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A plant intron enhances the performance of an infectious clone *in planta*

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ABSTRACT

Although infectious clones are fundamental tools in virology and plant pathology, their efficacy is often reduced by the instability of viral sequences in *Escherichia coli*. In this study, we constructed an infectious clone of PepMoV (pPepMoV) in a bacterial binary vector (pSNU1); the clone induces symptoms of PepMoV in agroinfiltrated plants. During its modification and maintenance in *E. coli*, however, the pPepMoV infectious clone was instable in the bacteria. Manipulation of this unstable clone in the bacterial strain DH10B led to the spontaneous formation of a recombined clone with high stability in the bacteria but with reduced infectivity due to an unwanted insertion of an *E. coli* sequence in the NIa-protease coding region. Replacement of this sequence with a plant intron restored infectivity and maintained plasmid stability. In addition to restoring plasmid growth in both *E. coli* and *Agrobacterium*, the presence of the intron in the PepMoV sequence enhanced the accumulation of PepMoV in agroinfiltrated leaves and resulted in symptom induction in upper systemic leaves that was nearly as strong as with PepMoV sap-inoculation. Plant introns have been previously used to stabilize plasmids in *E. coli* without any effect or with an unexpected lag in symptom development. In contrast, the current results demonstrated the in vivo enhancement of an infectious clone by a plant intron.

1. Introduction

Infectious clones, double-stranded DNA copies of viral genomes in bacterial plasmids, are fundamental tools in virology. Such clones are useful for stably maintaining the molecular characteristics of RNA viruses because of the lack of proof-reading activity of RNA-dependent RNA polymerase during RNA replication (Steinhauer et al., 1992). Following the invention of DNA recombination technology and the development of enzyme reverse transcriptase, infectious clones were reported for RNA viruses infecting bacteria (Taniguchi et al., 1978), animals (Racaniello and Baltimore, 1981), and plants (Ahlquist et al., 1984). These first-generation infectious clones of RNA viruses were delivered to the host in the form of RNA transcripts generated by in vitro transcription using DNA-dependent RNA polymerase T7 or SP6, which are costly and require strict experimental conditions. The next generation of infectious clones used an in vivo expression system in which the viral genome is under the control of a strong promoter such as the cytomegalovirus promoter for animal viruses or the cauliflower mosaic virus promoter (35S) for plant viruses (Maiss et al., 1992; Naldini et al., 1996). With the advance of *Agrobacterium*-mediated transient expression, the delivery of viral infectious clones to plants has been simplified (Grimsley et al., 1987). This technique, known as agroinoculation or agroinfection, is inexpensive and well-established (Vaghchhipawala and Mysore, 2008).

Several technical challenges remain, however, in the construction and delivery of infectious clones. First, construction of infectious clones may be hampered by plasmid instability in *Escherichia coli,* especially for viruses belonging to the genus *Potyvirus* (Bedoya and Daròs, 2010; Gao et al., 2012; González et al., 2002; Joshi and Jeang, 1993; Junqueira et al., 2014; Lopez-Moya and García, 2000; Mendez et al., 1998; Tuo et al., 2015). Second, symptom development is often slower with agroinoculation than with inoculation by infectious sap (Sandra et al., 2017). A new method for constructing stable infectious clones with enhanced infectivity would be very useful.

Pepper mottle virus (PepMoV) is a plant RNA virus in the genus *Potyvirus* in the family *Potyviridae* with a positive-sense, single-stranded RNA of 9640 bases (Vance et al., 1992). Different isolates of PepMoV with distinct symptoms in tested plants have been isolated from different crops in Korea (Kim et al., 2009). However, the biological characteristics of these isolates have not been investigated by in vivo experiments because of the lack of a low-cost infectious clone. Although infectious transcripts of PepMoV were recently made by in vitro transcription of the full-length cDNA clone driven by the SP6 promoter (Lee et al., 2011; Song and Ryu, 2017), development of a PepMoV infectious

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clone delivered via agroinoculation has yet to be reported.

In this study, we developed an infectious clone of PepMoV using a binary vector modified from pSNU1. When agroinfiltrated into *Nicotiana benthamiana*, this clone induced typical symptoms of PepMoV, but symptom induction was slower than with sap inoculation. We found that, during plasmid maintenance and propagation in *Escherichia coli*, instability of this clone led to an unwanted structure modification in the NIa-protease coding region of PepMoV. A replacement of the inserted sequence by the intron 2 of potato ST-LS1 increased the stability of the infectious clone and resulted in symptom induction with agroinfiltration that nearly as rapid and strong as that with sap inoculation.

