TALES DA SILVA MENDES

SYSTEMIC INFECTION OF PLANTS BY A GEMYCIRCULARVIRUS (FAMILY *Genomoviridae*)

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Magister Scientiae*.

Orientador: Francisco Murilo Zerbini Junior
Coorientadora: Anelise Franco Orílio

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Tales da Silva Mendes
(Autor)

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Francisco Murilo Zerbini Junior
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BIOGRAFIA

TALES DA SILVA MENDES, filho de Rosa de Lima da Silva e Luiz Carlos Mendes, nasceu em Ubá, Minas Gerais, no dia 24 de dezembro de 1991.

Residiu e concluiu o ensino fundamental e médio na cidade de Senador Firmino, Minas Gerais. Em 2010 iniciou o curso de Ciências Biológicas na Universidade Federal de Viçosa, Minas Gerais, graduando-se em julho de 2015. Ingressou, em agosto de 2017, no curso de Pós-graduação em Genética e Melhoramento em nível de mestrado na mesma universidade, submetendo-se à defesa de dissertação em 29 de agosto de 2019.

RESUMO

MENDES, Tales da Silva, M.Sc., Universidade Federal de Viçosa, Agosto de 2019. **Infecção sistêmica de plantas por um gemycircularvírus (fam. *Genomoviridae*)**. Orientador: Francisco Murilo Zerbini. Co-orientadora: Anelise Franco Orílio.

Membros da família *Genomoviridae* são vírus não envelopados, com genomas de ssDNA circular que variam de 2 a 2,4 kb, contendo duas ORFs separadas por uma região intergênica não codificadora. Uma das ORFs, localizada na fita viral, codifica a proteína do capsídeo (CP), e a outra, na fita complementar, codifica a proteína associada à replicação (Rep), semelhante à encontrada em membros da família *Geminiviridae*. Atualmente, a família *Genomoviridae* inclui 73 espécies virais classificadas em nove gêneros, sendo o gênero *Gemycircularvirus* o mais diverso. Membros dessa família foram identificados em mamíferos, aves, invertebrados, em amostras ambientais e em plantas. No entanto, apesar desse grande número de relatos e de sua presença disseminada no ambiente, o conhecimento sobre a infectividade da maioria dos genomovírus permanece escasso. O gemycircularvírus SsHADV-1 é o único genomovírus que apresenta um hospedeiro conhecido, um fungo. O gênero *Gemycircularvirus* inclui todos os genomovírus associados a plantas encontrados até o momento. É possível que os gemycircularvírus desempenhem papéis ecológicos relevantes em associação com plantas. Neste trabalho, relatamos o primeiro caso de infecção sistêmica de plantas por um gemycircularvírus, o *Euphorbia heterophylla associated gemicircularvirus* (EuaGmV). Um clone dimérico (aprox. 4,4 kb) do EuaGmV foi utilizado para a inoculação via biobalística de plantas de *Euphorbia heterophylla* e *Nicotiana benthamiana*. Plantas controle das duas espécies foram inoculadas com o geminivírus *Euphorbia yellow mosaic virus* (EuYMV) ou com água. Aos 21 e 28 dias após a inoculação (dpi), folhas superiores não inoculadas (que não apresentavam sintomas) foram coletadas para análise da presença do vírus via PCR. Oito plantas de *E. heterophylla* e todas as 10 plantas de *N. benthamiana* foram positivas para a presença de EuaGmV. Amplicons (612 bp) obtidos de *E. heterophylla* foram sequenciados e mostraram identidade de 99,6% com a sequência do clone de EuaGmV. Estes resultados demonstram, pela primeira vez, que um gemycircularvírus (família *Genomoviridae*) é capaz de infectar plantas sistemicamente.

ABSTRACT

MENDES, Tales da Silva, M.Sc., Universidade Federal de Viçosa, August, 2019. **Systemic infection of plants by a gemycircularvirus (family *Genomoviridae*)**. Advisor: Francisco Murilo Zerbini Junior. Co-advisor: Anelise Franco Orílio.

The *Genomoviridae* family includes non-enveloped, ssDNA viruses with circular genomes ranging from 2 to 2.4 kb, containing two ORFs separated by an intergenic, non-coding region. One of the ORFs, located in the virion-sense strand, encodes the putative coat protein (CP), and the other, in the complementary-sense strand, encodes a putative replication-associated protein (Rep) similar to the Rep protein found in members of the family *Geminiviridae*. Currently, the *Genomoviridae* family contains 73 viral species classified into nine genera, with *Gemycircularvirus* as the largest genus. Members of the family have been identified in mammals, birds, invertebrates, in a variety of environmental samples, and also in plants. However, despite the large number of reports and their pervasive presence in the environment, the infectivity of genomovirids to specific hosts remains largely unknown. The gemycircularvirus SsHADV-1 remains the only genomovirid with a known (fungal) host. The genus *Gemycircularvirus* includes all plant-associated genomovirids found so far. It is possible that gemycircularviruses play relevant ecological roles in association with plants. Here, we report the first case of systemic infection of plants by a gemycircularvirus, *Euphorbia heterophylla associated gemycircularvirus* (EuaGmV). A dimeric clone (~4.4 kb) of EuaGmV was used for the biolistic inoculation of plants of *Euphorbia heterophylla* and *Nicotiana benthamiana*. Control plants (of both species) were inoculated with the geminivirus *Euphorbia yellow mosaic virus* (EuYMV) or with water. At 21 and 28 days after inoculation (dai), non-inoculated upper leaves of the EuaGmV-inoculated plants (which did not show any symptoms) were collected for PCR-based analysis of the presence of the virus. Eight plants of *E. heterophylla* and all 10 plants of *N. benthamiana* were PCR-positive for the presence of EuaGmV. Amplicons (612 bp) obtained from *E. heterophylla* were sequenced, and a 99.6% identity with the sequence of the EuaGmV clone was obtained. These results demonstrate, for the first time, that a gemycircularvirus (family *Genomoviridae*) is capable of systemically infecting plants.

SUMÁRIO

GENERAL INTRODUCTION

Viruses are obligatory parasites present throughout the biosphere, with representatives that can infect the most diverse host types in all life domains (Ignacio-Espinoza *et al.*, 2013). Many are considered pathogens of great economic, clinical, and ecological importance (Krupovic, 2013). It is estimated that there are approximately 10^{31} viral particles on Earth, most of them infecting bacteria (Hendrix, 2002), totaling around 200 million tons of biomass across the globe (Suttle, 2005).

