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Short communication

Construction and biological characterization of an *Agrobacterium*-mediated infectious cDNA of squash mosaic virus

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ABSTRACT

Squash mosaic virus (SqMV), a member of the species *Squash mosaic virus* in the genus *Comovirus* (family *Comoviridae*), is an important seed-borne virus that causes serious economic losses in cucurbit crops. Here, we constructed infectious cDNA clones of SqMV genomic RNAs (RNA1 and RNA2) under the control of the cauliflower mosaic virus (CaMV) 35S promoter by Gibson assembly. The infectious cDNA clones of SqMV could infect zucchini squash (*Cucurbita pepo*) plants systemically by agrobacterium-mediated inoculation. The virus progeny from the infectious clones showed no difference from the wild type in terms of pathogenicity and symptom induction. It could be mechanically transmitted to zucchini squash (*Cucurbita pepo*), pumpkin (*Cucurbita moschata*), cucumber (*Cucumis sativus*), and muskmelon (*Cucumis melo*) but not watermelon (*Citrullus lanatus*) or *Nicotiana benthamiana*. This is the first report of construction of a SqMV infection clone and will facilitate the investigation of viral pathogenesis and host interactions.

Squash mosaic virus (SqMV) belongs to the species *Squash mosaic virus* in the genus *Comovirus* of the family *Comoviridae*. It has icosahedral particles ~ 30 nm in diameter which can be transmitted by mechanical means, seeds and beetles (Freitag, 1956; Thomas, 1973). A range of plants in the genera *Cucurbita*, *Citrullus* and *Cucumis* can be infected by SqMV and display mottling and malformed foliage with raised, blister-like areas, or vein-banding, leaf distortion and prominent serrations of the leaf margin, or small raised domelike projections or chlorotic patches on the surface of fruit, which seriously affects the growth of cucurbit crops and reduces the yield and quality of cucurbit fruits (Nelson and Knuhtsen, 1973; Thomas, 1973). SqMV was first reported in California in 1934 and subsequently in Cuba, Brazil, Greece, Spain, Morocco, Japan, Iran, Jordan, Indonesia, China, Turkey and Australia (Al-Musa et al., 1994; Avgelis and Katis, 1989; E. L. Lockhart, 1982; Edreira Alencar et al., 2016; Freitag, 1956; Gu et al., 2008; Izadpanah, 1987; Li et al., 2015; Lima and Vieira, 1992; Maina et al., 2017; Sevik, 2008; Yoshida et al., 1980). The biological properties and distribution of SqMV suggest that it poses a threat to the production of cucurbit crops worldwide.

The construction of infectious cDNA clones of plant RNA viruses aims to maintain their molecular and biological characteristics, and enables investigation of the host range, pathogenicity, gene functions and virus-host interactions (Govind et al., 2012; Kang et al., 2015; Klein et al., 2014; Lu et al., 2001; Stevens and Viganó, 2007). To date, infectious cDNA clones of 4 of the 15 comoviruses, including cowpea mosaic virus(CPMV), bean pod mottle virus(BPMV), radish mosaic virus (RaMV), and bean rugose mosaic virus(BRMV), have been constructed under the control of the T7 RNA polymerase promoter or cauliflower mosaic virus (CaMV) 35S promoter (Bijora et al., 2017; Gu and Ghabrial, 2005; Komatsu et al., 2013; Liu and Lomonossoff, 2002). Serological and molecular biological assays for SqMV have been developed, the seed transmission rate in different cucurbits has been assessed, and X-ray analysis and chitosan and plant growth promoting rhizobacteria(PGPR) have been used to control disease (Edreira Alencar et al., 2016; Firmansyah et al., 2017; Ling et al., 2011; Rosminim Purba et al., 2017). However, no infectious cDNA clone of SqMV has been constructed, which hampers reverse genetic studies.

