Journal of Virological Methods 263 (2019) 20–23

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

New approach for the construction of infectious clones of a circular DNA plant virus using Gibson Assembly

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ARTICLE INFO

Keywords:
Geminiviridae
Seamless cloning
Gibson Assembly
Begomovirus
Infectious clones

ABSTRACT

Viruses belonging to the genus *Begomovirus* (family *Geminiviridae*) have circular single-strand DNA genomes encapsidated into quasi-icosahedral particles, and are transmitted by whiteflies of the *Bemisia tabaci* complex. Biological and molecular properties of begomoviruses have been studied efficiently with infectious clones containing dimeric genomic components. However, current approaches employing enzymatic digestion and ligation to binary vectors are laborious, mostly due to many cloning steps or partial digestion by restriction enzyme. Here, an infectious clone of the bipartite begomovirus *Bean golden mosaic virus* (BGMV) was obtained using PCR and Gibson Assembly (GA). Common bean (*Phaseolus vulgaris*) seedlings displayed severe yellow mosaic and stunt symptoms 15 days after agroinoculation with DNA-A and DNA-B of BGMV. The approach based on PCR-GA protocol is a fast and useful tool to obtain infectious clones of a circular DNA plant virus.

Viruses belonging to the genus *Begomovirus* (family *Geminiviridae*) infect cultivated and wild plants in tropical and subtropical regions, causing serious economic losses (Navas-Castillo et al., 2011; Rojas et al., 2018). Bipartite begomoviruses have two genomic components known as DNA-A and DNA-B, while the genomes of monopartite begomoviruses consist of only one component, resembling the DNA-A. Both mono and bipartite begomoviruses are transmitted by whiteflies of the cryptic species group *Bemisia tabaci* (Homoptera: Aleyrodidae) (Zerbini et al., 2017). *Bean golden mosaic virus* (BGMV) is one of the main begomoviruses infecting leguminous plants, being considered the most important constraints to crops of the genus *Phaseolus* in Brazil (Rojas et al., 2018).

The conventional approach to construct infectious clones of begomoviruses employs restriction enzymes to generate more than one copy of the viral genomic component including two replication origins (v-ori) (Buragohain et al., 1994; Bang et al., 2014; Nagata and Inoue-Nagata, 2015). The duplicated v-ori allows the release of the full-length viral genome, increasing the infectivity of the genomic components via agroinoculation (Stenger et al., 1991; Nagata and Inoue-Nagata, 2015). A method based on rolling circle amplification (RCA) of viral genomic components followed by partial digestion with endonucleases has been described and largely used to obtain infectious clones of begomoviruses (Ferreira et al., 2008; Wu et al., 2008; Bang et al., 2014; Nagata and Inoue-Nagata, 2015). However, the partial enzymatic digestion requires a potentially laborious process of adjustments in conditions of the target DNA and enzyme concentration. Here, a new and simple approach to obtain infectious clones of begomoviruses employing PCR-Gibson Assembly (GA) is described.

For this study, DNA-A and DNA-B genomic components of the begomovirus BGMV isolate 173 A L (GenBank accession KJ939749 for DNA-A, MH925107 for DNA-B; Ramos-Sobrinho et al., 2014), previously cloned into pBluescript KS + plasmid vectors were used as PCR templates.

For the assembly of geminivirus infectious clones, dimeric constructs containing two replication origins are necessary for each genomic component. For this purpose, primers were designated to amplify two slightly different units (1 and 2) with a 20–22 nucleotides (nt) overlap region between units 1 and 2, and 20 nt overlap between genome segments and plasmid (Fig. 1, Table 1). Primer pairs BGMV-A-U1-For/BGMV-A-U1-Rev or BGMV-A-U2-For/BGMV-A-U2-Rev were used to amplify units 1 and 2 of the BGMV DNA-A (Fig. 1a), respectively, while BGMV-B-U1-For/BGMV-B-U1-Rev or BGMV-B-U2-For/BGMV-B-U2-Rev were used for BGMV DNA-B (Fig. 1b). Primer pair pJL89-Nos-For/pJL89-no35S-Rev was used to amplify the pJL-89

