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BRIEF REPORT

Genome sequence and characterization of a new virus infecting *Mikania micrantha* H.B.K.

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Abstract The complete RNA genomic sequence of a new virus infecting *Mikania micrantha*, designated as Mikania micrantha wilt virus (MMWV), has been determined. The genomic sequence and the predicted gene products of MMWV were similar to those of the other viruses of the genus *Fabavirus*. The MMWV nucleotide sequence showed 75.6% identity to that of gentian mosaic virus, 56.6 and 57% identity to those of two *Broad bean wilt virus 1* isolates, and between 55.7 and 58% identity to those of seven *Broad bean wilt virus 2* isolates. Our results suggested that MMWV represents a distinct isolate of the candidate species Gentian mosaic virus.

Mikania micrantha (Compositae), a fast-growing vine species originating from South and Central America, is one of the world's most aggressive weeds [25]. *M. micrantha* competes for water and nutrients, smothers other plant species and finally kills them by cutting off their sunlight. Its rampant growth characteristics and potential allelopathic effects [19] can devastate most native species populations [18] and cause substantial damage to natural ecosystem and biodiversity. *M. micrantha* is listed as one of the top hundred worst invasive species in the world and is considered to be the second-most serious weed in the South Pacific Region [31].

To better understand its fast growth, the molecular mechanism of its allelopathic effects, and eventually to find an effective way to control *M. micrantha*, we constructed the cDNA library from *M. micrantha* leaves. The material was collected from the mangrove forest in Qi Ao island, Zhuhai (N 21°48′, E 113°3′) and then maintained in a glasshouse in Guangzhou (N 23°8′, E 113°17′).

Total RNA was isolated from the leaves of *M. micrantha* using the guanidine thiocyanate method [6] and further purified using silica particles according to a method described before [8]. Double-stranded cDNA was synthesized using a SMART™ cDNA amplification Kit (Clontech). PCR products were purified using a QIAquick PCR purification kit (Qiagen) and then inserted into pGEM®-T Easy Vector (Promega) and finally transformed into *E. coli* Top10 (Invitrogen). The positive clones were verified by PCR with primers T7 and SP6 and sequenced using an ABI 3730 sequencer. A total of 783 clones were isolated and sequenced, two of which expressed sequence tags (ESTs), named WGJ20 (507 bp) and WGJ527 (856 bp) (Fig. 1a), that showed moderate homology with nucleotides (nts) 4,870–5,347 and nts 2,133–2,987 of gentian mosaic virus (GeMV) RNA1, respectively [21], and three ESTs, named WGJ9 (634 bp), WGJ555 (824 bp) and WGJ684 (567 bp) (Fig. 1b), showed moderately homology with nts 2,533–3,145, nts 2,353–3,145 and nts 2,587–3,145 of GeMV RNA2, respectively [21], and this inspired us to do further analysis.

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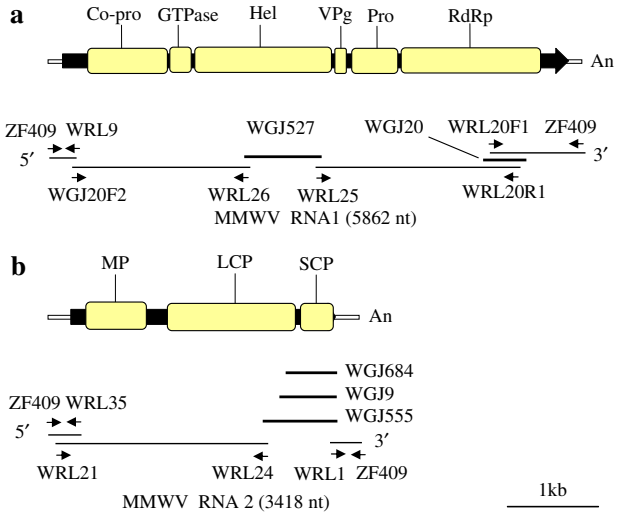

Fig. 1 The approximate positions of Co-pro, GTPase, Hel, VPg, Pro and RdRp within the ORF of RNA1 (**a**) and MP, LCP and SCP within the ORF of RNA2 (**b**). *Co-pro* a cofactor required for the proteinase, *Hel* RNA helicase, *VPg* viral protein genome-bound, *Pro* proteinase, *RdRp* RNA-dependent RNA polymerase, *MP* movement protein, *LCP* large coat protein, *SCP* small coat protein. The positions of ESTs (WGJ527, WGJ20, WGJ9, WGJ684 and WGJ555) and the PCR primers for the amplification of MMWV RNA1 (**a**) and RNA2 (**b**) are indicated in the *lower panel*

