J. Phytopathology **152**, 580–588 (2004)

ISSN 0931-1785

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Etiology of Leaf Chlorosis and Deterioration of the Fruit Interior of Watermelon Plants

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Received June 3, 2004; accepted September 1, 2004

Keywords: *Cucumber green mottle mosaic virus*, Tobamovirus, watermelon, reverse transcriptase-polymerase chain reaction, weed hosts

Abstract

A severe disease with combined symptomatology affecting only grafted watermelon plants appeared in several regions of northern and central Greece during the summers of 1999 and 2000. Disease symptoms included chlorotic mottling followed by pedicel necrosis at a later more mature fruit stage, and finally decomposition of fruit interior. Electron microscopy, double antibody sandwich enzyme-linked immunosorbent assay tests and reverse transcriptase-polymerase chain reaction assays identified *Cucumber green mottle mosaic virus* (CGMMV) as the causal agent. Our studies indicated that the CGMMV isolates from watermelon are closely related to the Israeli variant of CGMMV (CGMMV-Is). However, bioassays revealed that the Greek isolates elicit symptoms of higher severity and have a wider host range, compared with other described CGMMV isolates. Nucleotide sequence comparison of the movement protein gene of the Greek isolates showed a 97–99% identity with other CGMMV strains. This paper reports the presence of the virus in arable weeds such as *Amaranthus blitoides*, *Amaranthus retroflexus*, *Heliotropium europaeum*, *Portulaca oleracea* and *Solanum nigrum*.

Introduction

In Greece, the cultivation of watermelon is of great economical importance. Because of space limitation farmers use the same fields continuously, a cropping practice that led to the appearance of destructive watermelon soil-borne diseases such as *Fusarium* wilt (*Fusarium oxysporum* f. sp. niveum). To solve this problem cultivated varieties are grafted onto different *Fusarium* wilt resistant rootstocks including *Langenaria siceraria* or *Cucurbita pepo*. However, this practice in Greece led to the establishment of a strain of *Cucumber green mottle mosaic virus* (CGMMV, *Tobamovirus*), which was first reported in southern Greece (Peloponnesus) in 1992 (Bem and Vassilakos, 2000). Three strains of CGMMV were isolated in Greece from watermelons and were identified as closely related to CGMMV-SH (98.8% at the protein level) (Varveri et al., 2002). Another strain of CGMMV strain was isolated in greenhouse-grown cucumbers in Crete in 1979 (Avgelis and Vovlas, 1986).

As a result of the use of certified rootstock seed, reoccurrence of CGMMV was not reported in Peloponnesus and therefore no further research was carried out. However, a severe disease with combined symptomatology appeared in several watermelon-growing regions of central Macedonia and Thessaly during the summers of 1999 and 2000. In these areas, watermelon grafting has only recently been adopted and in many cases the farmers produce their own seed.

This disease affected grafted watermelon crops exclusively. Three types of symptoms were observed: the first type consisted of symptomless leaves, and fruits with otherwise regular appearance, except for the presence of dark brown, rod-shaped necrotic lesions on the pedicel surface (Fig. 1a). When these fruits reached maturity and were dissected, they exhibited the following symptoms: a whitish-yellow discoloration of the peripheral region and water-soaked inner fruit pulp with crescent shaped crevices (Fig. 1b). These symptoms rendered the fruits unmarketable. Mosaic-like green elevated areas were rarely observed on the surface of the fruit. These symptoms resemble those caused by the previously reported CGMMV-W and CGMMV(SH) in Japan and southern Greece (Komuro et al., 1971; Bem and Vassilakos, 2000; Varveri et al., 2002). The second type of symptoms

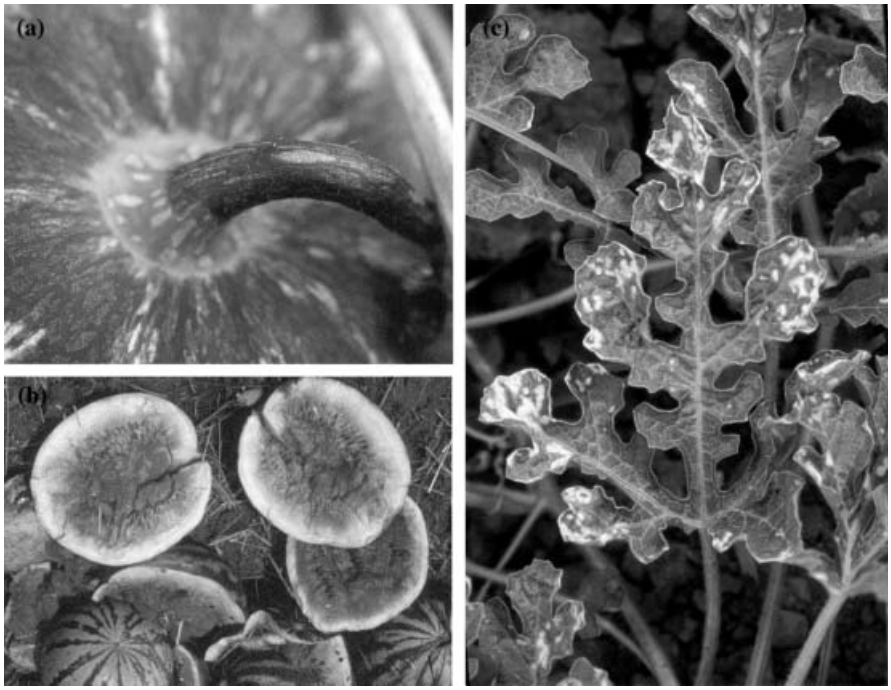

Fig. 1 (a) Watermelon fruit showing necrosis on the pedicel surface; (b) Water-soaked inner pulp of watermelon; (c) Characteristic chlororic patterns on watermelon leaf lamina

