Cucumber mosaic virus Isolates from Greek Legumes are Associated with Satellite RNAs that are Necrogenic for Tomato

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Abstract

Worldwide, *Cucumber mosaic virus* (CMV) is the causal agent of many economically important diseases. Based on immunological or molecular analysis, three distinct subgroups of CMV isolates can be identified (IA, IB, and II). In addition, some CMV isolates are associated with satellite RNAs (satRNAs), a type of noncoding transcript that may alter the symptoms of CMV infections. This study presents an analysis of CMV isolates occurring in legumes in Greece in respect to their genetic diversity, and the presence and diversity of their satRNA. Phylogenetic analysis of the CMV coat protein sequence of 18 legume and 5 tomato CMV isolates collected throughout Greece classified them

within subgroups IA and IB, with a limited genetic diversity. The CMV satRNAs found in nine field legumes exhibiting mild symptoms and in one tomato with a necrotic syndrome contained a functional necrogenic motif; therefore, they were grouped within the necrogenic group of CMV-satRNAs. The necrotic phenotype was expressed in all legume CMV isolates containing necrogenic satRNAs when mechanically inoculated onto tomato plants. To our knowledge, this is the first observation that legumes host necrogenic CMV-satRNAs. The possible role of legumes in the epidemiology of CMV and necrogenic satRNA complex is discussed.

Cucumber mosaic virus (CMV; genus *Cucumovirus*, family *Bromoviridae*) is an evolutionarily successful virus with probably the broadest natural host range, comprising over 1,200 species in more than 100 families (Jacquemond 2012; Palukaitis et al. 1992). It causes numerous diseases worldwide and its epidemics severely affect crops of agricultural importance such as tomato, tobacco, cucurbits, and legumes, resulting in devastating yield losses (Gallitelli 2000). CMV is spread by a large number of aphid species in a non-persistent fashion, while its perpetuation in nature is facilitated by seed transmission in several of its hosts and infection (usually latent) of a number of wild and weed species serving as a reservoir (Gallitelli 2000; Gildow et al. 2008; Jacquemond 2012; Palukaitis et al. 1992).

The tripartite genome of CMV consists of three single-stranded RNA (1, 2, and 3), which are encapsidated by the coat protein (CP) in virions of 29 nm in diameter. RNA1 and -2 code for the viral replication machinery (proteins 1a and 2a). The bicistronic RNA2 produces also a subgenomic RNA (RNA4A; encapsidated in the virions of the group II isolates) and encodes for a multifunctional protein (2b), which is involved in viral local and systemic spread, suppression of gene silencing, and symptom expression. RNA3 encodes for the movement protein (3a) and the CP, the latter via a subgenomic RNA4 encapsidated in the virion (Jacquemond 2012; Mochizuki and Ohki 2012; Palukaitis and García-Arenal 2003; Palukaitis et al. 1992).

The cosmopolitan nature of CMV, the flexibility of its nonpersistent transmission due to the large number of vector species, and its large host range result in a rich epidemiology (Jacquemond 2012; Palukaitis et al. 1992). Several studies have focused on the genetic structure of the CMV population, with emphasis on either specific genes or, recently, the entire genome. The CP gene is the most common target of these studies due to its role in the assembly of virions, cell-to-cell spread, long-distance movement, and aphid-mediated viral transmission (Mochizuki and Ohki 2012).

CMV isolates are classified into two quite distinct groups (I and II), reflecting sequence differences in the CP gene among the different isolates (with 70 to 75% nucleotide identity) (Jacquemond 2012; Roossinck 2002), although genetic recombination and reassortment events may lead to discrepancies in this classification (Ohshima et al. 2016). Group I is further divided in two subgroups (IA and IB, with 92 to 95% nucleotide identity). With regard to their geographical spread, CMV IA and II strains are found all over the world, while IB strains originated in Asia, with a few reports outside Asia (Jacquemond 2012; Palukaitis and García-Arenal 2003; Roossinck 2002). Interestingly, only CMV II isolates contain an additional RNA encapsidated (RNA5), corresponding to the 3' nontranslated region of RNA2 and -3 playing an important role in recombination (Jacquemond 2012).

CMV isolates of both groups I and II support the replication of satellite RNAs (satRNAs) as an extra genomic segment. These short noncoding sequences are encapsidated in the virions (Palukaitis 2016) and, consequently, spread with the associated CMV isolate (Alonso-Prados et al. 1998). SatRNAs may enhance (by inducing chlorosis or necrosis) or attenuate the symptoms induced by a CMV strain, depending on the host plant. Phylogenetic analysis showed that CMV satRNAs causing different symptoms on infected tomato plants fall into distinct groups (García-Arenal and Palukaitis 1999; Kouadio et al. 2013; Masuta 2014; Palukaitis and García-Arenal 2003; Roossinck et al. 1992). The tomato necrotic syndrome involves the activation of a programmed cell death mechanism (Xu and Roossinck 2000) and has been correlated with the presence of a conserved sequence domain (Devic et al. 1990) and specific structures near the 3' end of the satRNA (Rodríguez-Alvarado and Roossinck 1997).