To obtain the full-length cDNA of the virus, we designed specific primers (WRL1, WRL20F1, WRL20R1, WRL24, WRL25 and WRL26) according to the EST sequences of WGJ20, WGJ527 and WGJ555. The adapter primers ZF407 (TS-long primer, 5′-CTAATACGACTCACTATAGGGCA AGCAGTGGTATCAACGCAGAGT-3′), ZF408 (TS-short primer, 5′-CTAATACGACTCACTATAGGGC-3′) and ZF409 (nested TS-PCR primer, 5′-AAGCAGTGGTATCA ACGCAGAGT-3′) were used to perform both 3′ rapid amplification of cDNA ends (RACE) and 5′ RACE. The semi-nested step-out PCR reaction [26] was enhanced by the employment of 5 M betaine solution [12, 28], GC buffer I, GC buffer II (TaKaRa) and LA Taq polymerase (TaKaRa). We designed the degenerate primers WGJ20F2 (5′-TCS ATGGGKTTACAATCCAYT-3′, nts 223–243), WRL21 (5′-TWAAACAAACAGCTTTCGT-3′, nts 16–34) and WRL9 (5′-ARTGGATTGTAAMCCCATSGA-3′, nts 243–223) using sequences that are conserved among members of the genus *Fabavirus* (GeMV, two *Broad bean wilt virus* 1 (BBWV-1) isolates and seven *Broad bean wilt virus* 2 (BBWV-2) isolates), which were analyzed using Clustal X 1.83. For RNA1, two internal fragments were amplified with the primer WRL25 (5′-CTGGGGAATATCTCTTTCGG AA-3′, nts 2,942–2,963) paired with WRL20R1 (5′-GCAA TCTGCCCAAGTGTCAGCAATCTAC-3′, nts 5,192–5,165) and WRL20F2 paired with WRL26 (5′-GGCACAGGCA AAGACGACAA-3′, nts 2,219–2,200). Finally, a 300-bp fragment was obtained by 5′ RACE with the degenerate primer WRL9 and the adapter primers ZF407, ZF408 and ZF409 [26], while a 990-bp fragment was obtained by 3′ RACE using the specific primer WGJ20F1 (5′-GCGGGCG TAACAGATGGAGTTGATAAGA-3′, nts 4,867–4,894) and the adapter primers ZF407, ZF408 and ZF409. For RNA2, we first obtained a 3,000-bp internal fragment with primers WRL21 and WRL24 (5′-GCCATTGTTGGGT AACCAGAGC-3′, nts 2,577–2,556). The 5′ end of RNA2 (350 bp) was subsequently obtained by 5′ RACE with the specific primer WRL35 (5′-TTTCGCTGTCAAAGGAG TAG-3′, nts 365–346) and the adapter primers ZF407, ZF408 and ZF409. The 3′ end of RNA2 (350 bp) was obtained by 3′ RACE with the specific primer WRL1 (5′-CCATGGGAT GGGATGTGCAAGGAGA-3′, nts 3,063–3,087) and the adapter primers ZF407, ZF408 and ZF409. All PCR fragments were sequenced using an ABI 3730 sequencer.

The obtained nucleotide sequences were assembled using the program Contig Express, a component of the Vector NTI Suite 9.0 (Invitrogen). The assembled cDNA sequences of RNA1 and RNA2 contained the intact SMART II oligo sequence at the 5′ end and a typical poly(A) sequence at the 3′ end, indicating that the intact cDNAs were successfully obtained by the template-switching procedure [26]. The nucleotide sequences of RNA1 and RNA2 and their putative proteins were analyzed using the Blast program on NCBI (http://www.ncbi.nih.gov/). The results showed the RNA1 and RNA2 were similar to those of the other viruses of the genus *Fabavirus* (GeMV, BBWV-1 and BBWV-2) [10]. We therefore propose the name Mikania micrantha wilt virus (MMWV) for this virus. An RNA-dependent RNA polymerase (RdRp) motif was found on MMWV RNA1 by Pfam analysis [3]. Phylogenetic trees (Fig. 2) were constructed using MEGA 4.0 [32] based on the multiple alignment of the amino acid (aa) sequences of RdRp motifs performed by Clustal X 1.83 [4], adopting either the N-J (neighbor joining) or ML (maximum likelihood) algorithm with the bootstrap values determined by 10,000 replicates. The result showed that MMWV was grouped within the genus *Fabavirus* (family *Comoviridae*) (Fig. 2a) and was diverged from a basal node shared by BBWV-1 and BBWV-2 (Fig. 2b).