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https://doi.org/10.1016/j.jviromet.2018.10.017
Received 1 August 2018; Received in revised form 20 September 2018; Accepted 22 October 2018
Available online 23 October 2018
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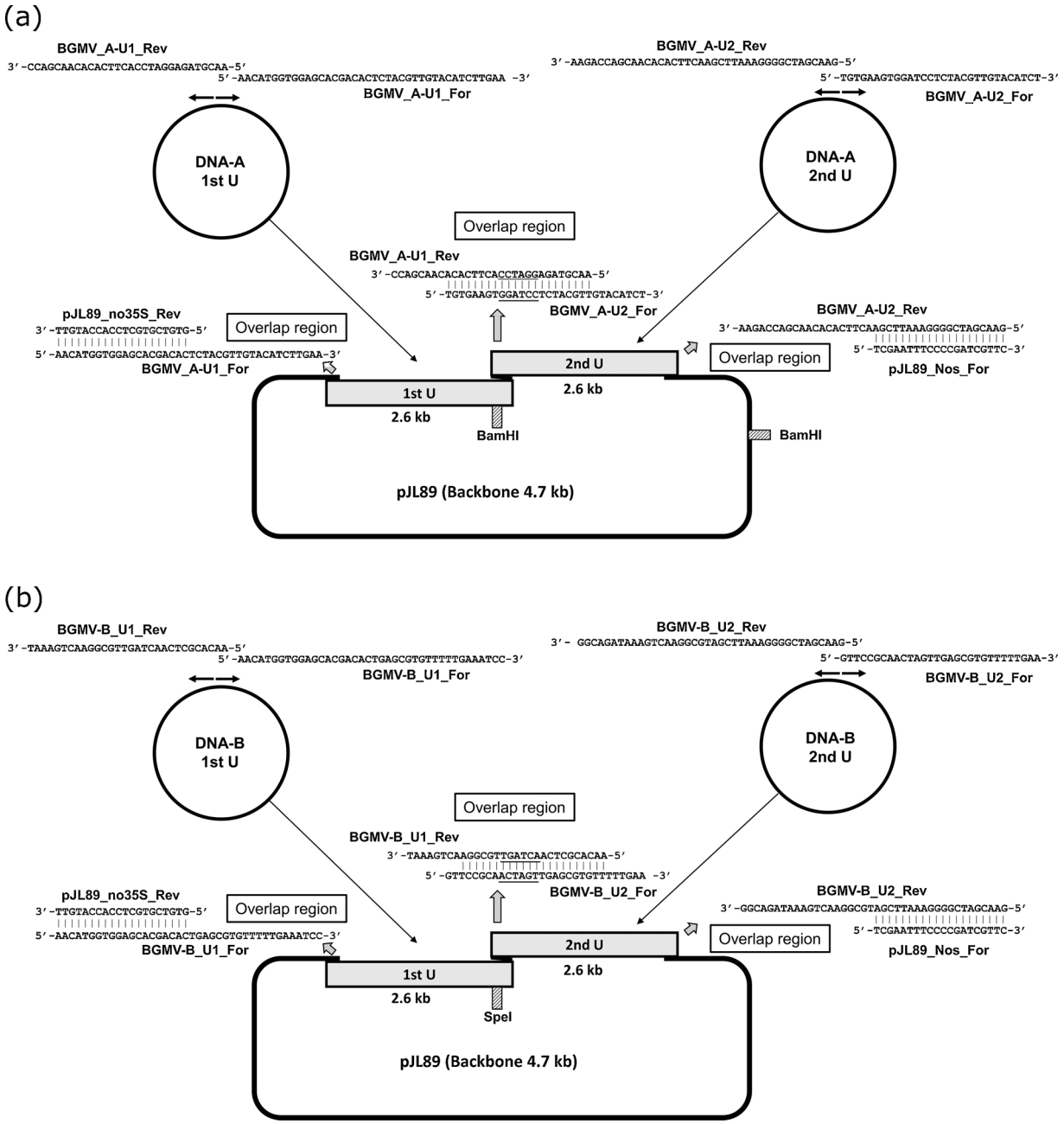

Fig. 1. Strategy to construct dimeric agroinfectious clone of BGMV into pJL-89 binary vector. (a) DNA-A genomic component; viral inserts (first and second units) are represented by grey rectangles; primer-pairs containing overlap sequences are indicated by grey arrows. (b) DNA-B genomic component; viral inserts (first and second units) are represented by grey rectangles; primer-pairs containing overlap sequences are indicated by grey arrows. *Bam*HI (DNA-A) and *Spe*I (DNA-B) restriction sites are represented by dotted rectangles.

