Acta virologica **62**: 202 – 207, 2018 doi:10.4149/av_2018_213

Two agroinfection-compatible fluorescent protein-tagged infectious cDNA clones of papaya leaf distortion mosaic virus facilitate the tracking of virus infection

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Received July 21, 2017; accepted August 22, 2017

Summary. – Papaya leaf distortion mosaic virus (PLDMV, the genus *Potyvirus)* is an emerging threat to papaya production. Here, agroinfection-compatible fluorescent protein-tagged PLDMV infectious cDNA clones driven by the *Cauliflower mosaic virus* 35S promoter were successfully constructed using one-step Gibson assembly. The clones were directly transformed into *Agrobacterium tumefaciens* to prevent potential problems such as plasmid instability during propagation in *Escherichia coli*. Ninety-five percent of papaya seedlings infected with PLDMV-GFP or PLDMV-mCherry developed systemic symptoms typical of those caused by wild-type PLDMV. Green and mCherry red fluorescence was observed in leaves, stems, and roots of infected papaya plants. The fluorescent protein-tagged agroinfectious PLDMV cDNA clones were stable in papaya for more than 90 days and during six serial passages at 30-day intervals. The availability of these infectious clones will contribute to research on PLDMV-host interactions and can be applied in the papaya breeding program for PLDMV resistance.

Keywords: PLDMV; fluorescent protein-tagged; agroinfectious clones

The construction of full-length infectious cDNA clones of plant viruses is a key step in developing a reverse genetic system for RNA viruses. Plant virus infectious clones tagged with fluorescent proteins and non-fluorescent genes have been used as highly sensitive probes to monitor the replication and the cell-to-cell and systemic movement of viruses in plants (Pasin *et al*., 2014; Tilsner *et al*., 2010). Potyviruses are one of the largest and most economically important groups of plant viruses. Foreign genes have been added to the potyviral genome via a pentapeptide insertion at the N-terminus of P1 (Rajamäki *et al.,* 2005) and two proteolytic sites between P1 and HC-Pro (Cui and Wang, 2016) or between the *NIb* and *CP* genes (Kelloniemi *et al*., 2006; Bedoya *et al*., 2012; Lee *et al*., 2011), and these additions have not affected viral infectivity. To date, the complex process of viral infection and plant-virus-vector interactions have been studied using more than 13 *in vitro*- or *in vivo*-transcribed, fluorescently tagged potyviruses including potato virus A (Rajamäki *et al*., 2005, Kelloniemi *et al*., 2006), tobacco etch virus (Majer *et al*., 2013), potato virus Y (Matevz *et al*., 2015), plum pox virus (Lansac et al., 2005; Cui and Wang 2016), tobacco vein banding mosaic virus (Gao *et al*., 2012), clover yellow vein virus (Masuta *et al*., 2000), pepper mottle virus (Lee *et al*., 2011), tobacco vein mottling virus (Dietrich and Maiss , 2003), lettuce mosaic virus (German-Retana *et al*., 2003; Bordat *et al*., 2015), turnip mosaic virus (Beauchemin et al., 2005), soybean mosaic virus (Seo *et al*., 2009), zucchini yellow mosaic virus (Kang *et al*., 2016) and papaya ringspot virus (Tuo *et al*., 2017). Recently, the *Antirrhinum majus* MYB-type Rosea1 transcription factor, a non-fluorescent marker that activates anthocyanin accumulation in infected tissues, has been used to tag several potyviruses (Cordero *et al*., 2017; Bedoya *et al*., 2012).

