Characterization of a *Cucumber mosaic virus* isolate infecting *Mandevilla sanderi* (Hemsl.) Woodson

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Summary. In 2004, virus-like symptoms consisting of yellow rings in older leaves were observed in the foliage of *Mandevilla sanderi* grown from shoots in the province of Catania (Sicily, Italy). The plants were tested for virus presence using DAS-ELISA, mechanical inoculations to the host, RT-PCR and SSCP. Serological tests detected *Cucumber mosaic virus* (CMV) in all symptomatic leaf samples. The virus was mechanically transmitted to 11 of the 15 host species tested, including some of the *Chenopodiaceae* and *Solanaceae* families; indicator plants showed symptoms typical of CMV subgroup I. RT-PCR, SSCP and cloning were employed to characterize this CMV isolate, and to make clear its genetic relationship with selected CMV-isolates from other countries. From this analysis, CMV-*M. sanderi* appeared closely related to AY153419 (CMV-PIM-SP-BR; subgroup IA) (nucleotide identities higher than 98%) from Brazil. The virus was transmitted by sap inoculation to healthy *M. sanderi* plants. As far as we know this is the first report of virus infection in *M. sanderi* and it then appears that this ornamental shrub is a new natural host of CMV.

Key words: new disease, phylogenetic analysis, SSCP.

Introduction

Cucumber mosaic virus (CMV) (genus *Cucumovirus*, family *Bromoviridae*) is one of the most economically important viruses causing hundreds of diseases worldwide in more than 1,000 botanical species (belonging to 100 families) (van Regenmortel *et al.*, 2000). It is transmitted by aphids (about 75 species) in a non-persistent manner and is also seed-borne in different host plants such as *Stellaria media* and *Ecballium elaterium*.

Isometric CMV virions are approximately 30 nm in diameter. CMV is a single-stranded positive-sense tripartite genome RNA virus: the genomic RNAs are designed as RNA1 (of 3,360 nt), RNA2 (of 3,050 nt) and RNA3 (of 2,200 nt). RNA1 and RNA2 are encapsidated separately, while RNA3 is encapsidated with another RNA (RNA4) having a subgenomic function. Some CMV isolates support the replication of small satellite RNA (satRNA), of 330–390 nt, that causes modifications of the symptoms induced by the virus (Palukaitis *et al.*, 1992). CMV has numerous strains which have been divided into two subgroups, I and II, based on serological data, peptide mapping of the coat protein, nucleic acid hybridization, RT-PCR

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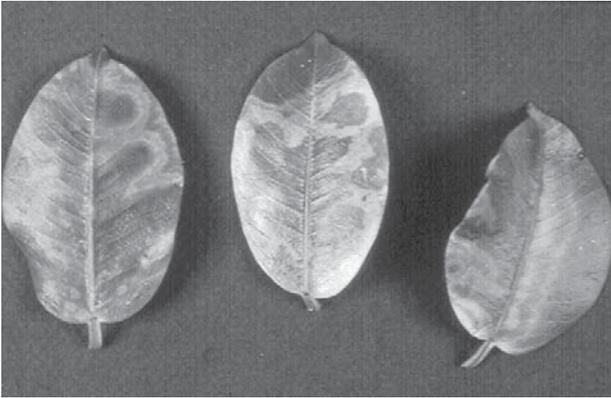Fax: +39 095 7147272
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Fig. 1. Yellow rings on old leaves of *M. sanderi* naturally infected with CMV.

combined with RFLP and nucleotide sequence similarities (Edwards and Gonsalves, 1983; Rizos *et al*., 1992; Singh *et al*., 1995). Other sequence data have shown that a number of CMV strains within subgroup I (called the "Asian strains") differ by 7–12% in sequence from other subgroup I strains; on this basis, Paulakaitis and Zaitlin (1997) proposed to place the Asian strains in a subgroup IB and the others in a subgroup IA.

During epidemiological surveys carried out in 2004 at a flower nursery in Catania province (Sicily, Southern Italy), almost all the 6,500 cultivated plants of *M. sanderi* (Hemsl.) Woodson. (Apocynaceae family) exhibited virus-like symptoms consisting of prominent yellow rings on the older leaves (Fig. 1), while the flowers were normal in colour and size. These plants, originally obtained from shoots received from Northern Italy, had been maintained under glasshouse conditions for two years, while their original native source was Brazil. In this study we report the results of research carried out to isolate, identify and characterize, at both the biological and molecular level, the causal agent of these symptoms, found to be cucumber mosaic virus, which is reported in this host plant for the first time.