occurred more frequently and consisted of chlorotic leaf spots on the leaves, which later coalesced to form characteristic irregular chlorotic patterns (Fig. 1c). This type resembles the symptoms caused by *Watermelon chlorotic stunt virus* (WmCSV, *Begomovirus*, Geminiviridae) (Jones et al., 1988). The third type of symptoms was observed on several plants, and consisted of the above chlorotic leaf patterns, followed by pedicel necrosis at a later time.

The aim of this work was to identify and characterize the causal agent of the disease that leads to 'leaf chlorosis and deterioration of the fruit interior of watermelon plant'.

Materials and Methods

Field surveys

Samples from different watermelon fields were collected at three different stages: (i) the beginning of summer, (ii) mid-summer and (iii) in fall. Fields were divided into three groups. The first group consisted of eight fields located in Thessaly (Larissa) and central Macedonia (Thessaloniki and Kilkis). These fields were planted with watermelon plants grafted on different rootstocks, usually bottlegourd (*Langenaria siceraria*). Watermelon plants in this group developed chlorotic patterns on leaves, fruit pedicel necrosis and decomposition of fruit pulp. The material sampled consisted of leaves, fruits, and fruit pedicels from symptomatic plants only. Bottlegourd that were planted for seed production on the borders of affected fields and showing mosaic symptoms were also sampled. The second group of fields consisted of three control fields in the same regions, which were planted with self-rooted watermelon plants. The third group consisted of four fields with grafted watermelon plants, in Trikala (Thessaly) and Ilia (Peloponnesus), where CGMMV-occurrence was reported earlier (Bem and Vassilakos, 2000). Plants of the last two groups did not exhibit any symptoms and therefore, leaf samples were collected randomly.

Virus isolates

Three isolates, designated CGMMV-NA, CGMMV-M and CGMMV-GGR (Table 1), were used in this study. All three isolates originated from watermelon plants exhibiting severe disease symptoms. Isolates CGMMV-WGR (Bem and Vassilakos, 2000) and CGMMV-CGR (Avgelis and Vovlas, 1986) previously isolated in Trifilia (Peloponnesus) and Crete, respectively, were also included in the study. Virus isolation was carried out using the local lesion assay (Walkey, 1991) involving three passages onto *Chenopodium amaranticolor* plants. Subsequently, virus isolates were maintained on cucumber (*Cucumis sativus* L. cv. 'Lungo') or melon (*Cucumis melo* L. cv. 'Gaglia F1') plants.

Host-range study

The isolates under study (Table 3) were mechanically inoculated (Hill, 1984) using 0.01 M phosphate buffer, pH 7.0 containing 0.1% sodium sulphate (buffer I), onto 18 indicator plant species, which belong to six different families. At least three plants from each species were inoculated and the experiment was repeated twice.

After inoculation, plants were transferred in an insect proof glasshouse (temperature, 20–24°C; L : D, 16 : 8) for symptom development for 3–6 weeks. Indicator plants with or without symptoms were analysed serologically.

Table 1
Cucumber green mottle mosaic virus (CGMMV) isolates used for host range and molecular studies

Isolate	Region	Original host	Provided by
CGMMV-WGR	Trifilia (Peloponnesus)	Watermelon	Dr F. Bem
CGMMV-CGR	Crete	Cucumber	Dr A. D. Avgelis
CGMMV-NA	Neo Agioneri (Macedonia)	Watermelon (pedicel necrosis)	This study
CGMMV-GGR	Giannouli (Thessaly)	Watermelon (chlorotic mottling and pedicel necrosis)	This study
CGMMV-M	Makrihori (Thessaly)	Watermelon (chlorotic mottling and pedicel necrosis)	This study

Presence of CGMMV in weeds

In the course of this investigation, several weeds from the surveyed watermelon fields affected by the disease were sampled as well. A total of 294 arable weeds, comprising 12 species and belonging to 10 genera and nine different families were randomly collected. Weed identification was carried out by Dr E. Drossos in the Laboratory of Systematic Botany and Phytogeography (Department of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece).

All weed samples were initially tested by enzyme-linked immunosorbent assays (ELISA; see ELISA tests) and ELISA positive samples were mechanically inoculated onto cucumber and melon to confirm virus presence. Three different inoculation buffers were used named buffer I, buffer II and buffer III. Buffers II and III consisted of buffer I supplemented with 2% PVP and 0.2% DIECA, respectively.