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Abbreviations: PLDMV = papaya leaf distortion mosaic virus; PRSV = papaya ringspot virus; GFP = green fluorescent protein; mCherry = mCherry red fluorescent protein

Papaya leaf distortion mosaic virus (PLDMV), a member of the genus *Potyvirus* in the family *Potyviridae*, was first reported from Okinawa Island of Japan in 1954 (Maoka *et al.*, 1996). PLDMV infection causes mosaic symptoms and distortion of leaves, water-soaking streaks on stems and petioles, and ring spots on papaya fruits, similar to the symptoms caused by papaya ringspot virus (PRSV, potyvirus), which is the most widespread and destructive disease affecting papaya (Bau *et al.*, 2008). An isolate of PLDMV-DF from China was recently identified in the commercialized PRSV-resistant

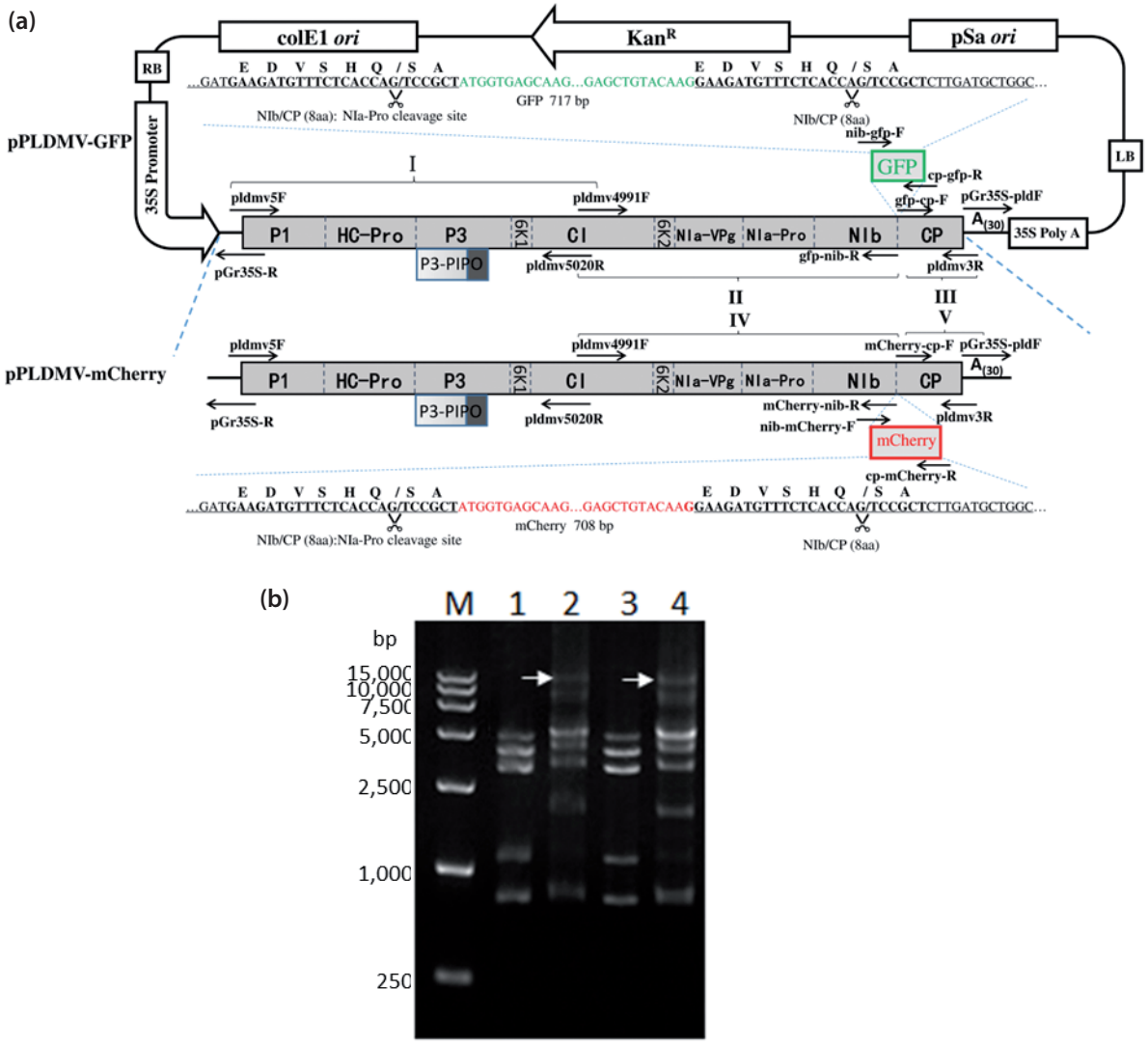

Fig. 1

Strategy to construct fluorescent protein-tagged PLDMV agroinfectious cDNA clones using Gibson assembly