Like other members of the genus *Comovirus*, SqMV has a bipartite single-stranded positive-sense RNA genome (RNA1 and RNA2) encapsulated separately with two capsid proteins into isometric particles. RNA1 encodes polyprotein 1, which is processed into a protease cofactor, helicase, viral protein genome-linked (VPg), protease, and RNA-dependent RNA polymerase (RdRp). RNA2 encodes polyprotein 2, which is processed into movement protein (MP), large capsid protein (LCP), and small capsid protein (SCP) (Hu et al., 2009). Both RNA molecules have a poly (A) 3′ tail. In China, mosaic symptoms on squash in Sanxi Province were caused by SqMV CH99/211 (SqMV – CH) (Gu et al., 2008). SqMV – CH was isolated from diseased leaves and both its

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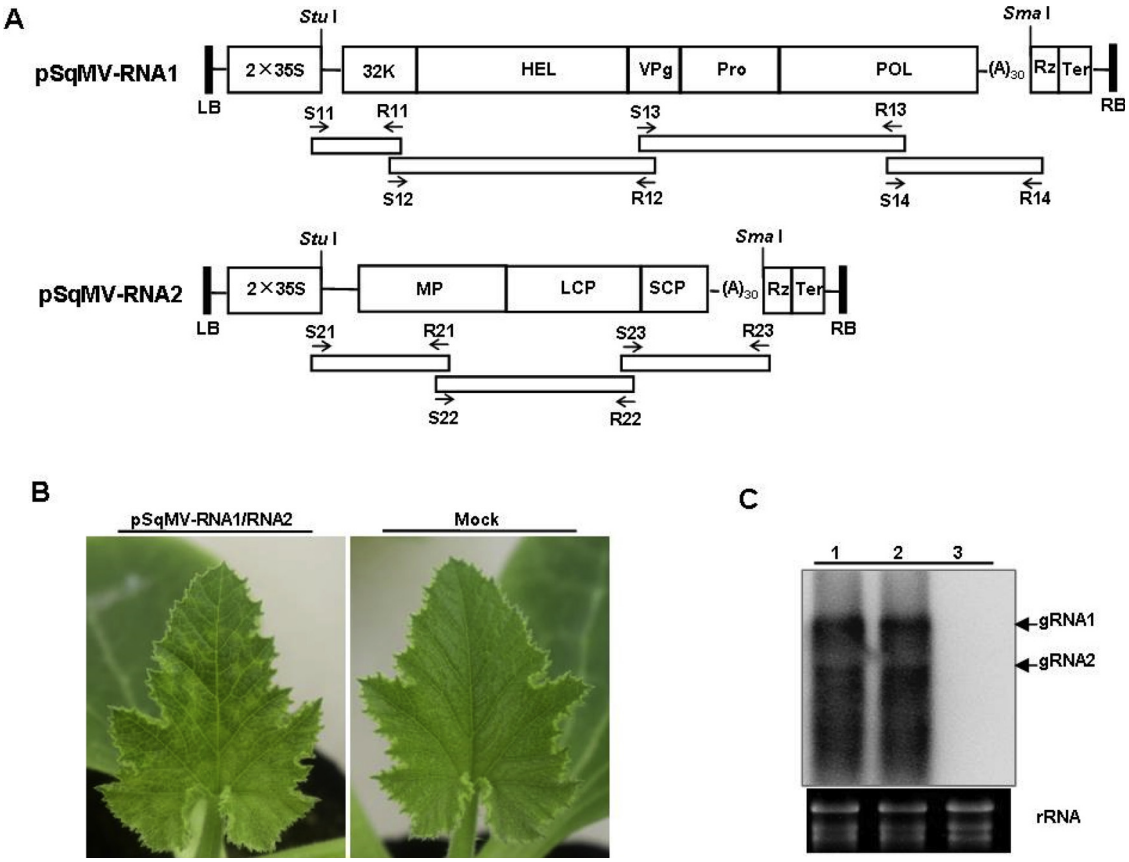