2. Materials and methods

2.1. Construction of a PepMoV infectious clone (pPepMoV) and introduction of intron 2 of ST-LS1 into pPepMoV

Total RNA from pepper leaf tissue infected by PepMoV isolate 134 (NCBI accession EU586123) was extracted by Triazol (RNAiso Plus, Takara) according to the manufacturer's instructions. The cDNA was synthesized by reverse transcription using oligodT (20 mer, Bioneer) and a reverse transcriptase (Goscripts, Promega). With the use of the flanking primers with appropriate restriction enzyme (RE) sites, the full-length cDNA clone of PepMoV (pPepMoV) was amplified and assembled in a modified pSNU1 vector (Vo Phan et al., 2014) by two consecutive cloning steps. In the first cloning step, the small fragment of PepMoV (1.4 kb) was amplified from the cDNA with the PepMoV *Eco*RI Fw primer (with *Eco*RI restriction enzyme site) and the PepMoV 1494 Rv primer (which binds to downstream of a *Bam*HI site in the viral sequence) using a *Taq* polymerase (EX *Taq*, Takara, Japan) according to the manufacturer's instructions. Briefly, PCR reaction was conducted in a total volume of 50 µl containing 1X EX buffer (Takara), 0.2 µM each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), and 0.25 µl of *Taq* polymerase. PCR reaction included an initial denaturing step at 94 °C for 1 min followed by 35 cycles of a denaturing step at 94 °C for 20 s, an annealing step at 55 °C for 30 s, and extension step at 72 °C for 1 min, and finally kept at 72 °C for 5 min. The PCR product was digested by *Eco*RI–*Bam*HI and ligated to a modified pSNU1 that was pre-digested by the same REs (Fermentas, USA). The short fragment containing pSNU1 was used as the vector for the second cloning step. The second fragment of PepMoV (8.2 kb) was amplified from cDNA with primers PepMoV 1220 Fw (upstream of the *Bam*HI site) and PepMoV oligodT *Mlu*I Rv using a high-fidelity DNA polymerase as described in Agilent manipulations (PfuUltra-II, Agilent). PCR reaction in a total volume of 50 µl containing 1X PfuUltra-II buffer (Agilent), 0.2 µM each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), and 1 µl of PfuUltra-II polymerase was conducted with an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 4 min, and finally kept at 72 °C for 10 min. The long PCR product was digested by *Bam*HI – *Mlu*I (Fermentas) and was cloned as described above. The pPepMoV infection clone with a multiple cloning site (pPepMoV-MCS) was then constructed: A 2 fragment overlapping PCR was used to make the 1.4-kb fragment with a multiple cloning site (MCS) containing REs *Kpn*I and *Hpa*I, and an extra sequence encoding for a PepMoV NIa protease recognition site (7 amino acids, YEVHHQS, from NIb-CP junction) between P1 and the HC pro coding region; the second fragment was constructed in the same way as described for pPepMoV construction. To make a GFP-expressing PepMoV infectious clone (pPepMoV::GFP), GFP was cloned to the MCS of pPepMoV-MCS via REs *Kpn*I and *Hpa*I. In each cloning step, sequencing analyses (Macrogen sequencing service) were done to select the cloned product with a consensus sequence of at least three different clones. Competent cells of *E. coli* strain Top10 (One Shot TOP10, Invitrogen, USA) were used in these cloning steps, except that DH10B was used for the cloning of GFP to make pPepMoV::GFP.

The *E. coli* strain Top10 tended to lose the pPepMoV plasmid in prolonged culture, i.e. the plasmid was unstable. To increase the stability of the plasmid, similar or other *E. coli* strains were tested including DH10B (Max efficiency DH10B, Invitrogen, USA; similar genotype to Top10), Stable 2, Stable 3, and Stable 4 (Stbl2, Stbl3 and Stbl4, NEB, England). Instability was also evident with DH10B and Stable 3. In the cloning step that introduced GFP into the PepMoV sequence, only the DH10 competent cells worked, but the latter competent cells generated a clone with an unwanted insertion of an *E. coli* sequence. None of the transformants at this stage survived in Top10, Stable 2, Stable 3, or Stable 4. To stabilize pPepMoV and pPepMoV::GFP, the region between the unique RE sites *Sac*I and *Mss*I of these clones was chosen for replacing the unwanted sequence with plant introns. Three-fragment overlapping PCRs using primers PepMoV 3371 Fw and PepMoV 7262 Rv were performed to fuse plant introns into PepMoV between nucleotides 6580 and 6581, the position of the unwanted insertion. Several plant introns of different sizes were tested including intron 2 (189 bp) of the light-inducible gene *ST-LS1* (X04753) from *Solanum tuberosum*, and intron 2 (842 bps) and intron 3 (1818 bp) of the tobacco virus-resistant gene *N* (U15605) from *N. glutinosa*. To match the natural splicing sequence of the introns, synonym mutations were introduced in the overlapped primers (for intron 2 of ST-LS1, for example, G rather than A was introduced at nucleotide 6581 of PepMoV). The fusion PCR products with plant introns were re-introduced into pPepMoV or pPepMoV::GFP via *Sac*I and *Mss*I (Fermentas). The bacterial strain Top10 was used in this step.

2.2. Determination of the viability, plasmid yield, and growth profile of bacterial cells containing the infectious clone

To evaluate the viability of bacterial cells carrying the infectious clone, 20 ng of each plasmid was chemically transformed into *E. coli*. An equivalent quantity of the plasmid was electrically transformed into *A. tumefaciens* with 15 pulses (100 ms each) at 1450 V using an electroporation device (ECM 830, BTX, USA). Following plate incubation (LB plate with kanamycin, 16 h at 37 °C for *E. coli*; YEP plate with rifamycine and kanamycin, 48 h at 28 °C for *A. tumefaciens*), the mean number of transformants per 1 µg of transformed DNA was determined for three biological replications.

To measure plasmid yield, batch cultures of single colonies were maintained in 5 ml of LB broth with kanamycin for 16 h at 37 °C. Plasmids were extracted using a DNA plasmid purification kit (Nucleospin Plasmid, MN, Germany) and were measured with a nanodrop spectrometer (Nanophotometer, Implen, Germany). The quantity of plasmid (µg) per volume (ml) of culture was calculated based on three biological replications.

To measure the kinetic growth of bacteria, 1 ml of a 5-ml overnight bacterial culture (OD_{600} of 2) was transferred into 50 ml of broth media and incubated at 37 °C for *E. coli* and 28 °C for *A. tumefaciens*. The OD_{600} values of three biological replicates of each culture during 9 h of incubation using an UV/visible spectrometer (Ultrospec 3100 Pro, Biochrom, England).

2.3. Agrobacterium-mediated delivery of the PepMoV infectious clone

Seedlings of *N. benthamiana* and *N. tabacum* were grown at 25 °C with 60% humidity and a 16-h light and 8-h dark photoperiod in a growth chamber. Two-week-old plants were used for the agroinfiltration. The infectious clones were transformed into *Agrobacterium tumefaciens* strain GV3101, and agroinfiltration was conducted as described previously (Tran et al., 2017). First, *Agrobacterium* strains harboring pPepMoV and pPepMoV-I clones were individually added to 1 ml of YEB broth containing 50 µg/ml of rifamycin and kanamycin. After the broth cultures had grown for 24 h at 28 °C and 200 rpm, 0.1 ml of each was transferred to 4.9 ml of YEP broth containing the same antibiotics and 200 µM acetosyringone. The 5-ml cultures were grown under the same conditions for 16 h before the *Agrobacterium* cells were collected

by centrifugation at 2000 × *g* for 10 min at room temperature. The cells were suspended and diluted in MMA buffer (pH 5.7, 10 mM MES, 10 mM $MgCl_2$, and 200 μM acetosyringone). The optical density of each bacterial suspension at OD_{600} was measured with a UV/visible spectrometer (Biochrom). The suspensions were then diluted to an OD_{600} concentration of 0.1. A syringe was used to gently infiltrate the suspensions into the abaxial sides of two fully expanded leaves of each 2-week-old plant. After infiltration, the infiltrated leaves and the whole plants were periodically photographed with a digital camera (7200, NIKON, Japan). For assessment of GFP expression under UV light, the camera was equipped with a long-pass filter (495 nm) combined with a green filter (G(X1), Hoya, Japan). GFP expression was also confirmed by immunoblotting as described previously (Tran et al., 2018) using anti-GFP (1:10,000, Santa Cruz, USA); plant rubisco was used as an internal control and was detected by anti-Rbcl antibody (1:50,000, Agrisera, USA).