Materials and methods

Biological characterization

Symptomatic leaf samples of *M. sanderi* were ground in cold 0.1 M phosphate buffer, pH 7.0, containing 3% polyethylenglycol 6,000 (PEG); the homogenate was immediately rubbed onto carbo-

rundum-dusted leaves of 15 botanical plant species belonging to 5 families: Chenopodiaceae, Cucurbitaceae, Lamiaceae (Labiatae), Fabaceae (Leguminosae) and Solanaceae. The plants inoculated were: *Capsicum annuum* L., *Chenopodium murale* L., *C. quinoa* Willd., *Citrullus vulgaris* Ser. "Crismon Sweet", *Cucumis melo* L., *Nicotiana benthamiana* L., *N. clevelandii* Gray, *N. glutinosa* L., *N. tabacum* L. "Samsun", *Ocimum basilicum* L., *Phaseolus vulgaris* L. "Bobis", *Pisum sativum* L. "Mezza Rama", *Solanum melongena* L., *Vicia faba* L. and *Vigna sinensis* Endl. All inoculated plants were maintained in an insect-proof greenhouse at a constant temperature of 24–25°C and checked for virus presence by back inoculations to *C. murale*. In addition, 10 non-CMV-infected *M. sanderi* plants (tested by ELISA) grown in another greenhouse were inoculated using the same technique as above.

Serology

In preliminary serological tests, 200 samples, each consiting of one piece of old and one piece of new vegetation from the same symptomatic plant, were randomly collected and tested by double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA). The sera to the following viruses were employed: *Tomato spotted wilt virus* (TSWV, BR01), *Impatiens necrotic spot virus* (INSV), *Tobacco mosaic virus* (TMV) and CMV (DTL/ToRS). Polyclonal antibodies came from the Loewe-Phytodiagnostica Biochemica (Sauerlach, Germany).

Molecular characterization

Fourteen *M. sanderi* leaf samples out of 198 (of the 200 tested) were found to be CMV-infected in DAS-ELISA tests and were used to extract total RNAs with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Total RNA was used as template for reverse transcription and polymerase chain reaction (RT-PCR) with primers CMV3R (5'-AGT GAC TTC AGG CAG T-3', genome position 198-2001, GenBank accession number Y16926), and CMV3F1 (5'-GCT TGT TTC GCG CAT TCA-3', genome position 1566-1583, GenBank accession number Y16926) (Bellardi *et al*., 2004). Non-CMV-infected *M. sanderi* samples were employed as a negative control and CMV from tomato grown in

greenhouse as the positive control. RT-PCR was performed in one-step in a 25 μl volume reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTPs, 1 μM of each primer, 4U of RNaseOut, 20 U of SuperScript II reverse transcriptase-RNaseH and 2U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). RT-PCR was carried out in a thermal cycler PTC 100 (M.J. Research, Waltham, MA, USA) using the following conditions and parameters: 42°C for 30 min, 94°C for 2 min, 35 cycles of 30s at 94°C, 30s at 56°C, and 30s at 72°C and a final elongation of 4 min at 72°C. In order to obtain finer molecular characterization of this CMV isolate, the RT-PCR amplified products were analysed with the single strand conformation polymorphism (SSCP) technique in a non-denaturing gel of polyacrylamide 8%. SSCP was carried out for 4 h at 4°C. Two SSCP patterns were detected allowing identification of two sequence variants (named in this work CMV-V1 and CMV-V2), which were cloned in an apCR2.1 – TOPO vector (Kit TOPO-TA Cloning, Invitrogen). Twenty-five plasmids for each molecular variant were extracted with the High Pure Plasmid Isolation Kit (Roche, Basilea, Switzerland), following manufacturer's instructions. After again performing the PCR with the above described primers, 20 plasmids containing CMV-V1 and 19 plasmids containing CMV-V2 fragments of the expected size were obtained. Two plasmids of each SSCP profile were sequenced in both directions using an ABI PRISM DNA 377 sequencer (Perkin-Elmer, Boston, MA, USA). Sequences were aligned with the program CLUSTAL W (Thompson *et al.*, 1994).