Fig. 1. Diagram and infectivity of pSqMV-RNA1/RNA2. A, Diagram of pSqMV-RNA1 and pSqMV-RNA2. LB, left border of T-DNA; 2 × 35S, double CaMV 35S promoter; 32 K, a protease co-factor; HEL, helicase; VPg, viral protein genome-linked, Pro, protease; POL, RNA-dependent RNA polymerase; RZ, *cis*-cleaving ribozyme sequence; Ter, nopaline synthase polyadenylation signal; RB, right border of T-DNA; MP, movement protein; LCP, large capsid protein; SCP, small capsid protein. B, Symptoms induced by pSqMV-RNA1/RNA2 in zucchini squash (*Cucurbita pepo*) at 7 days post-inoculation (dpi). C, Northern blot analysis of total RNAs extracted at 7 dpi from the systemic leaves of virus- or mock-inoculated plants. Total RNAs (2.5 μg) for each sample were hybridized with probes for RNA1 and RNA2. SYBR-SAFE staining was performed as a loading control. Viral genomic RNA1 and RNA2 are shown as gRNA1 and gRNA2, respectively. Lane 1, SqMV − CH wild-type-infected leaves; lane 2, pSqMV-RNA1/RNA2-infected zucchini squash leaves; lane 3, mock-inoculated zucchini squash leaves.

RNA molecules were sequenced (Gu et al., 2008; Hu et al., 2009). In this study, we constructed an infectious cDNA clone of SqMV − CH and evaluated its virulence and symptoms on a variety of hosts.

To construct the infectious cDNA clone of SqMV, the plant binary vector pXT1 was used (Yao et al., 2011). The genome sequence of SqMV − CH (accession numbers EU421059 and EU421060) was determined by Hu et al. (2009), but there were nine nucleotides in both its RNA molecules longer than the other isolates of SqMV and other comoviruses available from the GenBank. Considering the importance of infectious cDNA clones in plant virology and the requirement of high fidelity of viral genome sequence in constructing infectious cDNA clone, we performed RACE experiments to further confirm the 5′ terminal sequences of SqMV − CH by using a SMARTer RACE 5′/3′ Kit (Clontech, Dalian, China), and the novel nine nucleotides were not detected in both 5′ terminal of RNA molecules. Therefore, we designed the primers (Appendix table 1) based on the genome sequence of SqMV − CH except the novel nine nucleotides termini. In addition, the primers used to amplify the termini of the viral genome sequences were lengthened by the addition of 20 nucleotides of the 3′ terminus of the CaMV 35S promoter and the 5′ terminus of the hepatitis delta virus (HDV) ribozyme to develop an infectious cDNA clone of SqMV by Gibson assembly. The genome of SqMV was ligated downstream of the CaMV 35S promoter and upstream of the HDV ribozyme in the infectious cDNA clone to ensure production of the authentic termini of the viral RNAs with no non-viral sequence.

SqMV − CH was propagated in zucchini squash (*Cucurbita pepo*) cv. Zhenyu 358 plants in a greenhouse. Total RNAs were extracted from diseased leaves using RNAiso plus (TaKaRa, Kyoto, Japan). cDNA templates were synthesized using an oligo dT antisense primer and Primescript II First-Strand cDNA Synthesis Kit (TaKaRa). To obtain full-length genomic RNA1, we amplified it in four fragments using S11/R11, S12/R12, S13/R13, and S14/R14 primers and Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). The vector pXT1 was treated with *Stu* I and *Sma*I and assembled with the four fragments of genome RNA1 using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs) to generate an infectious cDNA clone of SqMV RNA1, named pSqMV-RNA1 (Fig. 1A). To obtain the full-length fragment of genomic RNA2, we amplified it in three fragments using S21/R21, S22/R22, and S23/R23 primers. Subsequently, the three RNA2 genome fragments were assembled using the *Stul*I and *Sma*I-treated pXT1 vector to generate an infectious cDNA clone of the SqMV RNA2 genome, named pSqMV-RNA2 (Fig. 1A). The presence of SqMV in pSqMV-RNA1 and pSqMV-RNA2 was confirmed by sequencing.

