Usta M *et al.* (2020)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca 48(2):523-534
DOI:10.15835/nbha48211822
Research Article

The molecular characterization of the coat protein sequence and differentiation of CMV- subgroup I on tobacco from native flora in Turkey

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Abstract

Cucumber mosaic virus (CMV) has a broad plant-host range and a wide ecological zone distribution. Virus-like symptoms were observed on tobacco fields of Adıyaman province (Turkey) showing conspicuous mottling, greenish mosaic patterns and severe malformations of leaves. A total of forty tobacco samples tested positive against CMV by reverse transcription polymerase chain reaction (RT-PCR) using coat protein gene specific primers. Five randomly chosen CMV isolates were cloned into pGEM T-Easy vector and transformed into *Escherichia coli* JM109 strain. The recombinant bacterial clones containing insert-DNA were further purified and sequenced bidirectionally. In multiplex-RT-PCR studies carried out, it was found that all 40 CMV isolates belong to Subgroup I by resulting a 593 bp long DNA fragments. CMV subgroup IA was found to predominate in 4 out of 5 tobacco samples and CMV subgroup IB was found in 1 out of 5 CMV-positive samples by comparing the isolates with CMV reference isolates in phylogenetic tree. However, no Subgroup II sequences were found by multiplex RT-PCR using discriminating primers. The nucleic acid sequences were analyzed for the investigation of diversity of coat protein (CP) sequences of 5 CMV isolates. The sequence similarity ranged from 94.2-100% with the CMV subgroup I isolates infecting diverse plants in other regions of the world. The evolutionary tree revealed that the CMV IA Adıyaman isolates exhibited a genetic affinity with Australian and Spanish isolates. However, the CMV IB Adıyaman isolate showed a close genetic relationship with only the Australian isolates. To our knowledge, this study shows for the first time the occurrence of CMV IA and IB isolates infecting cultured tobacco plants in Adiyaman province.

Keywords: characterization; cloning; cucumber mosaic virus; phylogeny; subgroup

Introduction

Tobacco (*Nicotiana tabacum* L.) is one of the substantial economic products and is grown in most countries such as Brazil, Turkey, Canada, USA, and China where it represents primary producing fields (FAO, 2013). Virus-borne diseases frequently lead to reduced quality of product and yield. Tobacco crops are attacked by numerous viruses such as potato Y potyvirus (PVY), tobacco ringspot nepovirus (TRSV), pepper mottle potyvirus (PeMoV), cucumber mosaic cucumovirus (CMV), alfalfa mosaic alfamovirus (AMV), tobacco leaf

Received: 20 Feb 2020. Received in revised form: 20 Apr 2020. Accepted: 21 May 2020. Published online: 30 Jun 2020.

curl geminivirus (TbLCV), tobacco etch potyvirus (TEV), tobacco mosaic tobamovirus (TMV), tomato spotted wilt tospovirus (TSWV) and become epidemic in many tobacco cultivated areas (Valand and Muniyappa, 1992; EPPO/CABI, 1996b; Chatzivassiliou, 2008; Chen *et al*., 2014; Akinyemi *et al*., 2016).

Adiyaman is traditionally one of the oldest tobacco planting provinces of Turkey which is well known for its local tobacco. Adiyaman's tobacco is an important source for rural income, ranking 5th with its 8% tobacco production of Turkey (TUIK, 2015). Single, double or multiple virus infections can potentially destroy the tobacco crops, if it is planted on a large scale. *Cucumber mosaic virus* (CMV) is considered important host of tobacco, which has a wide array of hosts capable of making epidemics in more than 1300 plant species (Garcia-Arenal and Palukaitis, 2008).

CMV, belonging to the *Bromoviridae* family, was firstly recorded in cucumber and melon cultivation areas in USA in 1916 (Doolittle, 1916), thereafter recognized in various agricultural and ornamental plants from other numerous countries such as Argentina, India, Korea, China in temperate and tropical regions (Rodríguez Pardina *et al*., 2013; Nagendran *et al*., 2018; Park *et al*., 2018).

