Characterization of a Novel Member of the Family *Closteroviridae* from *Mentha* spp.

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ABSTRACT

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While characterizing the agents involved in symptomatology of a variegated mint, *Mentha* × *gracilis* 'Variegata', a nursery plant with atypical symptoms was examined. This plant, unlike 'Variegata', did not exhibit yellow vein banding symptoms but instead had distorted and crinkled leaves. Molecular tests for the three viruses found in 'Variegata' clones failed to detect any of these viruses in the plant. Double-stranded RNA was extracted and cloned, disclosing the presence of two unknown viruses. One of the viruses was a novel member of the family *Closteroviridae*. The complete nucleotide sequence of the virus, designated as Mint virus 1, has been obtained. A detection test was developed, and revealed the presence of the virus in several other mint clones and species. Genomic regions from three additional isolates were examined to investigate the genetic diversity of the virus. Genome and phylogenetic analysis placed Mint virus 1 in the genus *Closterovirus* and transmission studies have identified the mint aphid, *Ovatus crataegarius*, as a vector for this new member of the genus *Closterovirus*.

Several viruses have been associated with disease symptoms in mint (*Mentha* spp.), including *Cucumber mosaic virus*, *Alfalfa mosaic virus*, *Tomato spotted wilt virus*, *Impatiens necrotic spot virus*, *Arabis mosaic virus*, *Strawberry latent ringspot virus* (SLRSV), and *Tobacco ringspot virus* (TRSV), among others (8, 14,37,46,47,54). Recently, two new viruses, together with SLRSV (37), were identified in clones of *Mentha* × *gracilis* 'Variegata' (also known as golden ginger mint) (52), an ornamental mint clone that displays striking yellow vein banding symptoms. While investigating the viruses that were detected consistently in 'Variegata' plants and trying to determine the causal agent or agents of the symptomatology, golden ginger mint was ordered from five nurseries in the United States. One plant received as golden ginger mint did not exhibit typical 'Variegata' symptoms; instead, it had slightly deformed and crinkled leaves (Fig. 1). Tests for a number of mint viruses revealed the presence of TRSV but none of the three viruses found in the other sources of golden ginger mint. TRSV also has been detected in symptomless mint plants at the National Clonal Germplasm Repository (NCGR) (Corvallis, OR) and, thus, it was unlikely that this virus alone was responsible for the symptomatology observed. Double-stranded RNA (dsRNA) was extracted and cloned. Sequence data revealed, in addition to TRSV, the presence of two previously unknown viruses in mint. One was a member of the family *Flexiviridae* (38). The other was a new closterovirus, designated Mint virus 1 (MV 1) and the subject of this study. This article reports the complete nucleotide sequence of MV 1 obtained from shotgun cloning and reverse-transcription polymerase chain reaction (RT-PCR). The sequence information was used to develop a detection test that was used to investigate the presence of the virus in other mint clones. Genomic regions of an additional three isolates in different mint species were studied in order to examine the genetic variability of the virus. Phylogenetic analysis revealed a close relationship of MV 1 with members of the genus *Closterovirus*, the aphid-borne genus in the family *Closteroviridae*. Aphid transmission studies demonstrated that the mint aphid (*Ovatus crataegarius*) can transmit the virus, thus confirming the results of the phylogenetic analysis.

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MATERIALS AND METHODS

Plant material. The isolate used to determine the complete nucleotide sequence of MV 1, designated as Oregon ginger mint (OGM), in addition to a 'Corvallis Mint' (*M.* × *piperita*) and a 'Mint Julep' (Kentucky Colonel Spearmint; *M. spicata*) isolates, were obtained from Oregon nurseries. The third isolate used for the genetic diversity study of MV 1 was a 'Redefined Murray' (*M.* × *piperita*) isolate obtained from a 3-year-old commercial field near Albany, OR.