Virus purification

Cucumber plants infected with the isolate CGMMV-GGR served as a virus source for purification based on the method described by Tung and Knight (1972), modified by the addition of a sucrose gradient step prepared as follows: Four sucrose solutions (10, 20, 30 and 40%) were prepared in 0.01 M phosphate buffer, pH 7.0, and gradients were formed by layering the solutions in ultracentrifuge tubes and incubating them at 4° C overnight. Samples were applied on gradients before ultracentrifugation (100 000 × ***g***, 90 min). Gradient fractionation was followed by EM analysis of the samples to detect the virus.

An extinction coefficient of $E^{0.1\%}_{260\text{nm}} = 3.18$ (Wetter et al., 1984) was used to estimate virus concentration spectrophotometrically. Purified viral coat protein preparations were separated on denaturing 10% polyacrylamide gels (Laemmli, 1970). Protein bands were visualized by staining with Coomassie brilliant blue.

Electron microscopy

Crude extracts from field watermelon symptomatic plants and purified virus preparations (CGMMV-GGR) were mounted on carbon-coated Formvar films, negatively stained with 2% aqueous uranyl acetate and observed under an electron microscope JEOL 1200 EX (Jeol Ltd., Tokyo, Japan).

ELISA tests

Double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) (Clark and Adams, 1977) were used to examine field samples for the presence of CGMMV, *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus* 2 (WMV 2), *Papaya ringspot virus* (PRSV), *Zucchini yellow mosaic virus* (ZYMV) and *Zucchini yellow fleck virus* (ZYFV), using flat bottom microtiter-plates (EIA/RIA Costar Corporation, Cambridge, MA, USA). A total of 386 bottlegourd seeds were collected from 11 plants, planted for seed production on the borders of four different heavily infected watermelon fields, and were tested for the presence of CGMMV in their coats using DAS-ELISA. Serological relationships of purified preparations of CGMMV-GGR, the Israeli isolate of CGMMV (CGMMV-Is) and *Cucumber fruit mottle mosaic virus* (CFMMV) (Antignus et al., 2001) were also investigated using antibodies against CGMMV-Is.

The concentrations of polyclonal antibodies and conjugated antibodies used, as well as their origins are presented in Table 2. Optical densities (OD) were recorded at 405 nm with a plate reader (Labsystems Multiscan RC (Labsystems, Helsinki, Finland)) and samples with an OD value twice higher than the average of the negative control, were considered as positive.

Oligonucleotide primers

Oligonucleotide primers (MWG AG Biotech, Anzinger, Germany) were designed for reverse transcriptase-polymerase chain reaction (RT-PCR) assays based on highly conserved regions of CGMMV movement protein (MP) and coat protein (CP) homologous genes (GenBank accession numbers: D12505, AJ243353, AB015146, J04322 and V0155). Sequence alignments were performed using the program WU-BLAST2 (EMBL,

Table 2
The origin and the concentrations of IgGs and conjugated IgGs used for the detection of watermelon viruses

	Concentration (μg/ml)		
Antisera	Antibody	Conjugated antibody	Origin
CGMMV	1	1	Dr F. Bem[a]
CGMMV-Is	0.5	1	Dr Y. Antignus[b]
CMV	1	0.5	Dr C. Varveri[a]
WMV2	1	0.5	Dr B. Falk[c]
ZYMV	1.5	0.75	Dr B. Falk
PRSV	1	0.5	Dr B. Falk
ZYFV	1	0.5	Dr H. Lecoq[d]

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[b]Department of Virology, ARO, The Volcani Center, Israel.
[c]Department of Plant Pathology, University of California, Davis, CA, USA.
[d]INRA, Station de Pathologie Vegetale, Montfavet Cedex, France.

European Bioinformatics Institute, Cambridgeshire, UK). Two different primer pairs (CGMMV sense: 5′-TGTA GTCGAG TCTGTCGTCTCTT-3′, CGMMV antisense: 5′-GGTACCTTGTGAAGCAAC TAGAA-3′ and CGMP sense: 5′-GATGTCTCTAAGTAAGTGT CAGTCGAG-3′, CGMP antisense: 5′-GCTAGGTGT GATCGGATTGTAG-3′) were designed for diagnostic and sequencing purposes, respectively. The first pair amplifies a product of approximately 332 bp (nt 5533–5865), while the second one amplifies a 795 bp-long fragment, encompassing the entire putative MP open reading frame (ORF).

Crude extract preparation and Immunocapture-RT-PCR assays

Crude plant extracts were prepared as described by Manganaris et al. (2003) and clarified by low speed centrifugation (10 000 × ***g***, 10 min, 4°C). Immunocapture assays were performed in 0.2 Thermowell® tubes (Costar Corning, New York, NY, USA) precoated with 50 μl of CGMMV IgG (1 μg/ml, carbonate buffer, pH 9.6), incubated for 3 h at 37°C and finally washed three times with PBS-Tween. Tubes were filled with 50 μl of a 1/20 dilution of crude extract and incubated at 4° C overnight, followed by two washes with PBS-Tween and one with diethyl pyrocarbonate-treated water, prior to RT-PCR. Immunocapture (IC)-RT-PCR was used exclusively for the detection of CGMMV in weed samples.