CMV is a destructive disease in *N. tabacum* with symptoms like mosaic, deformation, dwarfing and sometimes necrosis in infected plants depending on the variety of plant and viral isolate, weather conditions, existence of satellite RNA, pathogenicity, and co-existing infections (Palukaitis *et al*., 1992). It possesses an isometric-shaped, tripartite (+) ssRNA genome of approximately 29-30 nm diameters, without envelopes. This genome is encapsidated in separate particles, which are RNA-4 (also referred to as subgenomic RNA), RNA-3, RNA-2 and, RNA-1 in increasing length (Palukaitis and Garcia-Arenal, 2003). CMV has been transmitted experimentally by plant sap and non-persistently in more than 75 species of a stylet-borne aphids from plant to plant, especially from *Myzus persicae* and *Aphis gossypii* (Kaplan *et al*., 1997).

CMV isolates are principally categorized into two parts as subgroup I and subgroup II (SI and SII) based on serological tests, triplet mapping of coat protein (CP) gene, RT- PCR followed by RFLP analyses, nucleic acid hybridization, and nucleotide sequence similarity. Furthermore, SI is subdivided into the subgroup IA (SI-A) and subgroup IB (SI-B) depending on the analysis of open reading frame gene, untranslated region sequences of RNA3 and a cladistic assay of the CP gene. Such a classification has been reported and supported by various researchers (Palukaitis *et al*., 1992; Roossinck, 2002; Lin *et al*., 2003).

The presence of CMV and its subgroups has been reported in many international and national studies with different tests in different hosts (Kaplan *et al*., 1997; Rodríguez Pardina *et al*., 2013). However, studies on subgroup discrimination of this virus nationwide are limited. Although it is a widespread virus in many crops, little is known about the molecular features of Turkish tobacco isolates and their subgroups they belong. This research has addressed the categorization and molecular analysis of 5 CMV strains at the genomic level in symptomatic tobacco plants from Adiyaman province of Turkey.

Materials and Methods

CMV isolates

A total of 40 CMV isolates included in this paper were obtained from our previous tobacco field survey performed during the period from August to September 2018 in Adiyaman province, located in southeast region of Turkey (Günay, 2019). Plant samples and their RNA preparations were maintained at -70 °C until processed. The all isolates were subjected to CMV subgroup discrimination assays resulting two fragments of 593 and 704 bp, specific to CMV subgroup I and CMV subgroup II, respectively (Chen *et al*., 2011). Five randomly selected Subgroup I CMV isolates were further characterized by molecular cloning

Before the discrimination subgroup, the cultures of CMV isolates were maintained on *N. tabacum*. Mechanical inoculation was carried out in two cotyledon leaf stages by rub procedure using phosphate buffer (PB), nicotine (2.5%) and carborundum powder as an abrasive. The systemically infected test plants kept in

the climate chamber at room temperature and daylight situations for further investigations. The symptomless tobacco plants were used as a negative control during all experimental processes.

Total RNA extraction, primer design, and cDNA synthesis

For all specimens, total RNA extractions were performed from about 0.1 g frozen leaf tissues, following the protocol described by Foissac *et al*. (2001) with minor modifications. As given in Table 1, specific upstream and downstream primer sets were adopted from previous studies (Nakazono-Nagaoka *et al*., 2005; Chen *et al*., 2011), which targeted the CP gen to detect and differentiate CMV SI and SII in tobacco. In all cDNA syntheses, random hexamer primers were used instead of oligo-dT as complementary to mRNA because the CMV nucleic acid was not polyadenylated.

The extracted RNAs were used in the first-strand cDNA synthesis (complementary DNA). In brief, into a nuclease-free microfuge tube, 1 µl of random hexamer primer (20 pmol/µl), 2 µl of extracted RNA as a template and 1 µl of dNTP (10 mM) were put then completed to 12 µl with nuclease free water. The mixture was held at 65 °C for 5 min and chilled on ice.