(**a**) Schematic representation of recombinant PLDMV clones: PLDMV-GFP and PLDMV-mCherry. GFP or mCherry fragment was assembled into NIb/CP junction of PLDMV genome with three PLDMV fragments (I, II, and III, or I, IV, and V) and pGreenII-35S PCR fragment to produce pPLDMV-GFP or PLDMV-mCherry vector. Adjacent sequences of NIb and CP are underlined. Octapeptide recognized by NIa protease is shown in large bold letters. Scissors indicate cleavage site of NIa. Artificially introduced NIa-Pro cleavage sites are marked in blue. White rectangles and arrows indicate elements on backbone of pGreenII-35S vector. Genome organization of PLDMV is indicated by grey rectangles. Green and red rectangles represent GFP and mCherry, respectively, and their nucleotide sequences are shown in italics. Black arrows indicate primers used to construct recombinant PLDMV clones (Table S1). (**b**) Agarose gel electrophoresis of Gibson assembly reaction products. Five PCR amplification products (fragment I, 5045 bp; II, 4091 bp; III, 1155 bp; GFP, 741 bp; pGreenII-35S, 3235 bp) (lane 1) used to create plasmid pPLDMV-GFP and recombination product (lane 2) obtained after Gibson assembly; five PCR amplification products (fragment I, 5045 bp; IV, 4091 bp; V, 1154 bp; mCherry, 733 bp; pGreenII-35S, 3235 bp) (lane 3) used to create the plasmid pPLDMV-mCherry and recombination product (lane 4) obtained after Gibson assembly. Arrows indicate position of recombination products obtained after Gibson assembly. M, DNA ladder.

transgenic papaya (Tuo *et al*., 2013) and co-infected with PRSV (Shen *et al*., 2014). PLDMV is becoming an emerging threat to papaya production in China (Bau *et al*., 2008; Shen *et al*., 2014). The PLDMV-DF isolate (GenBank Acc. No. JX974555) has a single-stranded positive-sense RNA genome of 10,153 nucleotides (nt) excluding the poly-A tail. Like other potyviruses, PLDMV-DF has a genome that encodes a long open reading frame (ORF) and another short ORF that results from slippage of the RNA polymerase at the P3 cistron (Tuo *et al*., 2013). The polyproteins are proteolytically processed by three viral protease domains into 11 mature viral proteins (P1, HC-Pro, P3, P3N-PIPO, 6K1, CI, 6K2, VPg, NIa-Pro, NIb, and CP). Phylogenetic analyses suggest that PLDMV-DF is most closely related to an isolate from Japan (Maoka *et al*., 1996; Tuo *et al*., 2013). Based on a previous study, the full-length cDNA of PLDMV is unstable during propagation in *E. coli* and that P3 and CI are the main regions of instability (Tuo *et al*., 2015; 2017). To overcome this problem, a stable *in vitro*-transcribed infectious PLDMV clone was produced by inserting an intron into

