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Mint virus X: a novel potexvirus associated with symptoms in 'Variegata' mint

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Summary. *Mentha* × *gracilis* 'Variegata', an ornamental mint clone first described about 200 years ago, exhibits virus-like vein banding symptoms. Double-stranded RNA and virion isolations revealed the presence of three viruses in a 'Variegata' plant. Cloning and sequencing disclosed that one of the viruses was a previously unidentified species with similarities to members of the *Flexiviridae* family, designated as Mint virus X (MVX). The complete nucleotide sequence of the virus was determined. Phylogenetic analysis divulged the close relationship of the virus with lily virus X and strawberry mild yellow edge virus, members of the *Potexvirus* genus. A reverse transcription-polymerase chain reaction protocol was developed and used for detection of MVX in other 'Variegata' plants. All clones tested, obtained from nurseries around the United States were infected with MVX, making the virus a possible causal agent of the variegated symptoms.

Introduction

Mint (*Mentha* spp.) belongs to the family *Lamiaceae* and includes about 30 species found in the temperate regions of the world [11]. Mint has been used for centuries for its medicinal properties and in the food and fragrance industries [3, 26, 34]. Unlike the importance of the crop and the virus-like symptoms exhibited by many mint clones, little is known about the virus diseases of the crop, and our

The nucleotide sequence data reported in this communication have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers AY789138 for the genome of MVX and AY921609–AY921611 for the 3′ terminal regions of three other isolates.