To assess the infectivity of the cloned SqMV − CH, pSqMV-RNA1 and pSqMV-RNA2 were transformed into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw transformation and agroinoculation was performed by infiltration of the cotyledons of zucchini squash (*Cucurbita pepo*) cv. Zhenyu 358 plants with bacterial suspensions mixed at a 1:1 ratio (OD600 1.0). At 7 days post-inoculation (dpi), the upper leaves of plants agroinfiltrated with pSqMV-RNA1/RNA2 began to develop mosaic symptoms (Fig. 1B). Northern blotting of SqMV RNAs in diseased leaves was performed using the DIG Northern Starter Kit according to the manufacturer's instructions (Roche, Mannhein, Germany). For

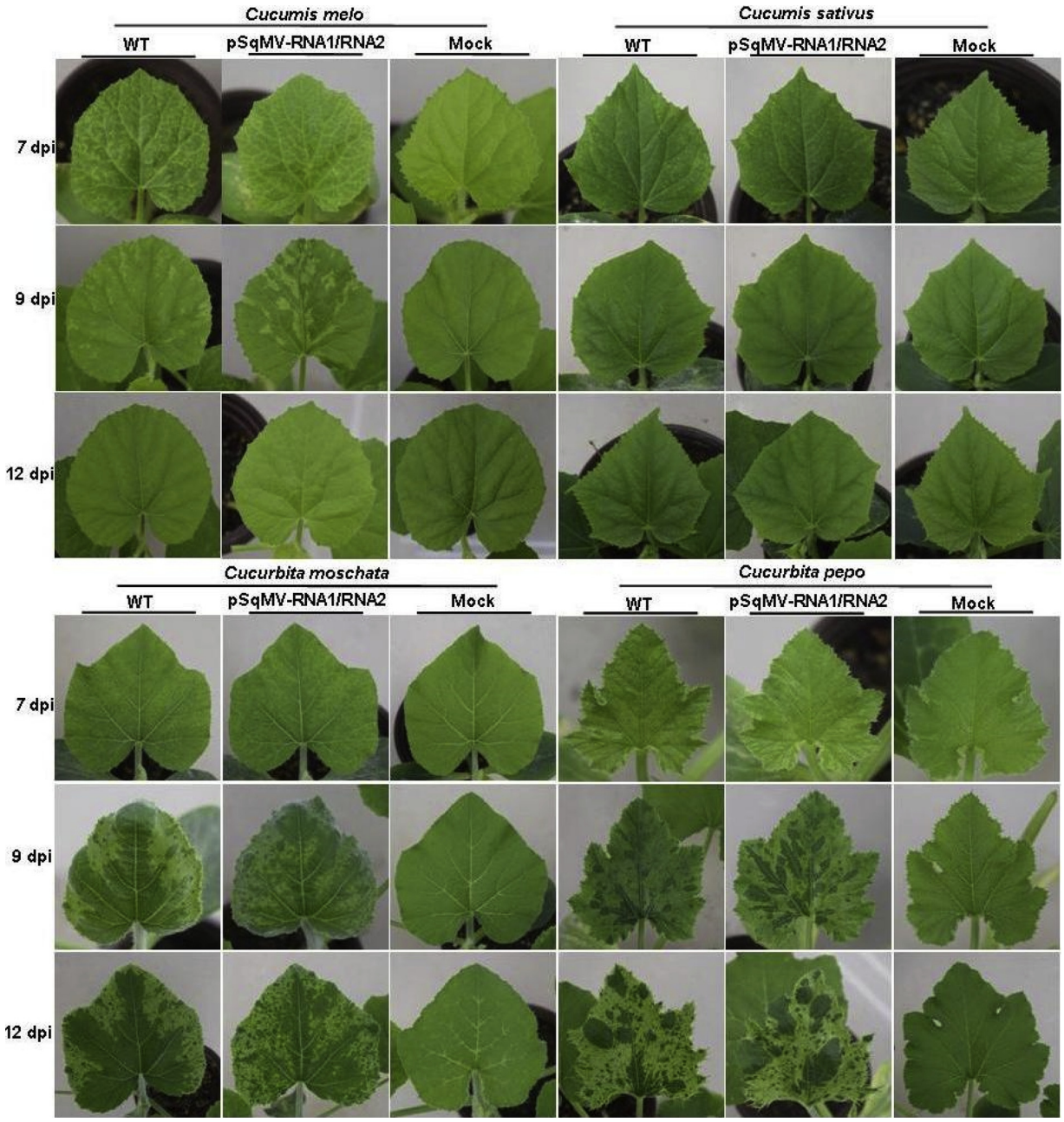

Fig. 2. Symptoms at 7, 9, and 12 dpi after mechanical inoculation of muskmelon (*Cucumis melo*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita moschata*), and zucchini squash (*Cucurbita pepo*) plants with sap from pSqMV-RNA1/RNA2-infected zucchini squash leaves. WT, SqMV − CH wild-type.

northern blot assays, 2.5 μg of total RNAs per sample were loaded, probes for SqMV complementary to the 641–1349 nucleotides of genomic RNA1 and the 212–911 nucleotides of genomic RNA2 were used, and images were captured using a Chemiluminescence Imaging System (Tanon, Shanghai, China). Northern blot hybridization showed that replication of the cloned SqMV produced genomic RNA1 (gRNA1) and genomic RNA2 (gRNA2), similar to wild-type (WT) SqMV − CH (Fig. 1C). Infection by SqMV was confirmed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) according to the manufacturer's instructions (Adgen, Auchincruive, UK) (data not shown). These results demonstrate the infectivity of SqMV infectious clones (pSqMV-RNA1/ RNA2) in plants.

