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Identification, detection and transmission of a new vitivirus from *Mentha*

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Summary

Mentha × *gracilis* 'Variegata' is an ornamental clone with a phenotype caused by virus infection. Several clones were ordered from mail-order nurseries in an attempt to identify a virus consistently associated with symptoms. One of these clones did not exhibit typical 'Variegata' symptoms, and steps were taken to identify any agents causing the 'off-type' symptoms. One of the viruses identified in the atypical 'Variegata' clone is a previously unknown virus, a member of the family *Flexiviridae*. Sequence and phylogenetic analysis indicate that the virus, designated as mint virus-2, is related to members of the species *Grapevine virus A*, *Grapevine virus B* and *Heracleum latent virus*, placing it in the genus *Vitivirus*. A detection protocol for the virus has been developed, and the mint aphid (*Ovatus crataegarius*) was able to transmit the virus in the presence of a helper virus but not from single infected plants.

Introduction

Mint (*Mentha* sp.) has been used for centuries for both medicinal and culinary purposes [5]. Several pathogens, including powdery mildew, rusts and verticillium wilt can cause significant losses [6, 8, 14, 21]. While mint fungal pathogens have been studied extensively, little is known about the viruses that infect the crop, other than reports identifying mint as a host [4, 20, 22]. A number of mint clones exhibit virus-like symptoms, and several are used as ornamentals because of those symptoms. One of the clones with striking phenotype is *Mentha* × *gracilis* 'Variegata', also known as golden ginger mint (GGM). GGM plants exhibit bright yellow vein banding symptoms that are lost during summer months and eliminated after heat therapy and apical meristem culture [29]. These observations led to the investigation of the putative virus etiology, also implied by Tucker and Fairbrothers [26]. Three viruses have been identified in a GGM clone [17, 29, 31]. Six GGM clones were acquired from mail-order nurseries in order to identify a virus consistently associated with symptoms and to identify the causal agent of the phenotype. One clone obtained from an Oregon nursery, marketed as GGM, did not exhibit the typical vein banding symptoms of GGM and was designated as Oregon ginger mint (OGM; Fig. 1). OGM had distorted leaves and no vein banding was observed. Tobacco ringspot virus (TRSV) was found in the plant, but another TRSV-infected mint clone in our collection was asymptomatic. In order to assess if other viruses caused OGM

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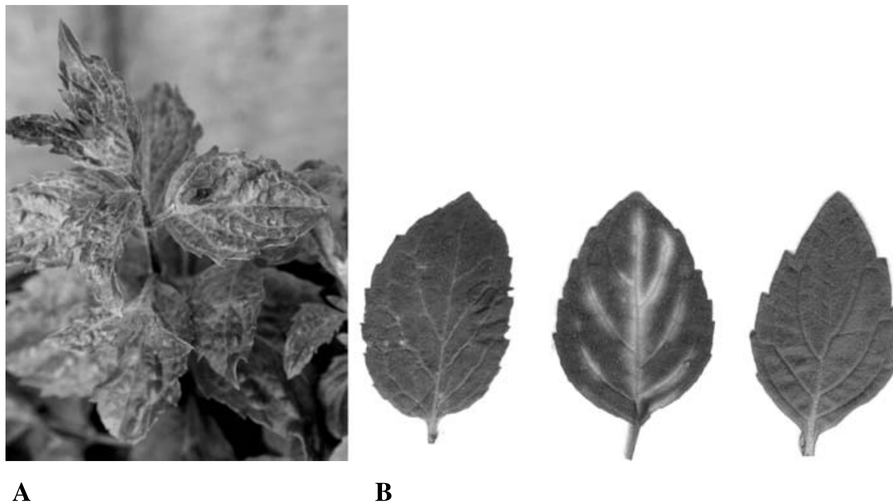

Fig. 1. (**A**) Crinkle symptoms on Oregon ginger mint; (**B**) Symptoms of Oregon and golden ginger mint. *Left* Symptoms of Oregon ginger mint; *middle* Symptoms of golden ginger mint; *right* Virus-free ginger mint NCGR 454.002 (healthy control)

symptoms, double-stranded RNA (dsRNA) was extracted and cloned. Two newly described viruses were identified: a virus belonging to the family *Closteroviridae* named mint virus-1 (MV-1) [30] and a virus belonging to the family *Flexiviridae*, designated mint virus-2 (MV-2), which is characterized in this communication.

Materials and methods

Virus purification and electron microscopy

MV-2 was purified according to the method of Martin and Bristow [15]. Virions were visualized after negative staining of the virus preparation with 2% molybdenum acetate.