RT-PCR

Complementary DNA (cDNA) was synthesized using 2.0 μl-aliquots of crude plant extract, or immunocaptured virions contained in a 50 μl-aliquot of plant extract. Reverse transcription was carried out in a 10 μl reaction mixture containing 0.2 μM antisense primer and 50 units Maloney Murine Leukaemia Virus Reverse Transcriptase (M-MLV, Life Technologies™ MD, USA (Life Technologies, Invitrogen, San Diego, CA, USA)), following the enzyme supplier's recommendations. An aliquot (1/5) of this preparation was amplified in a 20 μl reaction mixture containing 2 mM $MgCl_2$, and 1.0 unit of *Taq* DNA polymerase (Life Technologies, Invitrogen, San Diego, CA, USA), according the enzyme supplier's recommendations. Amplification profile included an initial denaturation step (94°C, 5 min), 35 cycles (1 min at 94°C, 1 min at 53°C and 1 min at 72°C) and a final extension step (72°C, 10 min). Amplifications were carried out in a Mastercycler gradient (Eppendorf) and the products were analysed by electrophoresis in 1.5% agarose gels in TAE (Tris–acetate EDTA) buffer, using standard procedures (Sambrook et al., 1989). Samples were considered as positive, if a clearly visible DNA band of the correct size was present. A 100 bp DNA ladder (Gibco-BRL) was used as a molecular weight marker.

Sequence analysis of PCR products

Sequence analysis of CGMMV MP genes from the isolates CGMMV-GGR, CGMMV-CGR and CGMMV-WGR (Table 1) was carried out directly on PCR products. Ethidium bromide-stained DNA bands of interest were excised and extracted with the Matrix Gel Extraction System (Life Technologies). The nucleotide sequence was determined in both directions by means of an ABI 310 DNA sequencer (Perkin–Elmer Applied Biosystems, Foster City, CA, USA).

Alignment analysis was carried out by means of the University of Wisconsin Genetics Computer Group BLASTN program (Stephen et al., 1997), while translation to amino acid sequence was performed using the online program TRANSLATE (SWISS PORT). The accession numbers of viral sequences retrieved from EMBL/GenBank Databases were: D12505 (CGMMV-SH), AJ243353 (CGMMV-Y), AB015146 (CGMMV-W), AF321057 (CFMMV), ZGR295949 (ZGMMV), KGR295948 (KGMMV-C1), AB015144 (CGMMV-C or KGMMV-C) and AB015145 (CGMMV-Yodo or KGMMV-Y).

Results

Virus incidence

None of the apparently symptomless plants collected from the second and third field-group were found infected throughout the growing period. On the contrary, plants collected from fields of the first group (symptomatic, grafted plants) were infected by CGMMV to a different extent, depending on the period of sampling, in the following manner: early in the season, the percentage of infected plants was approximately 5%, while 1 month later the percentage was 50% and by the end of the growing season the whole crop was infected. The timely manner of visible symptoms appearance exhibited the following pattern: chlorotic mottling was the only symptom to be recorded until the end of the summer but at fruit maturity, necrosis appeared on the pedicels and finally, the fruit interior decomposed. DAS-ELISA tests revealed that 100% of these plants were infected solely by CGMMV. Early in September, mosaic and/or leaf malformation were also observed, and in this case the aphid-borne WMV 2 was also detected serologically. The percentage of CGMMV-infected bottlegourd plants was 75% (34 out of 45).

Virus purification, CP characterization and serological relationships

An average virus yield of 0.6 mg/g of fresh tissue was obtained when virus was purified from infected cucumber leaves. No contamination with host proteins was observed, when virus preparations were analysed on polyacrylamide gels (Fig. 2, lanes 5 and 6). The CP subunits of CGMMV-Is and CGMMV-GGR showed a similar mobility after SDS-PAGE, which was greater than the one of CFMMV's CP. DAS-ELISA analysis showed that an antiserum raised against CGMMV-Is failed to react with CFMMV, while it reacted strongly with isolate CGMMV-GGR indicating a close serological relationship between the two isolates, as well as the specificity of CGMMV-GGR as an isolate of CGMMV (data not shown).

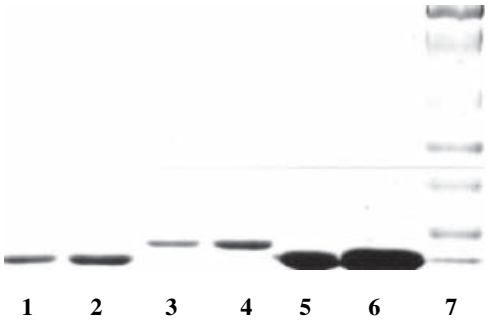

Fig. 2 Electrophoresis of purified coat proteins of CGMMV-Is (lanes 1 and 2), CFMMV (lanes 3 and 4) and the CGMMV isolate CGMMV-GGR (lanes 5 and 6) on 10% sodium dodecyl sulphate-polyacrylamide gels stained with Coomassie brilliant blue. The migration of standard proteins is shown in lane 7

Electron microscopy

Electron microscope observations of viral preparations and field samples revealed the presence of a large number of rigid, rod-shaped virus particles with a modal length of 300 nm, typical of those described for tobamoviruses (Fig. 3).