Advances in culture-independent molecular techniques such as rolling-circle amplification (RCA) and viral metagenomics coupled with high throughput sequencing (HTS) are expanding our understanding of viral diversity, making it possible to detect and discover a variety of viral genomes (Comeau *et al.*, 2006; Breitbart *et al.*, 2007; Culley & Steward, 2007). Nowadays, the International Committee on the Taxonomy of Viruses (ICTV) accepts metagenomic data in viral taxonomy, which is now based not only on biological properties, particle morphology, genome architecture and replication mechanisms (Fenner, 1976; King *et al.*, 2011), but also on viral sequences in the absence of any biological information (Simmonds *et al.*, 2017).

Twelve out of 14 families of single-stranded (ss) DNA viruses currently recognized by the ICTV have circular genomes, with the *Parvoviridae* and *Bidnaviridae* families being the exceptions with linear ssDNA genomes. Eight out of these 12 families comprise circular ssDNA viruses that infect eukaryotic organisms, with *Anelloviridae* being the only family whose members do not encode a protein homologous to the replication-associated protein (Rep). Members of the other seven families, *Bacilladnaviridae* (Kazlauskas *et al.*, 2017), *Circoviridae* (Rosario *et al.*, 2017), *Geminiviridae* (Zerbini *et al.*, 2017), *Genomoviridae* (Krupovic *et al.*, 2016), *Nanoviridae* (King *et al.*, 2011), *Redondoviridae* (Abbas *et al.*, 2019) and *Smacoviridae* (Varsani & Krupovic, 2018) encode a Rep and are referred to as circular Rep-encoding ssDNA (CRESS-DNA) viruses.

Viruses of the family *Circoviridae* are known to cause economically important animal diseases, while members of the *Nanoviridae* and *Geminiviridae* encompass plant pathogens that cause diseases of economic importance primarily in tropical and subtropical regions of the world. By contrast, *Bacilladnaviridae, Genomoviridae, Redondoviridae* and *Smacoviridae* are four families recently discovered that need to be better studied. Bacilladnaviruses have been identified infecting diatom microalgae, and so far there is no information on possible hosts of members of *Smacoviridae* and

Redondoviridae. Although members of these two families are suspected to be associated with animals, the association of smacoviruses with methanogenic *Archaea* has been suggested (Díez-Villaseñor & Rodriguez-Valera, 2019). *Genomoviridae* is also one of the newly established ssDNA genome virus families for which the known hosts of its members are largely unknown (Krupovic *et al.*, 2016; Adams *et al.*, 2017).

Genomovirids are non-enveloped, circular, ssDNA viruses with genomes ranging from 2 to 2.4 kb, containing two ORFs separated by an intergenic non-coding region (IR). One of the ORFs, located in the virion-sense strand, encodes the putative coat protein (CP), and the other, in the complementary-sense strand (often having an intron of 166 to 226 nucleotides) (Kraberger *et al.*, 2015a), encodes a putative replication-associated protein (Rep) similar to the Rep protein found in members of the family *Geminiviridae* (Schalk *et al.*, 1989; Mullineaux *et al.*, 1990; Dekker *et al.*, 1991; Wright *et al.*, 1997; Gutierrez, 1999). Genomovirids have a conserved stem-loop structure for initiating replication through the Rep protein which is analogous to those found in members of the families *Geminiviridae*, *Circoviridae* and *Nanoviridae* (Rosario *et al.*, 2012b; Kraberger *et al.*, 2015a). The structure, located at the origin of replication, includes a conserved nonameric sequence which is recognized by the Rep protein during the initiation of virion DNA replication. In genomovirids this nonanucleotide is variable (5'-TAWWDHWRN-3'), with the sequence 5'-TAATWYTAT-3' being the consensus for members of the genus *Gemycircularvirus* (Varsani & Krupovic, 2017), the most diverse genus of the family.

The genomovirid Rep is a multifunctional protein with well characterized functional domains. They consist of an His-hydrophobe-His (HUH) endonuclease domain in the amino (N) terminal portion and a superfamily 3 (SF3) helicase domain comprising a region of 120 nucleotides in its carboxy (C) terminal portion (Gorbalenya *et al.*, 1990; Ilyina & Koonin, 1992; Koonin, 1993). The N-terminal region also contains conserved motifs I, II and III involved in the initiation and termination processes of rolling-circle replication (RCR) (Ilyina & Koonin, 1992; Vega-Rocha *et al.*, 2007; Krupovic, 2013). The presence of one or two tyrosine residues in motif III separates Rep proteins into two superfamilies. The presence of a single catalytic tyrosine in motif III classifies genomovirid, geminivirid, bacilladnavirid, circovirid and nanovirid Reps (all families whose members infect eukaryotes) as superfamily II (Ilyina & Koonin, 1992; Krupovic, 2013). Superfamily I Reps have two tyrosine residues, and includes the Reps of bacteriophages and plasmids. The conserved motif I is related to the recognition of specific regions in the origin of replication of these viruses, while motif II, which

resembles that found in geminivirids, has catalytic histidine residues (Ilyina & Koonin, 1992; Laufs *et al.*, 1995a). A fourth conserved motif, GRS (geminivirus Rep sequence), is found only in genomovirids and geminivirids. Nash *et al.* (2011) demonstrated that GRS motifs are essential for the replication of tomato golden mosaic virus (TGMV). The presence of the GRS motif in members of *Geminiviridae* and *Genomoviridae* suggests an evolutionary relationship between these two families, reinforced by phylogenetic analyses (Krupovic *et al.*, 2016). In the C-terminal portion, Walker A, Walker B, and motif C are present, which give Rep its helicase activity during RCR (Gorbalenya *et al.*, 1990; Gorbalenya & Koonin, 1993) and catalyze the circularization of new strands following replication (Laufs *et al.*, 1995b).

Unlike the high degree of homology found among Reps from the eukaryotic CRESS-DNA virus families, the CP is highly divergent. For example, the *Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1* (SsHADV-1) CP bears no similarity to geminivirid proteins, or to the CPs of any other viral families (Yoon *et al.*, 2011).

Genomoviridae was initially comprised of a single viral species, SsHADV-1, identified infecting the phytopathogenic fungus *Sclerotinia sclerotiorum* (Yu *et al.*, 2010). Due to the genetic similarity between SsHADV-1 and geminiviruses, it was named a "gemycircularvirus" (gemini-like, myco-infecting, circular virus). As additional similar viruses were discovered and characterized, the group was named "genomovirus" (gemini-like, no movement protein).

