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A severe Hellenic CMV tomato isolate: symptom variability in tobacco, characterization and discrimination of variants

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Abstract

A severe strain of *Cucumber mosaic virus* (CMV) originating from an infected tomato plant (Gastouni-Olympia, Greece) was isolated in tobacco (*Nicotiana tabacum* cv. Xanthi nc), after three serial local lesion passages in *Chenopodium quinoa* and designated CMV-G. CMV-G induces yellow mosaic (YM) symptoms in tobacco. When CMV-G was passed mechanically through *C. quinoa*, phenotypic variants inducing YM or green mild mosaic (MM) in tobacco were isolated. Aphid transmission, from different hosts, appears to be an effective approach for separating MM variants of CMV-G from YM variants. In particular, aphid transmission from zucchini proved to be very efficient in selecting for MM variants. In contrast, aphids transmitted only YM variants from tomato plants. Molecular characterization of CMV-G and its progeny resulted in their classification in the CMV subgroup IB, free of satellite RNA, being the first discovery of the subgroup IB in Greece. In the *Solanaceae* family (tobacco, tomato, pepper) YM variants induced more severe symptoms than the MM variants. YM and MM phenotype was stable in tobacco for all seven passages tried using the obtained YM and MM variants. Cross-protection experiments showed that an isolated MM variant was able to protect tobacco plants against a challenge infection by a YM variant.

Introduction

Cucumber mosaic virus (CMV), the type species of the genus *Cucumovirus* in the family *Bromoviridae*, has a worldwide distribution infecting more than 1000 plant species in 100 families and can be transmitted by at least 86 aphid species in a non-persistent manner (Edwardson and Christie, 1991). CMV, first described in cucumber in 1916 by Doolittle, was known to induce symptoms such as fern leaf/shoestring and mottle in tomato. However, new severe forms of the disease in tomato have occurred since the 1970s in the Mediterranean region, causing severe outbreaks; the disease in tomato displays leaf and plant shrinkage, upward leaf curling, vein purpling, chlorosis, pericarp hardening and discolouration, plant and fruit necrosis and even plant death (Putz et al., 1974; Kyriakopoulou et al., 1991, 2000a; Jorda et al., 1992; Gallitelli, 1998, 2000). The common symptoms of CMV on tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) are stunting, deformations and mild mosaic (MM) on leaves; however, a few CMV strains induce yellow mosaic (YM) on tobacco leaves (Shintaku, 1991).

CMV maintains a large and diverse population of field isolates (Rodriguez-Alvarado et al., 1995), which can be divided into two groups,

namely groups I and II (Owen and Palukaitis, 1988). Group I and II isolates can be differentiated by serology, molecular hybridisation and RT-PCR/RFLP analysis (Gallitelli, 2000; Finetti-Sialer et al., 1999). A number of CMV isolates within group I, suggested to have originated from Asia, can be placed in the subgroup IB, whereas the rest compose the subgroup IA (Finetti-Sialer et al., 1999; Palukaitis and Zaitlin, 1997). Twenty-nine tomato-infecting CMV isolates obtained during 1995–1996 in Greece were found to belong to subgroup I (Varveri and Boutsika, 1999).

The genome of CMV consists of three plus sense single-stranded RNA species (RNA 1, RNA 2, RNA 3), and a subgenomic RNA (RNA 4). Many CMV isolates contain an additional, fifth, satellite RNA (satRNA), 333–405 nucleotides long, which is not essential for CMV replication, has little or no homology to viral genomic RNAs, does not encode any known proteins, is dependent on CMV for its replication and is encapsidated along with the viral genomic RNAs (Gallitelli, 2000). The presence of satRNA can modify the symptoms of CMV, depending on the host species, the CMV strain and the satellite sequence (Kaper and Waterworth, 1981; Roossinck, 2002; Gallitelli, 2000).

CMV disease outbreaks in tomato, pepper or cucurbits result in great economic losses for many countries (Gallitelli, 2000), including Greece (Kyriakopoulou et al., 1991, 2000a). The means of resistance to CMV depends on the use of transgenic plants, resistant or tolerant varieties and cross-protection (Gallitelli, 2000). In 1998, one of the most severe CMV outbreaks in Greece occurred in field-grown tomatoes in Gastouni-Olympia, as well as in other regions of Greece (Kyriakopoulou et al., 2000b). Three serial local lesion passages on *Chenopodium quinoa* were employed for obtaining an isolate of the virus from a field-grown tomato plant from this outbreak. This isolate induced yellow mosaic (YM) symptoms in tobacco and was designated CMV-G. The aim of this work was to characterize CMV-G, isolate and study its phenotypic variants. In particular, we focused on ways to isolate mild field CMV variants by employing different hosts and means of transmission, and the use of a mild variant obtained in cross-protection studies.

Materials and methods

Virus isolates, viral propagation, viral transmission and plant inoculation methods

CMV-S, CMV-D, and CMV-P6 isolates were a kind gift of Drs. J. Kaper and M. Tousignant (USDA Agricultural Research Service, Beltsville, Maryland, USA). These isolates were supplied as dried tobacco leaf samples in $CaCl_2$ and propagated in *Nicotiana tabacum* cv. Xanthi nc. An isolate of CMV, named CMV-G, was obtained from tomato (*Lycopersicon esculentum*) exhibiting leaf malformation and mottle, during a serious CMV disease outbreak in the industrial tomato area of Eleia prefecture (Gastouni-Olympia), in 1998. Three single local lesion passages in *C. quinoa* were employed to obtain this isolate of CMV. Tobacco (*N. tabacum* cv. Xanthi nc) was used as the basic virus source plant in our experimentation. Upper leaves from this tobacco plant were harvested, dried on $CaCl_2$, stored at 4 °C, and used as the source of inoculum (mother isolate).