Host range

Experimental host range

Virus isolates from watermelon plants showing either pedicel necrosis (CGMMV-NA) or pedicel necrosis and chlorotic mottling (CGMMV-GGR and CGMMV-M) (Table 1), produced similar symptoms in all indicator plants tested. All cucurbit species were infected systemically. Two weeks after inoculation, mottling appeared on cucumber, melon and *Luffa acutangola* plants. Interveinal chlorosis was observed in addition to mottling on bottlegourd plants, while mild mosaic appeared on zucchini plants. Fifty days after inoculation, watermelon leaves showed chlorotic mottling similar to that observed in the field. *Gomphrena globosa* and *Chenopodium amaranticolor* showed only local necrotic lesions. In *Datura stramonium*, *Hibiscus esculentus*, *Lycopersicum esculentum* and *Nicotiana benthamiana* virus was detected by ELISA only in inoculates leaves, but plants remained otherwise symptomless. On the other hand, *Chenopodium quinoa*, *Phaseolus aureus*, *Nicotiana glutinosa*, *Nicotiana tabacum* cv. 'Samsun', *Physalis floridana* and *Solanum melongena* were immune. CGMMV-WGR did not infect *Cucurbita pepo* cv. 'Jedida', *D. stramonium* and *N. benthamiana*, while CGMMV-CGR, in addition to these species, was also unable to infect *Citrullus lanatus* cv. 'Grimson sweet', *C. melo* cv. 'Gaglia', *G. globosa* and *C. amaranticolor* (Table 3).

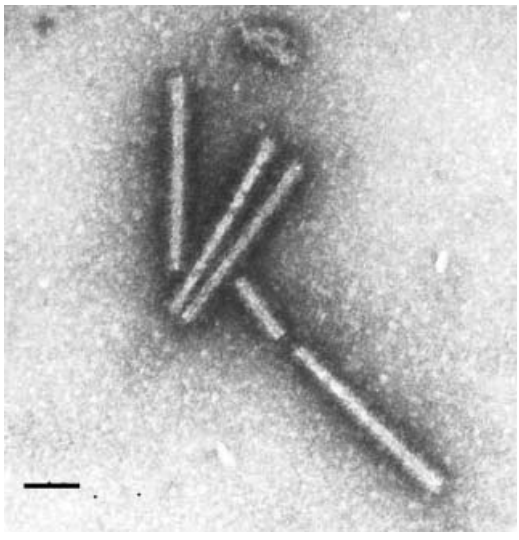

Fig. 3 Electron micrograph of CGMMV particles from purified preparation, stained in 1% unanyl acetate, Bar = 250 nm

Weeds as hosts of CGMMV

DAS-ELISA revealed the presence of CGMMV in six plant species: *Amaranthus retroflexus* (Amaranthaceae), *A. blitoides* (Amaranthaceae), *Heliotropium europaeum* (Boraginaceae), *Chenopodium album* (Chenopodiaceae), *Portulaca oleracea* (Portulacaceae) and *Solanum nigrum* (Solanaceae). Fourteen infected samples were also tested using IC-RT-PCR, which confirmed the presence of the virus in all samples except one (*A. retroflexus*). Inoculation tests yielded different results depending on the type of extraction buffer used. Using buffers I and II, the virus was transmitted only from a limited number of *H. europaerum*samples (Table 4). When 0.2% DIECA was added to buffer I (buffer III) the effectiveness of inoculation tests improved substantially and the final results were almost identical with those of DAS-ELISA and IC-RT-PCR (Table 4). Infected weed samples were found in all sampling regions, but more frequently in the Kilkis (central Macedonia) area. None of the infected weed species in the field showed any visible symptoms, which could have been attributed to a viral infection.

Molecular virus detection

Attempts to detect WCSV in plants showing chlorotic mottling, by PCR (Dr R. Briddon, Department of Virus Research, John Innes Institute) using degenerate genus-specific primers for the detection of *Begomoviruses*, proved fruitless (results not shown). To the contrary, analysis of the RT-PCR products utilizing CGMMV sense/CGMMV antisense primers, showed a specific DNA product 332 bp, which was absent in extracts of the respective healthy controls. CGMMV was detected both in watermelon plants showing pedicel necrosis and/or chlorotic mottling of leaves and also in all bottlegourd plants collected from the same affected fields (Fig. 4).

Sequence characterization of CGMMV isolates

The nucleotide and the corresponding predicted amino acid sequences of MP genes from the isolates, CGMMV-GGR (GenBank accession number: AY584530) isolated in this study, CGMMV-WGR (AY584528) and CGMMV-CGR (A484529) isolated previously from watermelon and cucumber plants,

Table 3
Experimental host range of the Greek isolates CGMMV-WGR and CGMMV-CGR and virus isolates from watermelon plants showing pedicel necrosis (CGMMV-NA) and pedicel necrosis plus chlorotic mottling (CGMMV-GGR and CGMMV-M)