Purification and dsRNA extraction. Purification of the virus was performed according to the protocol of Klaassen et al. (21), using 50 g of tissue from OGM-infected tissue. Virions were visualized after negative staining with 2% ammonium molybdate. dsRNA was extracted from 20 g of OGM-infected tissue as described previously (49) and visualized on a 2% agarose gel containing ethidium bromide at 100 ng/ml.

Cloning and genome acquisition. cDNA synthesis and cloning were performed as described elsewhere (50). Recombinant plasmids were screened for large inserts using PCR with the M13 primers and *Taq* polymerase (New England Biolabs, Beverly, MA) utilizing a program consisting of 8 min of denaturation at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 3 min at 75°C, followed by a 10-min extension at 75°C. Sequencing reactions were performed at the Macrogen Inc. facilities (Seoul, South Korea) in an ABI3730 XL automatic DNA sequencer.

Blastn and blastx (2) were utilized to identify MV 1-specific sequences that were used for development of oligonucleotide primers. These primers were used for RT-PCR amplification of the virus genome (Table 1) using Takara LA polymerase (Takara MirusBio, Madison, WI) according to the manufacturer's instructions. The 5′ end of the virus was acquired using commercial kits for 5′ random amplification of cDNA ends (RACE) and adenosine tailing utilizing the *Escherichia coli* poly-A polymerase (Invitrogen, Carlsbad, CA); whereas, for determination of the 3′ end, only adenosine tailing was performed (51). The consensus sequence was obtained with the CAP3 software (16) using at least four clones of each of the PCR products, the sequences obtained by shotgun cloning where applicable, and the PCR amplicons for an at least 5× coverage. The GenBank accession numbers are AY792620 for the genome of MV 1 (isolate OGM), AY902479 to AY902481 for the heat shock protein 70 homolog (HSP70h), and AY902482, AY902483, and AY902484 for the major coat protein (CP) genes for the 'Redefined Murray', 'Corvallis Mint', and 'Mint Julep' isolates, respectively.

Sequence and phylogenetic analyses. The open reading frames (ORFs) were identified using the FGENESV0 and ORF finder softwares, respectively. Conserved protein domains were identified with CDD (conserved domain database) (26). The secondary structure of the proteins, used to define accurately the enzymatic motifs of the proteins, was performed using the PROF software (40). Alignment of the nucleotide and amino acid sequences was done with ClustalW (48). The same software using the neighbor-joining algorithm, Kimura's correction, and bootstrap analysis consisting of 1,000 pseudoreplicates was applied for phylogenetic analysis. Trees were viewed on TreeView (30). The putative transmembrane domains of the proteins were predicted with TMHMM (24).

Detection. The methodology used for MV 1 detection was essentially identical to the one used for *Mint vein banding associated virus* (MVBaV) (53). The oligonucleotide primers used for detection were MV 1 CPF (5′-GTTTTAGCCACACTTTAACAT-3′) and MV 1 CPR (5′-GACATCAGTTGCGTTTTCCACCTCCG-3′) that amplify a 638-nucleotide region of MV 1 genome, including the complete CP ORF. Validation of the specificity of the technique was done by direct sequencing of several amplicons from different plants. All sequenced products were MV 1 specific.

Transmission studies. Five plants from each of the following eight herbaceous species (*Nicotiana benthamiana*, *N. tabacum*, *Cucumis sativus*, *Chenopodium quinoa*, *C. amaranticolor*, *Tetragonia tetragonioides*, *Phaseolus vulgaris*, and *Vicia faba*) in addition to 15 virus-free *M.* × *gracilis* clones (53) were mechanically inoculated with crude sap from OGM-infected leaves homogenized in 0.05 M phosphate buffered saline (PBS), pH 7.4, with the addition of 2% nicotine at a ratio (wt/vol) of 1:10. Carborundum (600 mesh) was dusted on the leaves of the herbaceous species and virus-free mint to facilitate infection. RT-PCR tests were performed 1 month after the mechanical inoculations.