binary vector (Lindbo, 2007) excluding the 35S region of the vector. DNA-A and DNA-B components were individually amplified using the Q5 High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, USA). Amplified fragments were analyzed by electrophoresis using agarose gels (1%), individually purified using the Gel Band Purification Kit (GE Healthcare, Uppsala, Sweden), and treated with *Dpn*I restriction enzyme for degradation of methylated plasmid DNA used as template. Dimers of DNA-A and DNA-B were assembled using Gibson Assembly Cloning Master Mix (New England BioLabs) following the manufacturer´s protocol. Briefly, 2.5 µL of pJL-89 amplified product (40 ng/µL), 2.2 µL of units 1 and 2 (DNA-A or DNA-B; 50 ng/µL), 1.0 µL of 10X CutSmart buffer, 1.1 µL of ultrapure H_2O, and 1.0 µL of *Dpn*I were mixed and incubated at 37 °C for 1 h, and then 10.0 µL of GA master mix was added and incubated at 50 °C during 1 h (an illustration of the PCR-GA approach is presented in Supplementary Fig. S1). Finally, GA products were dialyzed for 15 min using VSWP 0.025 µm cellulose membrane (Merck-Millipore, Darmstadt, Germany), and used to transform electrocompetent cells of *Escherichia coli* DH10B strain.

Colonies of transformed *E. coli* DH10B were grown on Luria-Bertani (LB) medium at 37 °C for 18 h, and plasmid DNA was extract using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, USA). In order to confirm the cloning process, assembled plasmid DNA was individually digested with *Bam*HI (DNA-A) or *Spe*I (DNA-B) (Fig. 1). For the DNA-A, fragments of about 3.0 and 6.9 kb, corresponding to the second copy of the viral genome (unit 2) plus part of the binary vector (~0.4 kb) and the first copy of the viral insert (unit 1) plus vector (~4.3 kb) were obtained. For the DNA-B, fragments of 9.9 kb corresponding to the linearized plasmid containing two copies

Table 1
Primer sequences used for amplification of BGMV DNA-A and DNA-B genomic components and pJL-89 binary vector.

Name	Sequence (5'–3')	Target
BGMV-A-U1-For	AACATGGTGGAGCACGACACTCTACGTTGTACATCTTGAA	DNA-A
BGMV-A-U1-Rev	AACGTAGAGGATCCACTTCACACAACGACC	
BGMV-A-U2-For	TGTGAAGTGGATCCTCTACGTTGTACATCT	DNA-A
BGMV-A-U2-Rev	GAACGATCGGGGAAATTCGAACTTCACACAACGACCAGAA	
BGMV-B-U1-For	AACATGGTGGAGCACGACACTGAGCGTGTTTTTGAAATCC	DNA-B
BGMV-B-U1-Rev	AACACGCTCAACTAGTTGCGGAACTGAAAT	
BGMV-B-U2-For	GTTCCGCAACTAGTTGAGCGTGTTTTTGAA	DNA-B
BGMV-B-U2-Rev	GAACGATCGGGGAAATTCGATGCGGAACTGAAATAGACGG	
pJL89-Nos-For	TCGAATTTCCCCGATCGTTC	pJL-89
pJL89-no35S-Rev	GTGTCGTGCTCCACCATGTT	

(units 1 and 2) of the genomic component plus the pJL-89 were obtained. Restriction enzyme sites used for the first cloning are shown in Fig. 1. Viral inserts were sequenced commercially (Macrogen Inc., Seoul, South Korea) and nucleotide sequences were analyzed using the Geneious program (Kearse et al., 2012).

Constructs of the DNA-A and DNA-B clones of BGMV (confirmed by enzymatic digestion and sequencing) were used to transform *Agrobacterium tumefaciens* GV3101:pMP90 strain (background C58; pTiC58DT-DNA), and infectivity was tested by agroinfiltration according to the protocol described in Hou et al. (1998). For agroinoculation of both viral components, DNA-A and DNA-B *A. tumefaciens* GV3101 cultures were adjusted to an OD of 0.5 and mixed in equal volumes. Then, 0.5 mL of these mixed cultures were used to agroinoculate 10 day-old seedlings of common bean (*Phaseolus vulgaris* L.) cv. 'Pérola' at four sites approximately 1 cm below the shoot apex. Control plants were agroinoculated with *A. tumefaciens* GV3101 containing empty pJL-89 vector. Inoculated plants were kept into whitefly-proof cages at 25 ± 2 °C and a photoperiod of 12 h during 30 days post agroinfiltration (dpa). Symptoms of severe yellow mosaic and stunting were observed in inoculated plants at 15 dpa (Fig. 2a). No symptoms were observed in the control plants (Fig. 2b).

