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Streamlined generation of plant virus infectious clones using the pLX mini binary vectors

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ABSTRACT

Recent metagenomic surveys have provided unprecedented amounts of data that have revolutionized our understanding of virus evolution and diversity. Infectious clones are powerful tools to aid the biological characterization of viruses. We recently described the pLX vectors, a set of mini binary T-DNA vectors (~3 kb) that includes strong bacterial terminators and a minimal replicon from the broad-host-range plasmid pBBR1, which replicate autonomously in both *Escherichia coli* and *Agrobacterium*. In this study, a workflow that encompassed pLX binary vectors, overlap-based assembly strategies, and sequencing-by-synthesis verification steps is described and applied for the streamlined generation of infectious clones suitable for *Agrobacterium*-mediated delivery. The pLX-based vectors herein assembled include the first infectious clone of *Wasabi mottle virus*, a crucifer-infecting tobamovirus, as well as binary vectors of positive-single-stranded RNA and single- and double-stranded DNA viruses from the *Potyviridae*, *Geminiviridae* and *Caulimoviridae* families, respectively. Finally, the clones generated were used to agro-inoculate the model plant *Arabidopsis thaliana* and infections were confirmed by a multiplex RT-PCR assay. This workflow facilitated the rapid generation of infectious clones which, together with agro-infection scalability, would allow the pursuit of systematic insights into virus biology and physiology of plant infections and the design of novel biotechnological applications.

1. Introduction

Full-length infectious clones of virus genomes are powerful tools for the study of plant viruses and their components, including transcripts, proteins and non-translated elements. They allow virus reverse genetic studies, systematic analysis of virus biology, as well as the evaluation of virus-virus and host-virus interactions (Nagyová and Subr, 2007; Ng et al., 2014; Massart et al., 2017).

Agrobacterium tumefaciens (hereafter *Agrobacterium*) can be used to initiate virus infections by the transient delivery of virus genome copies into plants (Peyret and Lomonossoff, 2015). Such *Agrobacterium*-mediated inoculation (agro-infection) was used to study DNA, positive- and negative-stranded RNA, mono- and multipartite viruses, as well as satellites and viroids (Grimsley et al., 1986; Prokhnevsky et al., 2002; Grigoras et al., 2009; Liou et al., 2014; Wang et al., 2015; Wrzesińska et al., 2016; López-Carrasco et al., 2017; Kushawaha and Dasgupta, 2018). Copies of virus genomes are inserted into the T-DNA of binary vectors to obtain virus clones suitable for agro-infection and referred to herein as "binary infectious clones", for simplicity. Large binary vectors make cloning of virus genomes and their subsequent modifications a challenge. During the assembly and propagation of infectious clones, sequence mutations and deletions can arise and hinder clone infectivity. Instability problems are intensified by promoter elements that drive unwanted expression of viral genes in bacterial hosts and/or by using plasmids with high-copy origins (Peremyslov and Dolja, 2007; Bedoya and Daròs, 2010).

The pLX vectors are a set of mini binary T-DNA plasmids suitable for the assembly of DNA constructs and their stable and transient delivery to plants *via Agrobacterium*-mediated transformation (Pasin et al., 2017). These vectors include a 1.5-kb minimal replicon from the broad-host-range plasmid pBBR1 (Silva-Rocha et al., 2013). The ~3.3-kb backbone of pLX vectors is smaller than the widely used pBIN- and

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Table 1
List of plant viruses and their binary infectious clones generated in the study.

Species		Family	Genome		Binary vector[a]		
			Type	Size	ID	Size	GenBank
Wasabi mottle virus	WMoV	*Virgaviridae*	ssRNA	6297 nt	pLX-WMoV	10508 bp	MH200603
Turnip mosaic virus	TuMV	*Potyviridae*	ssRNA	9835 nt	pLX-TuMV	14423 bp	MH200604
Turnip curly top virus	TCTV	*Geminiviridae*	ssDNA	2981 nt	pLX-TCTV	7625 bp	MH200605
Cauliflower mosaic virus	CaMV	*Caulimoviridae*	dsDNA	8030 bp	pLX-CaMV	12857 bp	MH200606

[a] pLX-TuMV includes a green fluorescent protein (GFP) sequence as a viral reporter gene.