2.4. Quantitative RT-PCR

Quantitative RT-PCR was carried out as described previously (Tran et al., 2018). Total RNAs from *N. benthamiana* leaves were extracted by Isol-RNA lysis reagent (Takara) and treated with RQ1 DNaseI (Promega, USA) to remove DNA contamination. The cDNAs were synthesized by using GoScript reverse transcriptase (Promega) and oligo(dT15) oligomers. Real-time PCR was then carried out with three biological replicates and three technical replicates in a real-time PCR system (CFX384, Bio-rad) using Sybr green master mix (IQ SYBR green supermix, Bio-rad, USA) as described by the manufacturer. Primers used for the qRT-PCRs are listed in Table 1. The geometric mean of the Ct values from Actin (AY594294) and EF1a (AY206004) were used as references to calculate the ΔCt value (Vandesompele et al., 2002). The samples from pPepMoV at 1 day post agroinfiltration (dpa) were used as the normalization control. Levels of PepMoV RNAs were interpreted as fold-changes. At each time point, the difference between pPepMoV and pPepMoV-I was accessed by an unpaired two-tailed *t*-test in Microsoft Excel 2010. At each time point, the difference between pPepMoV and the pPepMoV-I group was accessed by an unpaired two-tailed *t*-test.

3. Results

3.1. Infectivity of the PepMoV infectious clone

The full-length PepMoV isolate 134 was constructed in a binary vector (named pPepMoV, Fig. 1A). The infectivity of pPepMoV was assessed via agroinfiltration into *N. benthamiana*. At 7 dpa, upper non-infiltrated systemic leaves had typical symptoms, including yellowing, mosaic, and mottle. At 12 dpa, necrosis appeared throughout the plant (Fig. 1B). The infected plants died at 14 dpa (data not shown). Symptoms induced by agroinfiltration of the infectious clone were identical to those induced by sap inoculation of the original PepMoV isolate 134, but symptom appearance was delayed about 3 days in the case of the infectious clone.

To track the movement of PepMoV in plants, we constructed the PepMoV infectious clone expressing GFP (pPepMoV::GFP, as diagramed in Fig. 1B). Following agroinfiltration of *N. benthamiana*, GFP signal was clearly evident in local leaves at 9 dpa. At 12 dpa, the expression of GFP extended to the upper leaves; at that time, however, the yellow fluorescence was widespread because of the systemic necrosis caused by pPepMoV agroinfiltration (Fig. 1B). In the other host, *N. tabacum*, GFP

Table 1
Primers used for the construction of the PepMoV infectious clone and for qRT-PCR.

Name[a]	Sequence[b] (5′ → 3′)	Accession[c]	Purpose
PepMoV *EcoR*I Fw	caattggaattcAAATTAAAACATAACATACAACATAAACGAAAGC	EU586123	Amplify short fragment 1.4 kb of PepMoV
PepMoV 1494 Rv	GTTGTCGAGCAGTCGACCATTCC		
PepMoV 1220 Fw	TTCGAGTGAATGCGTGCAAGAGTTA		Amplify long fragment 8.2 kb of PepMoV
PepMoV polyT *Mlu*I Rv	cgacgcgtTTTTTTTTTTTTTTTGTCTCTCTCATGCCAACTACG		
P1-*Kpn*I-*Hpa*I-HC-Fw	TCGggtaccgttaacTATGAGGTTCATCACCAGTCAACACCTGAAGCATTTTGG	EU586123	Amplify 2 fragments for overlapping PCR to construct multiple cloning site
P1-*Kpn*I-*Hpa*I-HC-Rv	GATGAACCTCATAgttaacggtaccCGAATACTGTTCCATATGAAGTACAGTTGC		
GFP *Kpn*I Fw	ggggtaccGTGAGCAAGGGCGAGGAGCTG	LC336974	Construct pPepMoV::GFP
GFP *Hpa*I Rv	ttggttaacCTTGTACAGCTCGTCCATGCCG		
PepMoV 3619 Rv	GAACCTAGTGAGCACACAACACCC	EU586123	Overlapping PCR to fuse introns to PepMoV NIa
PepMoV 7262 Rv	TGCGTCGCAATCGACTACTCCT		
PepMoV NIa-IntronN2 Fw	CAACAGACAGTAAGTAAGCTAAATAATGCAATAA	U15605	Amplify 3 fragments for overlapping PCR to fuse intron 2 of N to PepMoV NIa protease
PepMoV NIa-IntronN2 Rv	TTTAGCTTACTTACTGTCTGTTGACGCAGGTTCT		
IntronN2-PepMoV NIa Fw	CTTCTTGTTACCAGGGGTGTGTCTCATTGGTTCA		
IntronN2-PepMoV NIa Rv	AGACACACCCCTGGTAACAAGAAGATCATTAGTC		
PepMoV NIa-IntronN3 Fw	GAGAACCAGGTACAATAGCTTGAATTCTATTTTG		Amplify 3 fragments for overlapping PCR to fuse intron 3 of N to PepMoV NIa protease
PepMoV NIa-IntronN3 Rv	AAGCTATTGTACCTGGTTCTCTGAATTTGAGTCG		
IntronN3-PepMoV NIa Fw	GTTCTAAACAGCATCAACAGACAGAGTGTGTCTC		
IntronN3-PepMoV NIa Rv	GTCTGTTGATGCTGTTTAGAACACAGACAGAATG		
PepMoV NIa-Intron2 ST-LS1 Fw	AACAGACAGGTTTGTTTCTGCTTCTACCTTTGAT	X04753	Amplify 3 fragments for overlapping PCR to fuse intron 2 of ST-LS1 to PepMoV NIa protease
PepMoV NIa-Intron2 ST-LS1 Rv	AAGCAGAAACAAACCTGTCTGTTGACGCAGGTTC		
Intron2 ST-LS1-PepMoV NIa Fw	GGTGATGTTTAGGGTGTGTCTCATTGGTTCAAAC		
Intron2 ST-LS1-PepMoV NIa Rv	GAGACACACCCTAAACATCACCATGTTTTGGTCA		
NbActin 784 Fw	CGCACCACCAGAGAGGAAAT	AY594294	Reference of real time RT-PCR
NbActin 840 Rv	AGGGAAGCCAAGATAGAGCCT		
NbEF1a 136Fw	ACTGTGCTGTCCTGATTATTGACT	AY206004.1	Reference of real time RT-PCR
NbEF1a 215Rv	TTTCCACACGACCAACAGG		
PepMoV CP 598 Fw	GGTCTGGCTCGATACGCATT	EU586123	Real-time PCR
PepMoV CP 690 Rv	TGCTGCTGCTTTCATTTGGA		