In Greece, CMV is often found infecting several economically important crops (Sclavounos et al. 2006; Vovlas and Di Franco 2004; Xanthis et al. 2015a,b). In particular, open-field tomato crops are severely affected by recurrent epidemics of lethal necrosis, but the epidemiology and inoculum sources remain unknown (Kyriakopoulou et al. 2000; Varveri and Boutsika 1999). In previous studies, satRNAs were detected in some CMV isolates from various hosts (Varveri and Boutsika 1999; Vovlas and Di Franco 2004) without performing any further characterization study. Because legumes represent major hosts for CMV and several legume landraces are cultivated throughout Greece, our objective was to investigate the presence of CMV satRNAs in Greek legumes and to study their molecular genetics and diversity. To type the subgroups of CP of the Greek

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CMV isolates, we analyzed and compared them against a world-wide collection of CMV isolates from several hosts and from the earlier characterized Greek CMV isolates.

Materials and Methods

Virus isolates and symptoms. CMV isolates were collected during field surveys made in legume production areas in northern,

central, and southern Greece (Chatzivassiliou et al. 2016) and from experimental fields on the farms of the Agricultural University of Athens and the Aristotle University of Thessaloniki. Plants showing any symptom suggestive of virus infection were sampled and tested for the presence of CMV with enzyme-linked immunosorbent assay tests using polyclonal antibodies (SRA44501; Agdia Inc.). In total, 18 field isolates were selected based on host plant, origin, and field

Table 1. Host plants, geographic origins, and sequences of *Cucumber mosaic virus* (CMV) isolates and their satellite (sat)RNA

Host origin and isolate	Geographic origin ^a	Target gene	Name	GenBank accession number
<i>Lens culinaris</i> Medik.				
Psi182_lentil	Thessaloniki (AUPh)	CMV CP	CPPsi182_lentil	KT734830
Psi220_lentil	Thessaloniki (AUPh)	CMV CP	CPPsi220_lentil	KT734831
Psi191_lentil	Thessaloniki (AUPh)	CMV CP	CPPsi191_lentil	KT734832
21_lentil	Karditsa	CMV satRNA	sat_21_lentil	KT382264
24_lentil	Attiki (AUA)	CMV satRNA	sat_24_lentil	KT382268
25_lentil	Attiki (AUA)	CMV satRNA	sat_25_lentil	KT382269
<i>Phaseolus vulgaris</i> L.				
43_bean	Viotia (Thiva)	CMV CP	CP43_bean	KT734835
		CMV satRNA	sat_43_bean	KT382261
43_bean_tobacco ^b		CMV CP	CP43_bean_tobacco	KT734836
		CMV satRNA	sat_43_bean-tobacco	KT382258
48_bean	Argolida (Drepano)	CMV CP	CP48_bean	KT734842
		CMV satRNA	sat_48_bean	KT382266
48_bean-tobacco ^b		CMV CP	CP48_bean-tobacco	KT734848
45_bean	Fthiotida (Tithorea)	CMV CP	CP45_bean	KT734834
49_bean	Preveza (Mitikas)	CMV CP	CP49_bean	KT734840
52_bean	Preveza	CMV CP	CP52_bean	KT734838
A46_bean	Pella (Megaplatanos)	CMV CP	CPA46_bean	KT734844
A47_bean	Pella (Aridaia)	CMV satRNA	sat_A47_bean	KT382263
B8_bean	Pierria (Elatochori)	CMV CP	CPB8_bean	KT734851
<i>Phaseolus coccineus</i> L.				
55_bean	Kastoria (Argos Orestiko)	CMV CP	CP55_bean	KT734843
		CMV satRNA	sat_55_bean	KT382267
57_bean	Florina (Prespes)	CMV CP	CP57_bean	KT734837
<i>Vigna unguiculata</i> L.				
46_bean-tobacco ^b	Argolida (Drepano)	CMV CP	CP46_bean-tobacco	KT734849
		CMV satRNA	sat_46_bean-tobacco	KT382262
47_bean	Argolida (Drepano)	CMV CP	CP47_bean	KT734841
		CMV satRNA	sat_47_bean	KT382265
47_bean-tobacco ^b		CMV CP	CP47_bean_tobacco	KT734852
<i>Solanum lycopersicum</i> L.				
A12_tomato	Argolida (Argoliko)	CMV CP	CPA12_tomato	KT734847
A12A tomato-tobacco ^b		CMV CP	CPA12A_tomato-tobacco	KT734845
A15_tomato	Argolida (Argoliko)	CMV CP	CPA15_tomato	KT734846
		CMV satRNA	sat_15_tomato	KT382259
A10_tomato-tobacco ^b	Argolida (Argoliko)	CMV CP	CPA10_tomato-tobacco	KT734839
44_tomato-tobacco ^c	Korinthia (Korinthos)	CMV CP	CP44_tomato-tobacco	KT734850

^a AUPh = farm of the Aristotle University of Thessaloniki and AUA = farm of the Agricultural University of Athens.

^b Mechanically inoculated on *Nicotiana tabacum* ‘Samsun’.