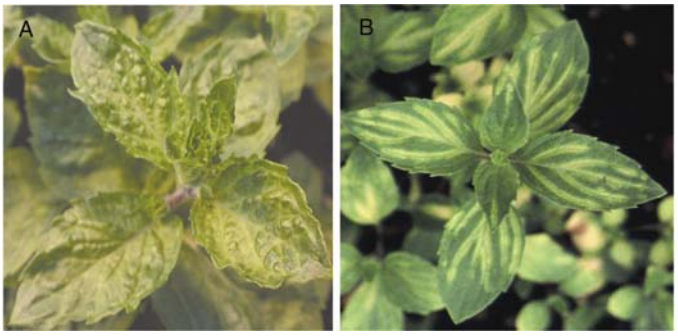

Fig. 1. A, Symptoms on Oregon ginger mint; **B,** typical symptoms on golden ginger mint.

Mint aphids were transferred from virus-free plants onto OGM-infected mint and given an acquisition period of 1 week. Approximately 10 aphids then were transferred onto virus-free *M.* × *gracilis* plants. After a transmission period of 1 week, plants were treated with Marathon, a systemic insecticide. Four trials for a total of 20 plants were used in the aphid transmission study. All plants were maintained with a 14-h photoperiod at 22°C. Plants were tested for the presence of MV 1 by RT-PCR at least 40 days after the application of the insecticide.

RESULTS

Purification and dsRNA extraction. The purification procedure described has yielded virions that could be visualized with an electron microscope with other clostero- and criniviruses (I. E. Tzanetakis and R. R. Martin, *unpublished data*). However, no MV 1 virus particles were discernible with this procedure, probably an indication of the low titer of the virus. dsRNA extractions revealed the presence of several bands on agarose gels, absent in virus-free mint plants (data not shown). The pattern was similar to those observed with other closteroviruses (13,15,27) due to the presence of a high molecular weight (MW) band of ≈15 kb, a feature present in a limited number of plant virus families, and the presence of several lower MW bands that may correspond to the subgenomic RNAs of the virus (Fig. 2).

Sequence analysis, genetic variability, and phylogeny. The complete nucleotide sequence of MV 1 was determined. The genome consists of 15,450 nucleotides and codes for nine ORFs (Fig. 3). The 5′ untranslated region (UTR) is 181 nucleotides long with no significant similarity with the other three closteroviruses that have been completely sequenced. The first AUG (nucleotides 182 to 184) is in excellent context for translation (23) and is predicted to be the start codon of a 2,511-amino-acid protein (1a). In *Beet yellows virus* (BYV), this protein is indispensable for replication (34) and contains three enzymatic motifs, a papain-like protease, a methyltransferase, and an RNA helicase, motifs similar to those found in other closteroviruses (28). The arrangement of the protein is more similar to that of BYV than the other two members of the genus that have been completely sequenced to date, *Citrus tristeza virus* (CTV) (19) and *Grapevine rootstock stem lesion associated virus* (GRSLaV) (41), because MV 1 encodes for one leader protease (L-pro) at the N terminus of the polyprotein. The catalytic Cys and His residues of the L-pro were found at amino acid positions 458 and 521, respectively, whereas the cleavage site is predicted to be between two Gly residues (amino acids 543 to 544). The 60-kDa protein is most closely

TABLE 1. List of the oligonucleotide primers used in the amplification of the genome of Mint virus 1

