure 2).

Detection and sequencing of satRNA

In the RT-PCR used to detect CMV satRNA, a band of approximately 350 bp was amplified from the potato sample 7-5 (Figure 3). The same band was amplified from the *N. tabacum* cv. White Burley and other hosts infected with the isolate PoCMV7-5 (Figure 3). SatRNA was detected with the CMV isolate PoCMV7-5 in all infected plants. Sequencing of PoCMV7-5 satRNA revealed five variable sequence species, namely PoSA1-5 with accession numbers AB448699-703, respectively, and had different length (estimated 333–336 nt including primers' sequences) due to nucleotide deletions in the central part (Figure 4). The DDBJ/EMBL/GenBank databases accession numbers are shown in Figure 3. PoCMV7-5 satRNA shared greatest similarity of 96.4–97.6% with those from tomato (T18; accession No. X86708) and tobacco (TO77; accession No. X86422) reported from Italy. PoCMV7-5 satRNA had several deletions of to-

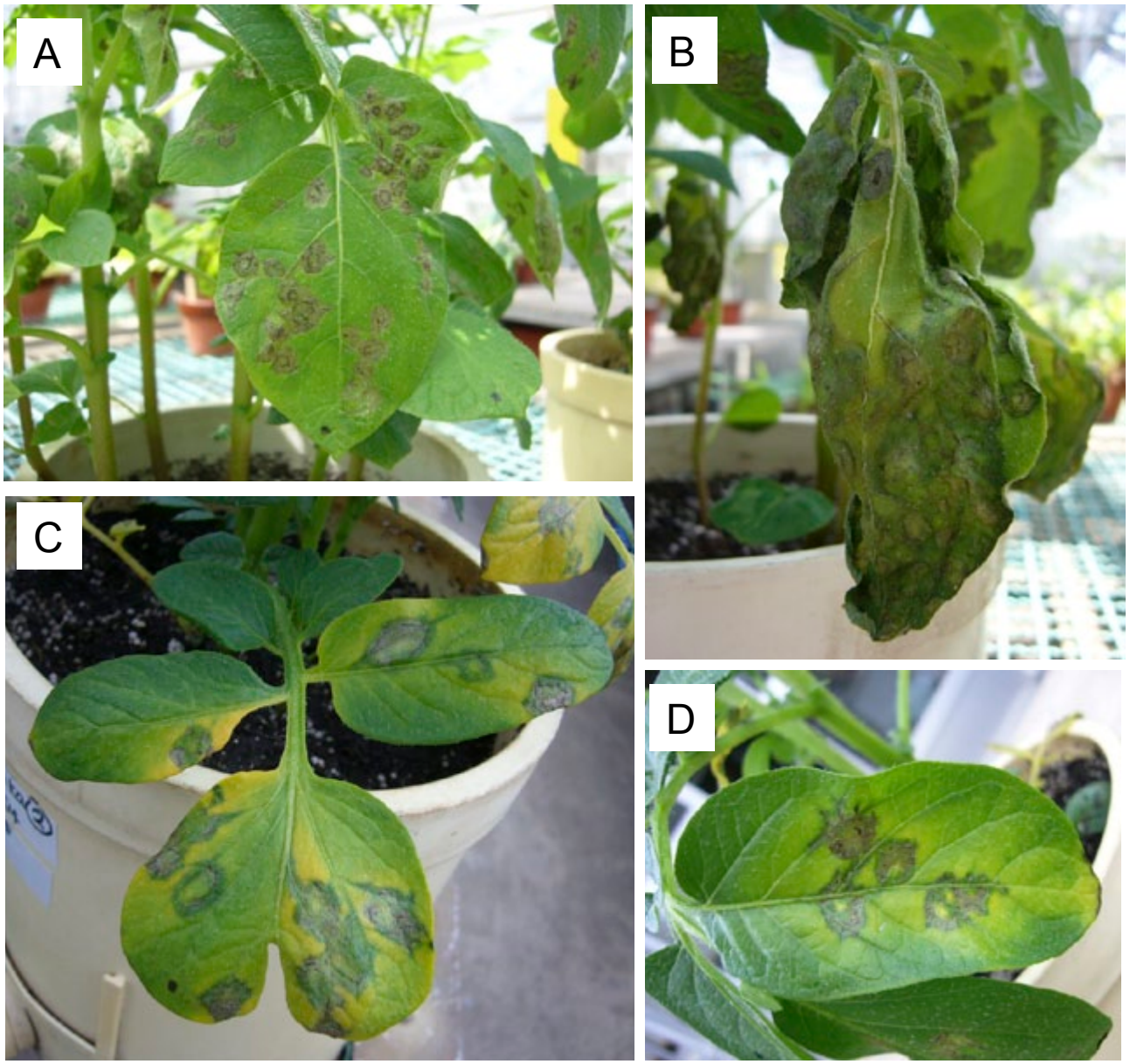

Figure 1. Local necrotic spots and rings induced by PoCMV7-5 in two potato cultivars: Maris Bard (A, B) and Nishiyutaka (C, D) which appeared about 1 week after inoculation.

tal 55–58 nt compared to the Japanese CMV satRNA 55-1 that consists of 391 nt.