Table 2. Sequences of primers used in polymerase chain reactions for the detection and characterization of Greek legume isolates of *Cucumber mosaic virus* (CMV) and its satellite (sat)RNAs^a

Primer name	Primer sequence (5' to 3')	Position on RNA3 (nt)	Amplicon size (bp)	Target
CMV_CP	F: AACCAGTGCTGGTCGTAACC R: GTTGGCTTGGACTCCAGATG	1,274–1,745 ...	472 ...	CMV CP ...
CMV_satRNA	F: GTTTTGTGTTGTGGAGAGTTGCG R: GGGTCCTGTAGAGGAATGTGACATT	Full length ...	340 ...	satRNA ...
CMV I ^b	F: GCCACCAAAAATAGACCG R: ATCTGCTGGCGTGGATTCTT	1,484–2,076 ...	593 ...	CMV CP (sub I) ...
CMV II ^b	F: AACCAGTGATTTACCATCGC R: CTACGTTTATCTTCC	970–1,674 ...	704 ...	CMV CP (sub II) ...
18S ^c	F: AAACGGCTACCATCCAAG R: CCCATCCCAAGGTTCAACTA		250 ...	18S rRNA ...

^a Abbreviations: nt = nucleotides and CP = coat protein.

^b Chen et al. (2011).

^c Giakountis et al. (2015).

symptoms for further analysis: 8 from common bean (*Phaseolus vulgaris* L.), 2 from scarlet runner bean (*P. coccineus* L.), 2 from cowpea (*Vigna unguiculata* L.), and 6 from lentil (*Lens culinaris* Medik.). Four isolates from field tomato (*Solanum lycopersicum* L.) in southern Greece were included as references for symptom expression in our studies; three isolates originated from plants showing a bushy appearance, fernleaf-shoestring symptoms, and internal browning of the fruit (A10, A12, and 44) and the fourth isolate (A15) from a plant showing lethal necrosis (Table 1). All field legume isolates were mechanically inoculated onto tomato (*Lycopersicon esculentum* Mill.) 'Ace 55VF' plants as well as tobacco (*Nicotiana tabacum* 'Samsun') plants to assay for the development of the necrotic syndrome, using 0.1 M phosphate buffer (pH 7.2). Lentil isolates were not included in this bioassay due to the poor quality of the infected plant tissues. The field isolates from tomato (A12a), common bean (43 and 48) and cowpea (47) were further molecularly analyzed after mechanical inoculation of tobacco plants designated as "tomato_tobacco" (from tomato) and "bean_tobacco" (from bean and cowpea) isolates, respectively. The CP sequences of tomato isolates A10 and 44 as well as the cowpea isolate 46 were only analyzed from the mechanically inoculated tobacco plants (Table 1).

Primer design. The complete reference nucleotide sequences of CMV CP (AY380533.1 and GU362669.1) and CMV satRNA (X69136.1 and D89673.1) were used to design polymerase chain

reaction (PCR) primers using the PrimerQuest Tool (Integrated DNA Technologies). Designed primers (Table 2) were further validated in silico in terms of target specificity using the Primer-BLAST tool of the National Center for Biotechnology Information against the viral and plant taxids and found to be only specific for the intended viral target sequences.

Total RNA extraction, DNase treatment, and cDNA synthesis.

Total RNA was extracted from liquid nitrogen powdered infected leaf tissue (100 mg) with TRI reagent (Molecular Research Center, Inc.), according to the manufacturer's protocol. Extracted RNA was quantified with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) and its integrity was examined in agarose gels. Total RNA (10 µg) was treated with DNase I (10U; New England Biolabs) for 1 h at 37°C. DNase-treated RNA (1 µg) was used in reverse transcription (RT) reactions with MML-V-reverse transcriptase (200 U) and random primers (Life Technologies). RT of an equimolar pool of all RNA samples without the addition of the respective enzyme was used as a negative control (no RT).

PCR. After RT, the cDNA template was diluted to 400 µl with double-distilled H₂O. The diluted cDNA template (4 µl) was amplified with PCR in a 20-µl final volume using 2.5 U of Taq polymerase (Invitrogen). The primers used are presented in Table 2. PCR conditions were initial denaturation at 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 58 to 63°C (depending on the primers) for 30 s, and extension at 72°C for 30

Table 3. *Cucumber mosaic virus* (CMV) strains or isolates (name, accession number, host, and origin) used in phylogenetic analysis of the coat protein sequences of the Greek legume and tomato isolates