To verify the biological activity of virus progeny from the cloned SqMV, the cotyledons of zucchini squash (*Cucurbita pepo*) cv. Zhenyu 358, pumpkin (*Cucurbita moschata*) cv. Zaolimiben, cucumber (*Cucumis sativus*) cv. Jinyan No. 4, watermelon (*Citrullus lanatus*) cv. Zhengkang No. 2 and muskmelon (*Cucumis melo*) cv. Baimei plants were inoculated with sap from the diseased leaves of zucchini squash plants agroinoculated with pSqMV-RNA1/RNA2. The inoculated muskmelon and cucumber plants began to develop chlorosis around or in the veins of new leaves at 5 dpi and more obviously at 7 dpi (Fig. 2). With time the symptoms in the new leaves weakened or disappeared (Fig. 2). In the inoculated zucchini squash and pumpkin plants, mild mosaic symptoms appeared between the veins of new leaves at 7 dpi. Subsequently, the leaves of zucchini squash and pumpkin plants developed the light green/dark green mosaic symptoms (Fig. 2). In addition, the new leaves of pumpkin plants curled slightly to the abaxial side at the beginning, whereas the leaves of diseased zucchini squash plants displayed vein-banding, raised, blister-like areas, and prominent serrations of the leaf margin over time (Fig. 2). Watermelon failed to show symptoms even at 21 dpi. SqMV − CH WT was also assayed in these hosts as a positive control, and its symptoms were similar to those of virus progeny from