Four µl of 5X RT Reaction buffer, 2 µl of 0.1M DTT, 1 µl of RNAse inhibitor and 1 µl of reverse transcriptase (RT) enzyme (Thermo Scientific, USA) were added to complete the reaction mixture to a final volume of 20 µl. The reverse transcription reaction was performed at 42 °C for 50 min. To inactivate the RT enzyme, the mixture was incubated at 70 °C for 15 min. The cDNAs were maintained at -20 °C until processed.

Identification of CMV SI and SII isolates by Multiplex-RT-PCR (M-RT-PCR) assays

The cDNAs were served as template in M-RT-PCR assay. For CMV SI and SII, the reagents were adjusted empirically in a total volume 50 µl, consisting of 34.6 µl of sterile distilled water, 2 µl of cDNA, 3 µl of $MgCl_2$ (25 mM), 1 µl of dNTPs (10 mM), 1 µl of downstream and upstream primers (20 pmol), 5 µl of 10X Taq buffer, 0.4 µl of Taq DNA polymerase (5 U/µl) (Thermo Scientific, USA) enzyme. The temperature cycles of M-RT-PCR reaction were as follows: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30s, annealing at 52 °C for 30 s, elongation at 72 °C for 45s. Final elongation occurred at 72 °C for 10 minutes.

Fifteen µl amplified target fragments were photographed under UV light after electrophoresis on 1.5% agarose gel containing ethidium bromide (EtBr). Healthy tobacco plants were used as negative control. A CMV SI isolate confirmed by previous sequence analysis was used as positive control.

Cloning, sequencing and cladistic analyses

Amplified-DNA fragments were separately excised from agarose gel and the amplicons were recovered using GeneJET Gel Extraction Kit (Thermo Scientific, USA) following the manufacturer's instructions. Five randomly selected CMV isolates were cloned into the pGEM T- Easy vector (Promega, USA) following the manufacturer's instructions and transformed into *E. coli* JM 109 strain. Recombinant plasmids were purified and sequenced bidirectionally in an automated sequencer (Sentebiolab Company, Turkey). Insert sequences were trimmed from raw sequences using CLC Main Workbench program (Version 6.7.1) and recorded to NCBI Database (www.ncbi.nlm.nih.gov).

As presented in Table 2, the cladistic analysis was performed with representative 16 CMV isolates belonging to Subgroup IA, IB, and Subgruop II published in the NCBI. The phylogenetic tree was constructed using Mega 7 program bootstrapped 100 times using the neighbor-joining algorithm (NJA) (Kumar *et al*., 2016). The nucleic acid sequence alignments were generated using CLC Main Workbench program (Version 6.7.1) to estimate similarity scores. Tomato aspermy virus (EF153735) sequence was used as an outgroup to root the phylogenetic tree.

Results

Symptoms produced by CMV isolates

Symptom development of CMV isolates was examined experimentally. The symptoms developed on *N. tabacum* in experimental studies were similar to the natural diseased *N. tabacum*, but were more evident and severe (Figure 1).

Figure 1. Symptoms of CMV-infected tobacco plants. Panel A and B: Foliar mosaic, leaf puckering in experimental tobacco plants in climate chamber, Panel C and D: Greenish mottle, leaf distortion in field detected tobacco plants

Identification of CMV subgroup I

Forty samples of nucleic acids were amplified by M-RT-PCR using discrimination primers (Table 1). The resulted amplicons were about 593 bp length indicating that the all isolates were belong to CMV Subgroup I. However, no 704 bp DNA fragments were observed when the Subgroup (SII) primers were used to investigate CMV Subgroup II members (Figure 2).

BLAST analyses and multiple alignments

Amplification products of five CMV Subgroup I, representing full length of coat protein gene of (657 bp), were successfully cloned into the pGEM T-Easy vector. The BLAST analyses confirmed the coat protein origin of five cloned sequences. The CMV-Subgroup I tobacco isolates were named TR41, TR54, TR93, TR128, and TR131 and submitted to GenBank with accession numbers of MK89142, MK890143, MK890144, MK890145, and MK890146, respectively. Multiple alignments revealed a high homology between Adiyaman and another Subgroup I isolate in the GenBank. Full-length CP gene sequences of Adiyaman CMV-Subgroup I isolates from tobacco growing region exhibited 94.2 to 100.0% indentity between five isolates. Multiple sequence alignments indicated that five CMV Subgoup I Adiyaman isolates and the isolates from other geographic origins had 75.1 to 99.7% identity at the nucleotide level (Table 3).