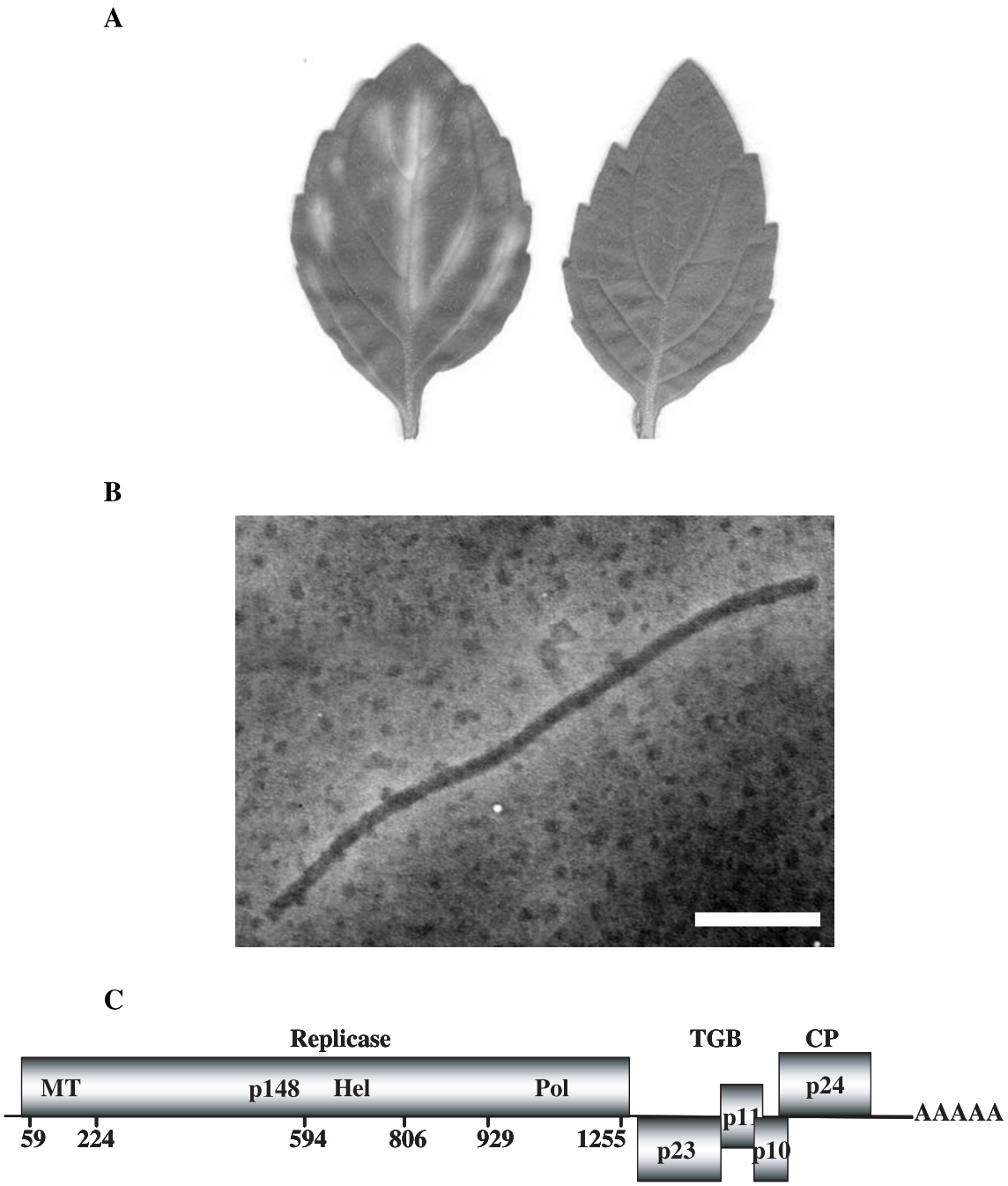

Fig. 1. A. *Mentha* × *gracilis* 'Variegata' phenotype. Left: National Clonal Germplasm Repository (NCGR) MEN 454.001, right: NCGR MEN 454.002, obtained from NCGR MEN 454.001 after heat therapy and apical meristem culture. **B.** Mint virus X virion. Bar represents 100 nm. **C.** Genome organization of Mint virus X. Abbreviations: *MT*: methyltransferase; *Hel*: helicase; *Pol*: RNA-dependent RNA polymerase; *TGB*: Triple gene block; *CP*: coat protein. The numbers indicate the amino acid coordinates of the enzymatic motifs of the replicase. Drawing is not in scale

knowledge is limited mainly to simple reports of viruses infecting mint rather than the importance of the viruses in disease development [32, 33, 42].

The National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, maintains the U.S. *Mentha* collection. Many clones in the collection have exhibited symptoms that could be attributed to viruses. One of these clones is *Mentha* × *gracilis* 'Variegata' (NCGR accession MEN 454.001). This taxon, known in the nursery trade as golden ginger mint (GGM), was first described more than 200 years ago [37] and is still grown throughout the world as an ornamental garden herb because of its "attractive" bright yellow patterns along the main leaf veins (Fig. 1).

Symptoms diminish during the summer months and are eliminated by heat therapy and apical meristem culture, a strong indication of the virus etiology of the symptoms. Double-stranded RNA (dsRNA) extracted from a GGM plant was cloned and three viruses were identified, strawberry latent ringspot virus [30], Mint vein banding associated virus (MVBaV) [40] and a previously unidentified virus with sequence similarity to members of the family *Flexiviridae*, designated as Mint virus X (MVX).

This communication reports the complete nucleotide sequence of MVX and the development of a detection protocol. The genome organization and phylogenetic analysis demonstrate that MVX belongs to the genus *Potexvirus*. The 3′ terminus of the MVX genome from three additional isolates of the virus was determined to evaluate the genetic variability of virus isolates obtained from different geographic regions. No vector for potexviruses has been identified and they are typically dispersed in nature by mechanical means, except for strawberry mild yellow edge virus (SMYEV) that is aphid transmitted, likely with the assistance of a yet unidentified helper virus [36]. The difficulty of mechanical transmission and the close relationship of MVX with SMYEV led to the investigation of aphid transmission of MVX utilizing the mint aphid (*Ovatus crataegarius*).

Materials and methods

Plant material

Six GGM clones were used in the study: NCGR MEN 454.001 and other GGM clones were obtained from mail-order nurseries in Maryland, Michigan, Nebraska, Ohio and Oregon. MEN 454.002, free of the three viruses present in MEN 454.001, was derived from MEN 454.001 after heat therapy and apical meristem culture [40].

Transmission studies

Mechanical inoculations were performed with either MEN 454.001 tissue or purified virions. Test plants were dusted with carborundum (600 mesh) prior to inoculation with tissue homogenized in 0.05 M phosphate buffered saline (PBS) pH: 7.4, containing nicotine (2% vol/vol), at a wt/vol ratio of 1:10 or purified virions at a concentration of about 10 ng/ml in PBS. The plant species inoculated were: *Chenopodium quinoa*, *Cucumis sativus*, *Glycine max*, *Nicotiana benthamiana*, *N. occidentalis*, *N. tabacum*, *Tetragonia tetragonioides* and NCGR MEN 454.002. Five plants per treatment of each indicator plant species were inoculated with plant tissue or purified virus, while 45 plants of NCGR clone MEN 454.002 were inoculated with diseased tissue and 30 were inoculated with purified virions.