For the mechanical inoculation method, single lesions on *C. quinoa* leaves were used to prepare sap in 100 μl 70-mM sodium phosphate buffer (pH = 7.2). Sap was spread on a Celite-dusted leaf of a *N. tabacum* cv. Xanthi nc. Plants were placed on a greenhouse bench and observed daily for symptom development. Each offspring variant was then designated as a YM or MM variant, according to the mosaic colour induced in tobacco. For the population analysis, 20–30 separate lesions from the local lesion host *C. quinoa* were cut out and ground individually in the above-mentioned buffer.

For the aphid transmission experiment, the green peach aphid *Myzus persicae* was used to transmit CMV-G from each one of the previously mechanically inoculated source plants, namely tobacco Xanthi nc, pepper (*Capsicum annuum*), tomato, zucchini (*Cucurbita pepo*) and eggplant (*Solanum melogena*). Single starved aphids were carefully deposited on a systemically infected leaf of each of the above mentioned inoculum source plants for virus acquisition (Castillo and Orlob, 1966). Each aphid individual was observed to make sure that an uninterrupted initial probe was made, carefully removed and deposited on a *N. tabacum* cv. Xanthi nc plant (indicator plant). Plants were covered with an insect cage, caged

plants were placed on a greenhouse bench and the following day they were sprayed with Confidor (Bayer, Germany) to kill the aphids. Tobacco plants, of the same age, that were not probed with aphids served as the negative control. The indicator plants were inspected daily for symptom expression. Each one of the viral offspring variants was further characterized as YM or MM variant according to the type of mosaic developed in tobacco.

Throughout the experimentation, plants were grown in an insect-proof greenhouse with temperature ranging between 22 and 27 °C. During the day, supplementary light (day light fluorescent lamps) was provided to plants, of approximately 16 h/day, to reach 20,000–25,000 lux.

Host range studies

For CMV-G and each one of its nine offspring variants (G1, 2, 3, 4, 5, 6, 7, 8, 10), 12 indicator plant species (five individual plants of each) were used in the host range experiments (Table 1). Plants were inoculated with sap prepared from infected tobacco Xanthi nc tissue, as previously described for CMV-G. Infections of experimental plants were confirmed by symptom observations and, in cases of lack of obvious symptomatology, by ELISA. The experiment was performed twice.

Discrimination methods of CMV-G variants and symptom phenotypic stability

Two approaches were used for analysis of CMV-G variants, direct single lesion analysis from mechanically inoculated *C. quinoa* and aphid transmission analysis. In the first approach, the inocula produced by single lesions in *C. quinoa* were sap-inoculated individually, in tobacco Xanthi nc to detect YM or MM variants of CMV. In the second approach, aphids were allowed only a single probe on the source plants before they were deposited on tobacco for virus transmission and symptom analysis. The original CMV-G and nine of its offspring variants, derived from the aphid transmission experiment (in tobacco), were analysed in tobacco following the single local-lesion assay in *C. quinoa*.

The stability of the YM or MM phenotype of CMV variants in tobacco was tested by seven serial passages in tobacco plants for a period of five months, in the greenhouse. To determine whether new phenotypic variants developed, each phenotypic type of isolate was analysed by mechanical inoculation in tobacco Xanthi nc.

Table 1. Symptoms of CMV-G and its offspring variants on different hosts

Host	CMV-G	Offspring variants of CMV-G								
		G1	G2	G3	G4	G5	G6	G7	G8	G10
N. tabacum Xanthi nc, Glurk, Samsun	YM, d, S	MM	YM, d, S	MM	MM	YM, d, S	YM, d, S	MM	MM	MM
N. benthamiana	YM, d, S	MM	YM, d, S	MM	MM	YM, d, S	YM, d, S	MM	MM	MM
L. esculentum 1001, 611, 609, 945	Ymot, d, S, lcu, lpu	Gmot	Ymot, d, S	Gmot	Gmot	Ymot, d, S	Ymot, d, S	Gmot	Gmot	Gmot
C. annuum 465, others	YM	MM	YM	MM	MM	YM	YM	MM	MM	MM
S. melogena[a]	mGmot	mGmot	mGmot	mGmot	mGmot	mGmot	mGmot	mGmot	mGmot	mGmot
C. sativus	YM	YM	YM	YM	YM	YM	YM	YM	YM	YM
C. pepo	YM	YM	YM	YM	YM	YM	YM	YM	YM	YM
C. vulgaris	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
C. melo	Gmot	Gmot	Gmot	Gmot	Gmot	Gmot	Gmot	Gmot	Gmot	Gmot
V. unguiculata	LL	LL	LL	LL	LL	LL	LL	LL	LL	LL
C. quinoa	LL	LL	LL	LL	LL	LL	LL	LL	LL	LL
Spinacea oleracea	B	B	B	B	B	B	B	B	B	B

Each index species or cultivar represented by five plants. The experiment was repeated twice. Positive infections confirmed by symptom observations.

YM = Yellow Mosaic, MM = Green Mosaic, Ymot = Yellow mottle, mGmot = mild Green mottle, lcu = leaf curling, lpu = leaf purpling, LL = Local Lesion, B = Blight, d = mild leaf deformation, S = stunting, NS = No Symptoms.