Until 2017, virus taxonomy was unable to accommodate all SsHADV-1-like viruses identified in recent years. Varsani & Krupovic (2017) defined a species demarcation criterion through Rep-based phylogenetic analyzes of 121 available genomes, since the Rep gene is the only one with high homology even among the most divergent ssDNA viruses (Simmonds *et al.*, 2017). Thus, using the guided phylogeny of Rep together with the taxonomy framework already established for *Geminiviridae*, it was possible to propose nine different genera within *Genomoviridae*. As a demarcation criterion for new species, it was defined that sequences with greater than 78% identity with another genomovirid belong to the same species. A nucleotide similarity of less than 78% identifies a new species.

Currently, according to the ICTV, the *Genomoviridae* family is composed of 73 viral species classified into nine genera. *Gemycircularvirus* is the largest genus with 43 species, and includes SsHADV-1, the first representative of the family. *Gemykibivirus* is the second most diverse genus, with 16 species. The genera

Gemygorvirus, *Gemykolovirus* and *Gemykrogvirus* have, respectively, five, two and three species, while *Gemyduguivirus*, *Gemykroznavirus*, *Gemytondvirus* and *Gemyvongvirus* are composed of one species each (Adams *et al.*, 2017). For naming purposes, all genera use the prefix "gemy" followed by "circular" in different languages to emphasize the circular structure of the genomes.

To date, classification of viral sequences in the family *Genomoviridae* has been made almost exclusively by *in silico* analysis of metagenomic data (Krupovic *et al.*, 2016; Simmonds *et al.*, 2017). Genomovirids have been identified in mammals, birds, human fluids (Zhou *et al.*, 2015; Macera *et al.*, 2019; Siqueira *et al.*, 2019), environmental sediment (Assis *et al.*, 2016), feces (Sikorski *et al.*, 2013; Male *et al.*, 2016; Schmidlin *et al.*, 2019b), sewage (Kraberger *et al.*, 2015a), invertebrates (Rosario *et al.*, 2011; Dayaram *et al.*, 2012; Rosario *et al.*, 2012a; Dayaram *et al.*, 2015; Steel *et al.*, 2016; Tijssen *et al.*, 2016; Kraberger *et al.*, 2018a; Kraberger *et al.*, 2018b; Kraberger *et al.*, 2019a; Kraberger *et al.*, 2019b; Schmidlin *et al.*, 2019a) and in plants (Kraberger *et al.*, 2015b; Lamas *et al.*, 2016; Rezende *et al.*, 2018; Chiumenti *et al.*, 2019; Richet *et al.*, 2019). However, despite the large number of reports and their pervasive presence in the environment, the infectivity of genomovirids to specific hosts remains largely unknown. The only member of the family with known hosts is SsHADV-1, which infects the fungi *Sclerotinia sclerotiorum*, *S. minor* and *S. nivali* (Yu *et al.*, 2010). Although all three hosts are associated with plants, infectivity assays clearly demonstrated that SsHADV-1 is unable to infect and move in plant cells (Yu *et al.*, 2013).

Viruses belonging to the *Genomoviridae* have never been reported as plant pathogens, but have been identified in several plants. Dayaram *et al.* (2012) described a new cassava-associated gemycircularvirus, named *Cassava associated circular DNA virus* (CasCV). Another gemycircularvirus was identified in association with plants of *Hypericum japonicum*, and named *Hypericum japonicum associated circular DNA virus* (HJasCV) (Du *et al.*, 2014). The presence of different gemycircularviruses associated with plants in the *Poacea* has also been reported (Kraberger *et al.*, 2015b; Male *et al.*, 2015). Ng *et al.* (2014) reported that a genomovirid was able to replicate in *Nicotiana benthamiana*, but without symptoms. Application of rolling-circle amplification (RCA) allowed the identification and sequencing of gemycircularviruses in association with olive trees (*Olea europaea*) in Italy (Chiumenti *et al.*, 2019), with a wild grass (*Brachiaria deflexa*) and sugarcane in Tonga (Male *et al.*, 2015), and with common bean (*Phaseolus vulgaris*) in Brazil (Lamas *et al.*, 2016). Rezende *et al.*

(2018) identified two new gemycircularviruses in non-cultivated plants, *Momordica charantia associated gemycircularvirus* (MoaGmV), detected in association with *Momordica charantia* (family Cucurbitaceae) and *Euphorbia heterophylla associated gemycircularvirus* (EuaGmV), detected in association with *Euphorbia heterophylla* (family Euphorbiaceae). The genus *Gemycircularvirus* includes all plant-associated genomovirids found so far. This is an indication that members of this genus may play relevant ecological roles in association with plants.

The objective of this work was to investigate the infectivity to plants of the two newly described gemycircularviruses, MoaGmV and EuaGmV.

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CHAPTER 1

SYSTEMIC INFECTION OF PLANTS BY A GEMYCIRCULARVIRUS (FAMILY *Genomoviridae*)

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Systemic infection of plants by a gemycircularvirus (family *Genomoviridae*)

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Abstract

Genomoviridae includes non-enveloped, ssDNA viruses with circular genomes ranging from 2.0 to 2.4 kb, containing two ORFs that encode a coat protein and a replication-associated protein (Rep) similar to the Rep of members of the family *Geminiviridae*. The family contains 73 species classified into nine genera, with *Gemycircularvirus* as the largest genus. Genomovirids have been identified in mammals, birds, invertebrates, in a variety of environmental samples, and also in plants. However, despite their pervasive presence in the environment, the infectivity of genomovirids to specific hosts remains largely unknown. The gemycircularvirus SsHADV-1 remains the only genomovirid with a known (fungal) host. The genus *Gemycircularvirus* includes all plant-associated genomovirids found so far. It is possible that gemycircularviruses play relevant ecological roles in association with plants. Here, we report the first case of systemic infection of plants by a genomovirid, Euphorbia heterophylla associated gemycircularvirus (EuaGmV). A dimeric clone (ca. 4.4 kb) of EuaGmV was used for the biolistic inoculation of *Euphorbia heterophylla* and *Nicotiana benthamiana* plants. Control plants were inoculated with the geminivirus *Euphorbia yellow mosaic virus* (EuYMV) or with water. At 21 and 28 days after inoculation (dai), non-inoculated upper leaves of the EuaGmV-inoculated plants (which did not show any symptoms) were collected for PCR-based detection of the virus. Eight plants of *E. heterophylla* and all 10 plants of *N. benthamiana* were positive for the presence of EuaGmV. Amplicons (612 bp) obtained from *E. heterophylla* were sequenced, and a 99.6% identity with EuaGmV was obtained. These results demonstrate, for the first time, that a gemycircularvirus (family *Genomoviridae*) is capable of systemically infecting plants.