For aphid transmission studies, mint aphids were given over 72 h acquisition period on MEN 454.001 and a GGM plant obtained from an Ohio nursery. Ten individual aphids were then relocated onto NCGR MEN 454.002 plants and given a 72 h inoculation access period before applying Marathon®, a systemic insecticide. Three trials for a total of 30 plants were used in the study, 20 inoculated with the NCGR MEN 454.001 isolate and 10 with the Ohio isolate. All plants were tested for MVX one month after the application of the insecticide.

Virus and nucleic acid purifications

MVX was purified from 200 g of NCGR MEN 454.001 leaf tissue according to the protocol of Martin and Bristow [25] and visualized after negative staining of the virus preparation with 2% molybdenum acetate. DsRNA and total RNA were extracted as described elsewhere [39].

Cloning and genome analysis

cDNA was synthesized and cloned as described previously [38] and compared against the blastn and blastx databases [2] to identify the genomic region cloned. The complete genome of the virus was acquired as described elsewhere [39] (without the tailing of the nucleic acids), using either dsRNA or viral RNA obtained from purified virus using the protocol for RNA extraction [39] as template for reverse transcription (RT) and Takara LA polymerase (Takara MirusBio, Madison, WI) in the polymerase chain reaction (PCR).

The consensus sequence of the MVX genome was obtained using the CAP3 software [15]. Open reading frames (ORF) were identified using the National Center for Biotechnology Information (NCBI) ORF finder. ClustalW [35] (neighbor-joining algorithm with Kimura's correction) and bootstrap of 1000 pseudoreplicates was used for phylogenetic analysis of the polymerase conserved motifs and coat protein (CP) of MVX. TreeView [28] was used for visualization of the cladograms. The comparisons between enzymatic domains and proteins of MVX and other potexviruses were performed on GatMat [5]. TMHMM [18] predicted the putative transmembrane domains of the proteins while RNA secondary structures were predicted on mfold [46].

Detection

The methodology described for MVBaV [40] was also applied in MVX detection. Primers MVX F (5′ GGCAATGGCACTGTCA 3′) and MVX R (5′ GGCGTTCAGATAGTAGCG 3′) were developed for RT-PCR amplification of a 332 base fragment of the virus replicase. Several RT-PCR amplicons were sequenced to verify the specific amplification of MVX sequences.

Results and discussion

A new potexvirus infecting mint, MVX, was identified. The filamentous virions of MVX are about 500×13 nm ($n = 30$) (Fig. 1), typical of potexviruses [1]. The complete genome of MVX consists of 5914 nucleotides (nt) (59% GC content), excluding the poly(A) tail, and contains at least five ORFs (Fig. 1). The genome starts with the sequence GAAAA, found in the majority of potexviruses and thought to be crucial for replication [45]. The 5′ untranslated region (UTR) is 69 nt long and could form a stem-loop structure [46] similar to that formed in other members of the genus such as SMYEV [36] and cymbidium mosaic virus [45].

The first AUG (nt 70–72) is in good translational context [20] and is potentially the start codon of a 1312 amino acid (aa) protein. Sequence analysis revealed that the 148 kDa protein is the virus replicase, and is most closely related to lily virus X (LVX) [8], SMYEV [17] and white clover mosaic virus (WClMV) [13]. The N-terminus of the protein contains methyltransferase conserved motifs between residues 59 and 224 [31] and the activity of the domain has been experimentally determined for bamboo mosaic virus (BaMV) [16]. The region shares highest aa sequence identity (71%) with the orthologous domain of LVX. An RNA helicase motif [6], was identified between residues 594–806 with 70% aa sequence identity with LVX. The Gly–Lys–Ser (aa 543–545), part of the Gly-rich A region of helicases involved in phosphate binding [14], was indispensable for replication of potato virus X [9]. Mutational analysis of the site for BaMV has determined its involvement in the RNA 5′-triphosphatase activity of the domain [21]. The most conserved region of the replicase is the polymerase domain of the virus. The domain shows 72% aa identity with the orthologous domain of LVX while it shares more than 50% aa identity and over 70% aa similarity with all potexviruses sequenced to date. The eight conserved motifs of RNA-dependent RNA polymerases [19] were found between residues 929 and 1255.