[a]only first systematic leaf showed a mild mottle.

Characterization of CMV-G offspring variants

In order to characterize CMV-G and its offspring variants from infected tobacco tissue, several methods were used. Firstly, for intact virion analysis in agarose gel electrophoresis, the method of Rodriguez-Alvarado et al. (1995) was used.

Secondly, restriction endonuclease analysis of amplified cDNA (RT-PCR/RFLP assay) of CMV RNA2 (Finetti-Sialer et al., 1999) was employed. Total nucleic acids were extracted from CMV-infected plants, according to the procedure described by White and Kaper (1989) with minor modifications. The forward primer (5′-GTTTAT TTACAAGAGCGTACGG-3′) and the reverse primer (5′-GGTTCGAAAGTATAACC GGG-3′) were used in PCR reactions with an initial denaturation cycle at 94 °C for 4 min, followed by 35 cycles (94 °C-30 s, 64 °C-60 s and 72 °C-2 min) with a final extension of 10 min at 72 °C using a PTC-100 thermocycler (MJ Research, England). The predicted length of the amplified DNA product was approximately 650 bp, and could be cleaved by the restriction enzyme *Mlu*I (Fermentas, Lithuania) to obtain a pattern that distinguishes CMV isolates in subgroups IA (one undigested fragment), IB (two fragments of approximately 470 and 160 bp) and II (three fragments of approximately 320, 170 and l50 bp) (Finetti-Sialer et al., 1999). DNA products were run in 1.2% agarose gels; gels were stained with EtBr and photographed.

Thirdly, satellite dsRNA and RT-PCR analyses were employed to identify the presence, if any, of satRNA in the CMV variants. DsRNA analysis was performed essentially as in Sayama et al. (1993). DNA samples were separated in a 5% polyacrylamide gel (acrylamide:bis-acrylamide = 39:1), the gel was stained with EtBr and photographed. To amplify a putative satRNA in these samples, the procedure of Grieco et al. (1997) was used. The primers used for the RT-PCR were: 5′-AAGGAT CCGTTTTGTTTG(AT)T(AG)GAGAATTGCG (CT)(AG)GAG-3′ (forward) and 5′-AAGGATC CGGGTCCTG(CGT)(AGT)(AGT)(AGT)GGAT TG-3′ (reverse). PCR was performed using the following programme: 2 cycles (94 °C-1 min, 42 °C-1 min, 72 °C-1 min), 35 cycles (94 °C-1 min, 55 °C-1 min, 72 °C-1 min), and a final extension at 72 °C for 10 min. Plasmid DNA (pSP65::satCMV-S) (Avila-Rincon et al., 1986) was used as a positive control. The amplification of the CMV polymerase gene was performed as a control as described above for the RT-PCR/RFLP assay. PCR products were run in 2% agarose gels; gels were stained with EtBr and photographed.

Nucleotide sequence analysis of the CP gene of CMV-G and its phylogenetic analysis

To classify CMV-G in a subgroup of CMV, the nucleotide sequence of CP was determined. The RT-PCR procedure of Rizos et al. (1992) was used in order to amplify the CP gene of a YM variant (CMV-G2) and two MM variants (CMV-G10, CMV-G3) (Table 1) using the proofreading *Pfu* DNA polymerase (Promega, USA). The 850 bp blunt-ended amplicon cDNA fragment was gel-purified (Concert gel extraction system, BRL, USA), modified by the A-tailing protocol and ligated in the pGEM-T Easy Vector (Promega, USA). *Escherichia coli* DH5a competent cells were transformed with the recombinant plasmid, bacterial colonies were screened and the recombinant plasmid was isolated with the Wizard Plus Miniprep kit (Promega, USA). Sequencing was performed in both directions (MWG, Germany) and sequence data were analysed using the Blast 2 software (NCBI, USA). The CMV-CP sequence obtained was compared to those of group IA, IB and II of CMV isolates, whose sequences are available in the Genbank (NCBI, USA). Phylogenetic analysis was performed with MEGALIGN programme (DNAstar, Madison, WI) utilizing selected existing DNA-sequences of CMV-CP from the GenBank, namely strains O (D00385), Fny (D10538), Sny (U66094), C (D00462), P6 (D10545), MY17 (AF103993), Mf (AJ276481), E5 (D42080), D8 (AB004781), Y (D12499), FT (D28487), Pepo 1 (AF103991), Pepo 2 (D28488), CS (D28489), N (D28486), KM (AB004780), SO (AF103992), M48 (D49496), L (D16405), M1 (AF268599), M (D10539), NT9 (D28780), Tfn (Y16926), C7-2 (D42079), K (AF127977), B2 (AB046951), Ly2 (AJ296154), Ix (U20219), IA (AB042294), Trk7 (L15336), Q (M21464), LS (AF127976), M2 (AB006813), Kin (Z12818), R (Y18138), Kor (L36251), WL (D00463). The obtained sequences were deposited in the GenBank with accession numbers: AY450854 (CMV/G2-CP), AY541691 (CMV/G10-CP).

Cross-protection experiment

For the cross-protection studies, the CMV-G2 and CMV-G10 offspring variants (Table 1) were sap-inoculated onto 10 tobacco Xanthi nc indicator plants each, whereas five indicators served as the non-treated control. At 10 dpi, half of the infected plants for each group were inoculated with sap containing the respective challenging variant of the opposite mosaic type. Symptom development was monitored daily for a month. The experiment was carried out twice.