Introduction

Advances in culture-independent molecular techniques such as rolling-circle amplification (RCA) and viral metagenomics coupled with high throughput sequencing (HTS) are expanding our understanding of viral diversity, leading to the discovery of a variety of new viral families (1-3), including the *Genomoviridae*.

Genomovirids are non-enveloped circular ssDNA viruses with genomes ranging from 2 to 2.4 kb, containing two ORFs separated by an intergenic non-coding region (IR). One of the ORFs, located in the virion-sense strand, encodes the putative coat protein (CP). The second ORF, in the complementary-sense strand (often having an intron of 166 to 226 nucleotides) (4), encodes a putative replication-associated protein (Rep) similar to the Rep protein found in members of the family *Geminiviridae* (5-9). Genomovirids have a conserved stem-loop structure for initiating replication through the Rep protein which is analogous to those found in members of the families *Geminiviridae*, *Circoviridae* and *Nanoviridae* (4, 10). The structure includes a conserved nonameric sequence (5'-TAWWDHWRN-3') which is recognized by the Rep protein during the initiation of DNA replication.

Genomoviridae was initially comprised of a single viral species, *Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1* (SsHADV-1), identified in the phytopathogenic fungus *Sclerotinia sclerotiorum* (11). Due to the genetic similarity between SsHADV-1 and geminiviruses, the virus was called a "gemycircularvirus" (gemini-like, myco-infecting, circular virus). As additional similar viruses were discovered and characterized, the group was named "genomovirus" (gemini-like, no movement protein). Currently, the *Genomoviridae* family includes 73 species classified into nine genera (12).

Classification of viral sequences in the family *Genomoviridae* has been made almost exclusively by *in silico* analysis of metagenomic data (13, 14). Genomovirids have been identified in mammals, birds, human fluids (15-17), environmental sediment (18), feces (19-21), sewage (4), invertebrates (22-31) and also in plants (32-36).

Despite the large number of reports and their pervasive presence in the environment, the infectivity of genomovirids to specific hosts remains largely unknown. The only member of the family with known hosts is SsHADV-1, which infects the fungi *Sclerotinia sclerotiorum*, *S. minor* and *S. nivali* (11). Although all three hosts are associated with plants, infectivity assays clearly demonstrated that SsHADV-1 is unable to infect and move in plant cells (37).

Viruses belonging to the *Genomoviridae* have never been reported as plant pathogens, but have often been identified in association with plants. Interestingly, the genus *Gemycircularvirus* includes all plant-associated genomovirids found so far. This is an indication that members of this genus may play relevant ecological roles in association with plants.

The objective of this work was to investigate the infectivity to plants of the two recently described gemycircularviruses, *Euphorbia heterophylla associated gemycircularvirus* (EuaGmV), detected in association with *Euphorbia heterophylla* (Euphorbiaceae), and *Momordica charantia associated gemycircularvirus* (MoaGmV), detected in association with *Momordica charantia* (Cucurbitaceae) (35).

Material and Methods

Viral isolates and plant material

Viral clones containing a complete genomic copy of *Euphorbia heterophylla associated gemycircularvirus* (EuaGmV; GenBank accession number MH047858) and *Momordica charantia associated gemycircularvirus* (MoaGmV; MH047857) (35) were used for the experiments. All experiments were conducted under greenhouse conditions. Seeds of the species *Euphorbia heterophylla*, *Momordica charantia* and *Nicotiana benthamiana*, available at UFV, were used to obtain plants for the infectivity assays.

Dimeric (infectious) clones of EuaGMV and MoaGmV were constructed as described by Ferreira et al. (38). The complete genomes (~2,200 nt) were released from the plasmid clones by digestion with *Apa*I (EuaGmV) and *Hin*dIII (MoaGmV). The genomes were recircularized with T4 DNA Ligase (Promega), followed by rolling-circle amplification (RCA) using the phi29 DNA polymerase (New England BioLabs) according to the manufacturer's instructions. EuaGmV and MoaGmV RCA products were partially digested with *Apa*I and *Hin*dIII, respectively, and fragments corresponding to dimeric genomes (4,400 nt) were cloned into the pBluescript KS+ plasmid vector (Stratagene). All dimeric clones obtained were confirmed by Sanger sequencing (Macrogen, South Korea).

Infectivity assays

Infectivity assays were performed using biolistics (39). The DNA of EuaGmV and MoaGmV dimeric clones was used as inoculum source. Positive control plants

were inoculated with the begomoviruses *Euphorbia yellow mosaic virus* (EuYMV) isolate BR:Cha510:10, known to infect *E. heterophylla* and *N. benthamiana* (40), and *Tomato yellow spot virus* (ToYSV) isolate BR:Bic2:99, known to infect *N. benthamiana* (41).

Plasmid DNA from all clones was isolated using the PureYield Plasmid Miniprep System (Promega) as recommended by the manufacturer. The integrity of the DNA was evaluated on 1% (w/v) agarose gels. Approximately 2 µg of DNA from each dimeric clone were precipitated in tungsten microparticles (M-10, Bio-Rad) in the presence of 1 M $CaCl_2$, 13 mM spermidine and 70% (v/v) ethanol and deposited on carrier membranes. The membranes were placed on the particle accelerator and delivered at 40 kgf/mmHg in each plant. As a negative control, healthy plants of each species were inoculated with tungsten particles without DNA.

A total of 8 *Euphorbia heterophylla* and 10 *Nicotiana benthamiana* plants were inoculated with EuaGmV, and 20 *Momordica charantia* and 20 *Nicotiana benthamiana* plants (10 plants of each species in two independent experiment) were inoculated with MoaGmV. One day after the experiment, the plants were transplanted to pots and transferred to the greenhouse.