pPZP-based binary vectors (*e.g.*, pCAMBIA series) and is equal to that of the pGreen plasmid (Murai, 2013). Although pGreen is the smallest available binary plasmid, its maintenance in *Agrobacterium* is not self-sufficient and it requires bacterial strains to host the helper plasmid pSoup that provides plasmid replicase functions (Murai, 2013). For its propagation in *Escherichia coli*, pGreen includes a high-copy origin that can promote deletions or alterations of large DNA inserts. In contrast, the pLX vectors contain a medium-copy origin and replicate autonomously in both *E. coli* and *Agrobacterium*, and pose no constraints on bacterium strain choices, thus simplifying experimental designs. The pCB301 vector and its derivatives (*e.g.*, pJL-89) are also mini binary vectors that use a self-sufficient replicon from the low-copy plasmid RK2 (Lindbo, 2007; Murai, 2013); their small sizes and versatility have facilitated the assembly of infectious clones from large plant viruses (Prokhnevsky et al., 2002; Wang et al., 2015; Rose and Maiss, 2018). The pBBR1 medium-copy origin of the pLX vectors is smaller than the RK2 replicon and outperformed it in transient expression assays (Pasin et al., 2017). An additional feature of the pLX vectors is the incorporation of synthetic bacterial terminators (Chen et al., 2013) that can avoid transcriptional read-through into the T-DNA cassettes and improve plasmid stability.

The pLX vectors and an overlap-based cloning strategy were used for the one-step assembly of an infectious clone of the 9.1-kb *Ugandan cassava brown streak virus* genome (Pasin et al., 2017). Overlap-based cloning overcomes major constraints linked to restriction enzyme use and was recently applied for virus reverse genetic studies and seamless assembly of infectious clones (Pasin et al., 2014b; Bordat et al., 2015; Wieczorek et al., 2015; Blawid and Nagata, 2015; Wang et al., 2015; Tuo et al., 2015). The present study describes a workflow utilizing the pLX vectors and advanced approaches that simplified the generation of binary infectious clones. Based on this strategy, pLX-based clones of the *Wasabi mottle virus* (Shimamoto et al., 1998) and members of diverse virus families were generated and delivered successfully by agro-inoculation into a model plant species.

2. Materials and methods

2.1. Design of an improved workflow for the one-step assembly of pLX-based binary infectious clones

An improved workflow to assemble binary infectious clones was devised and included steps as follows. (i) Cloning design: to amplify virus genomes, primers are based on the exact or reliable consensus sequences of genome termini. Primers that anneal to internal genome regions are designed to amplify multiple fragments that span the entire genome of large or circular viruses. Primers are also designed to linearize the pLX binary vector backbone (Pasin et al., 2017) and to include overlaps at fragment ends. Regulatory elements (such as ribozymes) that promote infectivity are included in the cloning design. (ii) Clone assembly: virus genome fragments and the pLX backbone are amplified. An enzyme with an error rate 280-fold lower than that of the *Taq* DNA polymerase is employed in all RT-PCR and PCR reactions (Potapov and Ong, 2017). Seamless assembly of amplified fragments is done *in vitro* by one-step, overlap-based cloning (Gibson et al., 2009) that uses an enzyme mix with improved fidelity. (iii) Clone validation: positive clones are screened by colony PCR and restriction endonuclease assays, transformed into *Agrobacterium* cells, and used in infectivity tests. A sequencing-by-synthesis (Illumina) approach is used to validate and obtain the full-length sequence of infectious clones.