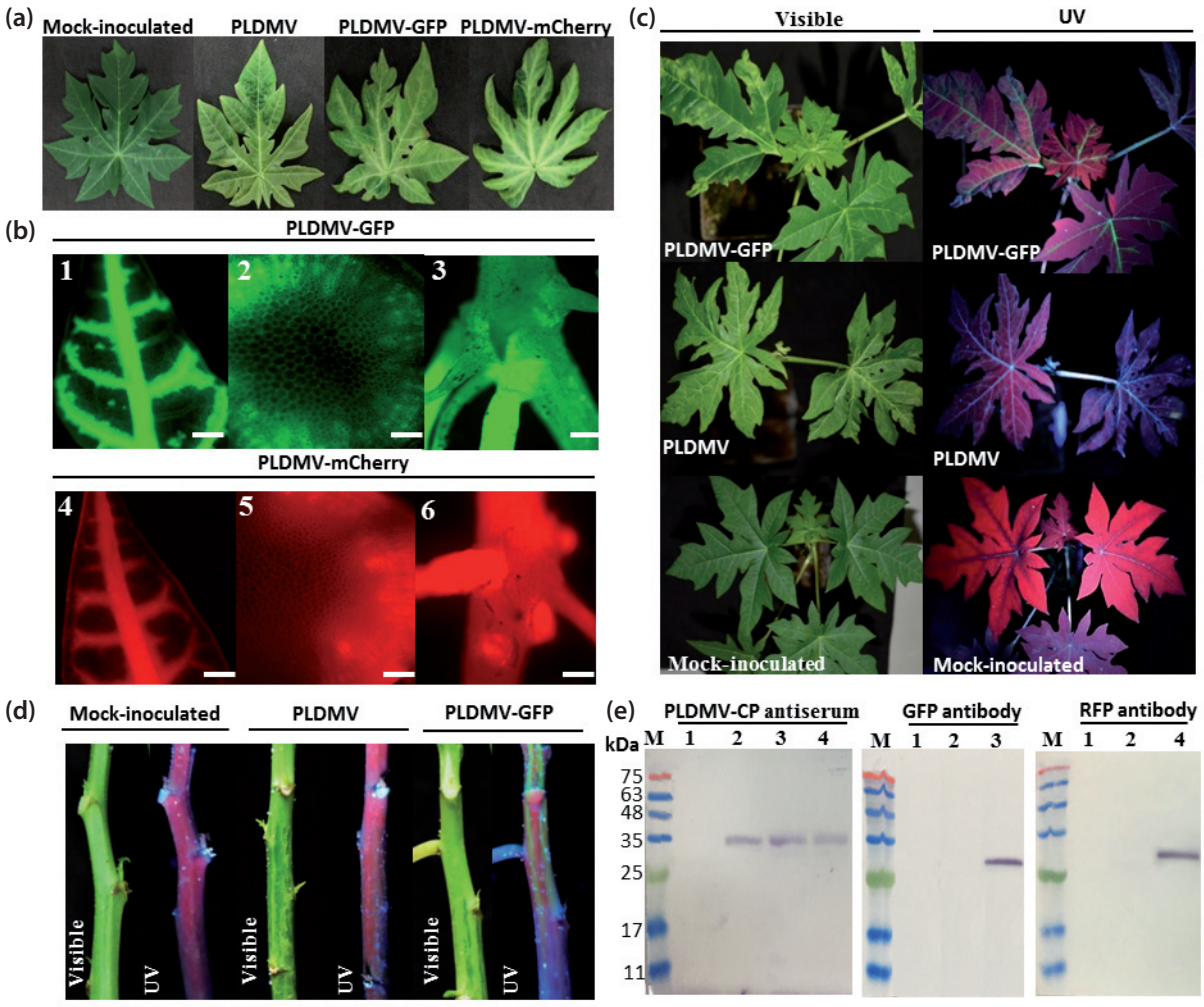

Fig. 2

Determination of fluorescence signals after agroinoculation with PLDMV-GFP, or PLDMV-mCherry infectious cDNA clones