Nucleic acid extractions and cloning

Total and dsRNA isolations and cloning were performed as described previously [27, 28]. Identification of recombinant plasmids with the largest inserts was performed using polymerase chain reaction (PCR) with *Taq* polymerase (New England Biolabs, Beverly, MA) and the M13 forward and reverse primers. Plasmids were sequenced at the Macrogen Inc. facilities (Seoul, South Korea) in an ABI3730 XL automatic DNA sequencer.

The MV-2 sequence deposited in Genbank under accession number AY91795 was acquired essentially as described for mint vein banding associated virus [29].

Detection

The methodology used for MV-2 detection was essentially identical to that described for MV-1 detection [30]. The primers used for routine detection of the virus were MV-2 F (5′ CCAGCAGACTTACAACTTGGT 3′) and MV-2 R (5′ TGGGGTCCGAATCTACATAGCA 3′), which amplify a 267-nt region of the polymerase domain of the virus. An internal control was added in the PCR reaction amplifying 721 nucleotide (nt) from the mRNA of the NADH dehydrogenase ND-2 subunits. The forward primer is the same as that of Thompson et al. [25] (5′ GGACTCCTGACGTATAC GAAGGATC 3′), while the reverse (5′ AGTAGATGCTATC ACACATACAAT 3′) was designed to amplify a larger fragment than the amplicons produced by detection primers used in our laboratory. These internal control primers have been used successfully with *Fragaria*, *Ribes*, *Rubus*, *Vaccinium* and all plant species listed here. Several amplicons were sequenced to verify the specific amplification of the expected MV-2 region.

The similarity of MV-2 with grapevine viruses A and B (GVA and GVB, respectively) led us investigate the possibility that antibodies raised against these viruses (antibodies provided by Dr. Adib Rowhani, UC Davis) could be used for immunological detection of MV-2 using DAS-ELISA [3].

Transmission studies

Thirteen herbaceous species (*Chenopodium quinoa, Ch. amaranticolor, Cucumis sativus, Nicotiana benthamiana, N. tabacum, N. occidentalis, Phaseolus vulgaris, Tetragonia tetragonioides, Vigna sinensis, Brassica rapa, Spinacia oleracea, Capsicum capsa, Gomphrena globosa*) and virus-free *M.* × *gracilis* clones were mechanically inoculated with tissue from OGM as described elsewhere [29]. At least five of each of the inoculated herbaceous plants and 15 plants of the virus-free mint were tested for the presence of MV-2 one month after inoculation by RT-PCR.

Mint aphids (*Ovatus crataegarius*) were left to feed on OGM or MV-2 single infected plants for at least one week. After the acquisition period, about 10 aphids for the OGM

and 20 aphids for the MV-2 single infected plants study were transferred onto virus-free *M.* × *gracilis* clones and were left to transmit the virus for at least one week before being treated with a systemic insecticide (Marathon®). Four trials of five plants each for the OGM and two trials of ten plants each for MV-2 single infected plants were used in the study and were tested for MV-2 after a period of about one month following Marathon® treatment.

Genome analysis

The open reading frames (ORF) encoded by MV-2 were identified with the FGENESV0 and ORF finder programs (http://www.softberry.com and http://www.ncbi.nlm.nih.gov/gorf/gorf.html, respectively). ClustalW [24] was used for nt and amino acid (aa) alignments. ClustalW was also used for phylogenetic analysis with bootstraps consisting of 1000 pseudoreplicates.

Results

Virus analysis

Few virus-like particles were visible under the electron microscope in purified preparations of MV-2 after negative staining. The width of the virions was about 13 nm, but the length varied, with the largest particles being approximately 550 nm.

The four-kilobase genomic sequence of MV-2 deposited in GenBank was acquired by a combination of shotgun cloning and PCR extension from the polymerase domain to the poly-adenosine tail of the 3′ terminus of the virus (Fig. 3). Phylogenetic analysis of the conserved motifs of the polymerase and the coat protein of the virus places it in the genus *Vitivirus* of the family *Flexiviridae* (Fig. 4).