The next three putative proteins known as the ‘triple gene block’ (TGB) are involved in virus movement [4, 27]. The first ORF encodes a 23 kDa protein (TGBp1), with NTP hydrolase and RNA binding motifs, typical of an RNA helicase. The NTPase activity may be directly involved in virus movement, as this function is proposed to be energy-driven [7]. Orthologous proteins participate in the ribonucleoprotein complex involved in cell to cell movement [23] and have been identified as suppressors of RNA interference (gene silencing) [43]. The other two members of the TGB group have hydrophobic motifs, predicted to form transmembrane domains [18], that could be integrated into cell membranes. The 11 kDa TGBp2 shares 49% aa identity and 60% aa similarity with LVX TGBp2. The protein has two predicted transmembrane domains between residues 13–30 and 68–87, with both N- and C-termini of the protein in the cytoplasm. MVX TGBp2 orthologs are localized at the endoplasmic reticulum (ER) and Golgi vesicles [27]. TGBp3 has a molecular mass of 10 kDa without significant sequence homology with other TGBp3 but is predicted to have a transmembrane domain between residues 33 and 52, a conserved feature of all potexvirus TGBp3. Both TGBp2 and TGBp3 may recruit TGBp1 to plasmodesmata and thus facilitate cell to cell movement of the virus [27].

The next ORF encodes the putative 24 kDa CP closely related to the CP of LVX with 50% aa identity and 64% aa similarity. The protein has a hydrophobic N-terminus with twelve Ala residues in the first 20 aa, a feature found in all sequenced isolates of the virus. The start codon is in excellent translational context [20] while the second aa is a Thr, a residue that prolongs protein turnaround in cells [41], a predicted CP trait. SDS-PAGE using purified virions

revealed the presence of a predominant 24 kDa and a minor 17–18 kDa band (data not shown), a possible expression of the protein from a second AUG in frame found at position 5269–5271. These findings suggest that MVX CP contains an unusual N-terminus. There at least two other examples of potexviruses with unusual CP N-termini. BaMV [22] contains multiple Gly and SMYEV [17] contains multiple Pro residues at the N-termini of the CP genes. The two residues, Arg and Asp probably involved in salt bridge formation [10] were found at positions 121 and 160 respectively. The CP, in addition to genome protection, is also involved in virus movement [12, 24].

Downstream from the putative start codon of the CP (+2 reading frame in comparison to the CP), there is an ORF that encodes a 89 aa putative peptide. The start codon is not found in good translational context [20]. The 10 kDa putative protein is Pro-rich (25%) and shares no significant similarity with any protein in the databases.

The 3′ UTR is 121 nt long excluding the poly(A) tail and is predicted to fold into three stem loop structures, similar to those identified in other potexviruses [36]. The conserved ACUUAA sequence essential for replication of potexviruses [29, 44], was also present in MVX (nt 5882–5887). The possibility that the hexanucleotide is essential for MVX replication is enhanced by its presence on the loop of the second hairpin, since the structural conformation is required for replication enhancement [29].

More than 1000 bases of the 3′ end of three additional isolates of MVX obtained from nurseries in Maryland, Ohio and Oregon were sequenced. The differences between isolates were minimal since they shared more than 99% nt and aa sequence identity.

Phylogenetic analysis using the conserved polymerase motifs and the CP (Fig. 2) revealed the close relationship of MVX with LVX and SMYEV.

The RT-PCR test was used to assay for MVX in GGM plants acquired from nurseries in five different states (Fig. 3). MVX was the only virus of the three found in MEN 454.001 that was found in all nursery plants. MVX was detected in *C. quinoa*, *N. benthamiana* and *N. occidentalis* inoculated with MEN 454.001 tissue. None of the 75 plants of MEN 454.002 that were inoculated with either purified virions or MEN 454.001 tissue became infected with the virus, however. In addition, grafting experiments using MEN 454.001 or *N. occidentalis* onto MEN 454.002 were unsuccessful at transmitting the virus although grafts remained alive for at least one month (data not shown). The aphid transmission studies using the mint aphid and two isolates of the virus were also unsuccessful.