(a) Symptoms induced by PLDMV (Tuo *et al*., 2017), PLDMV-GFP, and PLDMV-mCherry infectious cDNA clones at 90 days post inoculation (dpi). **(b)** Detection of GFP and mCherry expression under epifluorescence microscope at 10 dpi in papaya leaves (1, green fluorescence; 4, red fluorescence), stem cross sections (2, GFP fluorescence; 5, mCherry red fluorescence), and roots (3, green fluorescence; 6, red fluorescence) of plants systemically infected with PLDMV, PLDMV-GFP, or PLDMV-mCherry, respectively. Bars = 20 µm. **(c)** GFP fluorescence under ultraviolet light in papaya plants infected with PLDMV-GFP at 30 dpi. **(d)** Water-soaking streaks on stems induced by PLDMV and PLDMV-GFP, and GFP fluorescence under ultraviolet light on stems infected with PLDMV-GFP at 30 dpi. **(e)** Western blot analysis of PLDMV (lane 2), PLDMV-GFP (lane 3), or PLDMV-mCherry (lane 4) infection in papaya plants using CP antiserum, GFP antibody, and RFP antibody, respectively. Mock-inoculated plants (lane 1) were used as negative control. M, prestained protein ladder.

the unstable P3 region (Tuo *et al.*, 2015). However, *in vitro* RNA transcription is time-consuming and inconvenient. Recently, a new cloning strategy based on one-step Gibson assembly and direct *A. tumefaciens* transformation was developed to generate stable infectious clones of PLDMV, which prevent potential problems such as plasmid instability during propagation in *E. coli* (Tuo *et al.*, 2017). In this study, we developed green fluorescent protein (GFP) and mCherry red fluorescent protein (mCherry)-tagged agroinfection-compatible cDNA clones using this new method for further studies on PLDMV–host interactions by *in vivo* tracking of the virus inside papaya plants.

To develop the GFP- or mCherry-tagged recombinant infectious PLDMV clones pPLDMV-GFP and pPLDMV-mCherry, GFP and mCherry coding fragments were amplified from the vector pVPH-GFP//mCherry (Cui and Wang, 2016). Then, each fragment was assembled into the NIb/CP junction of PLDMV genome with three PLDMV fragments (I, II, and III or I, IV and V) and the mini-binary vector pGreenII-35S PCR fragment (Hellens *et al.*, 2000) by Gibson assembly (Fig. 1a) (Gibson *et al.*, 2009). All overlapping primer pairs shared 25-36 homologous bases at each end (Table S1). The synthesized first-strand cDNAs from total RNA of papaya leaves infected with PLDMV-DF were used as the template to amplify the PLDMV fragments (Tuo *et al.*, 2015). The PCR amplification reactions were performed with Phusion® High-Fidelity DNA polymerase (New England BioLabs; NEB, USA) and PCR products were purified with the MiniBEST agarose gel DNA extraction kit (TaKaRa, Japan). The purified PCR products were assembled according to the instructions in the manual of the Gibson Assembly® Cloning master mix (NEB). Briefly, 100 ng each purified PCR fragment and 5 µl 2× Gibson mix (NEB) was incubated at 50°C for 1 h. Agarose gel electrophoresis analysis confirmed the high efficiency of the Gibson assembly *in vitro* recombination reaction and revealed the high-molecular-weight fragments corresponding to pPLDMV-GFP and pPLDMV-mCherry obtained by the Gibson assembly reaction (Fig. 1b). Then the Gibson assembly reaction products were directly transformed into *A. tumefaciens* strain C58C1, respectively (Tuo *et al.*, 2017). Sequencing of the PCR products from *A. tumefaciens* transformants confirmed that the full-length viral sequences in pPLDMV-GFP and pPLDMV-mCherry were identical to that of the PLDMV-DF isolate, and that