Results

CMV-G was analysed in YM and MM phenotypic variants

We investigated the possible isolation of variants from a field CMV isolate, named CMV-G that induces yellow mosaic in tobacco. For this purpose aphid transmission and mechanical inoculation using various host plants were utilized to isolate naturally existing mild strains of CMV.

Offspring variants of CMV-G derived from the aphid transmission experiment from CMV-G-infected tobacco, pepper, tomato and zucchini were grouped into those which induce YM and those which induce MM in tobacco. Population analysis in *C. quinoa* by single-local lesion assay of CMV-G and three of its YM offspring variants, CMV-G2, CMV-G5 and CMV-G6, produced not only YM but also MM variants. However, population analysis of six MM offspring variants, CMV-G1, CMV-G3, CMV-G4, CMV-G7, CMV-G8, and CMV-G10, produced only MM symptoms in tobacco.

Host range of CMV-G and its offspring variants

Host range and symptomatology were studied for CMV-G and nine of its offspring variants (Table 1). The systemic symptoms induced by CMV-G on different hosts were yellow mosaic in *N. tabacum* (Figure 1a), upward leaf curling and deformation in *L. esculentum* (Figure 1d (i and ii)), a yellow mosaic in *C. pepo* (Figure 1e), and a yellow mosaic in *C. annuum* (Figure 1f). Both YM and MM variants did not differ in symptomatology in hosts belonging to the families *Cucurbitaceae, Leguminosae* and *Chenopodiaceae*. In contrast, in the *Solanaceae* family, YM variants (including CMV-G) could be easily differentiated from MM variants in *N. tabacum, N. benthamiana* and *L. esculentum*; symptoms of YM variants were more intense than those of MM variants. In *S. melogena* symptoms of all variants were minimal (very mild mottle) and therefore no differentiation between YM and MM variants was possible.

CMV-G and its offspring variants belong to subgroup IB of CMV free of satRNA

Intact virion electrophoresis analysis showed that virions of YM and MM variants of CMV-G and its offspring variants do not suffer from an instability problem (data not shown), i.e. they exist as particles, a characteristic which is important for aphid transmission.

The RT-PCR/RFLP assay showed that the CMV-G and its offspring variants had the same restriction endonuclease pattern of the first 600 nt of RNA 2 (Figure 2), producing two fragments of approximately 470 and 160 bp and thus belong to the subgroup IB. In addition, CMV-G offspring variants did not contain a satRNA based on the satellite dsRNA (Figure 3a) and RT-PCR analyses (Figure 3b).

CP sequences of a YM and MM variants of CMV-G were obtained to determine the genetic relationship to other CMV strains. CP sequences of CMV-G2 and CMV-G10 were identical with only a single nucleotide substitution that results in an amino acid difference at position 128, where Ile in CMV-G2 substitutes Val found to be the prevalent amino acid for this position for the 300 CMV sequences studied. Ile at position 128 was also found in CMV isolates from chrysanthemum (DQ028777), tomato (DQ141675), banana (DQ152254) and Amaranthus (AF198622), all characterized in India. Phylogenetic analysis (Figure 4) showed that the yellow CMV-G variant was classified in the CMV subgroup IB.

An MM variant cross-protected tobacco plants against an YM variant

Attenuated virus strains have been used as a pre-treatment on plant seedlings, in order to prevent closely related virulent strains from infecting them