The MMWV genomic RNA1 was 5,836 nts long excluding the 3′ poly(A) tail, and contained 177 nts at the 5′ UTR and 97 nts at the 3′ UTR (Fig. 1a). A single ORF, nts 178–5,736 of RNA1, encoded a predicted polyprotein of 1,853 amino acids and contained a cofactor required for proteinase (Co-pro), GTPase, RNA helicase, the viral cysteine protease domain (C3 protease), RNA-dependent RNA polymerase (RdRp) core domain and a possible viral protein genome-bound (VPg) (Fig. 1a) [11]. The domain arrangement of MMWV RNA1 polyprotein (Fig. 1a) was similar to those of the other members of the genus *Comoviridae*. The region of aa 439–513 encoded signal

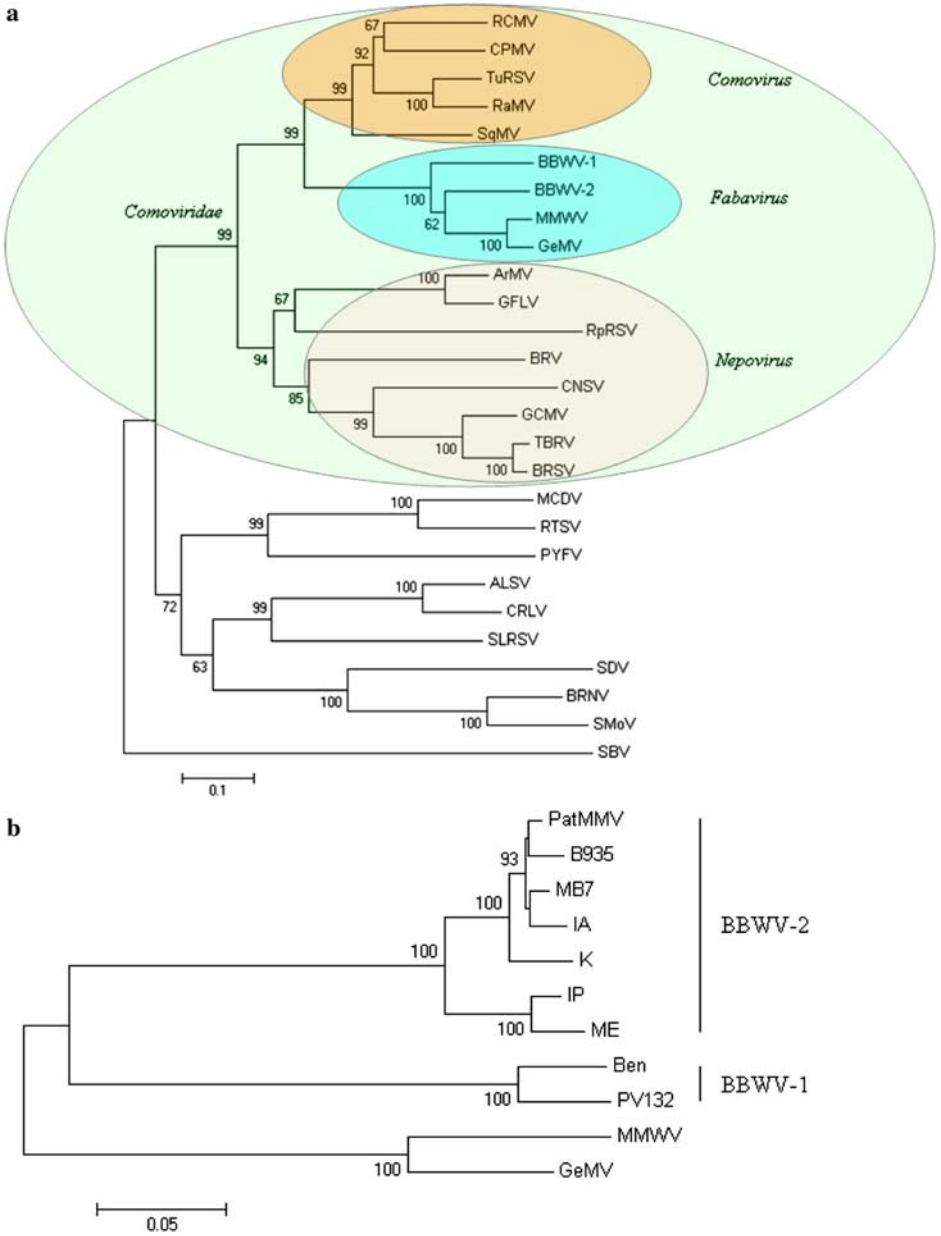

Fig. 2 **a** Phylogenetic analysis of the aligned amino acid sequences of RdRp to show the position of MMWV. The tree was constructed by the neighbor-joining method using MEGA 4.0. Values of the bootstrap support of the particular branching calculated for 10,000 replicates are indicated at the nodes. Bootstrap values greater than 60% are indicated at the *branch points*. The *bar* shows the branch length. Abbreviations and GenBank accession numbers: *SBV* sacbrood virus, AAL79021; *RCMV* red clover mottle virus, CAA46104; *CPMV* cowpea mosaic virus, CAA25029; *TuRSV* turnip ringspot virus, ABG56388; *RaMV* radish mosaic virus, AAY32935; *SqMV* squash mosaic virus, BAB62139; *GeMV* gentian mosaic virus, BAD99001; *BBWV-1* broad bean wilt virus 1, AAX12375; *BBWV-2* broad bean wilt virus 2, BAB40439; *ArMV* Arabis mosaic virus, AAQ73821; *GFLV* grapevine fanleaf virus, BAA00761; *RpRSV* raspberry ringspot virus, AAQ73822; *BRV* blackcurrant reversion virus, AAL36026; *CNSV* Cycas necrotic stunt virus, BAB89369; *GCMV* grapevine chrome mosaic virus, CAA33405; *TBRV* tomato black ring virus, AAN72830; *BRSV* beet ringspot virus, BAA00234; *MCDV* maize chlorotic dwarf