Cladistic analysis

Based on molecular relationships and nucleotide sequence similarity, five CMV isolates were classified into two major subgroups. The four isolates (MK890144, MK890146, MK890143, and MK890142) were clustered in SI-A and one isolate (MK890145) in SI-B, fortified by supporting values as shown in Figure 3.

Table 1. Product size and nucleotide sequences of primers utilized in RT-PCR and M-RT-PCR for detection and discrediting the subgroups of CMV

CMV primer types	Upstream	Downstream	Amp. size
Characterization	ATGGACAAATCTGAATCAAC	TCAGACTGGGAGCACTCCAG	657 bp
Subgroup I (SI)	GCCACCAAAAATAGACCG	ATCTGCTGGCGTGGATTTCT	593 bp
Subgroup (SII)	CTACGTTTATCTTCC	AACCGGTGATTTACCATCGC	704 bp

Table 2. CP gene nucleotide sequences of various CMV strains used for phylogenetic tree

No	Origin	Accession no	Strain names	Subgroup	Host
1	Hungary	L15336	trk7	SII	-
2	Australia	M21464	Q	SII	-
3	USA	AF127976	LS	SII	-
4	Japan	AB006813	m2	SII	-
5	India	AJ585086	Indian	SII	Lilium
6	Australia	U22821	Ny	SIA	-
7	Hungary	AJ517802	Rs	SIA	*Raphanus sativus*
8	Spain	AM183119	Ri-8	SIA	Tomato
10	Israel	U66094	Sny	SIA	*Cucurbita pepo*
11	Italy	Y16926	Tfn	SIB	Tomato
12	Taiwan	D28780	NT9	SIB	Tomato
13	USA	D00462	C	SIA	-
14	Japan	D42079	C7-2	SIB	-
15	India	AF281864	-	SIB	*Datura innoxia*
16	Turkey	KY474380	CWP17	SIB	Cowpea
17	India	EF153735	Kolkata	TAV	*Chrysanthemum morifolium*

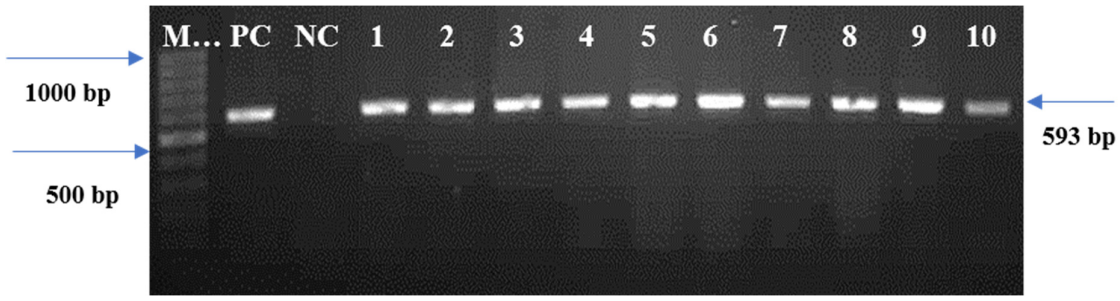

Figure 2. Analysis of CMV-Subgroup I from individually infected tobacco plants using multiplex RT-PCR; Lane M, 1-kb DNA ladder; PC, Positive control; NC, Negative control; Lane 1-10, CMV Adiyaman isolates

Table 3. Chart showing multiple alignment analysis of CMV isolates generated with CLC Main Workbench program