The polymerase conserved domain, as identified by Koonin [9], is 236 aa long and shares homology with the homologous domains of GVA and GVB, exhibiting more than 75% aa sequence identity and almost 90% aa similarity. The next open reading frame (ORF) encodes a putative 24-kDa protein lacking significant homology with any protein found in the database. A 329-aa ORF is found downstream from the putative 24-kDa protein. The 37-kDa putative protein shows significant homology with the movement proteins (MP) of other vitiviruses [18, 19], exceeding 46% aa sequence identity and 70% aa similarity with the MP of Heracleum latent virus (HLV). The coat protein (CP) of the virus follows the MP [2]. The ORF has two potential in-frame start codons. The first AUG lacks the optimal context for translational initiation [10], unlike the second AUG found 36 nt downstream, but the initiation signal remains to be determined. The protein is very similar to the CP of other vitiviruses,

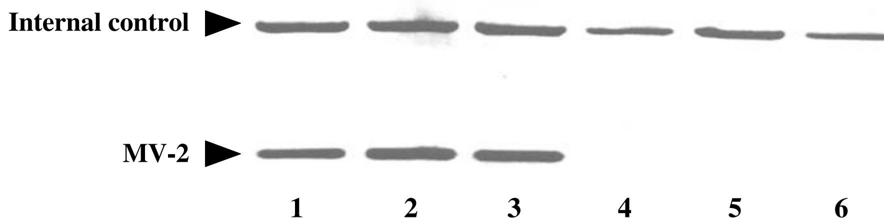

Fig. 2. Reverse transcription-polymerase chain reaction detection of mint virus-2. *1* Oregon ginger mint; *2*, *3* plants infected with mint virus-2 after aphid transmission from Oregon ginger mint; *4* mint virus-1-infected plant; *5* mint vein banding associated virus-infected plant; *6* NCGR 454.002 (healthy control)

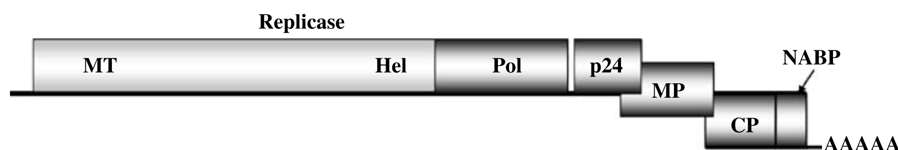

Fig. 3. Putative genomic organization of mint virus-2. The darker region indicates the region sequenced. *MT* Methyltransferase; *Hel* helicase; *Pol* RNA-dependent RNA polymerase; *MP* movement protein; *CP* coat protein; *NABP* nucleic-acid-binding protein. Drawing is not to scale

with about 60% aa sequence identity and 80% aa similarity. The last ORF identified in MV-2 encodes a putative 111-aa protein. The highly polar protein has significant similarity with the nucleic-acid-binding proteins (NABP) found in vitiviruses, allexiviruses and Citrus tristeza virus (CTV) [1, 7, 12, 23]. The 3′ untranslated region (UTR) of the genome is 85 nt long excluding the poly-adenosine tail of the virus, similar in size to the 3′ UTRs of the sequenced vitiviruses [1, 16, 19].