[a] The fused enzyme sites are included in the primer names in italics; Fw stands for forward primer and Rv stands for reverse primer; the number indicates the start nucleotide position from the start codon, based on the corresponding accession.
[b] The lowercase letter indicates fused sequence for restriction enzyme digestion.
[c] The NCBI gene accession numbers from which the primers were designed.

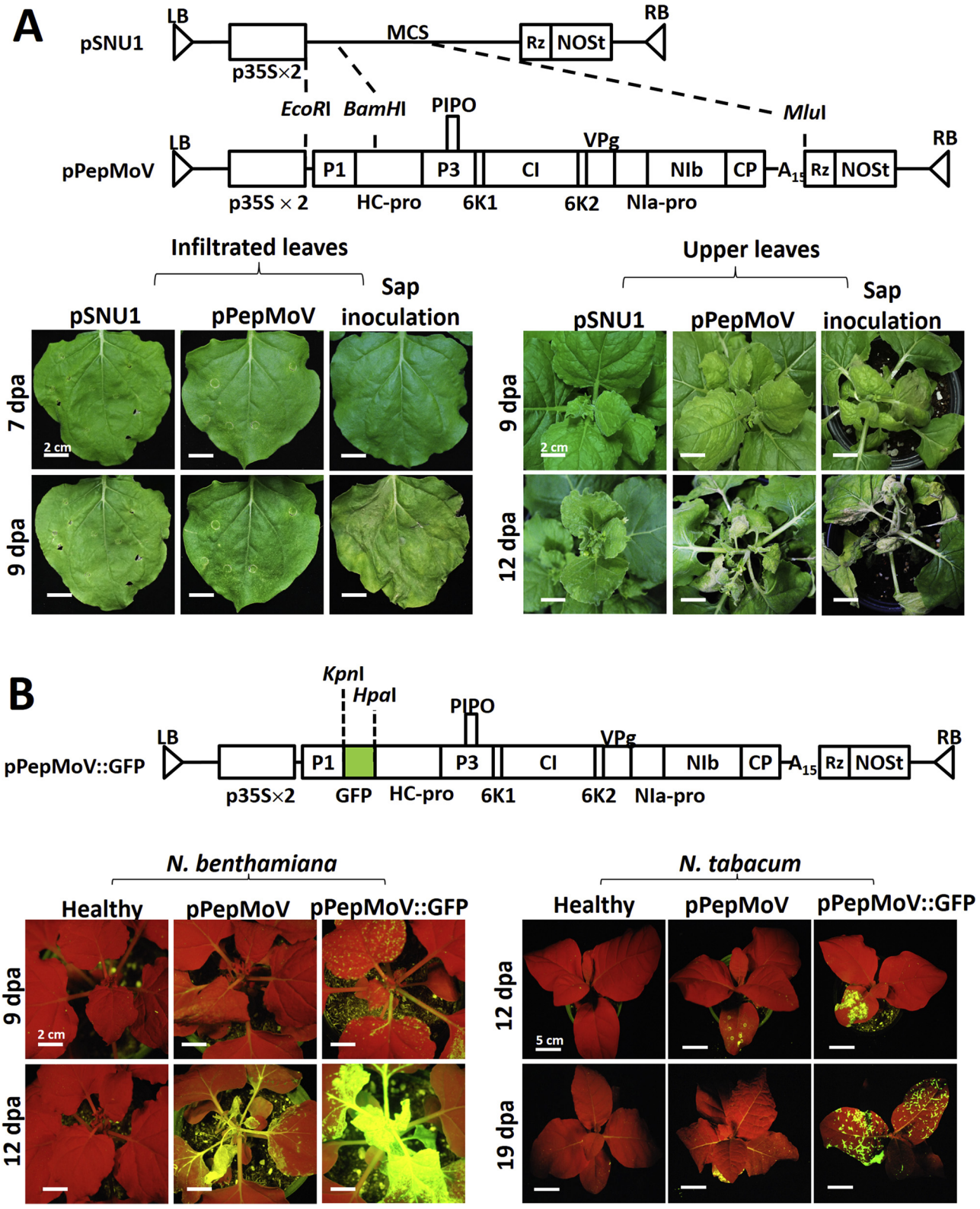

Fig. 1. Construction of the PepMoV infectious clone. (A) Schematic representation of the PepMoV infectious clone (pPepMoV) in the binary vector pSNU1 and its symptom induction in *N. benthamiana*. pSNU1 contains, in sequential order, a left border of T-DNA (LB), a double 35S promoter (35S), a multiple cloning site (MCS), a cis-cleaving ribozyme sequence (Rz), a NOS terminator (NOSt), and a right border of T-DNA (RB). (B) Schematic representation of the PepMoV infectious clone expressing GFP (pPepMoV::GFP) and its expression in *N. benthamiana* and *N. tabacum.* Scale bars are indicated.