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
U60694	1		99.85	99.70	99.54	99.54	99.39	99.24	98.93	96.93	95.43	95.13	94.22	94.22	91.17	92.85	81.43	76.52	76.21	76.36	75.45	75.00
U28221	2			99.85	99.70	99.70	99.54	99.39	99.09	99.09	95.59	95.28	94.37	94.37	91.32	93.00	81.58	76.67	76.36	76.52	75.61	75.00
AJ517802	3				99.54	99.54	99.39	99.24	98.93	98.93	95.74	95.43	94.52	94.22	91.17	93.15	81.43	76.67	76.36	76.52	75.61	75.00
AM183119	4					99.39	99.24	99.39	99.09	99.09	95.28	94.98	94.67	94.22	91.32	93.69	81.43	76.82	76.52	76.67	75.76	75.15
MK890144	5						99.24	99.39	99.09	99.09	95.59	95.28	94.06	94.37	91.32	93.00	81.43	76.97	76.67	76.82	75.91	75.30
D00462	6							98.93	98.63	98.63	95.13	94.82	93.91	93.91	90.87	92.54	81.13	76.21	76.91	76.06	75.15	75.55
MK890146	7								99.09	99.09	94.98	95.13	94.06	94.22	91.48	93.00	81.28	76.82	76.52	76.67	75.76	75.15
MK890142	8									100	94.98	94.98	93.76	94.52	91.32	93.30	81.74	76.97	76.67	76.82	75.91	75.30
MK890143	9										99.70	94.98	93.76	94.52	91.32	93.30	81.74	76.97	76.67	76.82	75.91	75.30
Y16926	10											99.70	94.98	93.61	92.85	93.76	82.65	77.88	77.58	77.73	76.82	76.21
D28780	11												94.98	93.30	92.54	93.76	82.34	77.58	77.27	77.42	76.52	75.91
D42079	12													93.30	91.78	92.54	81.28	76.97	76.67	76.82	75.91	75.30
MK890145	13														94.06	93.15	84.17	76.82	76.52	76.67	75.76	75.15
AF281864	14															92.39	82.19	76.30	75.00	75.15	74.24	75.94
AJ271416	15																80.97	76.82	76.52	76.67	75.76	75.45
KY474380	16																	67.58	67.27	67.42	66.52	66.21
M21464	17																		99.54	99.39	98.63	97.72
AF127976	18																			99.54	98.78	98.17
AB006813	19																				98.78	97.72
L15336	20																					96.96
AJ585086	21																					