Molecular diagnosis

To confirm the presence of the gemycircularviruses in inoculated plants, young upper leaves of all inoculated plants were collected at 14, 21 and 28 days after inoculation (dpi). Total DNA was extracted according Doyle and Doyle (42). Approximately 100 mg of leaf tissue were used for extraction of total DNA, which was used as a template in PCR reactions using specific oligonucleotides. EuaGmV-F (5'-TCC CTC GAG AAC TTT CAA TG-3'; nt 1,853 to 1,872) and EuaGmV-R (5'-TAC TGT CGA TCT CCG GTA AG-3'; nt 340 to 321) oligonucleotides directed the amplification of a 612 bp fragment of the EuaGmV genome. MoaGmV-F (5'-GCA TAG TGT GCT CTC TGA AG-3'; nt 2,158 to 2,177) and MoaGmV-R (5'-GTT GCC TGA GGA AAT AAT GC-3'; nt 783 to 764) oligonucleotides directed the amplification of a 0,8 kb fragment of the MoaGmV genome. Amplification reactions were conducted on a Bio-Rad C1000 Touch thermal cycler. All amplified PCR products were confirmed by Sanger sequencing (Macrogen).

Results

Construction of dimeric clones

Three dimeric clones were obtained for EuaGmV (EuaGmV18, EuaGmV19 and EuaGmV24) and one for MoaGmV (MoaGmV04). Restriction analysis with *Apa*I, *Bam*HI, *EcoR*V *and Hin*dIII showed the expected *in silico* pattern, confirmed by electrophoresis (Figure 1; Figure 2). Sequencing of clones EuaGmV18 and MoaGmV04 indicated identity with the original EuaGmV and MoaGmV isolates, respectively.

Infectivity assays

Twenty plants of *Momordica charantia* and 20 of *N. benthamiana* were inoculated with the MoaGmV04 dimeric clone (Figure 3), but none of the plants were PCR-positive for MoaGmV detection (Figure 4). DNA barcoding confirmed *N. benthamiana* and *M. charantia* DNA quality (data not shown). On the other hand, hosts for EuaGmV were successfully identified. All eight plants of *E. heterophylla* and ten of *N. benthamiana* were systemically infected with dimeric EuaGmV18 clone, even though they were asymptomatic (Figure 5). PCR-based detection using specific oligonucleotides confirmed the presence of EuaGmV in upper, non-inoculated leaves of all plants (Figure 6). The plants were maintained in the greenhouse for an additional two months to verify the possibility of symptoms appearing later in the infection, but remained asymptomatic during this period. Negative control plants bombarded with tungsten particles without EuaGmV or MoaGmV DNA showed no symptoms and were PCR-negative for both viruses.

The amplicon (612 bp) obtained with primer pair EuaGmV-R/EuaGmV-F from one infected *E. heterophylla* plant was directly sequenced, and sequence comparisons confirmed a 99.6% nucleotide sequence identity with the corresponding sequence of EuaGmV (MH047858). Together these results indicate that the EuaGmV dimeric clone is fully infectious and biologically active in both *E. heterophylla* and *N. benthamiana*, even though no symptoms were observed in either host.

Discussion

The first report of a gemycircularvirus dates back to 2010, when Yu et al. (11) described a virus able to induce hypovirulence in the fungus *Sclerotinia sclerotiorum*

(*Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1*, SsHADV1). Since then, related viruses (now classified in the family *Genomoviridae*) have been isolated from a variety of sources, but no other study has identified hosts of a gemycircularvirus, or in fact of any member of the family, with one exception. A genomovirid (now classified in the genus *Gemykrogvirus*) isolated from 700-years old frozen Caribou feces (*Rangifer tarandus caribou*) was able to cause a non-symptomatic infection in *N. benthamiana* (43). Demonstrating that a gemycircularvirus infecting plants systemically is a significant finding, considering the pervasiveness of these viruses in the environment.

Despite the absence of evidence, it has been generally assumed that gemycircularviruses are either fungal or plant viruses. Chiumenti et al. (32) characterized a gemycircularvirus found in olive trees, and tested the hypothesis that it could be a fungal virus by amplifying PCR fragments from total DNA extracted from the trees with "panfungal" primers. Sequence analysis of amplicons indicated similarity with fungi belonging to a range of genera, including *Alternaria*, *Arthrocaldium, Dactylonectria, Hormonema, Isaria, Mycosphaerella*, *Pleospora* and *Toxicocladosporium*. However, due to the wide diversity of fungal genera, the authors were unable to suggest a potential host for the virus.

In a previous work, our laboratories identified two gemycircularviruses in non-cultivated plants, *Euphorbia heterophylla* and *Momordica charantia* (35). Both plants had virus-like symptoms, and although infection by the geminivirus *Euphorbia yellow mosaic virus* (EuYMV) was confirmed in the *E. heterophylla* sample, no DNA virus was detected in the *M. charantia* sample. Here, we demonstrate that the EuaGmV isolate is capable of systemically infecting *Euphorbia heterophylla* and *Nicotiana benthamiana* plants efficiently, strongly indicating that *E. heterophylla* is a natural host of EuaGmV. All infected plants remained asymptomatic for three months after inoculation. Non-symptomatic infections have been observed previously in *N. benthamiana* when inoculated with geminiviruses for which it is an experimental host (44). The symptomless phenotype in inoculated *E. heterophylla* plants may reflect the simplicity of the EuaGmV genome, with only two genes encoding a capsid protein and a replication-associated protein. Symptoms of viruses in plants are often associated with viral proteins acting in the suppression of host defenses and viral movement (45-47), both of which seem to be lacking in genomovirids. The fact that the original sample from which EuaGmV was isolate was symptomatic could be due to co-infection with

EuYMV, since the symptoms were identical to those normally induced by this virus (40, 48).

The cucurbit weed *M. charantia* seems not to be the natural host of MoaGmV. Several studies have demonstrated *Momordica charantia* as a reservoir host of begomoviruses (49-51) and potyviruses (52-54). Our attempts to detect begomoviruses and potyviruses in the *M. charantia* sample yielded negative results (data not shown). MoaGmV sequence comparisons with other gemycircularviruses indicated a maximum nucleotide identity for the complete genome of 74% with Plant associated genomovirus 16 (GenBank access number MH939396), 73% with Plant associated genomovirus 17 (GenBank access number MH939397) and 73% with Hypericum associated gemycircularvirus 1 (55), all of them isolated from plant extracts.