Name	Accession	Host	Origin
ABI	L36525	Gladiolus	South Korea
AMA	AJ131625	<i>Amaranthus</i>	Taiwan
As	AF013291	Unknown	South Korea ^a
B2	AB046951	<i>Musa sapientum</i>	Indonesia
Ban	U43888	<i>M. acuminata</i>	Israel
BKD257	AJ131621	Ornamental crop	The Netherlands
C	D00462	<i>Cucurbita pepo</i>	United States
C7-2	D42079	Unknown	Japan
CS	D28489	<i>Limonium sinuatum</i>	Japan
D8	AB004781	Japanese radish	Japan
DEL	AJ131626	Larspur	Taiwan
Dasheen	AF535156	Dasheen	China
E5	D42080	Unknown	Japan
EUS	AJ131627	<i>Lisianthus</i>	Taiwan
Fny	U20668	Unknown	United States (New York)
FT	D28487	Field tomato	Japan
GPP	AJ131623	Gladiolus	The Netherlands
Hi	U31219	<i>Musa</i>	United States
HL	AB049568	Lily	Japan
IA	AB042294	Unknown	Indonesia
IPO	AJ304399	<i>Alstroemeria</i>	The Netherlands
Ix	U20219	<i>Tomato</i>	Philippines
K	AF127977	Unknown	China
Kin	Z12818	Unknown	UK
Km	AB004780	Melon	Japan ^a
Kor	L36251	Unknown	Korea
LBO	AJ131615	Lily	The Netherlands
Leg	D16405	Cowpea	Japan
LiNB	AJ131618	Lily	Taiwan
LiSR	AJ131617	Lily	The Netherlands
LiTW	AJ131619	Lily	Taiwan
LK	AJ495841	Lily	Korea
Ly2	AJ296154	<i>Lilium longiflorum</i>	Korea
M	D10539	Unknown	UK
M2	AB006813	Unknown	Japan ^a
M48	D49496	Unknown	Taiwan ^a
Mf	AJ276481	Unknown	South Korea

(Continued)

Table 3. (Continued)

Name	Accession	Host	Origin
Musa	U32859	Banana	Colombia ^a
MY17	AF103993	Unknown	Japan
N	D28486	<i>Limonium sinuatum</i>	Japan
N11	AJ276587	<i>Alstroemeria</i>	The Netherlands ^a
Nd-Ca	AY429432	<i>Arachis hypogaea</i> L.	China
Nd-Cs	AY429437	<i>A. hypogaea</i> L.	China
Nd-S	AJ131620	Ornamental crop	China
Ns	AJ511990	<i>Nicotiana glutinosa</i>	Hungary
NT9	D28778	Unknown	Taiwan ^a
Ny	U22821	Unknown	Australia ^a
O	D00385	Unknown	Japan
Oahu	U31220	<i>Musa</i>	United States
PE	AF268597	Passionflower	China
Pepo	AF103991	Squash	Japan
Pf	AJ237850	Unknown	United States
Plant	U32858	Plantain	Colombia ^a
PNPL	AJ304402	<i>Alstroemeria</i>	The Netherlands ^a
PR6A	AJ304403	<i>Alstroemeria</i>	The Netherlands ^a
Q	M21464	Unknown	Australia
R	Y18138	<i>Ranunculus</i>	France ^a
Rs	AJ517802	<i>Raphanus sativus</i>	Hungary
SD	AB008777	Unknown	China ^a
Si	AB063333	<i>Silene armeria</i>	Japan
SN	U22822	Unknown	Australia ^a
SO	AF103992	Unknown	Japan
SP103	U10923	<i>Spinacia oleracea</i>	United States
SP104	U10924	<i>S. oleracea</i>	United States
Tfn	Y16924	Tomato	Italy
TN	AB176847	Tomato	Japan
Trk7	L15336	Clover	Hungary ^a
Wem	U61285	Unknown	South Africa ^a
WL	D00463	Unknown	United States
Xb	AF268598	Banana	China
Ane1	LN810056	<i>Anemone</i> sp.	Greece
Ane2	LN810057	<i>Anemone</i> sp.	Greece
G10	AY541691	<i>Nicotiana tabacum</i>	Greece
G2	AY450854	<i>N. tabacum</i>	Greece
GRc11	LN810059	<i>Citrullus lanatus</i>	Greece
GRc12	LN810060	<i>C. lanatus</i>	Greece

^a Country of study without reported origin.

s; followed by a final elongation step of 72°C for 10 min. PCR annealing conditions and concentration of MgCl₂ were optimized for each primer pair separately. PCR products were analyzed by electrophoresis in 1% agarose gels coupled to ethidium bromide staining. DNA bands were gel extracted (PureLink gel extraction kit; Life Technologies) and sent for PCR sequencing. PCR with primers against the 18S gene sequence were used as a positive control to check cDNA quality. Samples without a template served as extra controls to evaluate excessive primer-dimer formation or as a measure of potential contamination of PCR assay components with the target template, together with the no-RT control and cDNA synthesized from healthy plants.

Sequencing and phylogenetic and recombination analysis. Sequencing of PCR products was performed in both VBC Biotech (<http://www.vbc-biotech.at/index.php/sequencing.html>) and CEMIA (<https://cemia.eu/company/>) facilities, with similar resulting quality of sequencing runs. Sequencing quality was inspected with DNASTar software (<http://www.dnastar.com>) and sequences were aligned with the CLC main workbench 7 software (<https://www.qiagenbioinformatics.com/products/clc-main-workbench/>). Phylogenetic analysis was performed using the maximum-likelihood method embedded in MEGA software, version 6 (Tamura et al. 2013), with Tamura-Nei as the substitution model, which is considered the most appropriate model of evolution (Nylander 2004), and the default parameter in MEGA and 100 rounds of bootstrapping. Percentage Identity Matrices were calculated with MUSCLE using the same software (MEGA 6) and trees were visualized with CLC main workbench 7. Recombination analysis was performed with the RDP4 package (<http://web.cbio.uct.ac.za/~darren/rdp.html>) following a multidisciplinary sequence analytic approach through the combinatorial use of RDP, Geneconv, Chimaera, MaxChi, Bootscan, SiScan, and 3Seq and standard settings (Bonferroni correction, highest acceptable *P* value set to 0.05) and 10,000 per mutations. The same aligned CP sequences were used for the construction of the phylogenetic tree and for the detection of recombination events.