2.2. Virus isolates and DNA constructs

A *Wasabi mottle virus* (WMoV; family *Virgaviridae*) isolate was obtained from wasabi plant samples collected in Taiwan (Deng et al., 2016). The *Turnip mosaic virus* (TuMV; *Potyviridae*) isolate UK1 (GenBank: AF169561) and its p35TuMV-GFP_vec01_DAE clone (Sánchez et al., 1998; Touriño et al., 2008), *Turnip curly top virus* (TCTV; *Geminiviridae*) isolate IR:Zaf:B11:06 (GenBank: GU456685) and its pGreen5.038 clone (Briddon et al., 2010; Razavinejad et al., 2013), *Cauliflower mosaic virus* (CaMV; *Caulimoviridae*) isolate Cabbage B-John Innes (GenBank: KJ716236) and its pGreen-35S-B-JI clone (Hull and Howell, 1978; Khelifa et al., 2010) have been described and clones were used as templates. The selected species are representative of plant virus families with different genome organizations (Table 1), and their sequences were assembled into a pLX-based mini binary vector (Pasin et al., 2017). Unless otherwise indicated, standard molecular cloning methods were used (Sambrook and Russel, 2001). The cDNA was synthesized using commercial kit components (SuperScript III reverse transcriptase, Thermo Fisher), RT-PCR and PCR reactions were performed with the Q5 high-fidelity 2× master mix (New England BioLabs), and PCR products were incubated with DpnI to remove plasmid templates. The overlapping DNA fragments were joined using a one-step isothermal assembly mixture (NEBuilder HiFi DNA assembly master mix, New England BioLabs). The diagrams of the infectious clone assembly strategies are included in Fig. S1 and the details of cloning materials, references and procedures are shown in Table S1. Primers were synthesized by Integrated DNA Technologies and their sequences are listed in Table S2; their Gibson assembly-overlapping regions are shown in Table S3. The *E. coli* DH10B strain was used for cloning and plasmid propagation. Plasmid stability assays were conducted in *E. coli* as described (Bedoya and Daròs, 2010). Transformed DH10B cells were selected in LB kanamycin plates and individual colonies were picked and grown in LB kanamycin liquid medium at 37 °C with constant shaking. After 24 h, each culture was refreshed by diluting with liquid medium and growth in liquid culture was continued to a total of six growth cycles. Plasmids were then purified, EcoRI digested, and subjected to agarose gel electrophoresis.

2.3. Sequence analysis

A sequencing-by-synthesis approach was used for binary vector sequencing (Shapland et al., 2015). Plasmid DNA was purified by silica column kits, adjusted to a final concentration of 40–65 ng/μL and a total amount of 1–1.5 μg was used for library preparation. Briefly, libraries were prepared from randomly sheared plasmids and sequenced (2 × 150 nt paired-end reads) in an Illumina MiSeq sequencer at the MGH CCIB DNA core facility (Cambridge, MA; https://dnacore.mgh.harvard.edu/). Plasmid sequences were determined by *de novo* read

assembly using the UltraCycler v1.0 (Brian Seed and Huajun Wang, unpublished). The coverage of a given contig base was computed by counting the number of read bases mapped to it. Among all mapped reads, the sequencing technical error rate of a given base was defined as the percentage of reads inconsistent (mismatch) with the consensus base. For phylogenetic analysis, the nucleotide sequences (Table S4) were aligned using MAFFT (Katoh et al., 2017) and a neighbor-joining tree was built in MEGA7 using 1000 bootstrap replicates (Kumar et al., 2016). RNA secondary structures were predicted using RNAfold (Lorenz et al., 2011) and visualized using Forna (Kerpedjiev et al., 2015).

2.4. Transient expression assays

Agrobacterium GV3101::pMP90, EHA105 and AGL1 strains (Hellens et al., 2000), kindly provided by Lin-Yun Kuang (Academia Sinica, Taiwan), were used for *Nicotiana benthamiana* transient expression assays. For expression of a red fluorescent protein (RFP), leaves were infiltrated with *Agrobacterium* strains harboring the pLX-based vector pLX-B3-TagRFP-T (Pasin et al., 2017). At 4 days post-agro-infiltration, plant samples were analyzed under an epifluorescence stereoscope using excitation and emission wavelengths of 545/25 and 605/70 nm, respectively. Fluorescence intensity was measured by placing leaf disks in 96-well flat-bottom plates and the signal was read in a monochromator-based plate reader (Synergy H1MF, BioTek), as reported (Pasin et al., 2014a).

2.5. Plant inoculation

For agro-infections of WMoV, TuMV, TCTV and CaMV, the binary infectious clones were transformed into *Agrobacterium* AGL1 cells by the freeze-thaw method and delivered to plants as described (Pasin et al., 2014b). *Arabidopsis thaliana* Columbia-0 (Col-0) or Wassilewskija-2 (Ws-2) ecotypes were used as model hosts (Fig. S2). Briefly, *Agrobacterium* cultures were centrifuged and bacteria were incubated at room temperature in 10 mM 2-(*N*-morpholino) ethane sulfonic acid, pH 5.5, 10 mM $MgCl_2$ and 150 μM acetosyringone for 3 h. The OD_{600} of the bacterial suspensions was adjusted to 2, forceps were dipped into the suspensions and used to pierce young leaves of soil-grown plants. Virus infections were confirmed using RT-PCR and PCR assays (see below and Table S5). The pLX-TuMV clone includes a green fluorescent protein (GFP) reporter gene. TuMV infections were also confirmed by GFP fluorescence imaging (425/60 and 505/40 nm) and immunoblot assays of the upper uninoculated leaf samples. Total protein extracts from plant samples were prepared and resolved by SDS-PAGE as described (Pasin et al., 2014b). Immuno-detection was conducted using a rabbit anti-TuMV coat protein serum as the primary antibody (Garcia-Ruiz et al., 2010). For mechanical inoculations of WMoV, *A. thaliana* plants were treated with *Agrobacterium* pLX-WMoV or mock cultures and used as inoculum sources. Briefly, the upper uninoculated leaf samples were collected at the times indicated, homogenized in 100 mM phosphate buffer, pH 7.2, and centrifuged (5 min, 14′000 rpm). Supernatants were mixed with silicon carbide (200 mesh particle size, Sigma Aldrich), and used to gently rub young leaves of *N. benthamiana* plants.