Huang et al. (56) reported 25 genera of fungi isolated from *Momordica charantia* in China, including endophytes belonging to potentially pathogenic species such as *Alternaria*, *Colletotrichum*, *Fusarium* and *Rhizoctonia*. *Alternaria alternata* was one of the most frequently isolated fungal species. It is reasonable to assume that MoaGmV could be associate to a fungal host associated with *M. charantia* plants. It is also possible that MoaGmV, and other genomovirids as well, are capable of infecting both plants and fungi. New evidence has shown that cross-kingdom virus transmission can occur experimentally and appears to occur in nature (57).

Plant viruses are frequently reported as disease-causing infectious agents that can cause a negative impact on their hosts and may sometimes be transmitted between crop and non-crop vegetation. In this context, viruses infecting non-cultivated hosts have the potential to start new diseases in cultivated plants (58). Bernardo et al. (59) demonstrated that the prevalence of viruses isolate from non-cultivated hosts can be high even in cultivated areas, since non-cultivated plants are frequently found in crop areas as weeds.

Besides their relevant economic importance as weeds, non-cultivated plants play an important role in ecosystems by acting/serving as viral reservoirs and inoculum sources (58, 60, 61). *E. heterophylla* plants are commonly found in crop areas (62, 63), and the begomovirus EuYMV is frequently found infecting *E. heterophylla* plants (40, 48). The detection of EuaGmV in EuYMV-infected *E. heterophylla* plants and the similarities between gemycircularviruses and begomoviruses in terms of genome architecture warrant further studies regarding a possible relation between EuaGmV and EuYMV. Nevertheless, despite what we report in this work, the nature of the interaction between gemycircularviruses and plants remains poorly understood and it

is not possible to affirm that these viruses are plant pathogens. It can be speculated that the pervasive presence and diversity of gemycircularvirus points to a relevant role, but this remains undetermined.

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Figure Legends

Figure 1. A. Restriction analysis of the *Euphorbia heterophylla associated gemycircularvirus* (EuaGmV) dimeric clone EuaGmV18 with enzymes *Apa*I, *Bam*HI, *Eco*RV *and Hin*dIII. **B.** *In silico*-predicted *Apa*I, *Bam*HI, *Eco*RV *and Hin*dIII restriction patterns of the EuaGmV18 dimeric clone. **M.** Size marker (1Kb Plus DNA Ladder, Invitrogen).

Figure 2. A. Restriction analysis of the *Momordica charantia associated gemycircularvirus* (MoaGmV) dimeric clone MoaGmV04 with enzymes *Apa*I, *Bam*HI *and Hin*dIII. **B.** *In silico*-predicted *Apa*I, *Bam*HI *and Hin*dIII restriction pattern of the MoaGmV04 dimeric clone. **M.** Size marker (1Kb Plus DNA Ladder, Invitrogen).

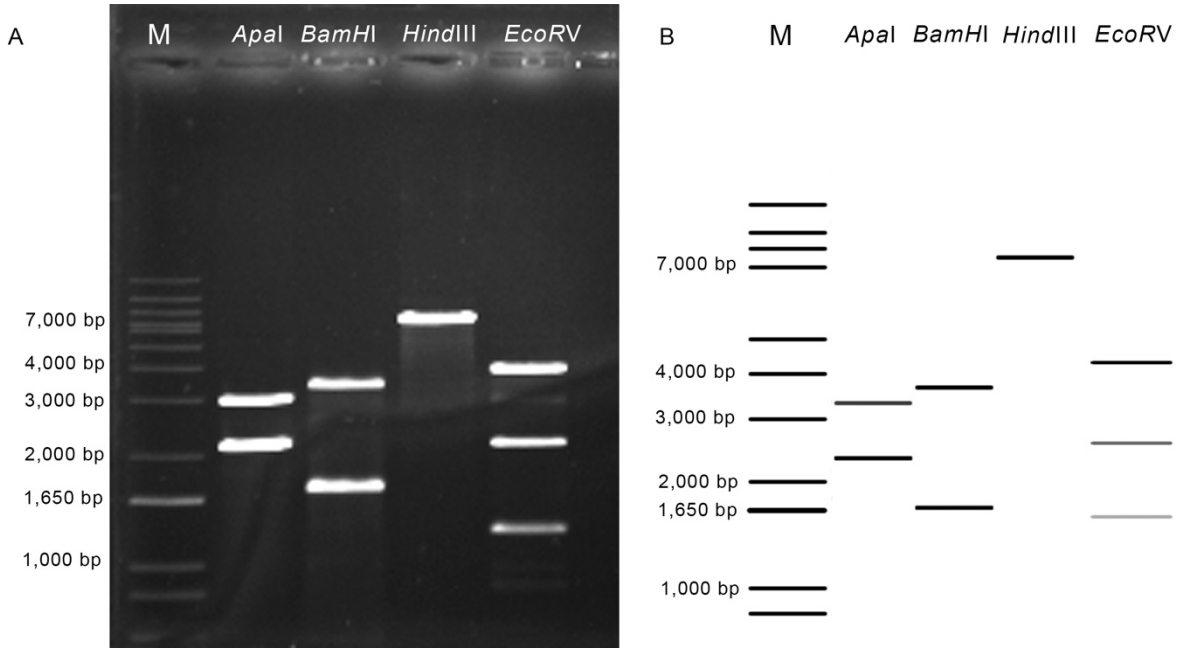
Figure 3. Plants of *Momordica charantia* and *Nicotiana benthamiana* at 28 days after biolistic inoculation with the *Momordica charantia associated gemycircularvirus* (MoaGmV) dimeric clone. **Mock**, plants inoculated with tungsten particles without DNA (as a negative control). **ToYSV**, plants inoculated with an infectious clone of the geminivirus *Tomato yellow spot virus* (as a positive control).

Figure 4. PCR-based detection of *Momordica charantia associated gemycircularvirus* (MoaGmV) in inoculated plants. **M**, size marker (1Kb Plus DNA Ladder, Invitrogen). **(-)**, no-DNA PCR control. **1-10**, *Momordica charantia* plants inoculated with the MoaGmV dimeric clone. **11-15**, *Nicotiana benthamiana* plants inoculated with the MoaGmV dimeric clone; **McH_2O, NbH_2O**, *M. charantia* and *N. benthamiana* plants inoculated with tungsten particles without DNA (as negative controls), respectively; **(+)**, PCR amplification from the MoaGmV dimeric clone (as a PCR positive control). The 800 bp band expected if samples were PCR-positive is indicated.