Results

Detection of CMV. With RNA extracted from plants infected with Greek CMV isolates, RT-PCR with the CMV_CP primers resulted in amplification of a single band of approximately 650 bp. Failure to amplify the CP gene from 21_lentil, 24_lentil, 25_lentil, and A47_bean may be due to some strain-specific variability; RNA quality issues cannot be excluded, although the necessary quality control tests were applied (18S amplification and agarose gel electrophoresis of RNA). All amplified products were successfully sequenced (Table 1), aligned against the RNA3 of CMV, and found to be specific for the CP gene (results not shown).

Phylogenetic and recombination analysis of CMV CP. These CP sequences were aligned with 76 CP sequences selected from GenBank, including 6 sequences already available from Greece from watermelon (LN810059 and LN810060), tobacco (AY541691 and AY450854), and *Anemone* spp. (LN810056 and LN810057) (Table 3), which represent the three known subgroups of CMV (IA, IB, and II) (Fig. 1). All recently collected Greek isolates clustered in subgroups IA and IB. Only, the already available isolates from *anemone* clustered in group II. Greek IA isolates shared an average 99% sequence identity with one another, and 95.5 and 94.5% sequence identity with the bean- and tobacco-infected IB isolates, respectively. The cowpea isolate A46 deviated slightly (4 to 5%) more from the average percentages of the other analyzed Greek IA isolates. Common bean, scarlet runner bean, and tomato isolates clustered within both IA and IB, while the two cowpea isolates (46 and 47) clustered within the subgroup IB. The three lentil isolates (PSi182, PSi220, and PSi191) originating from the same area clustered in subgroup IA. At the level of host-plant comparison, bean isolates shared a slightly higher sequence identity with the lentil isolates (97%) when compared with the tomato strains (94%). Within IA and IB, some Greek CMV isolates were genetically close or identical to isolates from distant countries such as IA isolates from the United States and Hungary and IB isolates from China and Taiwan (Fig. 1).

In order to validate the classification of our isolates, we performed RT-PCR analysis with primers specific for CMV groups I and II

Fig. 1. Phylogenetic analysis comparing Greek *Cucurbit mosaic virus* (CMV) capsid protein (CP) sequences and sequences from GenBank. Bootstrap values greater than 60/100 are indicated on the corresponding branches. All Greek isolates analyzed cluster together with isolates from subgroups IA and IB. Hosts origins for Greek CMV isolates are denoted by (i) triangles for bean (common bean, scarlet runner, and cowpea); (ii) squares for lentil; (iii) arrow for tomato; (iv) circles for bean passed through tobacco; and (v) diamonds for tomato passed through tobacco. The tomato Greek isolate associated with lethal necrosis in the field (A15) is shown with a star.

(Table 2) (Chen et al. 2011). The PCR analysis confirmed the phylogenetic tree results, because bands were detected only with group I-specific primers, whereas the respective reactions with primers specific for group II were negative (results now shown). In conclusion, both the phylogenetic tree analysis of CMV CP gene and the RT-PCR using specific primers confirmed that all Greek CMV isolates in this study belong to group I. Finally, recombination analysis did not detect any significant recombination events among the CMV strains from either the Greek or the global cohort (data not shown).

Detection and analysis of CMV satRNA. RT-PCR analysis was performed on satRNAs associated with the CMV-infected Greek legume isolates. Gel electrophoresis detected a single band of approximately 300 bp in 6 of 12 field bean isolates and 3 of 6 field lentil isolates. Only A15 of the tomato isolates showing lethal necrosis contained a satRNA. The field plants infected with CMV isolates 43 (common bean) and 46 (cowpea) were infected with satRNA, as were the respective tobacco plants infected with these isolates. However, when the 47_bean (cowpea) and 48_bean (common bean) isolates were mechanically inoculated onto tobacco plants, only CMV without satRNA was detected, even though tobacco is believed to be a suitable host for CMV satRNAs (Zahid et al. 2015).

Phylogenetic analysis of CMV satRNA. The sequences of the DNA amplified from satRNAs (Table 1) were aligned together with 35 selected full length CMV satRNA sequences from GenBank (Table 4), which represent all reported satRNA groups (non-necrogenic, including benign A and B, and necrogenic), and which were used to construct a phylogenetic tree (Fig. 2). The Greek satRNA isolates from CMV-infected legumes and the one from tomato clustered together with the isolates of the necrogenic group, sharing 86.5% nucleotide identity. Sequence identity within the bean CMV satRNAs and between bean and lentil satRNAs was very high at 95 and 90%, respectively.