virus, NP_734456; *RTSV* rice tungro spherical virus, AAA66056; *PYFV* parsnip yellow fleck virus, BAA03151; *ALSV* apple latent spherical virus, BAA90870; *CRLV* cherry rasp leaf virus, YP_081454; *SLRSV* strawberry latent ringspot virus, AAW63128; *SDV* satsuma dwarf virus, BAA76746; *BRNV* black raspberry necrosis virus, ABC71319; *SMoV* strawberry mottle virus, NP_733954 and *MMWV* Mikania micrantha wilt virus, EU158250. SBV was used as the outgroup. (**b**) Phylogenetic relationship between MMWV and members of the genus *Fabavirus*. The phylogenetic tree was generated from the deduced amino acid sequences of RdRp with MEGA 4.0 (N-J method) after bootstrapping for 10,000 replicates. The branch lengths are proportional to genetic distances. Bootstrap values greater than 60% are indicated at the *branch points*. The viruses included in this analysis are as follows, with accession number in parentheses: GeMV (BAD99001); isolates Ben (AAX12375) and PV132 (BAD00183) of BBWV-1; isolates B935 (AAD39217), IA (BAB18312), IP (BAB40439), K (AAD38152), MB7 (BAA34928), ME (AAK27841) and PatMMV (BAB83045) of BBWV-2

recognition particle GTPase, which might mediate viral fusion of the cell [30], while the region of aa 518–572 encoded RNA helicase, which might be involved in duplex unwinding during viral RNA replication [5]. The region of aa 973–1,129 encoded the peptidase C3, a cysteine protease which has high P1 specificity for Q and is involved in the processing of the polyprotein. The region of aa 1,186–1,659 encoded RdRp, an essential protein which catalyzes the synthesis of the complementary strand of the virus RNA [1]. A 26-amino-acid VPg peptide containing a tyrosine residue as the physical linkage to the RNA and responsible for stabilizing the 5′ end of the genomic RNA during replication and translation [23] was located immediately before the C3 protease [37].