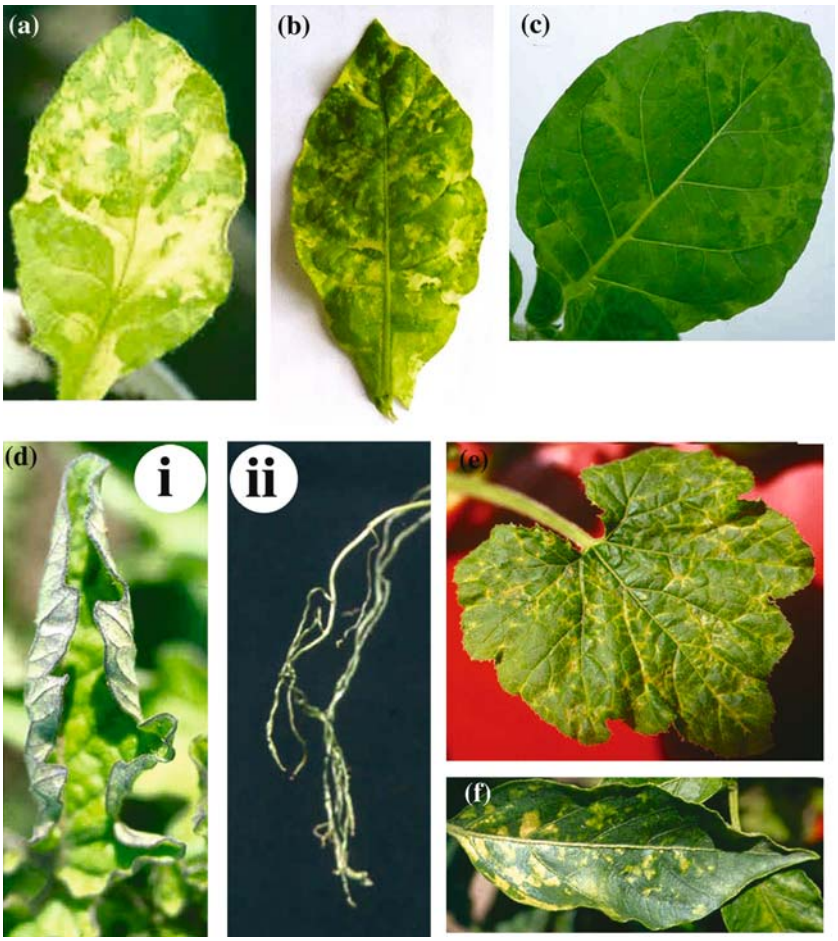

Figure 1. Disease symptom development: (a) CMV-G inducing yellow mosaic on tobacco; (b) CMV-G2 inducing yellow mosaic on tobacco (offspring variant from tobacco); (c) CMV-G3 inducing mild green mosaic on tobacco (offspring variant from tobacco); (d) CMV-G inducing upward curling, purpling under intense light (i), and shoestring under low light (ii) on tomato; (e) CMV-G inducing yellow mosaic on zucchini; (f) CMV-G inducing yellow mosaic on pepper.

and causing more severe disease symptoms; a strategy referred to as cross-protection (Pennazio et al., 2001). Cross-protection studies were made to investigate whether the MM variant has a potential use for protection against the YM variant. CMV-G10 (MM variant) cross-protected tobacco plants from a challenging infection by CMV-G2 (YM variant), since all plants challenged remained with the initial MM symptoms. Similarly, none of the YM manifesting tobacco plants, due to CMV-G2 (YM variant) infection, changed to MM after a second challenging infection with the MM variant CMV-G10. Furthermore, after seven serial passages in tobacco, CMV-G and its offspring variants kept their respective symptom phenotypes of yellow or green mild mosaic.

Discussion

CMV-G, an isolate inducing YM in tobacco, was isolated from an infected tomato plant from Gastouni-Olympia, Greece; it was characterized (symptomatology, aphid transmissibility, virus classification) and analysed for YM and MM variants, either by mechanical inoculation in the local lesion host *C. quinoa* or by aphid transmission after a single probe.

Various means for the isolation of mild strains of plant viruses have been employed like heat treatment (Oshima, 1975), cold treatment (Kosaka and Fukunishi, 1997) and mutagen (Yeh and Gonsalves, 1984). In addition, spontaneous emergence (Lecoq and Lemaire, 1991), the presence of

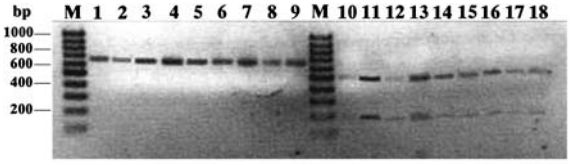

Figure 2. Electrophoretic mobility of RT-PCR/RFLP products of CMV-G and its offspring variants using the method of Finetti-Sialer et al. (1999). Lanes 1–9: RT-PCR products of CMV-G (lane 1) and its offspring variants; lanes 10–18: Digestion of RT-PCR products for CMV-G (lanes 1) and its offspring variants with *Mlu*I generating two fragments approximately 470 and 160 bp, respectively; lanes M: Molecular marker (100 bp ladder, BRL, USA).

satRNA (Montasser et al., 1991) and the host passage (Yarwood, 1979) could influence the production of mild plant viral strains. In our study, both transmission methods used were able to isolate MM variants of CMV-G from its YM phenotypic variants. In particular, *M. persicae* transmitted YM phenotypic variants from tomato and only MM variants from zucchini under our experimental conditions. Therefore, zucchini could be used to obtain a MM variant of CMV from a mixed population of YM and MM variants using aphids. Such a selection of MM variants from the YM by aphids could not be explained since the transcapsidation phenomenon and/or phenotypic mixing events may enable any CMV-variant RNA to be encapsidated by any functional CMV capsid protein, thus lowering the possibility of selection of a genome (YM or MM) through aphid transmission. Although a low percentage of reversion of YM to MM could also explain such a phenomenon, it is considered to be unlikely. In addition, intact virion electrophoretic analysis (data not shown) suggested that virions of YM and MM variants of CMV-G do not suffer from an instability problem, i.e. they exist as particles, a characteristic which is important for aphid transmission. The present data could indicate that the host plant remains the main factor affecting the relative distribution of YM and MM offspring variants in mixed populations.

All the MM variants exhibited only MM symptoms in tobacco indicating a stable symptomatological character that persisted for seven passages in tobacco, an important trait for cross-protection studies. In a total of 150 trials, no YM variant derived from any MM variant. In contrast, YM variants CMV-G2, -G5 and -G6 were always