Primer name	Nucleotide sequence (5′–3′)
AP	GGCCACGCGTCGACTAGTAC$(T)_{18}$
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
5′RC	GTGGAACCGACAGGTTGCCG
5′R	CGGCCATGAGGATCGAGTG
vbeg F	CCCTGCGTGTTTGGTTTTTTCGCT
ProR	TTCGACACCCGGAAAAGCAC
ProF	GTGCTTTTCCGGGTGTCGAAAGTTTCT
1a midR	ACCAGTGCTTAAACGAGTCGAAGAATTTAACG
1a midF	CCGTCGCTCCTGGATCTGTCAA
HelR	GCCGCAGTGAACTGTATTAGAGGTGGGAT
HelF	ATCCCACCTCTAATACAGTTCACTGCGGC
PolR	ACGAGAAATTAGACGCCAAACAATGAATG
PolF	CCACGCTGAAGCGATCTGTATCGAAAC
HSPR	ACCACGAAAGTGCTGTTTCTAACAGAGAC
HSPF	AGAAACAGCACTTTCGTGGTTAAAGCGTC
CPR	GACATCAGTTGCGTTTTCCACCTCCG
CPF	AGCTCGGAGGTGGAAAACGCAACTGA
3′R	TGATCATCTAGTACAGTCATTATACCG
p20F	CGACATCTCCGCTAACACGTTCAGGCAG

related to the L2-pro rather the L1-pro of CTV and GRSLaV (data not shown), an indication that the MV 1 protease functions in a mode similar to that of BYV (33) and is likely involved in long-distance transport and replication enhancement (31,32). After the predicted autocleavage of the L-pro from 1a, a 222-kDa protein with the methyltransferase and RNA helicase motifs involved in viral replication (11) is released. The further processing of the 222-kDa protein is probable because two peptides having either the methyltransferase or the helicase domains have been identified in BYV-infected plants (12), but no cleavage sites could be identified in MV 1. The methyltransferase is predicted between residues 633 and 963 (42) and the helicase between residues 2,133 and 2,390 (7). The methyltransferase domain is most closely related to that of GRSLaV and *Grapevine leafroll associated virus 2* (GLRaV 2), exceeding 55% amino acid sequence identity and 72% similarity, whereas the helicase is closely related to orthologous domains of CTV and BYV, with more than 53% amino acid sequence identity and ≈70% similarity. No enzymatic motif was identified in the more than 1,000-amino acid interdomain region between the methyltransferase and helicase domains (26), and the function of the region remains unknown.

The RNA-dependant RNA polymerase (RdRp) of the virus is probably expressed using a +1 ribosomal frameshift, a closterovirus characteristic that is unique among plant viruses (1,19). The +1 frame shift can produce a protein of 2,970 aa. The 52-kDa RdRp domain has the eight conserved RdRp polymerase motifs identified by Koonin (22) between residues 2,620 and 2,860 of the fusion 1a/1b polyprotein. The protein has nearly 60% amino acid sequence identity and over 70% similarity to orthologous regions of BYV, GRSLaV, GLRaV 2, and CTV, being the most conserved *Closterovirus* protein.

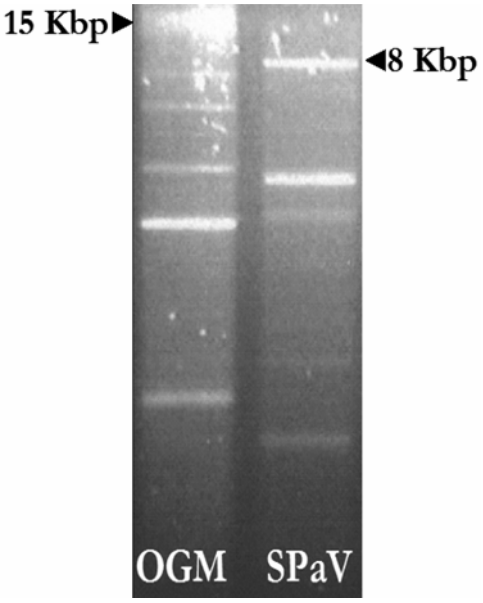

Fig. 2. Double stranded RNA (dsRNA) pattern of Oregon ginger mint (OGM) clone. Left: Multiple dsRNA bands of the OGM. Arrowhead indicates a band of ≈15 kb. Right: dsRNA pattern of Strawberry pallidosis-associated virus (SpaV). Arrowhead indicates the bands corresponding to the genomic RNAs of ≈8 kb.