Discussion

The phylogenetic tree of the CP gene of CMV isolates consisted of two groups, I and II, of which group I is divided into two subgroups IA and IB (Figure 2). This is consistent with phylogenetic analysis reported previously for the CP coding region of CMV (Roossinck *et al*., 1999; Lin *et al*., 2003). IA and II subgroups contained isolates of worldwide distribution while isolates of IB subgroup include CMV isolates from Asia (Figure 2; Roossinck *et al*., 1999;

Lin *et al*., 2003). All Syrian CMV isolates belonged to the subgroup A of the strain group I based on the CP coding region and were closely related regardless of the original host (i.e. potato or tobacco) or site of collection. This is in agreement with the results reported from other regions of the world, which have concluded that genetic structure of CMV population is not correlated with host plant species or location (Garcia-Arenal *et al*., 2000; Lin *et al*., 2003). When compared with CMV isolates from the DDBJ/EMBL/GenBank databases, Syrian CMV isolates were closely related to those from Europe and USA.

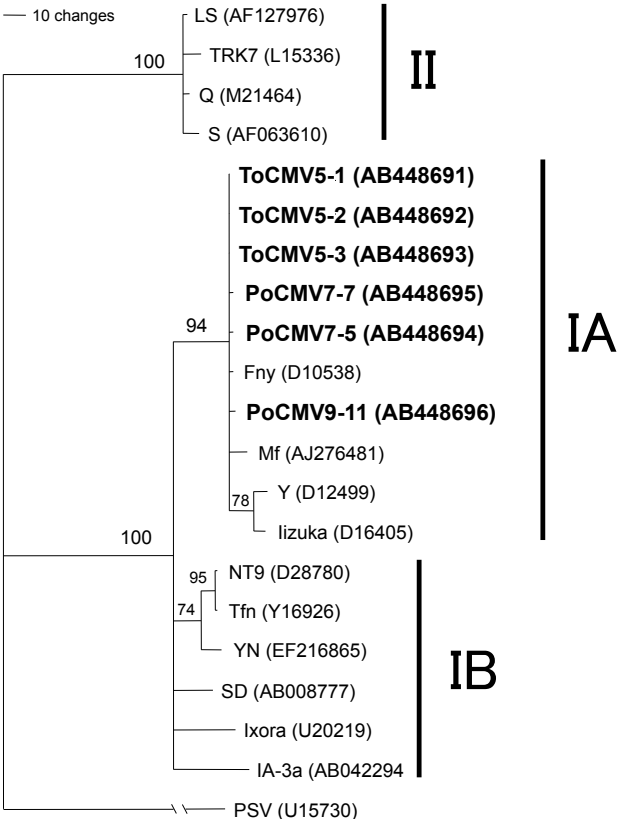

Figure 2. Maximum parsimony tree of the coat protein (CP) nucleotide sequences of CMV isolates. Percentage of bootstrap values in which each node was recovered is given when >70%. The tree was constructed using the CP nucleotide sequence of PSV as an outgroup. Syrian CMV isolates are shown in bold.

A range of host species belonging to four plant families was inoculated with the potato CMV isolate PoCMV7-5 which infected all solanaceous species/cultivars tested systemically except *D. stramonium* and potato, in which only local infection was detected. In legumes, PoCMV7-5 induced local but not systemic infection and the same was found in *C. amaranticolor*. Except for *Cucurbita pepo* cv. Diner, in which PoCMV7-5 induced local infection, PoCMV7-5 could not induce any local or systemic infection in the cucurbits tested. These results could be due to host adaptation (Tan *et al*., 2005), since PoCMV7-5 had adapted to solanaceous hosts and became less infectious to other hosts, including CMV common hosts such as those of *Leguminosae* and *Cucurbitaceae*.

PoCMV7-5 infection restricted to the inoculated leaves of potato cultivars, Maris Bard, King Edward, Desiree and Nishiyutaka inducing necrosis of various types without any systemic infection. The mixed infection with various PVY strains at variable temperatures did not lead to systemic infection with CMV in all inoculations carried out in the present study. It was reported that under high temperature, CMV overcomes potato resistance and induces systemic infection (Celebi-Toprak *et al*., 2003). At the beginning of the autumn growing season in Syria the temperature is as high as 30°C which in a combination with certain potato cultivars might enhance the systemic potato

Table 3. Nucleotide sequence comparison of the coat proteins of Syrian CMV isolates from potato and tobacco.