MVX is a novel virus infecting mint. Phylogenetic analysis, genome organization and features found in both the 5′ and 3′ UTR of the virus are typical of a potexvirus. The minimal diversity between the four isolates of the virus sequenced (>99% nt identity) may indicate that one or very few ‘Variegata’ clones are propagated in the United States and shared among nurseries and some, such as clone MEN 454.001, may have accumulated additional viruses over time.

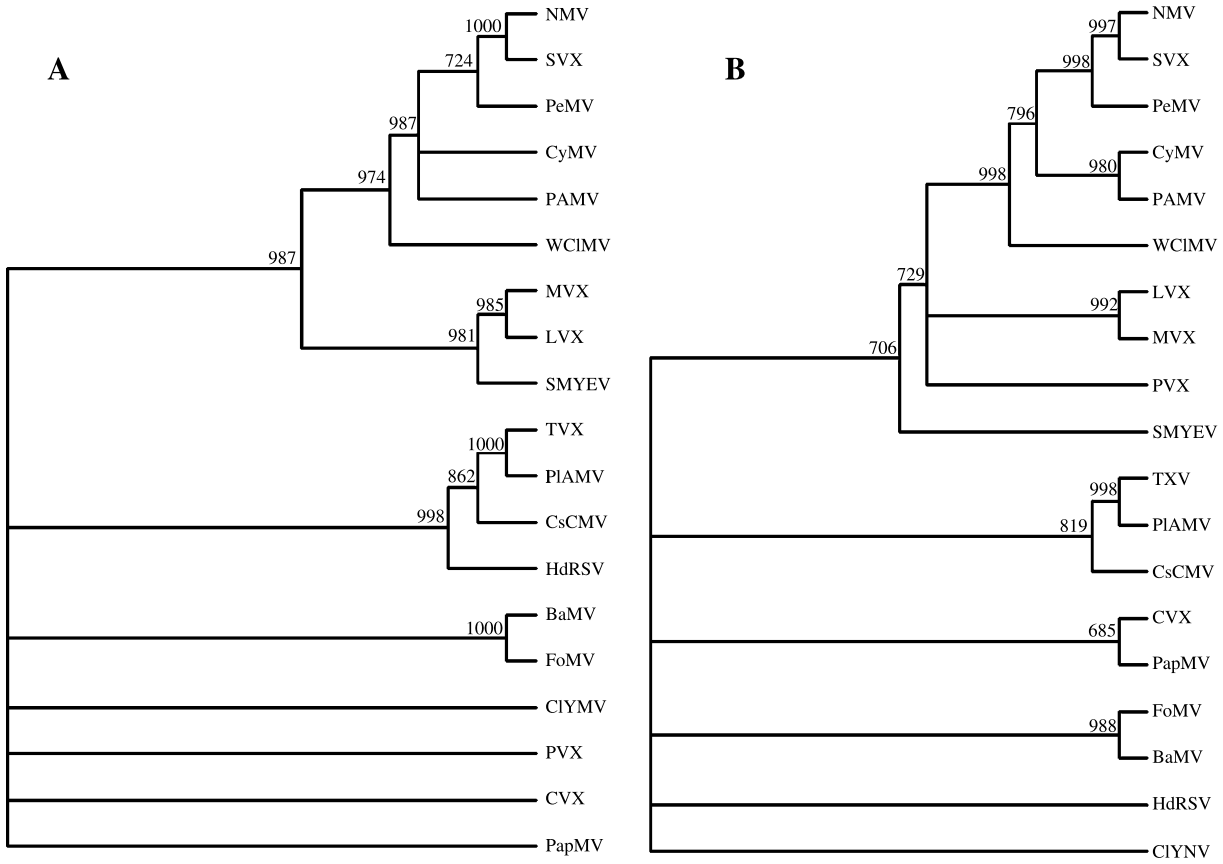

Fig. 2. A. Cladogram of Mint virus X and other potexviruses polymerase motifs. Abbreviations and Genbank accession numbers: bamboo mosaic virus, BaMV, NP042582; clover yellow mosaic virus, ClYMV, NP077079; cassava common mosaic virus, CsCMV, NP042695; cactus virus X, CVX, NP148778; cymbidium mosaic virus, CyMV, NP054025; foxtail mosaic virus, FoMV, NP040988; hydrangea ringspot virus, HdRSV, AAW30448; lily virus X, LVX, pending; Mint virus X, MVX, AAW67746; narcissus mosaic virus, NMV, NP040778; papaya mosaic virus, PapMV NP044330; pepino mosaic virus, PeMV, NP663724; plantago asiatica mosaic virus, PlAMV, NP620836; potato aucuba mosaic virus, PAMV, NP619745; potato virus X, PVX, NP056753; strawberry mild yellow edge virus, SMYEV, NP620642; scallion virus X, SVX, NP570726; tulip virus X, TVX, NP702988; white clover mosaic virus, WClMV, NP620715. Nodes with bootstrap values of less than 60% collapse and numerical values are not shown **B.** Cladogram of the coat proteins of Mint virus X and other potexviruses. Abbreviations and Genbank accession numbers: BaMV, NP042587; ClYMV, NP077083; CsCMV, NP042699; CVX, NP148784; CyMV, NP054029; FoMV, NP040992; HdRSV, AAW30452; LVX, CAA33396; MVX, AAW67750; NMV, NP040782; PapMV, NP044334; PeMV, NP663728; PlAMV, NP620840; PAMV, NP619750; PVX, NP056757; SMYEV, NP620646; SVX, NP570730; TVX, NP702992; WClMV, NP620719. Nodes with bootstrap values of less than 60% collapse and numerical values are not shown