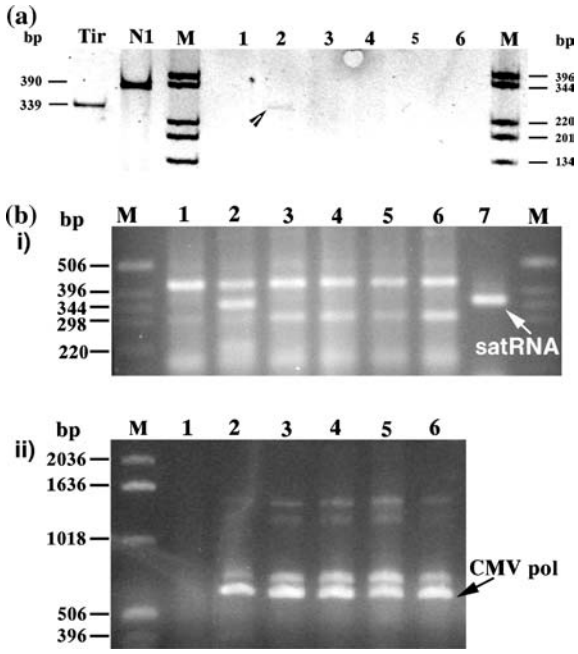

Figure 3. (a) Analysis of dsRNA by polyacrylamide gel electrophoretis of infected plants with various strains of CMV. Lane Tir: CMV strain from Tirintha, Greece; lane N1: CMV strain NDN1; lane 1: non-infected tobacco; lane 2: tobacco infected with CMV-P6; lane 3: tobacco infected with CMV-G2; lane 4: tobacco infected with CMV-G5; lane 5: tobacco infected with CMV-G10; lane 6: tobacco infected with CMV-G3; lanes M: Molecular marker (1 kb ladder, BRL, USA). (b) RT-PCR analysis for the presence of satRNA in CMV strains: (i) RT-PCR for CMV satRNA, and (ii) RT-PCR for CMV polymerase gene. In both panels RT-PCR reaction templates were as follows: lane 1: non-infected tobacco; lane 2: tobacco infected with CMV-P6; lane 3: tobacco infected with CMV-G2; lane 4: tobacco infected with CMV-G5; lane 5: tobacco infected with CMV-G10; lane 6: tobacco infected with CMV-G3; lane 7: plasmid DNA containing satRNA of CMV-S; lanes M: molecular marker (1 kb ladder, BRL, USA). The expected RT-PCR products for portion of CMV RNA 2 (CMV polymerase) and CMV-satRNA are indicated by arrows.

found as YM and MM variants in tobacco suggesting the presence of a mixture of YM and MM variants following the passage through *C. quinoa*. These data together suggest that MM is a rather stable, prevalent phenotype in contrast to YM.

CMV-G and its variants showed that these isolates possess the CMV-D type virion based on intact virion electrophoresis (data not shown). RT-PCR/RFLP analysis of CMV-G and its offspring variants (Figure 2), and phylogenetic analysis of the CMV-G2 CP sequence (Figure 4)

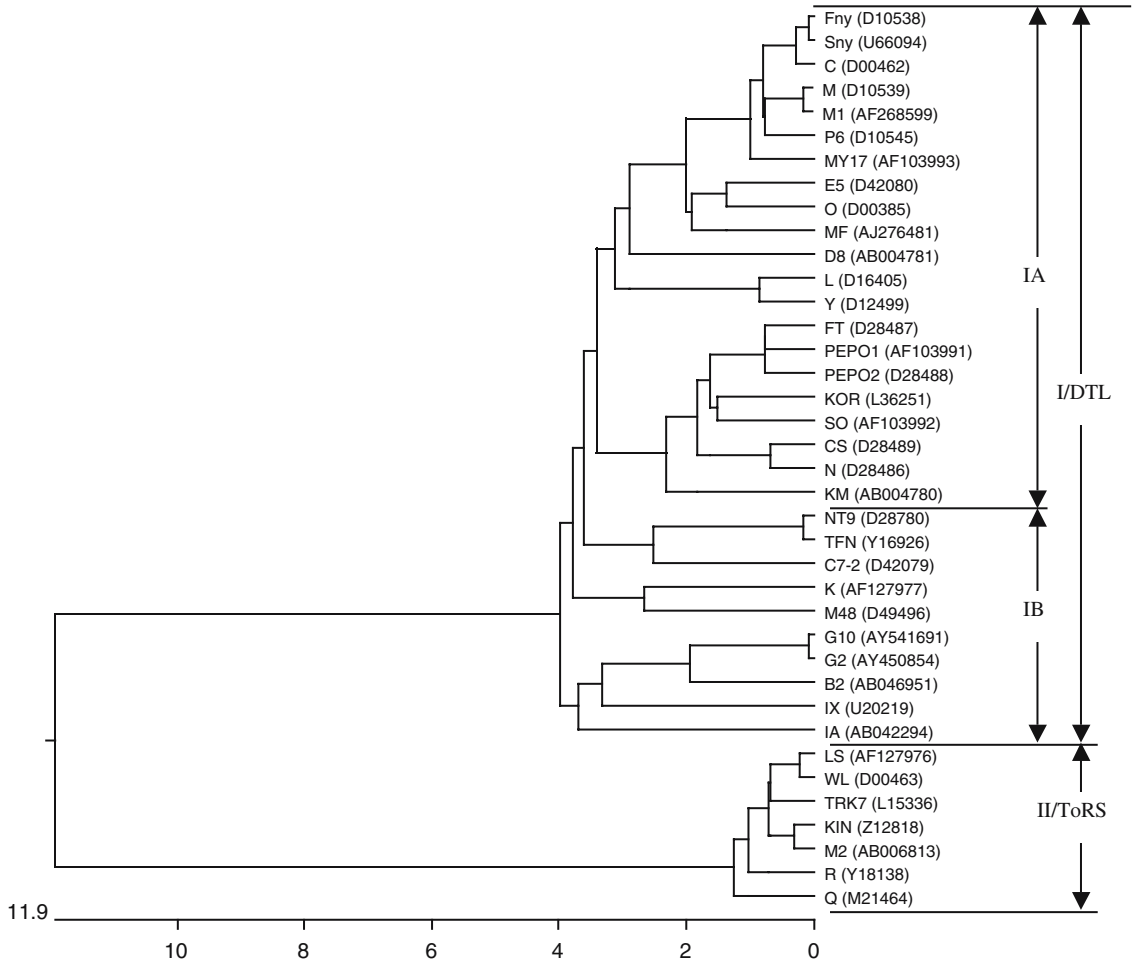

Figure 4. Phylogenetic analysis of CP nucleotide sequence of CMV-G2. The nucleotide sequences of the reference CMV strains were taken from the NCBI Genbank. The analysis was performed using the MEGALIGN programme (DNAstar, Madison, WI).