2.6. Multiplex RT-PCR assay for virus RNA detection

A multiplex RT-PCR assay was designed for the simultaneous detection of target viruses. At one month post-agro-inoculation, total RNA was purified from the upper uninoculated leaf samples using the plant total RNA mini kit (Geneaid). To remove DNA contaminants, 1 μg of the purified RNA was treated with ezDNAse enzyme (Thermo Fisher) before being used for cDNA synthesis reactions that included the Q2_R, Q4_R, Q6_R, Q8_R, Q10_R primers and the MMLV reverse transcription kit components (Protech). The cDNAs were used as templates in PCR reactions that included the 2× HotStart PCR master mix (Tools-biotech) and the Q1_F, Q2_R, Q3_F, Q4_R, Q5_F, Q6_R, Q7_F and Q8_R primers for simultaneous detection of the WMoV and TuMV genomes and the CaMV and TCTV transcripts. The PCR reactions consisted of a 10-min activation step at 95 °C, 20 cycles of amplification at 92 °C for 10 s followed by 58 °C for 3 s, and a final 2-min extension step at 72 °C. Independent PCR reactions with the Q9_F/Q10_R primers for the plant actin transcript were used as template control. Reaction products were resolved in 3.5% agarose gels in 0.5 × TBE buffer; the expected amplicon sizes are listed in Table S5.

2.7. PCR assays of CaMV and TCTV genomic DNA

At one month post-agro-inoculation, total DNA was purified from the upper uninoculated leaf samples using the Geno Plus genomic DNA extraction miniprep system (Viogene) and used as a template in PCR reactions that included the 2 × HotStart PCR master mix (Tools-biotech) and the Q3_F/Q4_R primers for CaMV detection, Q7_F/Q8_R for TCTV and Q9_F/Q10_R for Actin 2. Reaction products were resolved in 3.5% agarose TBE gels and the expected amplicon sizes are listed in Table S5.

2.8. Statistical analysis

A one-way ANOVA with Tukey's honestly significant difference (HSD) test was used to assess significance between more than two groups; significance levels of *p* values are indicated in the figures. Error bars indicate standard deviation of the mean from at least three biological replicates. Box plots were generated using the Tableau Public software.

2.9. Supporting information

Supporting information includes: (i) Supplementary Figs. S1–S6 and Tables S1–S5; (ii) Supplementary data supporting the Illumina sequencing results shown in Figs. 1 and 2. Sequences of the pLX-based infectious clones generated and the WMoV genome were deposited in GenBank under accession numbers MH200603 to MH200607. The raw Illumina sequencing data are available under the SRA accession number SRP139686 (https://www.ncbi.nlm.nih.gov/sra/SRP139686).

3. Results

3.1. One-step assembly of a pLX-based binary infectious clone of Wasabi mottle virus

The designed workflow was used to assemble an infectious clone of WMoV (genus *Tobamovirus*, Table 1). A pLX-based mini binary vector that includes the 1.5-kb pBBR1 origin, CaMV 35S promoter and nopaline synthase terminator sequences was linearized by inverse PCR, DpnI treated and used as backbone. Two RT-PCR fragments that span the entire 6.3-kb WMoV genome and have overlapping ends were amplified from the total RNA samples of infected plants (Fig. 1A, S1; Table S1). Virus infectivity can be impaired by the presence of non-viral nucleotides at genome ends. The junction of CaMV 35S promoter was designed to initiate transcription at the 5′ end of WMoV cDNA, and a synthetic hammerhead ribozyme was inserted downstream of the WMoV 3′UTR to remove non-viral sequences derived from the T-DNA expression cassette. To improve ribozyme folding and cleavage, the ribozyme 3′ end was extended with nucleotides complementary to the WMoV genome terminus (Fig. 1A; Table S3). Due to its small size (46 nt), the ribozyme sequence was directly incorporated into the amplification primers (Table S3). The amplified fragments had overlapping termini (Table S3) and were joined in a one-step isothermal reaction. The clone pLX-WMoV obtained (Fig. 1B; Table 1) was checked by comparing the observed restriction endonuclease fragment profiles to those computationally predicted. The Illumina sequencing was applied for validation of the 10.5-kb binary clone of WMoV (pLX-WMoV, Fig. 1B). An automated read assembly pipeline allowed us to retrieve the complete pLX-