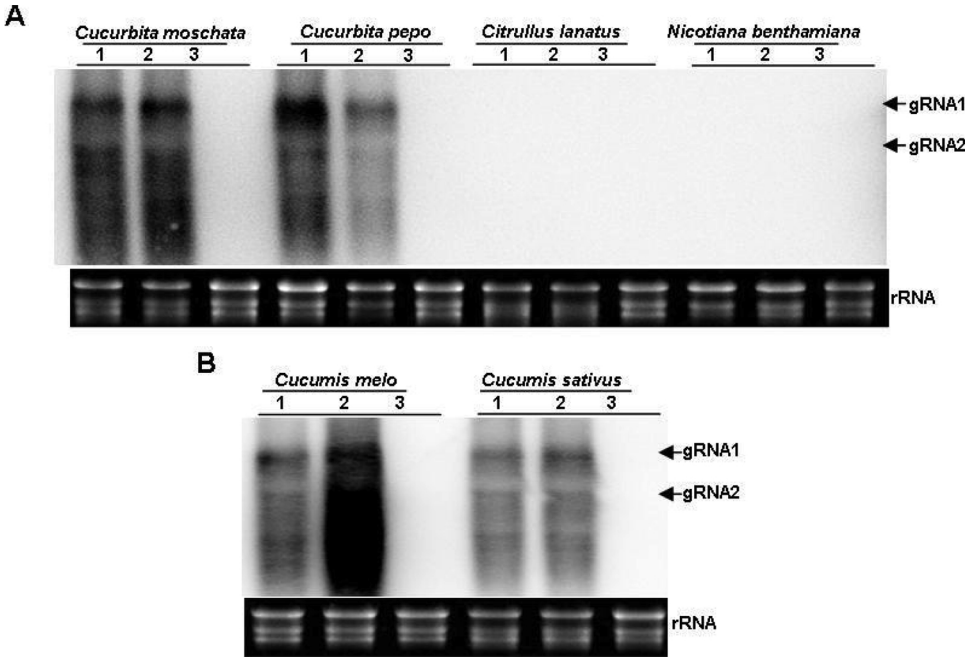

Fig. 3. RNA accumulation of pSqMV-RNA1/RNA2 in six mechanically inoculated hosts. A, Northern blot analysis of total RNAs extracted at 7 dpi from the systemic leaves of virus- or mock-inoculated zucchini squash (*Cucurbita pepo*), pumpkin (*Cucurbita moschata*), watermelon (*Citrullus lanatus*) and *Nicotiana benthamiana* plants. B, Northern blot analysis of total RNAs extracted at 7 dpi from the systemic leaves of virus- or mock-inoculated muskmelon (*Cucumis melo*) and cucumber (*Cucumis sativus*) plants. 2.5 μg of total RNAs were loaded for each sample. Lane 1, SqMV－CH wild-type-infected leaves; lane 2, pSqMV-RNA1/RNA2-infected leaves; lane 3, mock-inoculated leaves.

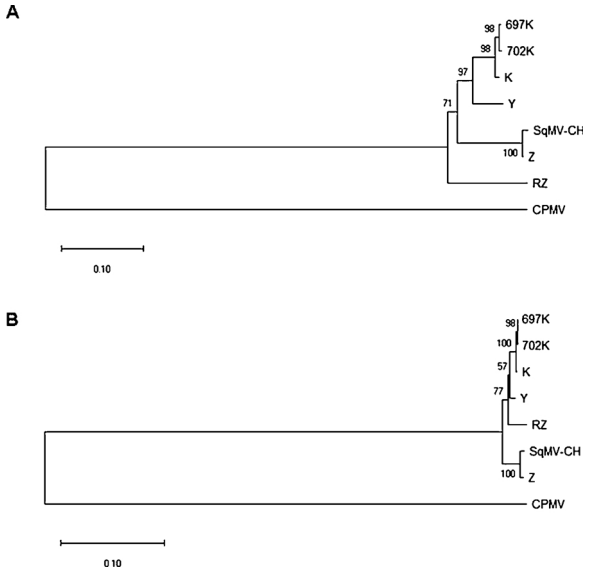

Fig. 4. Phylogenetic trees based on the full-length RNA2 sequences (A) and the deduced amino acid sequences of polyprotein 2 (B) of several SqMV isolates with CPMV as an outgroup. The GenBank accession numbers for isolate 697 K, 702 K, K, Y, RZ, SqMV－CH, Z and CPMV are MF166755, MF166754, AF059533, AB054689, KP223324, EU421060, AF059532 and NC_003550, respectively. Phylogenetic analyses were carried out by using the neighbor-joining method with the MEGA X (Kumar et al., 2018). Bootstrap analyses were performed by using 1000 replicates and the values are indicated at the branch points.

the cloned SqMV. Furthermore, SqMV gRNA1 and gRNA2 were detected by northern blot in the systemic leaves of zucchini squash, pumpkin, cucumber, and muskmelon plants inoculated with progeny virions from pSqMV-RNA1/RNA2 or SqMV－CH WT, but not in watermelon plants, whereas no viral RNA was detected in non-inoculated plants (Fig. 3A and B). The northern blot results were consistent with the symptoms. Therefore, progeny virions from pSqMV-RNA1/ RNA2 were biologically active and able to systemically infect zucchini squash, pumpkin, cucumber, and muskmelon, but not watermelon, similar to the virulence and symptoms of SqMV－CH WT.