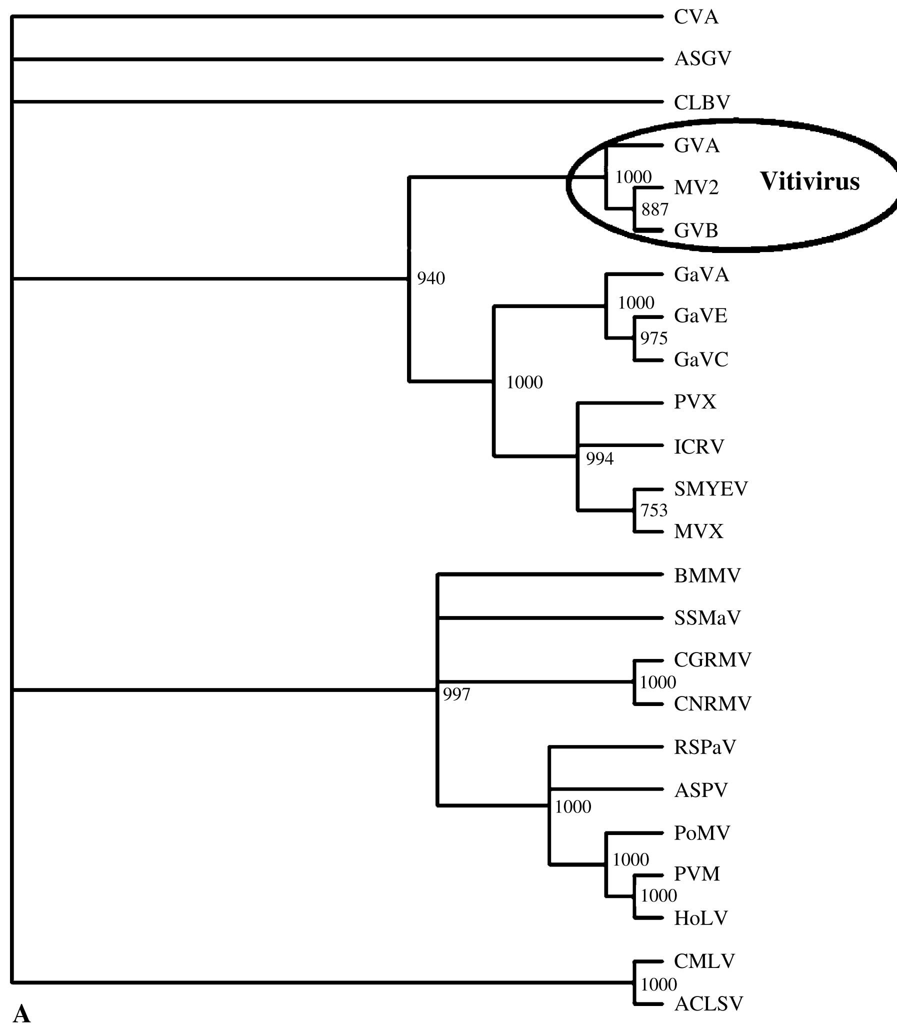

Fig. 4. Cladogram of the (**A**) polymerase conserved motifs and (**B**) coat protein of mint virus-2 and other members of the family *Flexiviridae*. Abbreviations and GenBank accession numbers: apple chlorotic leaf spot virus, ACLSV, NC 001409; apple stem grooving virus, ASGV, NC 001749; apple stem pitting virus, ASPV, NC 003462; banana mild mosaic virus, BMMV, NC 002729; cherry green ring mottle virus, CGRMV, NC 001946; cherry mottle leaf virus, CMLV, NC 002500; cherry necrotic rusty mottle virus, CNRMV, NC 002468; cherry virus A, CVA, NC 003689; Citrus leaf blotch virus, CLBV, NC 003877; garlic virus A, GaVA, NC 03375; garlic virus C, GaVC, NC 003376; garlic virus E, GaVE, NC 004012; grapevine virus A, GVA, NC 003604; grapevine virus B, GVB, NC 003602; grapevine virus D, GVD, Y 07764; Heracleum latent virus, HLV, X 79270; hop latent virus, HoLV, NC 002552; Indian citrus ringspot virus, ICRV, NC 003093; lily virus X, LVX, NC 007192; mint virus-2, MV-2, AY 913795; mint virus X, MVX, NC 006948; poplar mosaic virus, PoMV, NC 005343; potato virus M, PVM, NC 001361; potato virus X, PVX, NC 001455; rupestris stem pitting-associated virus, RSPaV, NC 001948; strawberry mild yellow edge virus, SMYEV, NC 003794; sugarcane striate mosaic associated virus, SSMaV, NC 003870. Nodes with bootstrap values of less than 70% collapse as they are not considered significant