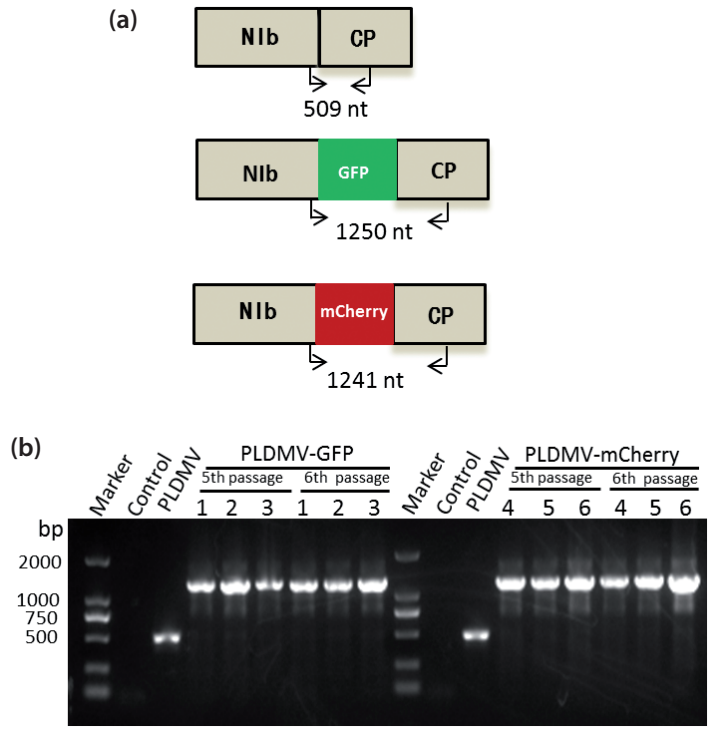

Fig. 3

Stability analysis of PLDMV-GFP and PLDMV-mCherry through serial passage in systemically infected leaves of papaya plants
(**a**) Schematic map of NIb/CP, NIb/GFP/CP, and NIb/mCherry /CP regions in PLDMV genome. Arrows indicate region amplified with primers flanking GFP and mCherry cistron in PLDMV genome. RT-PCR yields ~509-, 1250-, and 1241-bp products from PLDMV-, PLDMV-GFP- and PLDMV-mCherry-infected papaya plants, respectively. (**b**) RT-PCR amplification profile from papaya plants infected with PLDMV-GFP and PLDMV-mCherry at 30 dpi of 5th serial passage (lanes 1–3) and 6th serial passage (lanes 4–6). Buffer-inoculated and PLDMV-infected papaya plants were used as controls.

no spontaneous mutations, deletions, or rearrangements of viral fragments had occurred. The sequencing results also confirmed that GFP or mCherry was introduced correctly between the NIb and CP cistrons flanked by original and artificial NIa-Pro cleavage peptides.

Two leaves of each of 20 papaya plants were agroinoculated with each *A. tumefaciens* transformant harboring the plasmid pPLDMV (Tuo *et al*., 2017), pPLDMV-GFP, or pPLDMV-mCherry. Ninety-five percent of the agroinoculated papaya seedlings developed systemic mosaic symptoms on leaves and water-soaking symptoms on the stem, with the streaks indistinguishable from the typical symptoms caused by wild-type PLDMV at 30 days post inoculation (dpi). Insertion of GFP or mCherry with octapeptide cleavage sequences did not affect the infection efficiency or symptom phenotypes of PLDMV in papaya (Fig. 2a). Moreover, from 10 dpi downwards, GFP or mCherry fluorescence was detected in different parts of systematically infected papaya plants (leaves, stem, and roots) under an epifluorescence microscope (Zeiss, Jena, Germany) (Fig. 2b). Using these analyses, we were able to track the movement of PLDMV-GFP and PLDMV- mCherry during the source-to-sink transportation of photoassimilates through the vasculature-associated tissues and phloem (Hipper *et al*., 2013). When symptomatic plants infected pPLDMV-GFP were illuminated with ultraviolet light, strong green fluorescence was observed in leaves and stem at 20 dpi and was still detectable at 90 dpi (Fig. 2c,d). Using western blot analyses, we were able to detect the 27 kDa GFP or mCherry from total proteins extracted from leaves of papaya infected with pPLDMV-GFP or pPLDMV-mCherry (Fig. 2e). These results confirmed that the inserted homologous artificial NIa-Pro cleavage sites at the C-terminus of GFP or mCherry released GFP or mCherry from the CP as expected.