The necrotic syndrome has been correlated with the presence of a “consensus” sequence (GA-GCUAAGGCUUA-UCUAUGCUGAU) and, more specifically, with G at position 318, U at position 323, or C at position 325, which are absent in the non-necrogenic isolates (Devic et al. 1990). Analysis of the full-length sequence alignment of the satRNAs showed that the Greek CMV satRNAs also varied in sequence outside of the necrogenic motif. A thorough inspection of the 3' end sequence of the aligned Greek and foreign satRNA isolates confirmed that all Greek CMV satRNA isolates carried an intact necrogenic motif, similar to other necrogenic isolates

Table 4. Isolates or strains used for the phylogenetic analysis of *Cucumber mosaic virus* satellite (sat)RNA, their original host and the symptoms observed, the characteristic symptoms associated with their experimental presence or absence on tomato, and the subgroup classification of the satRNA and its associated virus (data as recorded in the GenBank or the respective citations)

Isolate name	Accession	satRNA subgroup ^a	CMV Group ^b	Origin	Natural infection ^c		Experimental infection (in tomato) ^c	
					Host	Symptoms	+satRNA	–satRNA
CE-satRNA	X86425	Necrogenic	I	Italy	Celery	bs, mo	ln	fi
PG-satRNA	X86426	Necrogenic	II	Italy	Tomato	ln	ln	fi
T2-satRNA	X86410	Necrogenic	II	Italy	Tomato	ln	ln	...
T6-satRNA	X86414	Necrogenic	II	Italy	Tomato	ln	ln	...
T7-satRNA	X86411	Necrogenic	II	Italy	Tomato	ln	ln	...
T8-satRNA	X86420	Necrogenic	I+II	Italy	Tomato	ln	ln	...
T12-satRNA	X86416	Necrogenic	I	Italy	Tomato	ln	ln	fi
T16-satRNA	X86413	Necrogenic	II	Italy	Tomato	ln	ln	...
T18-satRNA	X86708	Necrogenic	II	Italy	Tomato	ln	ln	...
T27-satRNA	X86419	Necrogenic	I	Italy	Tomato	ln	ln	...
T57-satRNA	X86418	Necrogenic	II	Italy	Tomato	ln	ln	fi
T59-satRNA	X86423	Necrogenic	I	Italy	Pepper	ss, mo	ln	fi
TO77-satRNA	X86422	Necrogenic	I	Italy	Tobacco	ns, mo	ln	fi
TO79-satRNA	X86417	Necrogenic	I	Italy	Tobacco	ns, mo	ln	fi
TO80-satRNA	X86421	Necrogenic	I	Italy	Tobacco	ns, mo	ln	fi
J876-satRNA	U31660	Necrogenic		United States	Tomato	...	ln	...
T43-satRNA	D10039	Necrogenic		Japan	Tobacco	...	sn	...
I17N-satRNA	M18867	Necrogenic		...	...	...	...	...
I17R(3)-satRNA	M18871	Unknown		...	...	...	...	...
D27-satRNA	U31661	Non-necrogenic		United States	Tomato	...	ns	fi
K8-satRNA	X53534	Non-necrogenic	I	Italy	<i>Cucumis metuliferus</i>	...	...	...
NG71-satRNA	X86424	Non-necrogenic	I	Italy	<i>Nicotiana glauca</i>	mo	fi	fi
Tfn-satRNA	X65455	Non-necrogenic	I	Italy	Tomato	ifn	ns	fi
TTS-satRNA	X69136	Non-necrogenic (benign B)	I	Italy	Tomato	ss	st	fi
T1-satRNA	X86409	Non-necrogenic (benign B)	II	Italy	Tomato	fi	fi	...
T4-satRNA	X86415	Non-necrogenic (benign B)	II	Italy	Tomato	fi	fi	...
T8A-satRNA	X86408	Non-necrogenic (benign B)	II	Italy	Tomato	fi	fi	fi
T22-satRNA	X86412	Non-necrogenic (benign B)	II	Italy	Tomato	fi	fi	fi
B1-satRNA	M16586	Benign B		United States ^d	...	...	...	...
B2-satRNA	M16587	Benign B		United States ^d	...	...	...	...
G-satRNA	M16585	Benign B		United States	Squash	...	...	...
Ra-satRNA	D00541	Benign B		...	...	...	...	...
S-satRNA	M14934	Benign B		...	...	...	...	...
WL2-satRNA	M16590	Benign B		United States	Tomato	...	...	...
OY2-satRNA	M20845	Benign A		Japan ^d	...	...	...	...

^a satRNA subgroups according to Grieco et al. (1997) (satRNA isolates CE, PG, T2, T6, T7, T8, T12, T16, T18, T27, T57, T59, TO77, TO79, TO80, K8, NG71, Tfn, TTS, T1, T4, T8A, and T22), Tousignant and Kaper (1993) (satRNA isolates J876 and D27), Devic et al. (1990) (satRNA isolates G, I17 N, B1, B2, Ra, S, WL2, and OY2), or GenBank information (satRNA isolates T43 and K8).

^b CMV grouping of the associated virus.

^c Abbreviations: bs = bushy stunt, fi = filiformity, ifn = internal fruit necrosis, ln = lethal necrosis, mo = mosaic, ns = no symptoms, sn = stem necrosis, ss = severe stunting, and st = stunting.