The MMWV genomic RNA2 was 3,387 nts long, excluding the 3′ poly(A) sequence (Fig. 1b). It contained a 193-nts 5′ UTR and a 284-nts 3′ UTR. A single ORF, from nts 194 to 3,100 of RNA2, encoded a predicted polyprotein of 969 amino acids. The polyprotein contained the movement protein (MP), large coat protein (LCP) and small coat protein (SCP). The viral MP was encoded from aa 115 to 293, whereas the large coat protein and the small coat protein were encoded from aa 423–797 and aa 874–969, respectively (Fig. 1b).

The consensus secondary structures (Supplemental Fig. 1) of the 5′ untranslated region (UTR) and 3′ UTR of MMWV RNA1 and RNA2 were generated using the Vienna RNA secondary structure servers (http://www.tbi.univie.ac.at/~ivo/RNA/) [13]. The stem-loop structures at the 5′ UTR of the MMWV RNA1 and RNA2 might act as an internal ribosome entry site (IRES) [29]. The secondary structures of the MMWV RNA1 and RNA2 could also protect virus from RNase degradation [2], although these complex secondary structures caused problems in RT-PCR of the MMWV RNA1 and RNA2 (data not shown).

Viruses of the genus *Fabavirus* (family *Comoviridae)* can infect a wide range of plants, including the most economically important crops and horticultural and ornamental species, potentially causing a huge amount of loss worldwide every year [10]. Presently, the genus *Fabavirus* includes three recognized species: *Broad bean wilt virus 1*, *Broad bean wilt virus 2*, *Lamium mild mosaic virus* (LMMV), and a new candidate species, Gentian mosaic virus, which has been proposed recently [21]. No genomic sequence data for LMMV are available yet. Patchouli mild mosaic virus (PatMMV), which had once been classified as a species of *Fabavirus* [9, 20], was proposed to be a BBWV-2 isolate [16, 17]. A comparison of nts and aa identity of RNA1 and RNA2 between MMWV and other viruses from the genus *Fabavirus* is shown in supplemental Table 1. The overall aa identities between polypeptides encoded by RNA1 and RNA2 of MMWV and GeMV were 88.1 and 83.8%, respectively, whereas identities with homologous polypeptides of the BBWV-1 and BBWV-2 isolates ranged from 57.1 to 59.6% and 53.5 to 58.3%, respectively. The aa identity in RdRp was the highest (87.5% between MMWV and GeMV, 63.5 and 64.2% between MMWV and BBWV-1 isolates, and 64.2 to 64.9% between MMWV and BBWV-1 isolates), and the aa identity in Co-pro was the lowest (30.7–39.8% between MMWV and BBWV-2). VPg was the most conserved protein between MMWV and GeMV, with only one conservative aa substitution. Identities with VPg of BBWV-1 isolates were 65.4 to 69.2%, but identities with VPg of BBWV-2 isolates were relatively low, ranging from 42.3 to 50%. In the 5′ UTRs, the nts similarity was 24.3–79.9% for RNA1 and 20.2–61.7% for RNA2. For the 3′ UTRs, they were 11.2–73% (RNA1) and 10.8–68.3% (RNA2), respectively. The results indicated that the 3′ UTR sequences were less conserved than the 5′ UTR sequences within the genus *Fabavirus*. The sequence of seven nucleotides preceding the poly(A) was identical in RNA1 and RNA2 among members of the genus *Fabavirus*, which might control the stability of the viral genome or be