Species	CGMMV-GGR	CGMMV-M	CGMMV-NA	CGMMV-WGR	CGMMV-CGR
Amaranthaceae					
Gomphrena globosa L.	L RS	L RS	L RS	L	–
Chenopodiaceae					
Chenopodium amaranticolor	L CS	L CS	L CS	L	–
Chenopodium quinoa	–	–	–	–	–
Cucurbitaceae					
Citrullus lanatus cv. Grimson sweet	C.P	C.P	C.P	S	–
Cucumis sativus cv. Lungo	G Mot, BL	G Mot, BL	G Mot, BL	G Mot, BL	G Mot, BL
Cucumis melo cv. Gaglia	Mo, BL	Mo, BL	Mo	Mo	–
Cucurbita pepo cv. Gedida F1	M Mo	S	S	–	–
Lagenaria siceraria	InV CL, Mo	InV CL, Mo	Mo	Mo	Mo
Luffa acutangola	Mo	Mo	Mo BL	Mo	Mo
Leguminosae					
Phaseolus aureus	–	–	–	–	–
Malvaceae					
Hibiscus esculentus	L	L	L	L	–
Solanaceae					
Datura stramonium	L6	L	L	–	–
Lycopersicon esculentum	L	L	L	L	–
Nicotiana benthamiana	L	L	L	–	–
Nicotiana glutinosa	–	–	–	–	–
Nicotiana tabacum cv. Samsun	–	–	–	–	–
Physalis floridana	–	–	–	–	–
Solanu melongena	–	–	–	–	–

BL, blisters; CM, chlorotic mottling; C.P, chlorotic patterns; CS, chlorotic spots; G Mot, green mottling; InV CL, interveinal chlorosis; L, local infection; Mo, mosaic; RS: red spots; S, Symptomless systemic infection; –, immune.

respectively, were obtained by direct sequencing of PCR products and compared with those of other CGMMV isolates. CGMMV-WGR seems to be almost identical (99.6%) to CGMMV-Y (AJ243353) from Korea (Table 5). Their MP amino acid sequences differ in only one site, at position 195 (E in CGMMV-WGR instead of D in CGMMV-Y). Furthermore, the MP sequence of CGMMV-WGR differs from that of CGMMV-Wa (AB015146), another CGMMV strain also isolated from watermelon, in three positions: 195, 229 and 256, where amino acids E, G and E in CGMMV-WGR replace

Table 4
Comparison of bioassays and immunocapture-reverse transcriptase-polymerase chain reaction (IC-RT-PCR) results with that of double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) concerning the control of weed samples

	ELISA	Mechanical inoculation			
Weed species	Optical density	Buffer I*	Bufffer II	Buffer III	IC-RT-PCR
Amaranthus blitoides	0.33	–	–	–	+
A. blitoides	0.67	–	–	+	+
A. blitoides (Healthy)	0.11	–	–	–	–
Amaranthus retroflexus	0.25	–	–	–	–
A. retroflexus	0.60	–	–	+	+
A. retroflexus (Healthy)	0.15	–	–	–	–
Chenopodium album	0.30	–	–	+	+
C. album (Healthy)	0.06	–	–	–	–
Heliotropium europaeum	0.67	–	–	+	+
H. europaeum	0.38	–	+	+	+
H. europaeum	0.38	–	+	+	+
H. europaeum	0.45	+	+	+	+
H. europaeum	0.35	–	+	+	+
H. europaeum (Healthy)	0.17	–	–	–	–
Portulaca oleracea	0.80	–	–	+	+
P. oleracea (Healthy)	0.18	–	–	–	–
Solanum nigrum	0.48	–	–	+	+
S. nigrum	0.57	–	–	+	+
S. nigrum	0.54	–	–	+	+
S. nigrum (Healthy)	0.17	–	–	–	–

Buffer I: 0.1 M phosphate buffer pH 7.0 + 0.01 M Na_2SO_3.
Buffer II: 0.1 M phosphate buffer pH 7.0 + 0.01 M Na_2SO_3 + 2% PVP.
Buffer III: 0.1 M phosphate buffer pH 7.0 + 0.01 M Na_2SO_3 + 0.2% DIECA.

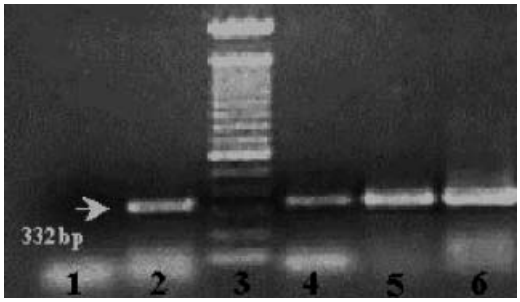

Fig. 4 Agarose gel electrophoresis of reverse transcriptase-polymerase chain reaction products obtained from *Cucumber green mottle mosaic virus*-infected watermelon plants. Lane 1: negative control, lane 2: positive control, lane 3: 100 bp molecular weight marker (Gibco-BRL), lane 4: watermelon plant showing pedicel necrosis, lane 5: watermelon plant showing chlorotic mottling and lane 6: watermelon plant showing chlorotic mottling and pedicel necrosis

D, S and D in CGMMV-Wa, respectively. CGMMV-CGR shows the lowest degree of nucleotide sequence identity (90–94.4%), although this resulted in only three amino acid substitutions compared with all other available CGMMV isolates.