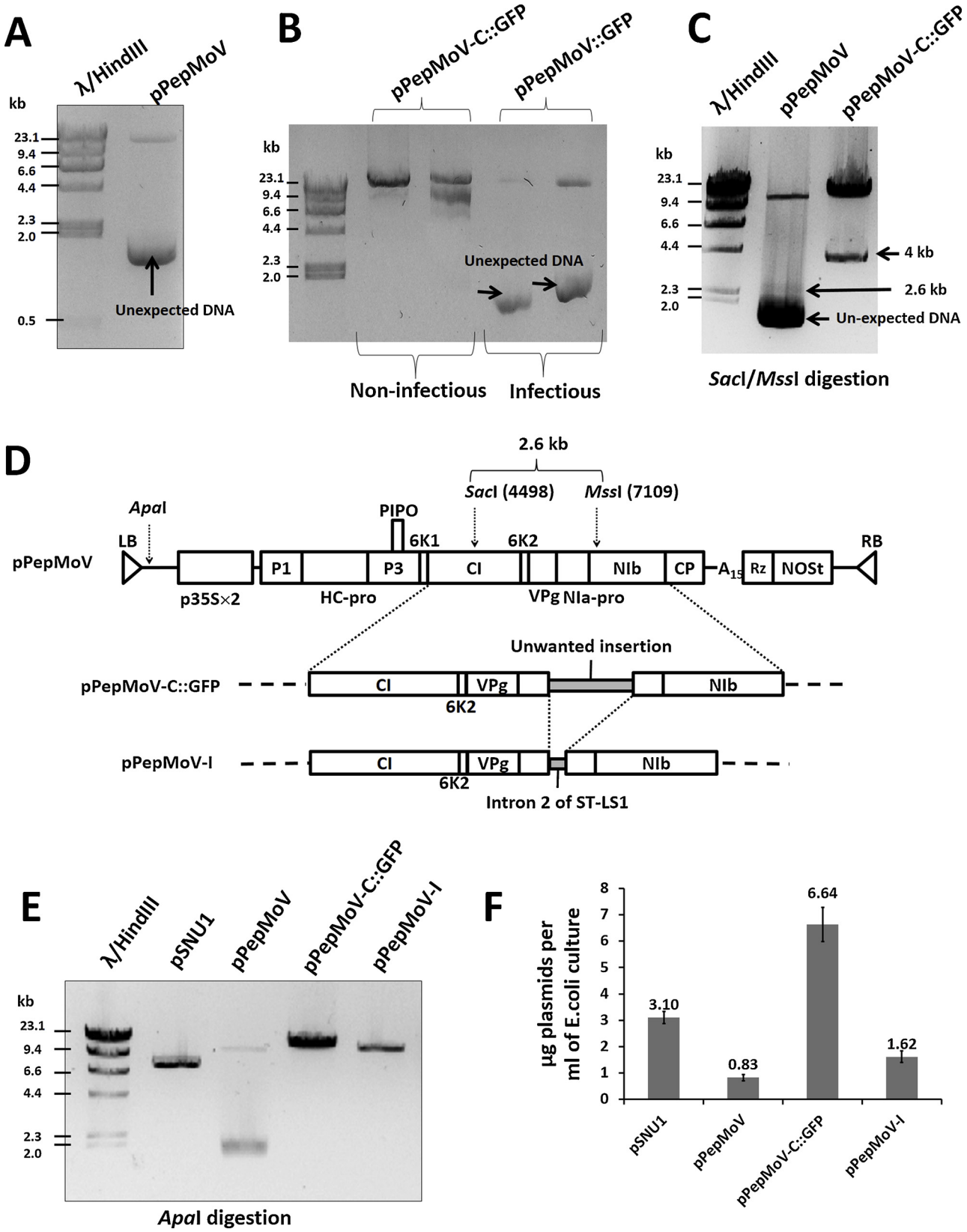

Fig. 2. Instability of the infectious clone pPepMoV. Stability of the infectious clones pPepMoV, pPepMV-C::GFP, and pPepMoV-I as indicated by agarose gel electrophoresis. (A) DNA plasmid of pPepMoV with high accumulation of an extra DNA fragment. (B) Plasmid of pPepMV-C::GFP without the extra DNA band (this clone lost infectivity). (C) pPepMoV-C::GFP was longer than pPepMoV in the region between RE sites *Sac*I and *Mss*I. (D) Schematic representation of pPepMoV-C::GFP with the unwanted insertion and its replacement by the intron 2 of ST-LS1 in pPepMoV-I. (E and F) Restored stability (single band) and increased yield of pPepMoV-I: errors bars in (E) are means + SD of three biological replications.

expression was evident in the inoculated leaves at 12 dpa. In the upper leaves of the *N. tabacum*, however, pPepMoV did not cause any necrosis or yellow fluorescence at 12 dpa. At 19 dpa of *N. tabacum*, systemic expression of GFP was evident in pPepMoV::GFP-inoculated plants (Fig. 1B).

3.2. Insertion of an intron increased pPepMoV stability and maintained its infectivity

In the *E. coli* strain Top10, a long incubation of the bacterial culture produced a low quantity of pPepMoV plasmid and a high quantity of an unexpected DNA that was < 2 kb in size (Fig. 2A). When we extracted the corrected plasmid band from the gel and re-transformed it into the *E. coli* strain DH10 which is similar genotype with the strain Top10 and a stable *E. coli* strain Stable3, the small unexpected DNA was still present (data not shown). None of the transformants that were transformed with only the extracted small DNA was viable (data not shown). We then looked for clones that lacked the unexpected DNA and maintained high plasmid yields. During construction of pPepMoV::GFP using *E. coli* DH10B, we found clones with high yield and without the unexpected DNA despite a prolonged culture (Fig. 2B). However, these clones (named pPepMoV-C::GFP; C stands for 'compromised') lost infectivity in *N. benthamiana*, while the clones with the unexpected DNA maintained their infectivity (data not shown). Restriction enzyme digestion showed that the plasmid was longer in the region between RE sites *Sac*I and *Mss*I in pPepMoV-C::GFP than in pPepMoV (Fig. 2C). Sequencing revealed an extra sequence of 1347 bp in the NIa-Pro coding region (Fig. 2D); this unwanted sequence has 96% identity to a region of *E. coli* genome assembly FHI29 (NCBI blast, nucleotide 38,559 to 39,906 in accession LM995867.1). This region contains an ORF encoding for 403 aa which is 93% identical to an IS4-like element ISVsa5 family transposase (WP_006250222.1). The transposon contamination might increase pPepMoV-C stability and reduce its toxicity to *E. coli.* To stabilize the pPepMoV, we replaced the unwanted insertion with several plant introns. Only the pPepMoV derivative with intron 2 of ST−LS 1 (named pPepMoV-I; I stands for 'intron'; represented in Fig. 2D) maintained the viability of the *E. coli* transformants. Due to unknown reasons, clones including intron 2 or intron 3 of gene N could not be obtained. When the *E. coli* transformants were cultured overnight, pPepMoV-I showed a single plasmid with higher yield than pPepMoV; the unexpected DNA band was not evident in pPepMoV-I (Fig. 2E and F).