suggested that they belong to the subgroup IB of CMV isolates. Based on satellite dsRNA (Figure 3a) and RT-PCR analyses (Figure 3b) CMV-G was found not to contain satRNA. This is the first discovery of subgroup IB of CMV, free of satellite, associated with devastating disease outbreaks of CMV on tomato in Greece during the last twenty years (Kyriakopoulou et al., 2000a). This work confirms in part previous work identifying some tomato isolates of CMV in Greece as belonging to a subgroup I (Varveri and Boutsika, 1999).

Symptomatology in different hosts showed that CMV-G and its YM offspring variants differ from the MM variants only in terms of symptom severity. YM variants, including CMV-G, showed a more deleterious effect on solanaceous plants, including stunting and leaf deformation (upward curling and purpling under intense light, severe shoestring under low light conditions) as compared to the MM variants that had a milder effect. Eggplant, known as a non-sensitive host of CMV, was an exception in the *Solanaceae* family, as it exhibited very mild green mottle to almost no symptoms for both groups of CMV variants tested. These results indicate that in *Solanaceae* there is a positive correlation between YM phenotype and disease severity.

It is known that the presence of the satRNA can modify the symptoms of CMV, depending on the host species, the CMV strain and the satellite clone present. In a previous study, 29 CMV isolates from tomato in Greece were molecularly characterized showing that only five did not encapsidate a satRNA (Varveri and Boutsika, 1999). Most importantly,

in that study, the severe mosaic symptoms in tomato correlated with the presence of CMV satRNA. In this study, CMV-G offspring variants inducing YM or MM were found free of satRNA (Figures 4a and b). Therefore, most likely, the mosaic phenotypic differences in tobacco could be attributed to the CMV genomes themselves requiring further investigation. It was reported previously, that chlorosis in tobacco is associated with CMV-CP amino acid 129 with Ser and Leu replacing Pro found in the MM phenotype inducing CMV strains (Suzuki et al., 1995; Shintaku et al., 1992). However, it could not be overlooked that gene 2b of CMV influences its virulence (Shi et al., 2002) and that plant encoded proteins were shown also to be involved in the exhibition of the YM symptoms in tobacco (Takahashi and Ehara, 1993); both factors could be involved in the symptom manifestation.

Cross-protection has been employed to reduce yield losses in several cultivated crops including tomatoes (Cassells and Herrick, 1977), squash (Wang et al., 1991), papaya (Yeh and Gonsalves, 1994) and citrus (Costa and Muller, 1980). Although the detailed mechanism of cross-protection remains unknown, the isolation of effective attenuated strains of viruses is of crucial importance for plant protection. In this study, cross-protection studies showed that tobacco seedlings pre-treated with a mild (MM) variant of CMV did not develop severe symptoms upon a subsequent infection by an aggressive (YM) variant of CMV. Therefore, this agronomically important plant protection method could be applied in the case of tobacco and CMV utilizing a mild variant obtained from a severe disease-causing field isolate.

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References

Avila-Rincon MJ, Collmer CW and Kaper JM (1986) *In vitro* translation of cucumoviral satellites. I. Purification and nucleotide sequence of Cucumber mosaic virus-associated RNA 5 from cucumber mosaic virus strain S. Virology 152: 446–454.

Cassells AC and Herrick CC (1977) Cross protection between mild and severe strains of tobacco mosaic virus in doubly inoculated tomato plants. Virology 78: 253–260.

Castillo MB and Orlob GB (1966) Transmission of two strains of cucumber and alfalfa mosaic viruses by single aphids of *Myzus persicae*. Phytopathology 56: 1028–1030.

Costa AS and Muller GW (1980) Tristeza control by cross protection: a U.S.-Brazil cooperative success. Plant Disease 64: 538–541.

Edwardson JR and Christie RG (1991) Comoviruses. In: Edwardson and Christie (eds.) Handbook of viruses infecting legumes (pp. 504) CRC, Boca Ratoon, Florida.

Finetti-Sialer MM, Cillo F, Barbarossa L and Gallitelli D (1999) Differentiation of cucumber mosaic virus subgroups by RT-PCR RFLP. Journal of Plant Pathology 81: 145–148.

Gallitelli D (1998) Present status for controlling Cucumber Mosaic Virus. In: Hadidi A, Khetarpal RK and Koganezawa H (eds.) Plant Virus Disease Control (pp. 507–523) The American Phytopathological Society, St. Paul, MN.

Gallitelli D (2000) The ecology of cucumber mosaic virus and sustainable agriculture. Virus Research 71: 9–21.

Grieco F, Lavane C and Gallitelli D (1997) Evolutionary dynamics of cucumber mosaic virus satellite RNA during natural epidemics in Italy. Virology 229: 166–174.