The stability of pPLDMV-GFP and pPLDMV-mCherry after infection was monitored during six serial passages in papaya plants by mechanical inoculation at 30-day intervals. In RT-PCR analyses of total RNA from passages 1 through 6 from papaya plants infected with pPLDMV-GFP or pPLDMV-mCherry, the intact GFP or mCherry were detected as products of the expected size. The primers used in these analyses were pldmv9000F: GAAATGTGTGCAAACG ATTTTAACG and pldmv9508R: CCATTCTCAATACA CCAAACCATT, which flanked the GFP or mCherry cistron at the 3' end of the NIb and at the 5' end of the CP (Fig. 3a). After six passages of the viral clones in papaya plants, the infectivity of the viral clones remained unchanged and GFP or mCherry fluorescence was detected in the systematically infected papaya plants (Fig. 3b).

Taken together, these results illustrate that stable fluorescent protein-tagged agroinfectious cDNA clones were successfully constructed by one-step Gibson assembly followed by direct transformation into *A. tumefaciens* without plasmid propagation in *E. coli*. These GFP and mCherry-tagged PLDMVs are certainly useful tools to facilitate research on PLDMV-host interactions and evaluation of PLDMV resistance in papaya breeding.

Acknowledgments. This work was supported by Innovative Research Team of the Natural Science Foundation of Hainan Province, China (grant No. 2018CXTD343). We thank Dr. Aiming Wang (London Research and Development Centre, Agriculture and Agri-Food Canada) for his linguistic assistance during the preparation of this manuscript.

Supplementary information is available in the online version of the paper.

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Acta virologica **62**: 202 – 207, 2018 doi:10.4149/av_2018_213

Supplementary information

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Received July 21, 2017; accepted August 22, 2017

Table S1. Primers used for amplification of various gene fragments

Primer	Sequence (5'-3')	PCR product
pGr35S-pldF	GGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATCGGTACGCTGAAATCACCAGTCTC	pGreenII-35S fragment
pGr35S-R	CCTCTCCAAATGAAATGAACTTCCT	
pldmv5F	AGGAAGTTCATTTCATTTGGAGAGGAAAAATATAAAAACTCAACAAAACTTATGC	fragment I
pldmv5020R	ACTATATCGGTCGAACCAATTTTCATGG	
pldmv4991F	CACCATGAAAATTGGTTCGACCGATAT	fragment II
gfp-nib-R	CCTTGCTCACCATAGCGGACTGGTGAGAAACATCTTCATC	
gfp-cp-F	CGAGCTGTACAAGGAAGATGTTTCTCACCAGTCCGCTCTT	fragment III
pldmv3R	CGATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCCTCCTTGCTTAGTCTGAAGTTCC	
pldmv4991F	CACCATGAAAATTGGTTCGACCGATAT	fragment IV
mCherry-nib-R	CCTTGCTCACCATAGCGGACTGGTGAGAAACATCTTCA	
mCherry-cp-F	GAGCTGTACAAGGAAGATGTTTCTCACCAGTCCGCTC	fragment V
pldmv3R	CGATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCCTCCTTGCTTAGTCTGAAGTTCC	
nib-gfp-F	CACCAGTCCGCTATGGTGAGCAAGGGCGAGGAGCTGT	GFP
cp-gfp-R	AGAAACATCTTCCTTGTACAGCTCGTCCATGCCGAGAGT	
nib-mCherry-F	CACCAGTCCGCTATGGTGAGCAAGGGCGAGGAGGAT	mCherry
cp-mCherry-R	GAGAAACATCTTCCTTGTACAGCTCGTCCATGCCGC	

All overlapping homologous bases are underlined.