Figure 5. Plants of *Euphorbia heterophylla* and *Nicotiana benthamiana* at 28 days after biolistic inoculation with the *Euphorbia heterophylla associated gemycircularvirus* (EuaGmV) dimeric clone. **Mock**, plants inoculated with tungsten particles without DNA (as a negative control). **EuYMV**, plants inoculated with an infectious clone of the geminivirus *Euphorbia yellow mosaic virus* (as a positive control).

Figure 6. PCR-based detection of *Euphorbia heterophylla associated gemycircularvirus* (EuaGmV) in inoculated plants. **M**, size marker (1Kb Plus DNA Ladder, Invitrogen). **1**, *Euphorbia heterophylla* plant inoculated with EuaGmV. **2**, *Nicotiana benthamiana* plant inoculated with EuaGmV. **(-)**, no-DNA PCR control. **(+)**, PCR amplification from the EuaGmV dimeric clone (as a PCR positive control). The 612 bp band corresponding to the EuaGmV fragment is indicated.

Figure 1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B
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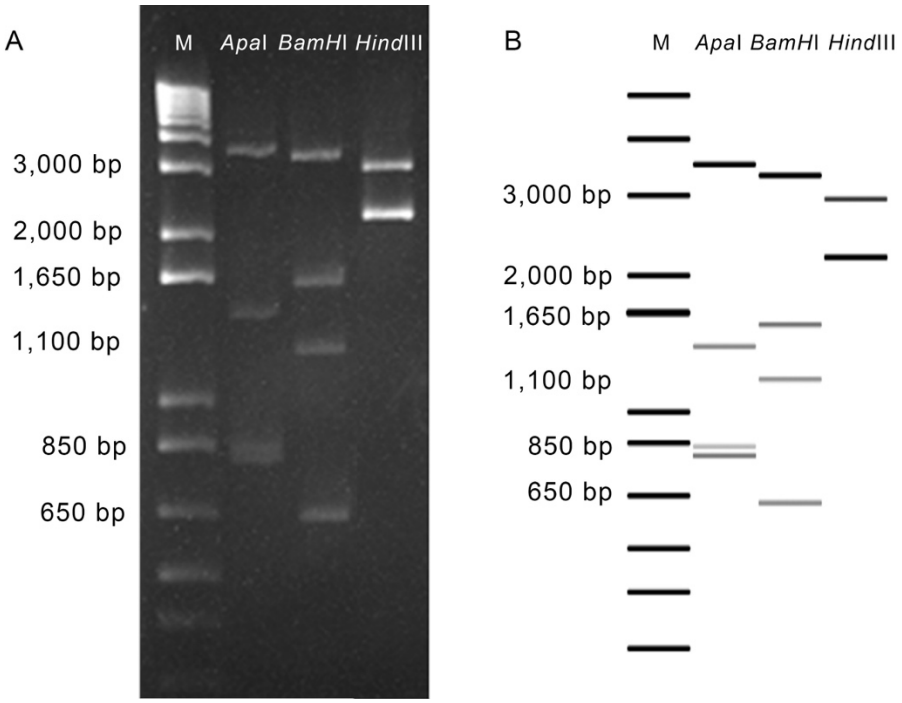
Figure 2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850 bp
650 bp

Figure 3

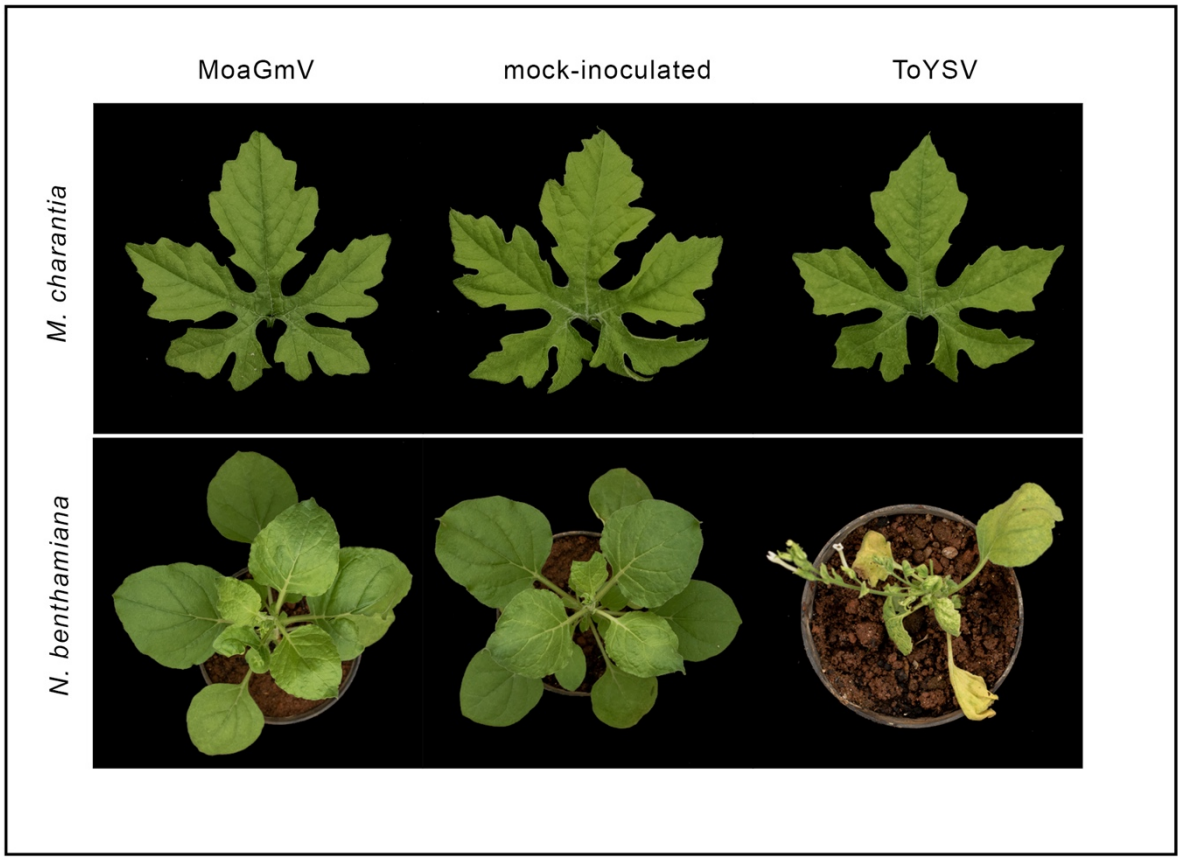


Figure 4


M. charantia + MoaGmV
N. benthamiana + MoaGmV
McH2O NbH2O
M
—
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
+
3,000 bp
800 bp
500 bp