The next ORF (nucleotides 9,106 to 9,306) codes a 7-kDa protein, similar in size and position to other small peptides encoded by closteroviruses. The peptide has a putative transmembrane motif between residues 7 and 29 (24). Similar closteroviral proteins are found associated with the endoplasmic reticulum and are involved in virus movement, being one of the five proteins involved in that function (9). The protein has a Cys residue at position 3, similar to that identified in BYV as being essential in disulfide bridge formation and dimerization of the protein which, in turn, are essential for virus movement (36).

Downstream from the small hydrophobic protein is an ORF (nucleotides 9,309 to 11,123) encoding a 67-kDa protein, which is the HSP70h of the virus, the hallmark gene of the family *Closteroviridae.* The HSP70s are chaperones with conserved ATPase domains at the N-terminus and substrate recognition domains near the C-terminus of the proteins (6). The five conserved motifs of the HSP70s (5) also are present in the MV 1 protein between residues 3 and 348. The *Closterovirus* HSP70h ATPase function is essential for virus movement, as shown for BYV (35). In addition to movement, the HSP70h also is needed for virion assembly (4,45). MV 1 HSP70h shows greatest similarity with orthologous proteins of BYV, *Beet yellow stunt virus* (BYSV) (20), and some isolates of CTV exceeding 40% amino acid sequence identity and 60% similarity.

ORF 4, found between nucleotides 11,126 and 12,745, codes for a 62-kDa protein. Database searches identified the protein as the CP homolog (CPh) of MV 1. CPh is another movement protein of closteroviruses and has been found associated with virions (3,45). The homology of the protein to the CPs is due to the identification of two conserved Arg and Asp residues (29) involved in virion assembly of filamentous virions (10,17) and the similarities of some closteroviral CPh with the CP of members of the family *Flexiviridae* (I. E. Tzanetakis, *unpublished data*). The two conserved residues of the MV 1 CPh are found at positions 410 and 449 for Arg and Asp, respectively. The protein is most similar to the CPh of BYV, with over 35% amino acid sequence identity and almost 60% similarity.

The next two ORFs (nucleotides 12,681 to 13,337 and 13,477 to 14,082) of MV 1 encode the two structural proteins of the virus, minor CP (CPm), and CP. The proteins protect the genome and are involved in virus movement (4). The 24-kDa CPm ORF is found upstream from the 22-kDa CP and has the 'CPm' box identified by Alzhanova et al. (4). The CPm orthologs of BYV and CTV have been shown to encapsidate ≈5% of the 5′ terminus of the genome (44,56) while the CP encapsidates the remaining 95%. Dolja et al. (10) identified conserved Ser, Arg, and Asp in the CPs of filamentous viruses. The CPm of MV 1 has all three

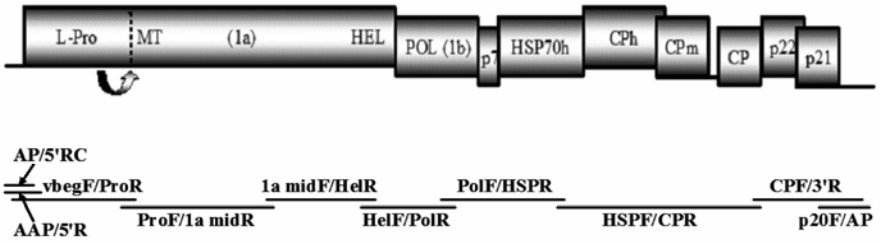

Fig. 3. Schematic representation of the genome organization of Mint virus 1. Abbreviations: L-Pro: leader papain-like protease; MT: methyltransferase; Hel: helicase; Pol: RNA-dependent RNA polymerase; HSP70h: heat shock protein 70 homolog; CPh: coat protein homolog; CPm: minor coat protein; CP: major coat protein. Bars represent the reverse transcription-polymerase chain reaction products used to acquire the genome of the virus. Primers are listed in Table 1. Open reading frames are not to scale.

conserved amino acids at positions 89, 132, and 173, respectively, but the CP has a substitution of Ser with Thr (amino acid 65). This amino acid substitution is present in all four isolates of the virus sequenced. The other two conserved amino acids of the CP are found at positions 112 and 154.