Sequence data analysis obtained for the PCR product of the CGMMV-GGR isolate revealed that its MP gene has a 97.3–99% of nucleotide identity compared with all other CGMMV isolates deposited in the EMBL/GenBank Databases. Comparison of the three Greek isolates revealed that the number of nucleotide substitutions between CGMMV-WGR and CGMMV-GGR is very small (99.5% identity) (Table 5). They differ although in two amino acids, at position 19 and 147, where Q and C in CGMMV-GGR, replaces K and S in CGMMV-WGR.

Detection of virus in seeds

DAS-ELISA tests revealed that all 11 bottlegourd plants used for seed sampling were infected by CGMMV and that the virus was also present in the seed coats of 102 out of 386 (27%) seeds, which were designated for rootstock production.

Discussion

During this survey, on grafted watermelon crops in central (Thessaly) and northern Greece (Macedonia) we recorded a disease, which combined the characteristic symptoms of 'Konnyaku' disease caused by CGMMV-W, with the chlorotic patterns on leaf lamina similar to those attributed to WmCSV (Jones et al., 1988). The present study is an attempt to investigate and partially characterize the causal agent of this disease.

In electron micrographs of infected plants, virions were observed typical for members of the *Tobamovirus* genus (Raychaudhuri and Varma, 1978; Avgelis and Vovlas, 1986; Antignus et al., 1990; Al-Shahwan and Abdalla, 1992). CGMMV was identified both serologically by DAS-ELISA and by molecular techniques such as RT-PCR and its presence was highly correlated with chlorotic mottling symptoms. All efforts to detect WmCSV in plants showing chlorotic mottling proved fruitless. In addition, the observation that the vector of WCSV, *Bemisia tabaci* (Gennadious) was absent from watermelon plants strengthens the hypothesis that WCSV is not the causal agent of the disease. Moreover, the results of inoculation tests lend further support to the involvement of CGMMV in this disease. These tests revealed that: (i) the agent of leaf chlorosis is mechanically transmitted, as this symptom was reproduced on watermelon plants inoculated with field isolates (CGMMV-GGR, CGMMV-M and CGMMV-NA) of CGMMV, and (ii) that the causal agent of this disease is CGMMV, as it was the only virus detected in the inoculated plants.

The results of the biological and molecular analysis of the CGMMV isolates, which induce chlorotic mottling and fruit internal decomposition on watermelon, prove that they constitute CGMMV isolates. However, these isolates elicit more severe symptoms and have a wider host range, when compared with all other virus isolates tested in this study (Table 3). For example, they do infect *C. pepo* like the CGMMV-Is (Antignus et al., 1990) but unlike the latter, they cause local lesions on *G. globosa*, a characteristic exhibited by strain CGMMV-SH (Ugaki et al., 1991). Interestingly, our isolates behave like *Kyuri green mottle mosaic virus*(KGMMV-Y) (Tan et al., 2000), *Zucchini green mottle mosaic* virus (ZGMMV) (Ryu et al., 2000), and *Cucumber fruit mottle mosaic virus* (CFMMV) (Antignus et al., 2001) and concomitantly, they all differ from CGMMV-W, regarding the infection of *D. stramonium*, *C. amaranticolor* and *N. benthamiana*. Furthermore, our study revealed that the isolate CGMMV-GGR is serologically identical to CGMMV-

Table 5
Percent identities for pairwise nucleotide and amino acid sequence comparisons of movement protein (MP) among *Cucumber green mottle mosaic virus* (CGMMV) isolates from Greece (CGMMV-GGR, CGMMV-WGR and CGMMV-CGR), Japan (CGMMV-SH and CGMMV-Wa) and Korea (CGMMV-Y)[a]

Isolates	CGMMV-GGR	CGMMV-WGR	CGMMV-CGR	CGMMV-Y	CGMMV-SH	CGMMV-Wa
CGMMV-GGR	100	–	–	99 (98.81)	98.9 (98.5)	97.6 (98.1)
CGMMV-WGR	99.5[b] (99.2)	100	–	99.6 (99.6)	99.4 (99.2)	98 (98.8)
CGMMV-CGR	93.7 (97.7)	94.3 (98.5)	100	94.4 (98.8)	94.2 (98.5)	90 (98.5)

[a]GenBank accession numbers of sequence data used for the calculation identity: CGMMV-GGR (AY584530), CGMMV-WGR (AY584528), CGMMV-CGR (AY584529), CGMMV-SH (D12505), CGMMV-Wa (AB015146) and CGMMV-Y (AJ243353).
[b]Numbers show nucleotide identity and numbers in parentheses show amino acid identity.

Is, a biological variant of CGMMV, which has been shown to be serologically similar to CGMMV-W and CGMMV-SH (Antignus et al., 2001). To further support findings originating from electron microscopy, serology and bioassays, we examined three different isolates (CGMMV-WGR, CGMMV-GGR and CGMMV-CGR) at the nucleotide sequence level. To this end, we determined the nucleotide and the predicted amino acid sequences of their MP-genes. Selection of this gene was based on two considerations: (i) the recent findings by other researchers (Varveri et al., 2002) that the divergence of the predicted amino acid sequences of the CP among CGMMV isolates is rather limited (98.8–99.4% identity), and (ii) our findings that CGMMV-GGR exhibits a wider host range compared with isolates CGMMV-WGR and CGMMV-CGR, which could be partly or exclusively attributed to alterations occurring within the MP-gene/protein.