In order to confirm viral infection by PCR, systemically infected leaf samples were collected at 15 and 30 dpa. Total DNA was extracted from each sample according to Doyle and Doyle (1987), and used as template for BGMV detection using the primer pairs BGMV-A-U2-For/BGMV-A-U2-Rev or BGMV-B-U1-For/BGMV-B-U1-Rev, specific to the DNA-A and DNA-B, respectively (Table 1). Expected fragments of about 2.6 kb (DNA-A or DNA-B) were observed only from symptomatic plants from both sampling times (Fig. 2c and d).

Although the construction of infectious clones of geminiviruses is a laborious process, this procedure has been successfully used, reducing hard-to-control steps such as partial digestion of genomic component by endonucleases. The single-step GA is a method used to easy assemble DNA molecules in an isothermal reaction (Gibson et al., 2009). Here, it has been shown that this approach can be used to construct infectious clones of circular DNA plant viruses such as begomoviruses.

Declarations of interest

None.

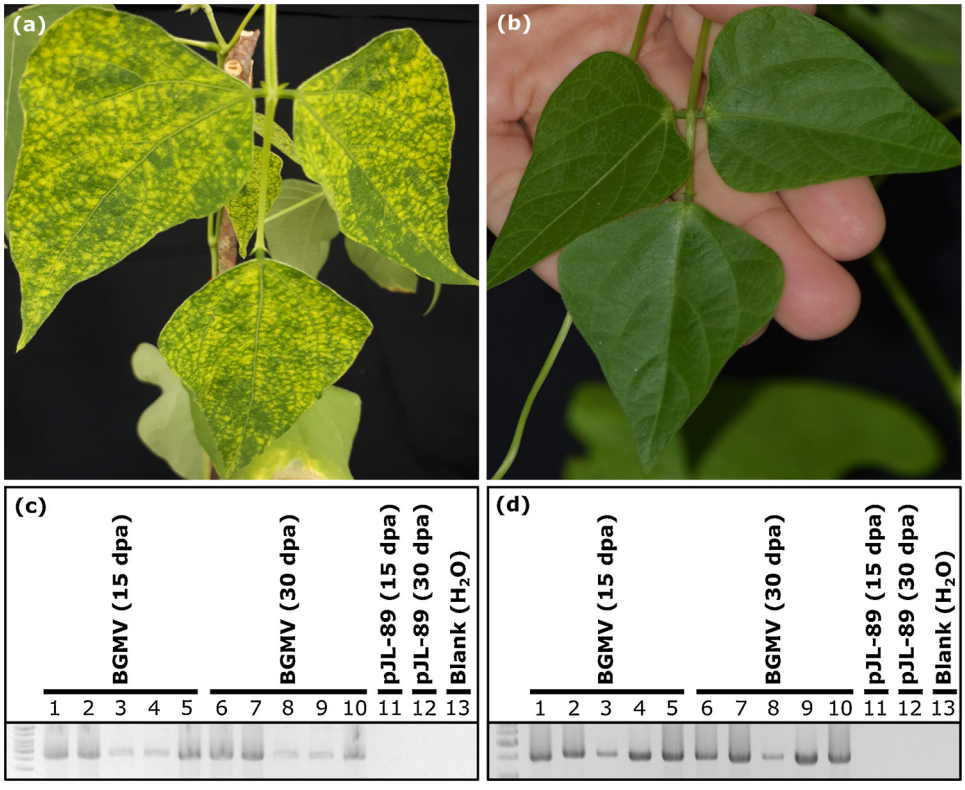

Fig. 2. Agroinoculation tests using infectious clone of BGMV. (a) common bean cv. 'Pérola' displaying yellow mosaic symptoms; (b) symptomless control plant; (c) BGMV DNA-A detection from symptomatic plants 15 dpa (lanes 1–5) and 30 dpa (lanes 6–10); no amplification fragment was observed from control plants (lanes 11 and 12); (d) BGMV DNA-B detection from symptomatic plants 15 dpa (lanes 1–5) and 30 dpa (lanes 6–10); no amplification fragment was observed from control plants (lanes 11 and 12) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Acknowledgments

The authors wish to thank Dr. Alice Inoue-Nagata (Embrapa) for the support during plant inoculation assays. M.M.M.F. was a CAPES doctoral fellowship. G.S.A.L. was supported by CNPq grants (431109/2016-8). T.N. is research fellow of CNPq (CNPq 306377/2014-4). I.P.A. was supported by CAPES/FAPEAL grants (60030000022/2014).

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.jviromet.2018.10.017.

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