The next ORF spans from nucleotide 14082 to 14675. Alignment with other *Closterovirus* proteins found at the same genome position did not reveal any significant homology. These proteins are involved in systemic transport (9). The putative 22-kDa protein does not have any significant similarity with any other proteins in the database.

The final ORF in the MV 1 genome (nucleotides 14,608 to 15,168) encodes a 21-kDa protein with similarities to a silencing suppressor protein found in other closteroviruses (25,39). Alignments of eight *Closterovirus* p21 orthologs (I. E. Tzanetakis, *unpublished data*) revealed the presence of a conserved motif near the C terminus of the proteins at a region of predominantly helical structure (39,40). The motif is αXXXXXXXαXXXXXXXXαXX S/TXXXαXXXXXL, where α stands for a hydrophobic amino acid (A, V, L, I, M, F, W, or Y) and X can be any amino acid.

The 3′ UTR is 282 nucleotides long and has a long adenosine-rich region of over 30 nucleotides. Similar sequences are found in all the *Closterovirus* 3′ UTRs found in the database other than that of CTV, and its role in replication remains unknown.

Alignment of the HSP70h and CP of MV 1 from OGM with three other isolates of the virus showed 96 to 99% nucleotide sequence identity for the HSP70h (98 to 99% amino acid sequence identity) and 97 to 100% nucleotide sequence identity for the CP (99 to 100% amino acid sequence identity).

The phylogenetic analysis using the complete amino acid sequences of the polymerase and HSP70h genes of all the members of the family *Closteroviridae* found in the database, places MV 1 within the genus *Closterovirus* (Fig. 4).

Transmission studies. None of the herbaceous hosts used for mechanical transmissions of MV 1 developed visible symptoms. All five plants from each species were tested by RT-PCR for the presence of the virus and none gave amplicons. All plants used for the aphid transmission studies remained asymptomatic during the course of the study, and RT-PCR was employed for evaluation of transmission of the virus. Seven aphid-inoculated plants (35%)