Phylogenetic relationships

Since the amplicons obtained were produced on a conserved area of CMV coat protein (Bellardi *et al.*, 2004), they were employed to study further the phylogenetic relationships of the two molecular variants identified in this CMV isolate. The program MEGA (Kumar *et al.*, 2001) was applied to estimate nucleotide distances between pairs of sequences (number of nucleotide differences per site) using the Kimura 2-parameter correction (Kimura, 1980). To compare the CMV-isolate found in *M. sanderi* with isolates from other countries, 20 nucleotide sequences were retrieved from the GenBank entries: D10538 (Strain Fny),

U22821 (Ny), AY153419 (PIM-SP-BR), AJ511990 (NS), D10539 (M), D49496 (M48), AJ304403 (PR6A-ALS), AJ304398 (NAK-ALS), L15336 (Trk7), AF127976 (Ls), AB042294 (IA), U20219 (Ixora), D16405 (Leg), AJ276481 (Mf), D28780 (NT9), M21464 (Q), AF063610 (S), AB008777 (SD), Y16926 (Tfn) and D12499 (Y). Phylogenetic relationships were inferred using the MEGA based neighbour-joining method with a 1,000 replicate bootstrap value.

Results

Biological characterization

Cucumber mosaic virus was transmitted by mechanical inoculation to 11 of the 15 species tested, which were infected, reacting with local and/or systemic symptoms. Local lesions developed in *C. murale*, *C. quinoa*, *P. sativum* and *V. sinensis* while *N. tabacum*, *N. benthamiana*, *N. glutinosa* and *N. clevelandii* reacted with systemic mosaic and reductions in leaf size; *C. annuum*, *C. melo* and *C. vulgaris* showed chlorotic mottle and malformations on younger leaves. No infection was observed on *O. basilicum*, *P. vulgaris*, *V. faba* and *S. melongena*. After six weeks the symptoms of the disease were also clearly visible on 9 of the 10 *M. sanderi* plants inoculated.

Serology

All the 200 samples tested negative for TSWV, INSV and TMV, while 198 tested positive for CMV.

Molecular characterization

When RT-PCR was applied to the 40 *M. sanderi* leaf samples (of the 198 that were CMV-infected), all samples yielded the expected 436 bp DNA fragment, as did the CMV isolate employed as positive control, whereas no amplification was obtained from non-CMV-infected plants of *M. sanderi* used as negative control. With SSCP, two patterns were detected, allowing identification of two sequence variants: CMV-V1 and CMV-V2 respectively, and confirming the sensibility of SSCP for nucleotide substitution detection (Rubio *et al.*, 2001).