Relative to pPepMoV, pPepMoV-I produced about four times more transformants in *E. coli* Top10 and about five times more transformants in *Agrobacterium* GV-3101 (Fig. 3A). The presence of the intron also slightly increased *E. coli* fitness and strongly increased the growth of *Agrobacterium* in liquid culture (Fig. 3B). Agroinfiltration into *N. benthamiana* was then conducted to determine whether pPepMoV-I retained its infectivity. At 5 dpa, the tissues from the infiltrated and upper leaves were harvested, and their total RNAs were quantified by RT-PCR using NIa-pro primers. Agarose electrophoresis and sequencing using NIa-pro primers showed that the intron was completely spliced out (Fig. 3C and D); none of the unexpected splicing was detected by the PCR or sequencing. These results indicated that the PepMoV infectious clone with intron 2 of ST-LS1 maintained its infectivity following agroinfiltration into *N. benthamiana*.

3.3. The intron increased the accumulation of PepMoV RNAs in inoculated leaves and enhanced symptom induction in plants

To determine whether the generation and accumulation of PepMoV RNA from the binary construct in infiltrated leaves was affected by the intron, we collected samples of the pPepMoV and pPepMoV-I constructs at early time points, i.e., from 1 to 5 dpa. qRT-PCR with PepMoV-specific primers revealed that pPepMoV generated 10 times more transcripts than pPepMoV-I at 1 dpa after infiltration (Fig. 4A). The number of transcripts generated by PepMoV then declined and did not begin to increase again until 3 dpa (Fig. 4A). The construct with the intron had a shorter lag phase than pPepMoV, i.e., it began to rapidly increase the viral RNA level at 2 dpa; at 5 dpa, the viral RNA level was about 40 times greater for pPepMoV-I than for pPepMoV (Fig. 4A). Accumulation of virus in infiltrated leaves was also monitored at the translation level by using PepMoV::GFP and pPepMoV-I::GFP. As illustrated in Fig. 4B, GFP expression at 4 dpa was strong with pPepMoV-I::GFP but not with pPepMoV::GFP; the difference in GFP expression was confirmed by immunoblotting with anti-GFP antibody at 5 dpa. These results proved that the intron strongly enhanced the early accumulation of PepMoV in infiltrated leaves.

The systemic development of PepMoV symptoms was monitored in upper, non-infiltrated leaves. The systemic development of symptoms in *N. benthamiana* was nearly as rapid and severe following agroinfiltration with pPepMoV-I vs. sap inoculation with PepMoV (Fig. 4C). At 9 dpa, symptoms induced by pPepMoV in *N. benthamiana* were much milder than those induced by pPepMoV-I (Fig. 4C). At 12 dpa, the *N. benthamiana* plants infiltrated with pPepMoV had more severe symptoms than at 9 dpa, but those infiltrated with pPepMoV-I or sap-inoculated with PepMoV were nearly dead (Fig. 4C). Similarly, the effect on *N. tabacum* of agroinfiltration with pPepMoV-I was nearly as strong as the effect of sap-inoculation with PepMoV (Fig. 4D). The movement of PepMoV::GFP was also monitored via GFP expression; following agroinfiltration with pPepMoV-I::GFP, the virus was evident in non-infiltrated *N. benthamiana* leaves at 9 dpa and in non-infiltrated *N. tabacum* leaves at 12 dpa (Fig. 4, red images at the bottom). These results consistently indicated that insertion of intron 2 of ST-LS1 into pPepMoV resulted in nearly "normal" symptom development, i.e., symptom development was nearly as severe and as rapid with pPepMoV-I agroinfiltration as with PepMoV sap-inoculation.

4. Discussion

4.1. The instability of the pPepMoV

Our results with pPepMoV are consistent with previous findings that instability can be a major problem in maintaining a large infectious clone in *E. coli* (Friehs, 2004; Oliveira et al., 2009; Smith and Bidochka, 1998). Such instability, which can be segregational or structural, may result from bacterial responses that minimize the cytotoxicity or metabolic burden generated by the viral sequence. In the case of segregational instability, the plasmid in the bacterial cells is not altered, but the plasmid-free cells rapidly outgrow the plasmid-containing cells (Lau et al., 2013). A theoretical model has recently suggested that even small changes in the fidelity of plasmid segregation can seriously reduce plasmid stability (Werbowy et al., 2017). However, this might not be the case for the PepMoV infectious clone since plasmids containing cells was selected by using antibiotics in the bacterial culture. Indeed, structural instability was evident in the current study in that pPepMoV contained a DNA contaminant. Working with another infectious clone of another potyvirus, soybean mosaic virus, we also detected the presence of an unknown, small DNA in the plasmid prep (unpublished data). The nature of this contaminating DNA of unexpected size is unknown. This may be the common problem in the development of potyvirus infectious clones. Further sequence identification is necessary to determine whether this DNA is derived from the bacterium or is modified from the vector sequence.

The contaminating DNA may have also caused structural instability by creating a favorable site for recombination in the PepMoV sequence. This indicated the existence of undesired product harmful to the *E. coli* cells and a favorable site for recombination in PepMoV sequence. Even though several possible prokaryotic promoters were predicted in the entire PepMoV sequence with high score (> 0.9, neuron network-based prediction (Reese, 2001), data not showed), no open reading frame (ORF) covering the recombination site was found in either strand except the one encoding the PepMoV polyprotein. Further deletion of