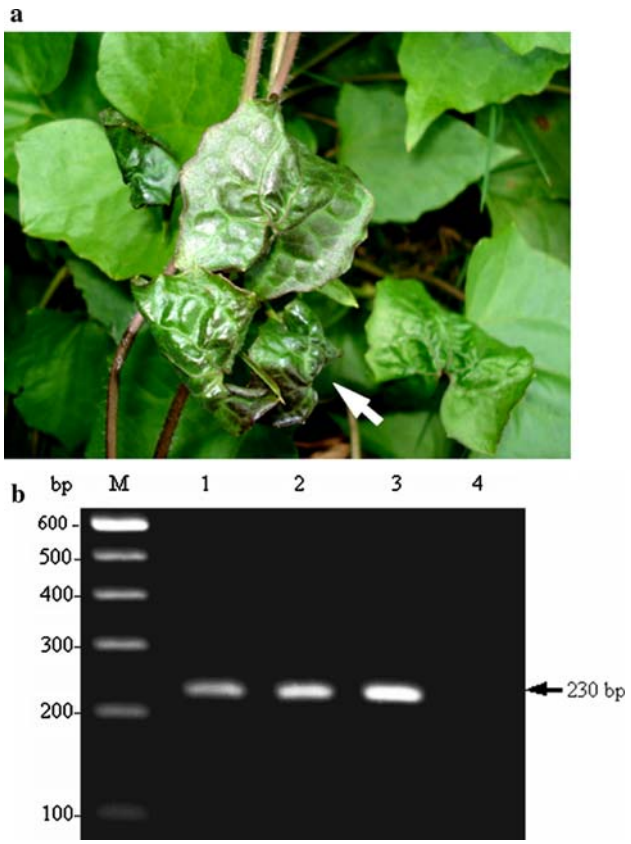

Fig. 3 **a** An *M. micrantha* plant showing wilt symptoms under field conditions is indicated by an *arrow*. **b** Detection of MMWV RNA by RT-PCR. Total RNA was extracted from aphids (*lane 1*), a vein of *C. campestris* (*lane 2*) and leaves of *M. micrantha* (*lane 3*). *Lane 4* is a negative control. *Lane M* is a DNA molecular marker

involved in signal recognition for a viral or host protein [20, 27, 38]. The 35 nts in 5′ end sequence of RNA1 and RNA2 was also highly conserved in members of the genus *Fabavirus*, which might reflect its critical roles in virus replication, translation [14, 15, 24] and the formation of the covalently linkage to VPg. Our data indicate that both MMWV and GeMV belong to a third subgroup of the genus *Fabavirus*, which provides evidence that GeMV is a member of a new species of *Fabavirus* (Fig. 2b) and MMWV is a new distinct isolate of GeMV.

Viruses in the genus *Fabavirus* are transmitted by aphids in a nonpersistent manner and can cause disease in ornamental plants and vegetables [21]. Since MMWV belongs to the genus *Fabavirus*, we hypothesized that MMWV might also be transmitted by aphids. Interestingly, we found aphids appearing on the leaves of the wild *M. micrantha* displaying the mild wilt symptoms. By electron microscopy, a few spherical virus particles approximately 30 nm in diameter could be found in samples of fresh leaves from *M. micrantha* with typical wilt symptoms (Fig. 3a). To further detect MMWV virus in *M. micrantha* plants, in aphids on the *M. micrantha* plants, and in *Cuscuta campestris*, a parasitic plant of *M. micrantha*, MMWV RNA2-specific primers WRL4 (5′-AA ATTCCACCAGATGTGGAA-3′, nts 3,158–3,177) and WRL82 (5′-GATATAAAACACAACATTATTTTACA TA-3′, nts 3,387–3,360) were used to amplify the MMWV SCP gene. A 230-bp fragment of the MMWV SCP gene, which was verified by sequencing, was amplified by RT-PCR from *M. micrantha* plants showed wilting symptoms, aphids and *C. campestris* (Fig. 3b). The presence of MMWV in *C. campestris* suggests that, in addition to the aphids, MMWV might also be transmitted by *C. campestris*, but this will require more evidence.

Although there are currently several ways to control the spread of *M. micrantha* [39], the discovery and utilization of new natural enemies could provide a much more effective approach for controlling this aggressive weed. Employing natural enemies to control the spread of *M. micrantha* began in 1978; however, this effort mainly focused on insect agents [7] and the parasitic plant *C. campestris* [31]. Recent studies have shown that viruses could be a feasible approach to control the pest [22, 33–36]. Although MMWV is not lethal for *M. micrantha*, it efficiently inhibits its growth. Therefore, the feasibility of using MMWV to control *M. micrantha* is worth exploring further.

In this study, a new distinct isolate of GeMV, MMWV, infecting *M. micrantha* was found, and the full-length sequence of MMWV genomic RNAs was determined. The genomic sequence of MMWV shared about 60% identity with BBWV-1 and BBWV-2, and it might be transmitted to important crops and cause serious problems for agriculture [9, 21]. Further studies are required to isolate the MMWV from *M. micrantha* plants and to verify its transmission mechanism, distributing range, potential host and impact on important crops in southern China.

The sequences presented in this paper have been submitted to the GenBank database and have been assigned the accession numbers EU158249 and EU158250.

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