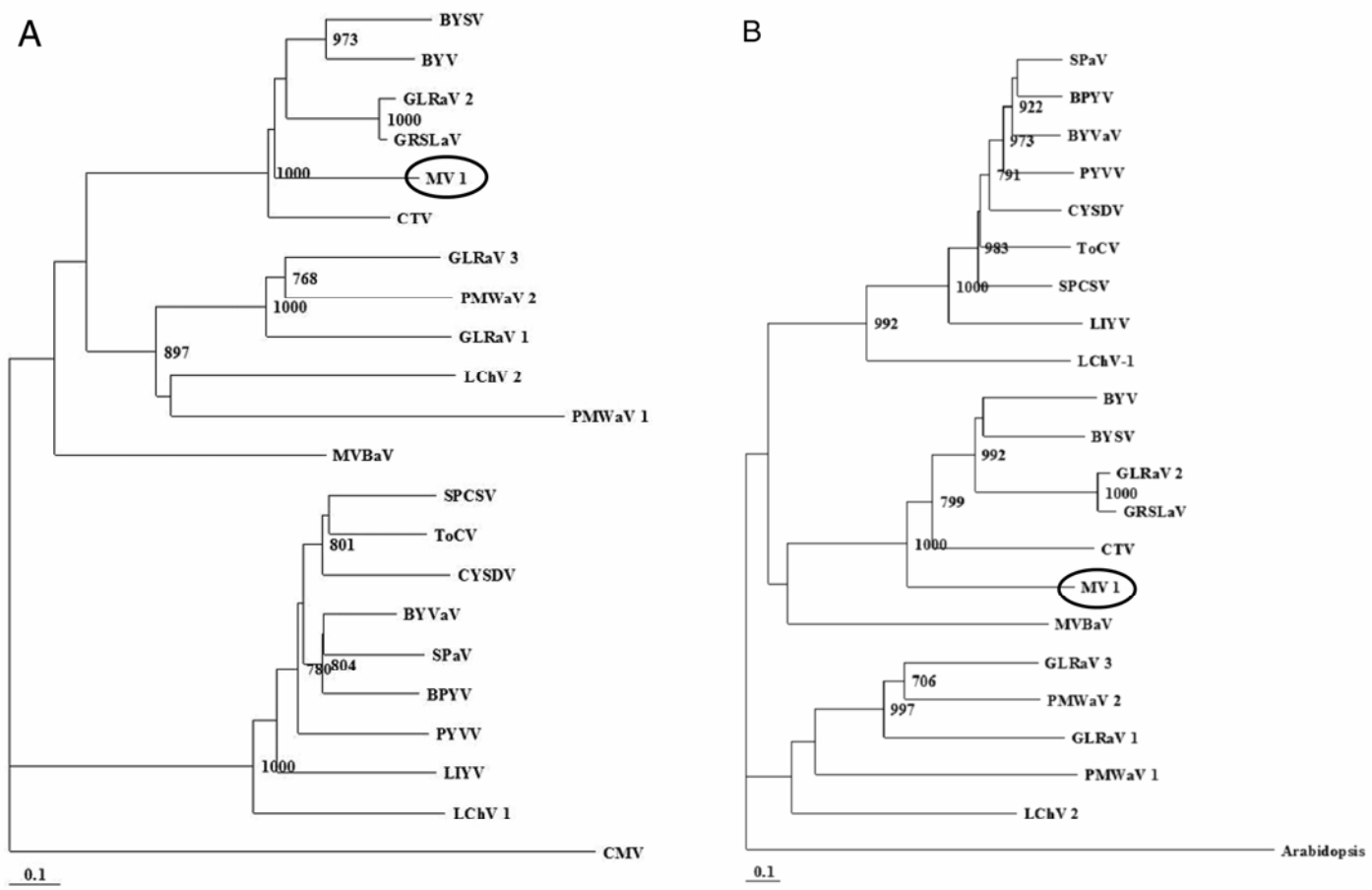

Fig. 4. A, Phylogram of the polymerase of Mint virus 1 and other closteroviruses. Abbreviations and GenBank accession numbers: BPYV, *Beet pseudo yellows virus*, NP940796; BYV, *Beet yellows virus,* NP733949; BYSV, *Beet yellow stunt virus*, AAC55659; BYVaV, Blackberry yellow vein-associated virus, AAV40963; CTV, *Citrus tristeza virus*, NP733947; CMV, *Cucumber mosaic virus,* NP049324; CYSDV, *Cucurbit yellow stunting disorder virus*, AAM73639; GLRaV 1, *Grapevine leafroll-associated virus 1*, AAF22738; GLRaV 2, *Grapevine leafroll-associated virus 2*, AAC40856; GLRaV 3, *Grapevine leafroll-associated virus 3*, AAC40705; GRSLaV, *Grapevine rootstock stem lesion-associated virus*, NP835244; LIYV, *Lettuce infectious yellows virus*, AAA61798; *Little cherry virus 1*, NP733945; LChV 2, *Little cherry virus 2*, AAP87784; MVBaV, Mint vein banding-associated virus, AAS57939; MV 1, Mint virus 1, AAW32893; PMWaV 1, *Pineapple mealybug wilt-associated virus 1*, AAL66709; PMWaV 2, *Pineapple mealybug wilt-associated virus 2,* AAG13939; PYVV, *Potato yellow vein virus*, CAD89680; SPaV, Strawberry pallidosis-associated virus, AY488137; SPCSV, *Sweet potato chlorotic stunt virus*, NP733939; ToCV, *Tomato chlorosis virus*, AAY21795; Bootstrap values are shown as percentage value and only the nodes over 70% are labeled. The bar represents 0.1 amino acid changes per site. CMV is used as the outgroup. **B,** Phylogram of heat shock protein 70 homolog of Mint virus 1 and other closteroviruses. Abbreviations and GenBank accession numbers: Arabidopsis, *Arabidopsis thaliana* putative heat shock protein 70, AAN71949; BPYV, AAQ97386; BYSV, AAC55662; BYV, NP041872; BYVaV, AAV40966; CTV, NP042864; CYSDV, NP851572; GLRaV 1, AAF22740; GLRaV 2, AAR21242; GLRaV 3, NP813799; GRSLaV, NP835247; LIYV, NP619695; LChV 1, NP045004; LChV 2, AF531505; MVBaV, AAS57941; MV 1, AAW32895; PMWaV 1, AAL66711; PMWaV 2, AAG13941; PYVV, CAD89682; SPaV, AAO92347; SPCSV, NP689401; ToCV, AF024630. Bootstrap values are shown as percentage value and only the nodes over 70% are labeled. The bar represents 0.1 amino acid changes per site. The *Arabidopsis* protein is used as the outgroup.