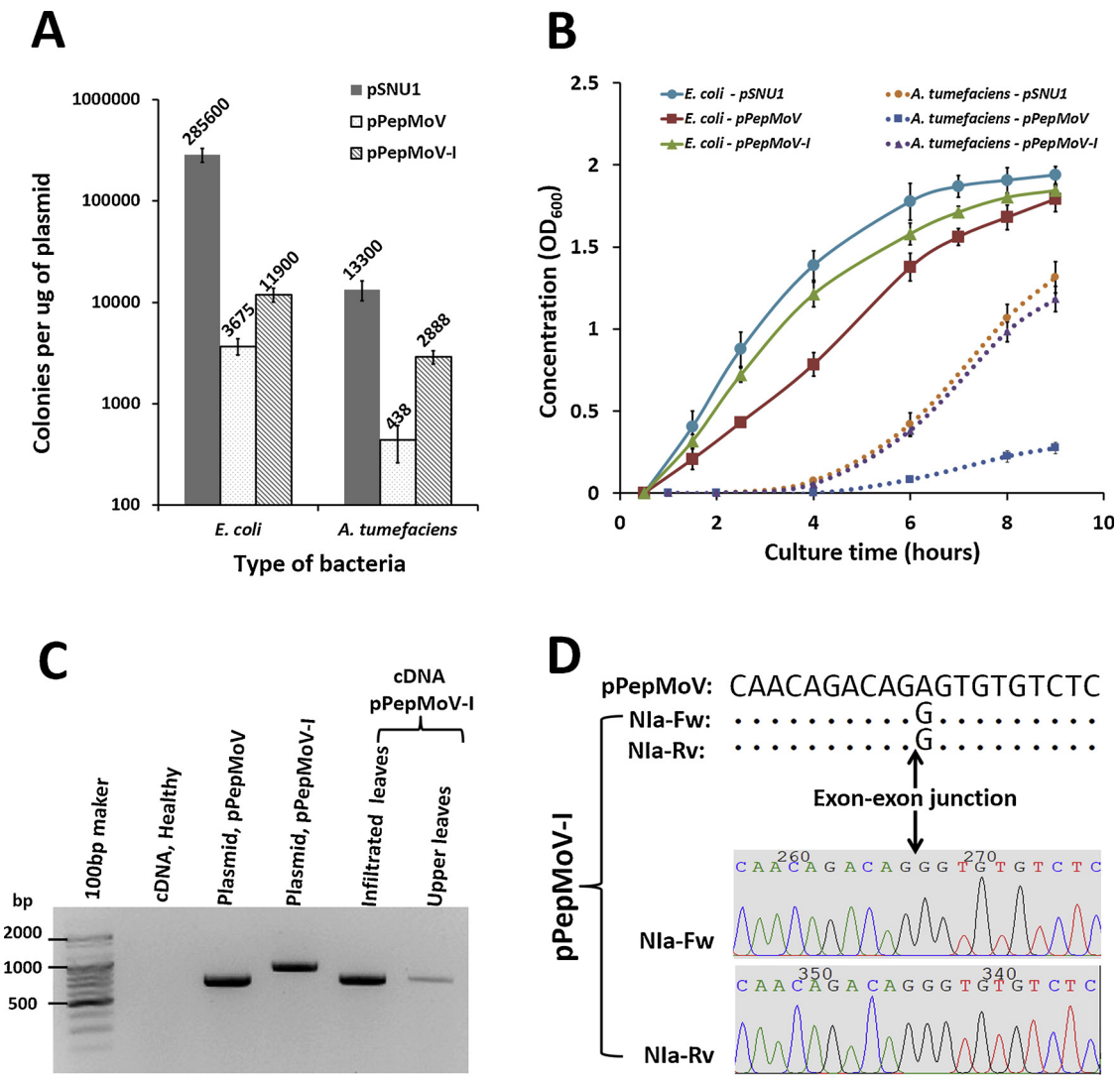

Fig. 3. Insertion of a plant intron into PepMoV restored plasmid fitness in bacteria and maintained clone infectivity. (A and B) Viabilities and growth profiles, respectively, of bacteria carrying the empty vector pSNU1 or PepMoV infectious clones; values are means + SD of three biological replications. (C) RT-PCR using the NIa-pro amplicon confirmed the infectivity of pPepMoV-I. (D) Sequencing of the NIa-pro RT-PCR product using NIa Fw and Rv primers confirmed the intron splicing.

cryptic promoters upstream of the PepMoV ORF is necessary to determine whether the potyvirus polyprotein is related to the cytotoxic effect in the bacterial cells.

4.2. Insertion of a plant intron restored pPepMoV stability and enhanced its infectivity

To reduce the instability of infectious clones, researchers have investigated several strategies such as decreasing the temperature of the bacterial culture (Joshi and Jeang, 1993; Junqueira et al., 2014), using stable bacterial strains (Junqueira et al., 2014; Mendez et al., 1998), destroying cryptic promoters upstream of the potentially toxic ORFs in the viral sequence by synonymous mutations (Chikh Ali et al., 2011), cell-free cloning and biolistic delivery (Fakhfakh et al., 1996), using Gibson assembly (Bordat et al., 2015), and inserting transposable elements (González et al., 2002; Watson et al., 2016). Low-copy plasmids were used to overcome toxicity/instability problems (Pasin et al., 2018; Peremyslov and Dolja, 2007). However, manipulating with a low copy plasmid is time consuming and need larger culture media to obtain sufficient amount plasmid for molecular cloning. In our current study, we used a modified version of pSNU1, a high copy binary plasmid which provide a high yield and cloning efficiency (Seo et al., 2009). To increase the stability of infectious clones in *E. coli*, researchers have also inserted plant introns into the viral genome (Johansen, 1996; Johansen and Lund, 2008; Lopez-Moya and García, 2000). In this study, we found that replacing the unwanted sequence with a plant intron restored the stability of the pPepMoV plasmid in *E. coli*. Like a spontaneous insertion, this insertion could reduce the side-effects caused by the PepMoV sequence.