^d Country of study without reported origin.

and in contrast to non-necrogenic isolates (Fig. 3). Even though the number of satRNAs analyzed is small, these results indicate that, thus far, no non-necrogenic isolates have been detected in Greece.

Legume CMV isolates and symptoms. Regardless of the presence of satRNA, all common bean, scarlet runner bean, and cowpea

plants infected by CMV in the field exhibited common mosaic or green mottling, leaf curling, deformation or roughness, while lentil plants had a bushy appearance with narrowed leaves. The tomato plants mechanically inoculated with the CMV isolates with satRNA from tomato (A15), common bean (43, 48, and A47), runner bean

Fig. 2. Phylogenetic analysis comparing Greek *Cucumber mosaic virus* (CMV) satellite (sat)RNAs and sequences from GenBank representing both necrogenic and non-necrogenic (including benign A and benign B) isolates. Bootstrap values greater than 60/100 are indicated on the corresponding branches. All Greek isolates clustered together with isolates from the necrogenic subgroup. Host origins of Greek CMV isolates supporting satRNA isolates are denoted by (i) triangles for bean (common bean, scarlet runner, and cowpea); (ii) squares for lentil; and (iii) circles for bean passed through tobacco. The tomato Greek isolate associated with lethal necrosis in the field (A15) is shown with a star.

(55), and cowpea (46, and 47) exhibited top necrosis 10 to 15 days after inoculation. The tomato plants infected with the mechanically inoculated isolates not associated with satRNAs exhibited mainly mosaic and fern leaf-shoestring symptoms but no necrosis. All tobacco plants infected with these isolates exhibited mosaic and leaf malformation symptoms.

Discussion

The existence of satRNAs and, especially, those which prompt necrotic symptoms forms a most interesting trait in CMV epidemiology (García-Arenal and Palukaitis 1999; Kouadio et al. 2013; Masuta 2014; Palukaitis and García-Arenal 2003). In natural CMV populations, necrogenic satRNAs seem infrequently to occur in non-tomato hosts but their incidence increases during epidemics in tomato as they accumulate in the developing parts of the plant and will be further spread within the crop. As soon as parts of the tomato plant become necrotic, the necrogenic satRNAs are no longer accessible for aphid transmission and, thus, reach an epidemiological dead end (Alonso-Prados et al. 1998; Aranda et al. 1997; Escribe et al. 2000a; Fraile et al. 1997; Grieco et al. 1997). Once the necrogenic satRNAs are at a disadvantage in tomato plants (Escribe et al. 2000a), their introduction to the tomato crop reflects at its onset a spread from hosts without necrotic symptoms (Betancourt et al. 2013; Escribe et al. 2003). Evolutionary studies suggest that crops rather than weeds are the primary sources for group I isolates of CMV and their satRNAs (Gallitelli 2000). Cucurbits, one of the major hosts of CMV, may harbor necrogenic or non-necrogenic satRNAs without any obvious difference in symptom expression (Betancourt et al. 2011; Moriones et al. 1991). Our study showed, for the first time, that necrogenic satRNAs are associated at a relatively high frequency (47.4% in this study) with CMV-infected legumes that exhibit only mild symptoms (Supplementary Fig. S1) but which express severe necrotic symptoms when transferred to tomato. In this study, only symptomatic field

plants were analyzed; therefore, it cannot be excluded that asymptomatic legumes may also harbor satRNAs. A more extended study may also reveal the presence of non-necrogenic satRNAs in CMV-infected legumes.

Host-plant species determine the effect of satRNA on symptom expression of its associated virus, which subsequently influences fitness parameters of the complex, thus playing a pivotal role in plant invasion and in vector-mediated spread (Escribe et al. 2000b; García-Arenal et al. 2000; Roossinck et al. 1992). This host effect has been thoroughly studied for cucurbits (Betancourt et al. 2011, 2013; Escribe et al. 2000a; Moriones et al. 1991). The effect of legumes on the evolution and epidemiology of CMV and its necrogenic satRNAs is not well studied; however, some implications can be made. One of the main features is the high seed-transmission rate, reaching up to 49% in some bean varieties (Davis and Hampton 1986). Taking into account that satRNAs are encapsidated with the genome of their associated virus, it is expected that the satRNAs are also seed transmitted with CMV. This key trait in their survival and long-distance spread requires more detailed study. Such a host-specific feature could limit the genetic diversity of CMV satRNAs generated in natural populations by point mutations and recombination (Alonso-Prados et al. 1998; Aranda et al. 1997; Grieco et al. 1997).