tested positive for MV 1. Attempts to use aphids to transmit MV 1 into herbaceous hosts (*N. benthamiana*, *N. tabacum*, *Cucumis sativus*, *Chenopodium quinoa*, *T. tetragonioides*, and *P. vulgaris*) were unsuccessful due to the inability to establish the mint aphid on any of the hosts.

DISCUSSION

Closteroviruses encode the largest positive-strand RNA genomes among known plant viruses and are important pathogens causing significant losses in world agriculture (18). Members of the family have been understudied, mainly because of the difficulty in mechanical transmission, the low virus titer, and their large genomes. Until recently, mint was not known to be infected by closteroviruses. After the identification of MVBaV (53), a second closterovirus, MV 1, has been identified in mint. The complete nucleotide sequence of the virus as determined in this study shows that MV 1 is a new member of the genus *Closterovirus*. Closteroviruses differ in genome organization, in both the number of the protease domains of ORF 1a and the number of ORFs encoded in the 3′ proximal end of the genome. MV 1 has genome organization identical to that of BYV, the type member of the genus, but sequence comparisons revealed that different regions of the genome show greater similarity with different closteroviruses, suggesting that recombination between orthologous genes may have a significant role in the *Closteroviridae* evolution. The placement of MV 1 within the aphid-borne genus *Closterovirus* by phylogenetic analysis (Fig. 4) was confirmed by the transmission studies showing the mint aphid tc can be very diverse, and there is evidence of continuous recombination that gives rise to new strains (43,55). Because MV 1 is a newly described virus, comparison of the HSP70h and CP genes from virus isolates obtained from different mint species was used to test for the existence of diverse strains. The variability observed was minimal, because similarity between the four isolates exceeded 97%.

M. × *gracilis* remained asymptomatic when solely infected with MV 1. The possible involvement of the virus in reduced yield or mint oil content cannot be ruled out. Studies are currently under way to reconstruct the symptoms observed in OGM by re-infecting healthy plants with various virus combinations.

Assorted mint clones and species from different sources have been tested for the presence of MV 1 by RT-PCR (I. E. Tzanetakis, *unpublished data*), and the presence of the virus has been confirmed in 15% of the 80 plants tested. In all cases, plants were asymptomatic or, when symptomatic, one or more additional viruses were present in the plants, leaving the role of MV 1 in symptomatology uncertain. The number of infected plants is significant for a previously unknown virus, and it may be that MV 1 is an under-recognized problem in the mint industry, especially when found in mixed infections with other viruses, since all three viruses found in OGM are latent when found alone in mint and symptoms develop only when MV 1 is found in mixed infections.

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