Nicotiana benthamiana is extensively used in plant virology due to its susceptibility to over 500 plant viruses (Clemente, 2006). One of two RdRPs derived from *N. benthamiana* contains a 72 nt insertion, which introduces tandem in-frame stop codons in the 5′ portion of the open reading frame compared to the salicylic acid-inducible RdRP derived from *Nicotiana tabacum*, and is the genetic basis of viral susceptibility of *N. benthamiana* (Yang et al., 2004). The type species of the genus *Comovirus*, CPMV, was infected to *N. benthamiana* by agroinoculation using a CPMV-based construct (Liu and Lomonossoff, 2002). Herein, the top three fully expanded leaves of *N. benthamiana* plants (6–8 leaf stage) were inoculated with pSqMV-RNA1/RNA2 or SqMV－CH WT infected leaves of zucchini squash plants, but failed to display symptoms, and viral RNA did not accumulate in the systemic leaves of inoculated *N. benthamiana* plants (Fig. 3A). Thus, *N. benthamiana* is not a host of SqMV, at least of SqMV－CH, which reacted with *N. benthamiana* differently from CPMV.

SqMV isolates from the Western hemisphere are classified into six biotypes based on their host range and symptomatology, but into only two serogroups (Nelson and Knuhtsen, 1973). Previous studies have grouped isolates K and Y into serotype I (Haudenshield and Palukaitis, 1998; Hu et al., 1993), isolate Z into serotype II (Haudenshield and Palukaitis, 1998), and full-length RNA2 sequences of several SqMV isolates, including isolate K, Y, Z, RZ, 697 K and 702 K, are available from the GenBank, so it permits us to establish a correlation of serogroup with the RNA2 sequence of SqMV. According to the phylogenetic analyses of full-length RNA2 nucleotide sequences of SqMV isolates, isolate RZ was assigned to be a third genotype of SqMV(Fig. 4A), which coincided with the results of sequence comparision of RZ with all known SqMV isolates (Li et al., 2015). However, the result of the amino acid phylogenetic analyses of polyprotein 2 that isolate 697 K, 702 K, and RZ were grouped with serotype I isolate K and Y and isolate SqMV－CH and Z were grouped into serotype II may better reflect the exist of two serogroups (Fig. 4B). It was confirmed that the serological specificity of SqMV isolates was related to their host reactions, and differences in symptoms between the two serogroups were evident in melon and squash (Nelson and Knuhtsen, 1973). Isolate Z has been reported to cause more severe symptoms in squash than isolate K and R (Haudenshield and Palukaitis, 1998), and SqMV－CH was confirmed to induce severe symptoms in squash and mild symptoms in melon in present study, which was typed to the characteristic of serogroup II

(Nelson and Knuhtsen, 1973). Therefore, it's possible to establish a correlation of virulence of two serogroups with polyprotein 2 sequence of SqMV. The amino acid sequences of polyprotein 2 of SqMV isolates were aligned by clustal W, and 18 special amino acids in polyprotein 2, including 7, 6, and 5 amino acids in MP, LCP, and SCP respectively, were found in both of SqMV − CH and Z comparing to isolate K, Y, RZ, 697 K and 702 K (Appendix table 2). Thus, these 18 amino acid sequence differences found in the polyprotein 2 may function in serological response and symptomatology of SqMV.

In this study, we developed an infectious cDNA clone of SqMV − CH. The virus progeny of the infectious clone was mechanically transmitted and exerted virulence in different hosts. Therefore, the developed system enables investigation of viral determinants, pathogenesis and virus-host interactions. To our knowledge, this is the first report of the construction of SqMV infectious clone and will facilitate reverse genetic studies of SqMV.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197766.

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