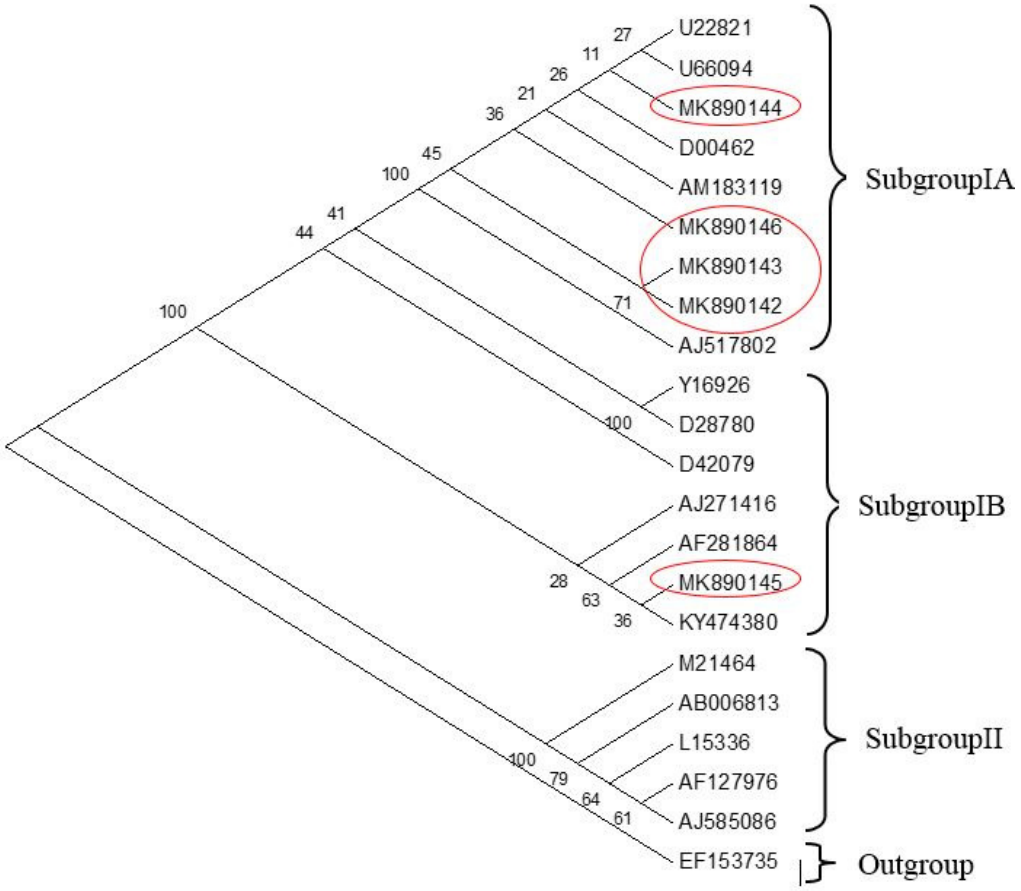

Figure 3. Phylogenetic dendrogram constructed with CMV-Adiyaman isolates and the isolates, retrieved from GenBank, created by the neighbour-joining algorithm. The sequence of tomato aspermy virus isolate (EF153735) was used as an outgroup to root the tree. Bootstrap values are shown on each branch

Discussion

Identification of CMV isolates is important to elucidate the ancestor of this virus and develop a control strategy. Although ELISA and RFLP assays are used (Haase *et al*., 1989; Shevchenko *et al*., 2015), Multiplex RT-PCR is a practical method for the simultaneous differentiation of CMV subgroup I and II (Rizos *et al*., 1992; Yu *et al*., 2005; Eyvazi *et al*., 2015). To differentiate CMV subgroups, various primers have been developed by various researchers based on the conserved sequence within the viral genomic segment. Primers used by Chen *et al*. (2011) produced 593 bp amplicons in RT-PCR tests, consistent with the present study.

SI-A, SI-B and SII groups of CMV have been reported in literature from numerous countries such as USA (D10538, IA), Japan (D16405, IA), South Korea (AJ27648, IA), Indonesia (AB042294, IB), Philippines (U20219, IB), Spain (AM183119, IA), Taiwan (D28780, IB), Italy (Y16926, IB), USA (AF127976, II), Australia (AF198103, II), South Africa (U37227, II), and Hungary (L15336, II) (Rodríguez Pardina *et al*., 2013; Arafati *et al*., 2013). Furthermore, historical records denoted that S-IA members were distributed throughout the world, while SI-B members were mostly reported in eastern Asia, considered to be the origin of this subgroup, although some have been found in Mediterranean region, Iran, California, Brazil, Australia and Greece (Sclavounos *et al*., 2006; Farzadfar *et al*., 2013). In this study, it was determined that one isolate classified in CMV SI-B and the others (four isolates) were in CMV SI-A based on the phylogenetic assay. None

of the isolates was classified in CMV subgroup II. The widespread of CMV subgroup I isolates in this region may be due to its severity and abundancy worldwide, compatible with previous reports (Singh *et al*., 1995; Tian *et al*., 2009). The absence of CMV SII isolates in our tests could probably be explained by inappropriate climate conditions in the surveyed areas and probably because of its tropical climate's adoption (Hord *et al*., 2001; Kumari *et al*., 2013).