Phylogenetic relationship

Using the MEGA program, the nucleotide distance between CMV-V1 and CMV-V2 was 0.0069,
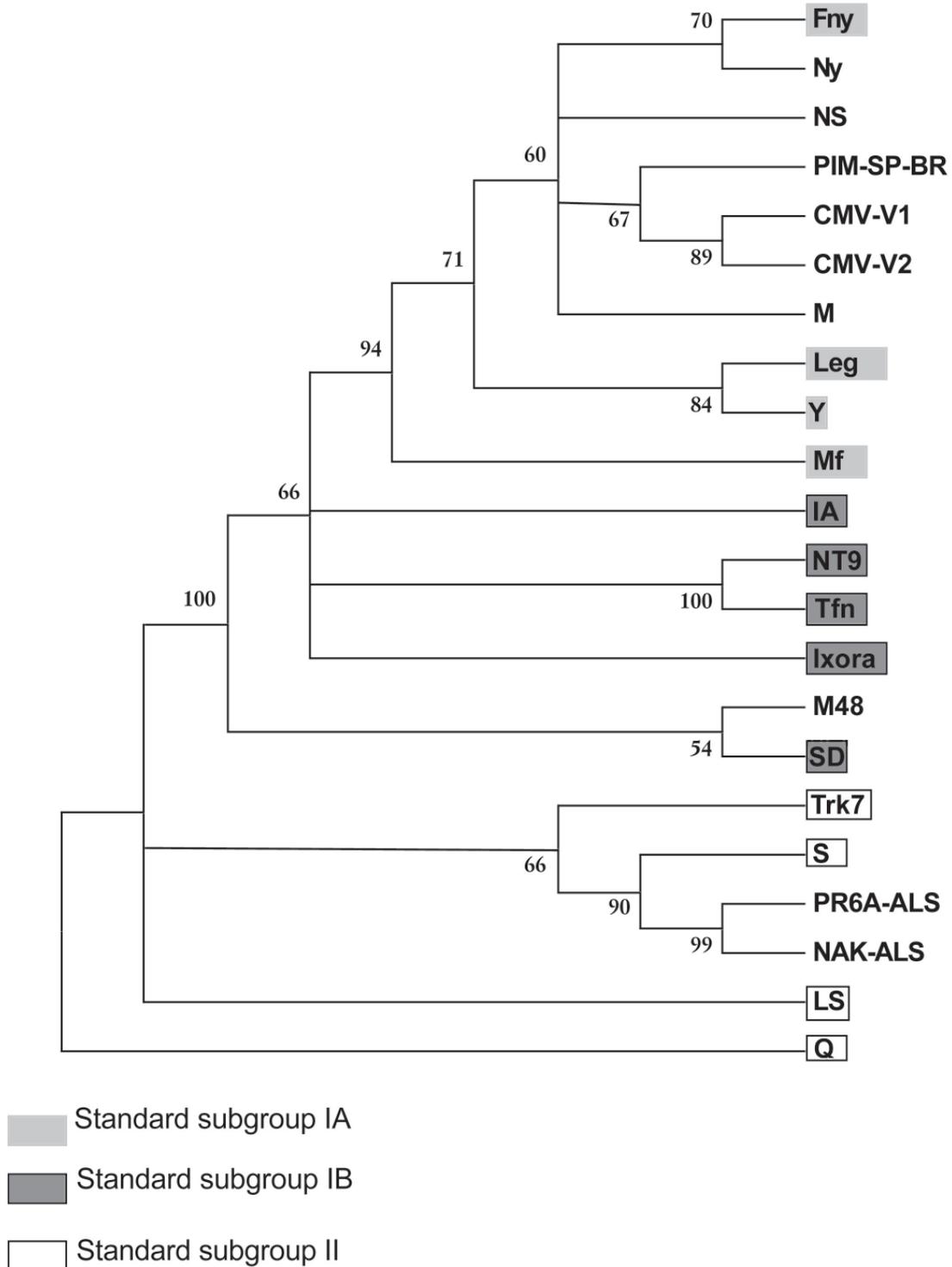
Fig. 2. Bootstrap consensus trees of coat protein (CP) of RNA3 from CMV-1 and CMV-2 partial sequences, constructed with the MEGA program according to the neighbour-joining method. Bootstrap values (percentage) are indicated in the nodes, strain acronyms are as in the text.

confirming their very close genetic relationship. Using the MEGA based neighbour-joining method with a 1,000 replicate bootstrap value, the CMV-V1 and CMV-V2 isolates appeared closely related to AY153419 (CMV-PIM-SP-BR; subgroup IA) (nucleotide identity greater than 98%) (Fig. 2).

Discussion

The tests showed that the yellow rings on the *M. sanderi* leaves were caused by an isolate of CMV belonging to subgroup I, possibly IA. The reactions on the inoculated test plants were similar to those induced by isolates of CMV subgroup I (Daniels and Campbell, 1992), even if it must be borne in mind that there is much variation in the host response, which depends mainly on the temperature and time of the year, the age of the indicator plants and the source of the inoculum.

From the molecular characterization this CMV isolate appeared closely related to AY153419 (CMV-PIM-SP-BR) also belonging to subgroup IA (nucleotide identity greater than 98%); in addition, the original source of imported *M. sanderi* is Brazil where, up to now, no CMV strains of subgroup II have been isolated, suggesting that the isolate probably originated in that country (Eiras *et al.*, 2004).

This is the first time that CMV is reported as a pathogen of *M. sanderi* and characterized. CMV is known to naturally infect other ornamental shrubs, including some belonging to the Apocynaceae family: golden trumpet (*Allamanda cathartica* L.) and oleander (*Nerium oleander* L.) on both of which it causes pronounced symptoms of mottle and yellow areas or rings on the leaves (Bellardi and Bertaccini, 1993; Bellardi *et al.*, 1996).

So far virus diseases have not been reported on *M. sanderi*, which is an important ornamental plant in Southern Italy, where every year more than 200,000 potted plants are produced in nurseries of Catania province and exported both to other Italian regions and to northern Europe. Infected *M. sanderi* plants could become a potential source of CMV inoculum for other cultivated species. The existence of CMV isolates that are genetically related but occur in geographically distinct areas, as noted in this work, suggests that they may move together with infected plant material between countries. The use of molecular characterization may help to detect such movements and also to prevent the spread of new pathogen isolates and/or strains to other host plants in other environments.

Acknowledgements

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