	ToCMV5-1	ToCMV5-2	ToCMV5-3	PoCMV7-5	PoCMV7-7	PoCMV9-11
ToCMV5-1	-					
ToCMV5-2	100	-				
ToCMV5-3	100	100	-			
PoCMV7-5	99.7	99.7	99.7	-		
PoCMV7-7	99.7	99.7	99.7	99.4	-	
PoCMV9-11	99.5	99.5	99.5	99.2	99.2	-

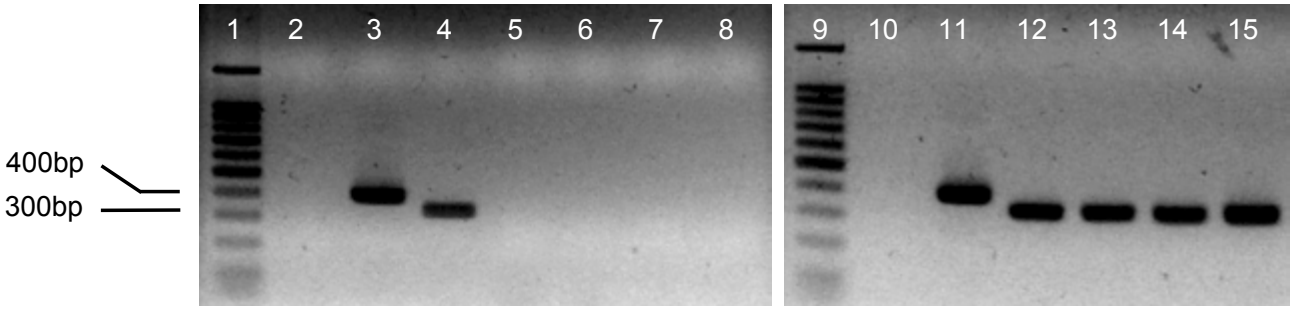

Figure 3. Detection of CMV satellite RNA in potato and tobacco samples infected with CMV. *Lane 1, 9*, 100bp DNA ladder; *2, 10*, healthy tobacco cv. White Burley; *3, 11*, tobacco cv. White Burley infected with SatRNA 55-1 (positive control); *4, 12*, original infected potato with PoCMV7-5; *5*, original infected potato with 7-7; *6*, original infected potato with 5-1; *7*, original infected potato with 5-2; *8*, original infected potato with 5-3; *13*, tobacco cv. Xanthi infected with PoCMV7-5; *14*, tobacco cv. White Burley infected with PoCMV7-5; *15*, potato cv. Maris Bard infected with PoCMV7-5.

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          201                                                  250
PoSA1     ACATGGTTCGCCGTTACCATGGATT--CGAAAGAAACA--CTCTGTTAGG
PoSA2     ACATGGTTCGCCGTTACCATGGACTT-CGAAAGAAACA--CTCTGTTAGG
PoSA3     ACATGGTTCGCCGTTACCATGGATTTTCGAAAGAAACA--CTCTGTTAGG
PoSA4     ACATGGTTCGCCGTTACCATGGATT--CGAAAGAAAAAAACTCTGTTAGG
PoSA5     ACATGGTTCGCCGTTACCATGGATTTTCGAAAGAAACA--CTCTGTTAGG
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Figure 4. The hypervariable part of SatRNA variants detected with the Syrian potato CMV isolate PoCMV7-5. Numbers above indicate nucleotide positions; -, nucleotide deletion.

infection by CMV and increase the significance of this virus in that region. The other factor that may contribute to the CMV systemic infection in potato is the synergy with other viruses such as PVY (Palukaitis and Garcia-Arenal, 2003). Indeed, all CMV infections detected in potato samples in this study were mixed with PVY infection. This would indicate possible synergy between these viruses in some cultivars of potato in Syria. Unfortunately, the original potato cultivar from which PoCMV7-5 was isolated is unknown, which makes it impossible to conduct the inoculation test under similar conditions, i.e. a similar virus-host-environment combination. Further studies are needed on the incidence of CMV in potato in Syria and the the role of PVY in CMV infection in potato.

The isolate PoCMV7-5 contained satRNA that was detected whenever CMV infection occurred. PoCMV7-5 and the satRNA could not be separated by local lesion isolation in *Vigna unguiculata* and *Chenopodium amaranticolor* (data not shown), hence the effects of this satRNA on the virulence and pathogenecity of PoCMV7-5 is yet to be determined. PoCMV7-5 satRNA comprised heterogeneous sequences that vary in length due to consecutive A and/or T deletion/insertion at approximate position 225–240. Similar heterogeneity at a similar site was reported for other satRNA sequences produced following serial passage in tobacco (Kurath and Palukaitis, 1990).

In conclusion, the present study has characterized and classified potato and tobacco CMV isolates for the first time from Syria. Regardless the host species or geographic origin, CMV isolates belonged to the IA strain subgroup and were closely related, which indicates the significance of potato CMV infection, not only for potato but for other crops nearby and *vice versa*. An isolate of CMV showed host preferences with higher infectivity in solanaceous hosts compared to other hosts including those of *Leguminosae* and *Cucurbitaceae* which might be explained by host adaptation. Moreover CMV satRNA

has been reported for the first time from potato, though the effects of this satRNA on potato yield are yet to be determined.

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