Jorda C, Alfaro A, Arranda MA, Moriones E and Garcia-Arenal F (1992) An epidemic of cucumber mosaic virus plus satellite RNA in tomatoes in eastern Spain. Plant Disease 76: 363–366.

Kaper JM and Waterworth HE (1981) Cucumoviruses. In: Kurstak E. (ed.) Handbook of Plant Virus Infections and Comparative Diagnosis (pp. 257–332) Elsevier/North Holland Biomedical Press, Amsterdam.

Kosaka Y and Fukunishi T (1997) Multiple inoculation with three attenuated viruses for the control of cucumber virus disease. Plant Disease 81: 733–738.

Kyriakopoulou PE, Bem F and Varveri C (1991) Tomato shrinkage and tomato fruit toughness, two new diseases in Greece, probably related to cucumber mosaic virus. Annales Institut Phytopathologique Benaki (N.S.) 16: 151–156.

Kyriakopoulou PE, Vassilakos N, Bem FP, Varveri C, Girkis MS and Skopelitis NG (2000a) Cucumber mosaic virus in industrial tomato of Eleia county and the new threat from tomato necrosis. Phytopathologia Mediteranea 39: 330.

Kyriakopoulou PE, Perdikis DCh , Sclavounos AP, Girgis SM, Lycouressis DP, Tsitsipis JA and Christakis PA (2000b) Cucumber mosaic cucumovirus incidence in open-field tomato in the Olympia area and trap captures of alate aphids. OEPP/EPPO Bulletin 30: 305–315.

Lecoq H and Lemaire O (1991) Control of zucchini yellow mosaic virus in squash by cross protection. Plant Disease 75: 208–211.

Montasser MS, Tousignant ME and Kaper JM (1991) Satellite-mediated protection of tomato against cucumber mosaic virus: greenhouse experiments and simulated epidemic conditions in the field. Plant Disease 75: 86–92.

Oshima N (1975) The control of tomato mosaic disease with attenuated virus of tomato strain of TMV. Review Plant Protection Research 8: 126–135.

Owen J and Palukaitis P (1988) Characterization of cucumber mosaic virus I. Molecular heterogeneity mapping of RNA 3 in eight CMV strains. Virology 166: 495–502.

Palukaitis P and Zaitlin M (1997) Replicase-mediated resistance to plant virus disease. Advances in Virus Research 48: 349–377.

Pennazio S, Roggero P and Conti M (2001) A history of plant virology. Cross protection. New Microbiologica 24: 99–114.

Putz C, Kuszala J, Kuszala M and Splindler C (1974) Variation du pathogene des isolats du virus de la mosaique du concombre associee a la necroce de la tomato. Annales de Phytopatholie 6: 139–154.

Rizos H, Gunn LV, Pares RD and Gillings MR (1992) Differentiation of cucumber mosaic virus isolates using the polymerase chain reaction. Journal of General Virology 73: 2099–2103.

Rodriguez-Alvarado G, Kurath G and Dodds A (1995) Heterogeneity in pepper population of cucumber mosaic cucumovirus. Plant Disease 79: 450–455.

Roossinck MJ (2002) Evolutionary history of Cucumber mosaic virus deduced by phylogenetic analyses. Journal of Virology 76: 3382–3387.

Sayama H, Sata T, Kominato M, Nastuaki T and Kaper JM (1993) Field testing of a satellite containing attenuated strain of cucumber mosaic virus for tomato protection in Japan. Phytopathology 83: 400–410.

Shi B-J, Palukaitis P and Symons RH (2002) Differential virulence by strains of cucumber mosaic virus is mediated by the 2b gene. Molecular-Plant Microbe Interactions 15: 947–955.

Shintaku M (1991) Coat protein gene sequences of two cucumber mosaic virus strains reveal a single amino acid change correlating with chlorosis induction. Journal of General Virology 72: 2587–2589.

Shintaku M, Zhang L and Palukaitis P (1992) A single amino acid substitution in the coat protein of cucumber mosaic virus induces chlorosis in tobacco. Plant Cell 4: 751–757.

Suzuki M, Kuwata S, Masuta C and Takanaki Y (1995) Point mutations in the coat protein of Cucumber Mosaic Virus affect symptom expression and virion accumulation in tobacco. Journal of General Virology 76: 1791–1799.

Takahashi H and Ehara Y (1993) Severe chlorotic spot symptoms in cucumber mosaic virus strain Y-infected tobaccos are induced by a combination of the virus coat protein gene and two host recessive genes. Molecular-Plant Microbe Interactions 6: 182–189.

Wang HL, Gonsalves D, Provvidenti R and Lecoq HL (1991) Effectiveness of cross protection by a mild strain of zucchini yellow mosaic virus in cucumber melon and squash. Plant Disease 75: 203–207.

White JL and Kaper JM (1989) A simple method for detection of viral satellite RNAs in small plant tissue sample. Journal of Virological Methods 23: 83–94.

Varveri C and Boutsika K (1999) Characterization of cucumber mosaic cucumovirus isolates in Greece. Plant Pathology 48: 95–100.

Yarwood CE (1979) Host passage effects with plant viruses. Advances in Virus Research 25: 169–189.

Yeh SD and Gonsalves D (1994) Practices and perspective of control of papaya ringspot virus by cross protection. In: Advances in Disease Vector Research. Vol. 10 (pp. 237–257) Springer-Verlag, New York.

Yeh SD and Gonsalves D (1984) Evaluation of induced mutants of papaya ringspot virus for the control by cross-protection. Phytopathology 74: 1086–1091.