Sequence analysis of the MP-gene from CGMMV-GGR revealed that it has a high percentage of sequence identity when compared with all other known CGMMV isolates, thus confirming its identity as a CGMMV isolate. In particular, CGMMV-GGR seems to be closely related to the CGMMV-WGR, as they exhibit a 99.2% amino acid identity of their predicted MP sequences. CGMMV-GGR and CGMMV-WGR differ in their experimental host range, as the latter in our studies did not infect *C. pepo*, *D. stramonium* and *N. benthamiana*, while the corresponding difference between CGMMV-GGR and CGMMV-CGR is substantially greater (Table 3). At this stage, we cannot rule out the possibility, that the observed difference between CGMMV-GGR and CGMMV-WGR regarding their host range could be, at least partly, attributed to the recorded amino acid differences between their MP sequences, as has been suggested by others (Ugaki et al., 1991) on a similar occasion. It is interesting to note, that the change from a positively charged (lysine, in CGMMV-WGR) to a polar amino acid (glutamine, in CGMMV-GGR), as well as the change from serine (CGMMV-WGR) to cystein (CGMMV-GGR), could lead to substantial alterations of the MP structure.

Several researchers have studied the experimental host range of CGMMV (Hollings et al., 1975; Horvath, 1985a,b) in the past, and its natural host range has been determined to include a few crop plants along with *Ecballium elaterium* as the only naturally infected weed (Antignus et al., 1990). In the present study, we report the survey, although on a small scale, for the presence of CGMMV in arable weeds. Using DAS-ELISA, mechanical inoculation and IC-RT-PCR, we were able to identify six weed species belonging to families Amaranthaceae, Boraginaceae, Chenopodiaceae, Portulacaceae and Solanaceae, which proved to be natural hosts of CGMMV (Table 5). All infected plants were symptomless, as has been previously observed for other viruses (Powell et al., 1984; Khan et al., 1991; Chatzivassiliou et al., 2000) and this is attributed, among others, to natural selection (Duffus, 1971).

Although arable weeds, generally play a critical role in the epidemiology of many important viral diseases (Duffus, 1971), their role in the case of CGMMV seems to be rather limited, especially when certified seed is used for watermelon production (Bem and Vassilakos, 2000). In spite of being certain that the virus survives and retains its infectivity in weed debris in the soil, this could not serve its spread in the field substantially.

In addition to the results obtained during the examination of weed species by serology and PCR, we were also able to demonstrate the presence and infectivity of CGMMV by means of mechanical inoculation. CGMMV is a stable virus reaching high concentrations in infected plants and consequently we did not encounter such problems as with other viruses like TSWV (Chatzivassiliou et al., 2000). In spite of its stability, the addition of DIECA (0.2% w/o) in the extraction buffer proved to be necessary, as it improved the transmission efficiency of the virus by a factor of 10 and three in comparison to buffers I and II, respectively. Presumably, DIECA's effectiveness resides in the removal of inhibitors such as phenols, polysaccharides and other substances present in the weed plant extracts (Cho et al., 1986).

During this study, we have found several bottlegourd plants, used by farmers for seed production, to be highly infected with CGMMV. Through testing seeds from these plants, we confirmed that CGMMV is seed-transmitted in this species (Nagai et al., 1974). As the appearance of CGMMV-W coincided with the use of grafting technique, it is suspected that the virus was imported to Greece through infected seeds of the *Fusarium* resistant rootstocks (Varveri et al., 2002). As long as farmers used only certified seeds, the disease was contained below detection level. On the other hand, as soon as they started producing and using their own rootstock seeds, most of the crops were severely infected, as it was determined in the course of this study.

The isolation of CGMMV from field infected samples, the reproduction of symptoms in watermelon plants grown in the laboratory and the re-isolation of the same virus, using ELISA and RT-PCR techniques, certainly fulfils the requirements of the Koch postulates and confirms that CGMMV is the causal agent of the disease 'leaf chlorosis and deterioration of the fruit interior of watermelon plants'.

Disease control should be focused on the use of certified seeds, because infected rootstocks combined with the grafting technique constitutes the main reason for the high disease incidence, as shown by this study.

Acknowledgements

The authors thank Dr F. Bem (Plant Virology Laboratory, Benaki Phytopathology Institute, Athens, Greece) and Dr A. D. Avgelis (Plant Protection Institute, Plant Virology Laboratory Heraklio, Crete, Greece) for providing CGMMV isolates. The authors also thank F. Bem and C. Varveri (Plant Virology Laboratory, Benaki Phytopathology Institute, Athens, Greece), H. Lecoq (INRA, Station de Pathologie Vegetale, Montfavet Cedex, France) and Prof.

B. Falk (Department of Plant Pathology, University of California, Davis, CA, USA) for providing antisera against CGMMV and other cucurbit viruses, Dr R. Briddon (Department of Virus Research, John Innes Institute) for testing watermelon samples for the presence of WCSV and Dr E. Drossos (Department of Biology, Aristotle University of Thessaloniki, Thessaloniki, Greece) for weed identification.

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