Insertion of a plant intron into an infectious clone resulted in a delay in the development of plant symptoms in one previous study (Yang et al., 1998) but not in another (Johansen, 1996). When agroinfiltration is used, the insertion of an intron can delay virus accumulation and symptom development in the plant because harboring a bigger plasmid may impose a fitness cost on *Agrobacterium* (Platt et al., 2012). Moreover, plasmid type and copy number affect the Ti plasmid transfer machinery and the plant transformation frequency, respectively (Steck, 1997; Zhi et al., 2015). In this study, the reduction in the viability and growth of the *Agrobacterium* carrying pPepMoV indicated a higher fitness cost for the plasmid with the viral sequence than for the empty vector pSNU1. However, a correlation between *Agrobacterium* fitness and *Agrobacterium*-mediated transient expression has not been reported. The construction and observation of a reporter cassette independent of the viral expression cassette in the same vector may clarify whether

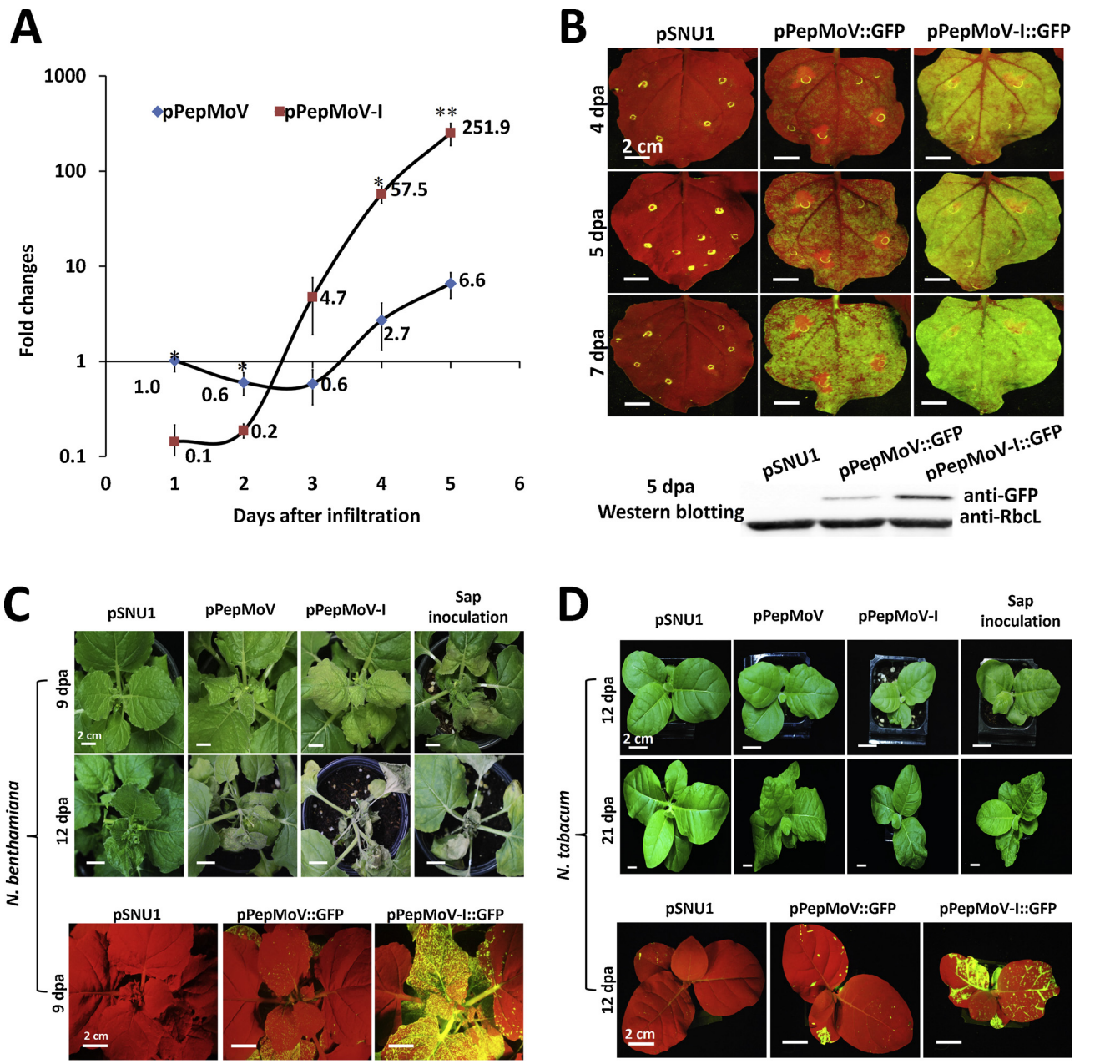

Fig. 4. Insertion of the plant intron enhanced the accumulation of PepMoV RNA, the accumulation of translation product in agroinfiltrated leaves, and symptom induction in upper systemic leaves. (A) qRT-PCR quantification of PepMoV RNA accumulation in agroinfiltrated leaves from 1 to 5 dpa; values are means + SD of three biological replications, and asterisks indicate significant *t*-tests (* $P < 0.05$, ** $P < 0.01$). (B) Expression of GFP delivered by the infectious clone in agroinfiltrated leaves at 4, 5, and 7 dpa. (C and D) Systemic symptoms and expression of GFP resulting from agroinfiltration of *N. benthamiana* and *N. tabacum* with pPepMoV and pPepMoV-I or sap inoculation with PepMoV. Scale bars are indicated.

Agrobacterium fitness affects transient expression in plant leaves.

Although they do not encode protein products, introns can enhance gene expression by a process known as intron-mediated enhancement (Gallegos and Rose, 2015). For example, an intron enhanced the level of mRNA in rice (Morello et al., 2010) and the efficiency of mRNA translation in *Arabidopsis* (Akua and Shaul, 2013). Moreover, introduction of an intron into a transgene strongly reduced RNA silencing in *Arabidopsis* (Christie et al., 2011). Also, a transgene mimicking an endogene with introns was resistant to DNA methylation and systemic silencing (Dadami et al., 2014). In a systemic agrotransfection system of TMV based vector, Marillonnet et al. (2005) demonstrated that addition of multiple introns in to the TMV genome provides efficient processing of the DNA information into active replicons in almost all cells (as high as 94%) of *Nicotiana benthamiana*, an up to 1,000-fold improvement over intronless vectors.

In the current study, insertion of intron 2 of *ST-LS1* into the infectious clone enhanced the early accumulation of PepMoV RNA and translational products, such that symptom development was nearly as rapid and severe with pPepMoV-I agroinfiltration as with PepMov sap inoculation. We suspect that the intron ST-LS1 enhanced the stability and translational efficiency of the PepMoV transcripts generated by the pPepMoV-I in the infiltrated leaves. The consistent enhancement of PepMoV RNA level and translation products (GFP) observed in our study supported this hypothesis. However, some of the viral RNA in inoculated leaves may also have been generated by the replication of the viral RNA. Determining whether the intron can enhance viral replication will require additional research using a non-replicable form of pPepMoV and intron-containing RNA transcripts of PepMoV.

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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.jviromet.2018.12.012.

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