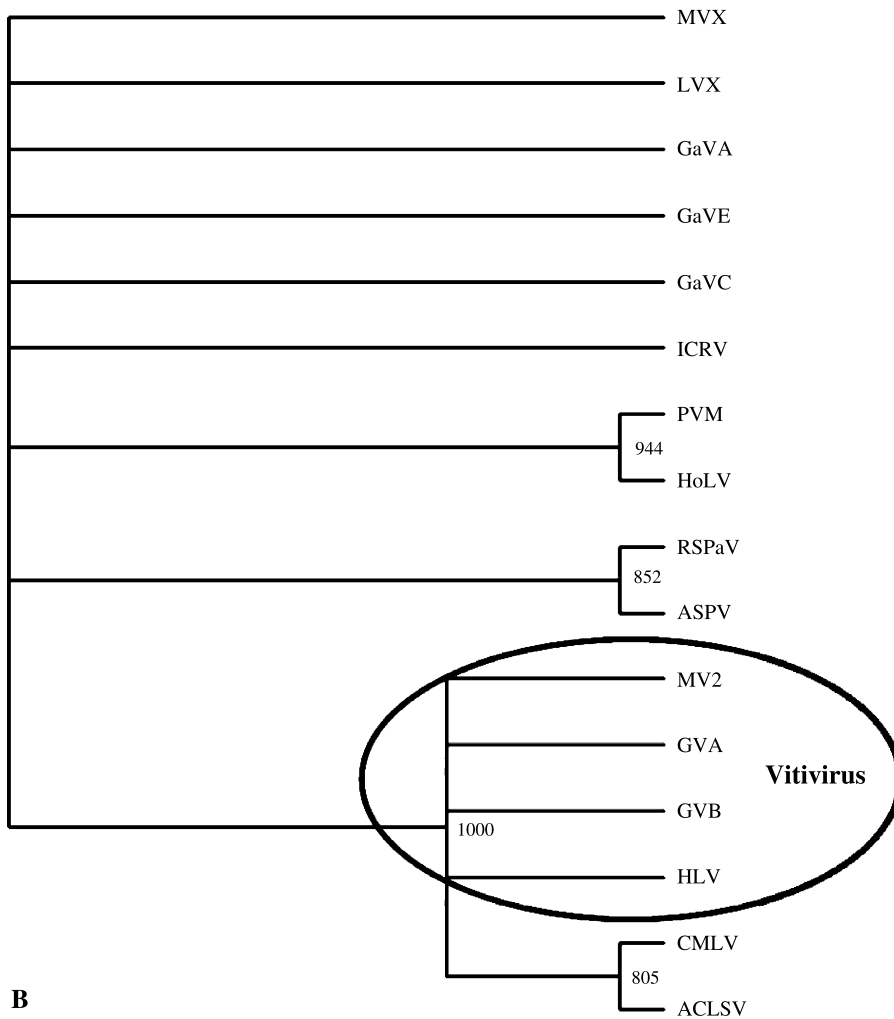

Fig. 4 (*continued*)

Detection

A multiplex RT-PCR test that detects both MV-2 and a host mRNA, used as an internal control, has been successfully employed in detection of MV-2 (Fig. 2). The ELISA test using a cocktail of GVA and GVB antibodies failed to detect the virus.

Transmission studies

None of the herbaceous hosts and mint plants that were mechanically inoculated tested positive for MV-2 by RT-PCR, indicating that the virus either is not mechanically transmissible or that it is transmitted with difficulty, as is the case with other vitiviruses [16]. When OGM was used as the source plant for the aphid transmission experiments, three out of twenty plants become infected with MV-2. The virus was not aphid-transmissible when one of the three single infected MV-2 plants was used as source material. The infected plants remained asymptomatic for a period of over three years after aphid transmissions.

Discussion

Only two members of the genus *Vitivirus* have been studied extensively [16, 19], and this communica-

tion adds to the limited knowledge about this group of viruses, identifying a new member of the genus that infects mint. Members of the genus have positive-sense single-stranded RNA genomes of about 7.5 kb and form filamentous particles of about 800×12 nm [2]. The purification protocol for MV-2, successfully used for mint virus X [31], yielded a few virus-like particles, 13 nm in width but of varied length, presumably due to virion breakage.

The MV-2 sequence encodes the virus RNA-dependent RNA polymerase, a putative protein of unknown function, the MP and CP and a NABP, a protein that shares significant homology with suppressors of silencing [13, 32]. Phylogenetic analysis using the polymerase conserved motifs and CP indicated the clustering of MV-2 with members of the genus *Vitivirus* and a more distant relationship with other members of the family *Flexiviridae* (Fig. 4).

Transmission studies showed that MV-2 can be transmitted by aphids in the presence of a helper virus, presumably MV-1, similar to what has been found for HLV [2]. Aphid-inoculated MV-2 plants were infected only with this virus and not with the putative helper virus MV-1. This was surprising since conditions used for transmission of MV-1 and MV-2 were identical. In order to minimize the possibility that MV-1 was not detectable at the time we tested for MV-2 transmission, we retested these plants three more times over a period of three years and we were unable to detect MV-1.

MV-2 is latent in single infections, as determined by the aphid transmission studies and thus it is not the causal agents of the symptoms observed in OGM.

Another vitivirus, peppermint stunt virus (PmSV) has been previously discovered in peppermint [11]. Data provided by Drs. T. Sit and S. Lommel (North Carolina State University) indicate that PmSV and MV-2 are closely related but distinct species, as they share about 70% nucleotide sequence identity (75% aa identity) in the majority of the coat protein region available for PmSV, including the two in-frame start codons in the coat protein gene. The two viruses share characteristics such as low titer in plants and difficulty to purify [11] but differ in pathogenesis since severe symptoms are observed in PmSV-infected plants.

The symptoms in OGM are caused by a combination of the viruses found in this clone or another, yet unknown virus, as each of the OGM viruses remains asymptomatic in single infections [30]. A limited number of mint clones (80) from North America and Asia have been tested for the presence of MV-2, and two were found to be infected, both of which were asymptomatic and infected with MV-1 (Tzanetakis, unpublished data).

Acknowledgments

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