The phylogenetic analysis of the CP gene of the Greek CMV isolates from legumes showed the presence of only group I isolates, which are considered the most virulent ones (Mochizuki and Ohki 2012; Roossinck 2002). Group I isolates were originally detected in Greek field tomato crops during surveys performed in 1995 and 1996 (Varveri and Boutsika 1999), while a tomato IB isolate was collected during epidemics in 1998 (Sclavounos et al. 2006) and two watermelon isolates (IA and IB) were recently identified (Xanthis et al. 2015b). The so-called “heat-sensitive” isolates of group II that are common in cooler areas or seasons in Northern latitudes (Jacquemond 2012) also occur around the Mediterranean area in Northern Tunisia, Spain, and Italy (Ben Tamarzizt et al. 2013; Bonnet et al. 2005; Davino et al. 2012; Fraile et al. 1997; Hsu et al. 2000). In Greece, group II isolates were only reported on *N. glauca* (Vovlas and Di Franco 2004) and *Anemone* spp. (Xanthis et al. 2015a). The lack of these isolates in our survey is not surprising because worldwide studies show that, on average, only 20% of CMV isolates belong to this group (Hsu et al. 2000). Our study detected only a limited genetic diversity among Greek legume isolates, with no apparent relationship with their specific field host or origin. In general, CMV populations show a rather limited variability (Ben-Tamarzizt et al. 2013; Bonnet et al. 2005; Eiras et al. 2004; García-Arenal et al. 2000; Masuta 2014; Nouri et al. 2014), without any obvious correlation between virus phylogeny and host, geographic location, and year of collection or surveys (Davino et al. 2012; Lin et al. 2003, 2004; Thompson et al. 2015). Only in natural CMV populations in Tunisia and California was a genetic differentiation observed in terms of haplotype frequencies and the presence of resistance-breaking strains (Ben-Tamarzizt et al. 2013; Lin et al. 2003). Evidence for recombination events that could increase genetic heterogeneity (Ben-Tamarzizt et al. 2013; Bonnet et al. 2005; Fraile et al. 1997; Lin et al. 2004; Ohshima et al. 2016) was not found in our isolates.

The Greek legume and tomato CMV CP sequences clustered within both subgroups IA (type strain Fny and C) and IB (type strain Tfn), and they all had great affinities to the previously characterized sequences from Greek watermelon and tobacco. CMV IA isolates are present all over the world but IB strains are thought to be of East Asian origin (Jacquemond 2012; Palukaitis and García-Arenal 2003; Roossinck 2002). In Europe, IB isolates of CMV occur in Italy and Spain (Aramburu et al. 2007; Bonnet et al. 2005; Fraile et al. 1997; Gallitelli 2000), possibly imported from Asia with infected pepper and tomato plants (Ben-Tamarzizt et al. 2013). CMV-infected legume seed (Fraile et al. 1997; Jacquemond 2012) may represent an efficient mean for global migration and possibly explain the presence of the IB isolates in the Greek legumes. The observed narrow genetic distance among the Greek isolates and isolates from other countries point toward a common ancestor, origin, or “founder effect”

Fig. 3. Alignment of the 3' termini, delimiting the necrogenic consensus according to Devic et al. (1990), of the Greek and reference isolates of *Cucumber mosaic virus* (CMV) satellite (sat)RNA sequences. Asterisks mark the specific nucleotides of the active domain in necrogenic satRNAs which differ from the non-necrogenic ones. Host origins of Greek CMV isolates are denoted by (i) triangles for common bean, scarlet runner, and cowpea; (ii) squares for lentil, and (iii) circles for bean passed through tobacco. The tomato Greek isolate associated with lethal necrosis in the field (A15) is indicated by the star.

(Fraile et al. 1997; Lin et al. 2004), or strong genetic bottlenecks such as host-plant- or aphid-mediated transmission (Ali et al. 2006; Betancourt et al. 2013; Li and Roossinck 2004; Moury 2004) and, possibly, seed transmission (Ali and Roossinck 2008). Several domains in the amino acid sequence of the CP are associated with CMV host adaptation (e.g., the bean-adapted CMV genotypes in New York) (Thompson et al. 2015).

Our results suggest that legumes may play a significant role in the epidemiology of CMV, as demonstrated for melon (Betancourt et al. 2011). This may be particularly true for legume landraces, crops of which are established with the use of noncertified seed. In legume production areas, the succession of winter (lentil) and spring legumes (bean and cowpea) may also provide this complex with the necessary hosts to build up a high population throughout the year. Legumes are usually not systematically targeted by insecticides so that, during mild winters, large aphid populations develop early in the season, a situation known to favor invasion of CMV populations by necrogenic satRNAs (Betancourt et al. 2016; Escriu et al. 2003). When such virus sources are cultivated in the vicinity of tomato crops, any factor triggering aphid migration may initiate a devastating epidemic. On the other hand, low temperatures early in the season favor multiplication of necrogenic satRNAs (White et al. 1995), which can infect tomato, resulting in a complete crop failure. Such a scenario could explain the erratic reemergence of the tomato necrotic syndrome as encountered in several areas of Greece.

The composition of the local flora contributes to a host-associated differential selection of satRNA genotypes (Betancourt et al. 2011, 2013; Moriones et al. 1991; Roossinck and Palukaitis 1991) affecting the spread and, consequently, the composition and evolution of the satRNA-CMV population complex in specific areas. Our results suggest that legumes may be an important component of this complex system affecting the development of the necrotic epidemics in tomato crops. Further studies are needed to fully explore their role. However, in the absence of a well-defined resistance and in light of the aforementioned results, the cocultivation of legumes with tomato should be avoided.

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