CMV SI isolates have been perfectly adapted to various plants such as cucumber, tomato, pepper, tobacco, pumpkin, bean, celery, musa crops, peanut, yam, and weeds from different locations in world, confirmed by serological and molecular tests (Eni *et al*., 2013; Ayo-John and Hughes, 2014; Zhu *et al*., 2018). The presence of CMV in tobacco has been reported by several researchers as both natural hosts (Chatzivassiliou *et al*., 2004; Dai *et al*., 2012; Zhang *et al*., 2013) and experimental host (Tian *et al*., 2009; Chikh Ali *et al*., 2012). It has a nationwide host range with the exception of tobacco, which includes: Myrtle leaf milkwort, globe artichoke, parsley, mint, broccoli, squash, olives, cabbage, peppers, lettuce, spinach, cowpea, gladiolus, tomatoes, zucchini, cucumber, ornamental plants (daffodils, hyacinths, lilies), bean and spinach (Gümüs *et al*., 2004; Beler and Acıkgöz, 2005; Sevik and Akcura, 2011; Culal Kılıc and Yardımcı, 2012; Erkan *et al*., 2013; Ergün *et al*., 2013; Culal Kılıc *et al*., 2015; Uzunoğulları and Gümüs, 2015; Sertkaya, 2015; Karanfil *et al*., 2016; Gökdag *et al*., 2016; Güngör *et al*., 2017; Karanfil and Korkmaz, 2017; Koc and Fidan, 2017; Sevik, 2012, 2017). Since its transmission to tobacco is not seed-borne (De Bokx and Huttinga, 1981), its presence and prevalence can stem from other virus sources like weeds, the suitable climate environment for active aphid populations throughout tobacco production season and acquired from other cultivated plants grown from CMV- infected seeds (Kaplan *et al*., 1997; Tsitsipis *et al*., 2001).

CMV exhibited characteristic symptoms more severely such as mottle, mosaic and leaf malformation in experimental transmission studies on tobacco. These symptoms were similar to those mentioned by Chikh Ali *et al*. (2012), Sclavounos *et al*. (2006) and Arafati *et al*. (2013) on tobacco leaves and on many hosts both native flora and experimental studies (Zhang *et al*., 1994; Jalender *et al*., 2017).

Analysis of phylogenetic tree showed that Turkish tobacco SI-A isolates (MK89142, MK890143, MK890144, MK890146) were clustered in the same branch, and well differentiated from the members of SI-B isolates. Within SI-A, one isolate (MK890144) was closely related to the Israelli-squash (U66094) isolate, one isolate (MK890146) was related to the Spanish-tomato (AM183119) isolate, the other two isolates (MK890142 and MK890143) were closely related to each other, which formed a separate cluster. The Adiyaman SI-B isolate (MK890145) was phylogenetically grouped with the Turkish cowpea SI-B isolate (KY474380). Multiple alignments further revealed that the sequence of CP of CMV SI-B isolate showed approximately 94% sequence similarity, whereas CMV SI-A isolates shared the maximum identity of 94.22-100% among themselves at the CP gene level (Table 2). These incidences coincide with the literature reported by Palukaitis and Garcia-Arenal, (2003). The highest homology (100%) was shared between MK890142 and MK890143 isolates (subgroup I-A), while the lowest homology (94.22%) was shared between MK890146 (subgroup I-A) and MK890145 (subgroup I-B) isolates. This variation among CMV I isolates can likely be due to reassortment occurring within the CP gene demonstrating the event of new strains due to proceeding evolvement (Moury, 2004).

Based on previous studies carried out in Turkey, CMV SI-A has been found in pepper, tomato and watermelon (Caglar, 2006), CMV SI and SII in pepper, tomato, cucumber, watermelon and melon (Sarı, 2015), CMV SI-B in cowpea (Karanfil and Korkmaz, 2017) and, CMV SI-A in spinach (Kurtoglu and Korkmaz, 2018). Even though CMV has been detected on a wide range of hosts in Turkey, subgroup categories and their classification of most of them were not currently completed at the nucleotide levels. Further molecular analyses are needed for classifying the CMV isolates into subgroups on diverse crops. The present study is the first report on the presence of CMV subgroup I-A and I-B in infected tobacco and their molecular characterizations in cultured tobaccos in Adiyaman province.

Acknowledgements

The study was supported by a grant from The Research Fund of Van Yüzüncü Yil University-YYU-BAP (Project no: FYL-2018-7339). The authors acknowledge YYU-BAP for their financial supports.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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