The virus can be mechanically transmitted, as shown by inoculations to various herbaceous hosts, but there was no transmission to mint using either infected tissue or purified virions. The virus could not be maintained in herbaceous hosts

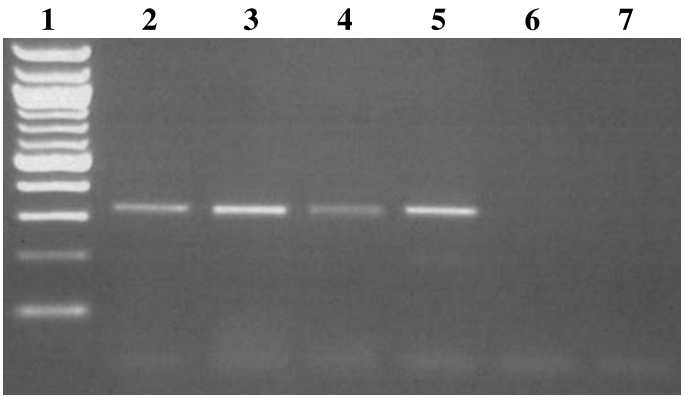

Fig. 3. Detection of Mint virus X. *1*: 100 base pairs marker (New England Biolabs, Beverly, MA); *2–5*: *Mentha* × *gracilis* 'Variegata' clones from Maryland, Michigan, Ohio and Oregon; *6*: National Clonal Germplasm Repository MEN 454.002; *7*: blank control

after mechanical passages, a feature atypical of a potexvirus. Another potexvirus, SMYEV, shares these properties of MVX (Martin, personal observation). SMYEV is the only known aphid transmitted potexvirus, probably due to the presence of a helper virus [36]. The possibility of MVX being aphid transmittable was examined using *Ovatus crataegarius*, the predominant species that colonizes mint, and two isolates of the virus, to minimize the possibility of negative results. However, aphid transmission of MVX was not observed.

The ORF found between nt 5123 and 5392 was found in all isolates sequenced and was very similar between isolates (over 99% nt identity). The high identity between the isolates sequenced does not allow any prediction whether the ORF is present in more diverse isolates or strains of the virus and thus possibly expressed. The position in the genome, translational context of the proposed start codon, and the predicted amino acid sequence indicate that this ORF is probably not expressed.

MVX was detected in symptomless mint clones at NCGR representing five different *Mentha* species: *M. cervina* L., *M. japonica* (Miq.) Makino, *M. longifolia* L., *M. pulegium* L., and *M. spicata* L. (Tzanetakis, unpublished data). These results are indicative of the different phenotypes observed in different mint species when infected with the same virus as in the case of MVBaV [40]. Tests are underway to determine whether the virus is also present in commercial mint producing areas of the United States.

Acknowledgments

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