Figure 5

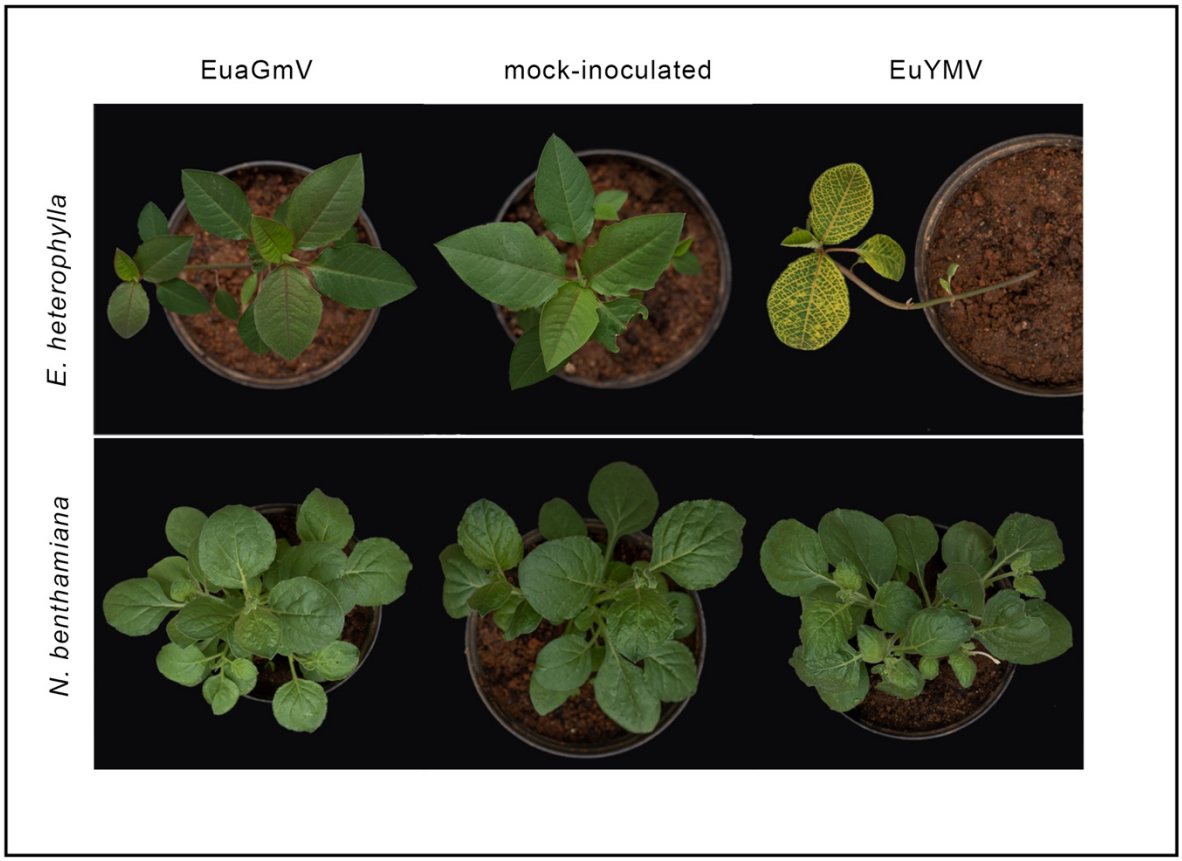
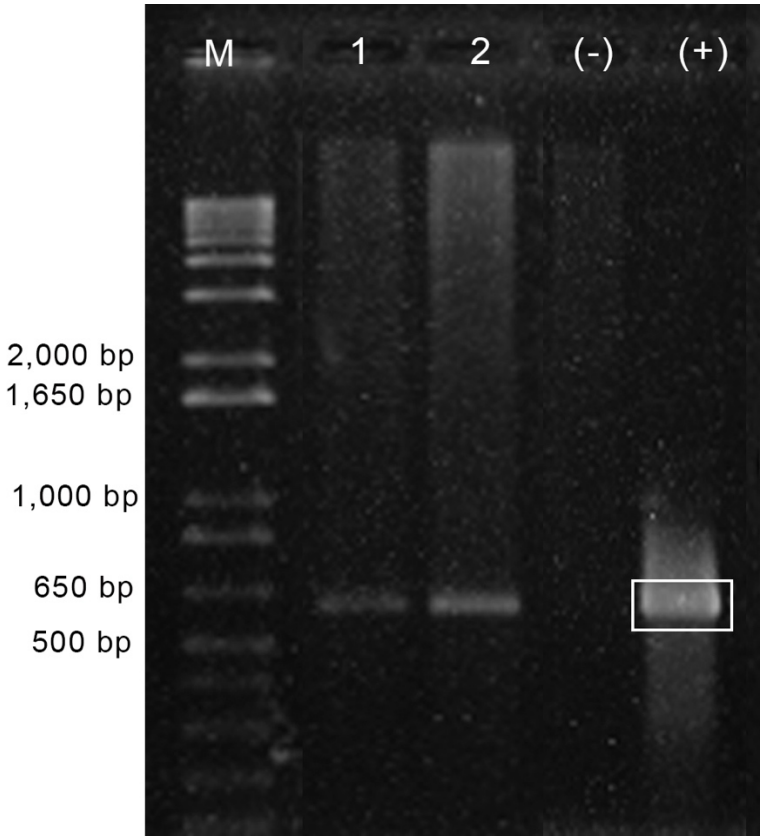

EuaGmV
mock-inoculated
EuYMV
E. heterophylla
N. benthamiana

Figure 6



GENERAL CONCLUSIONS

1. The gemycircularvirus *Euphorbia heterophylla associated gemycircularvirus* (EuaGmV; fam. *Genomoviridae*) infects plants systemically, and its host range includes *Euphorbia heterophylla*, the plant from where it was originally isolated, and *Nicotiana benthamiana*.
2. The dimeric clone MoaGmV04, of the gemycircularvirus *Momordica charantia associated gemycircularvirus* (MoaGmV; fam. *Genomoviridae*), was not able to infect *Momordica charantia* or *Nicotiana benthamiana* plants.