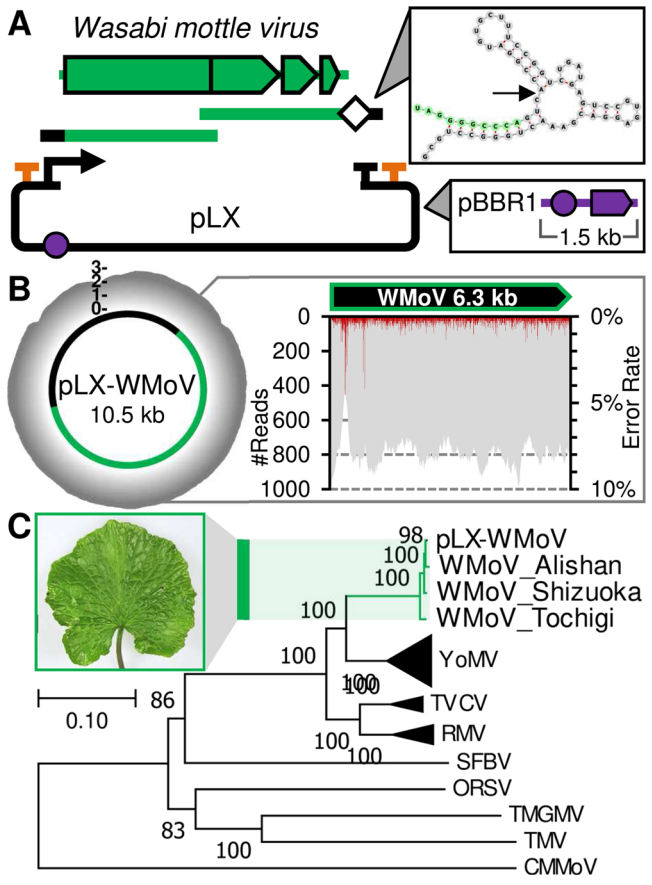

Fig. 1. Assembly of an infectious clone of *Wasabi mottle virus* (genus *Tobamovirus*). (A) *Wasabi mottle virus* (WMoV) genome organization and clone assembly strategy. Two RT-PCR fragments (green boxes) spanning the entire WMoV genome were assembled in a linearized binary vector (pLX); synthetic bacterial terminators (orange) and the pBBR1 vector origin including the *oriV* and *rep* gene (inset) are indicated. The hammerhead ribozyme sequence was included (diamond; Table S3); inset, folding prediction of the ribozyme sequence and its up- and downstream nucleotides: WMoV 3′UTR terminal nucleotides (green) and ribozyme cleavage site (arrow) are indicated. The ribozyme 3′ end was extended with six nucleotides complementary to the WMoV 3′UTR terminus; the extra G–U pairing originates from the vector backbone sequence. (B) The pLX-based binary clone of WMoV was generated (pLX-WMoV). The full-length plasmid sequence was verified by Illumina; left, read coverage (log10) of the sequence assembled; vector backbone and WMoV sequences, black and green, respectively. Right, plot of read number (gray) and error rate in percentage (red) of assembled 6.3-kb WMoV sequence. (C) Phylogenetic relationship of the WMoV sequence cloned (pLX-WMoV; GenBank: MH200607), reported WMoV isolates (Shizuoka, Tochigi and Alishan) and reference tobamoviruses. The WMoV group is indicated; inset, leaf symptoms of WMoV-infected wasabi. CMMoV, *Cactus mild mottle virus*; ORSV, *Odontoglossum ringspot virus*; RMV, *Ribgrass mosaic virus*; SFBV, *Streptocarpus flower break virus*; TMGMV, *Tobacco mild green mosaic virus*; TMV, *Tobacco mosaic virus*; TVCV, *Turnip vein-clearing virus*; YoMV, *Youcai mosaic virus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

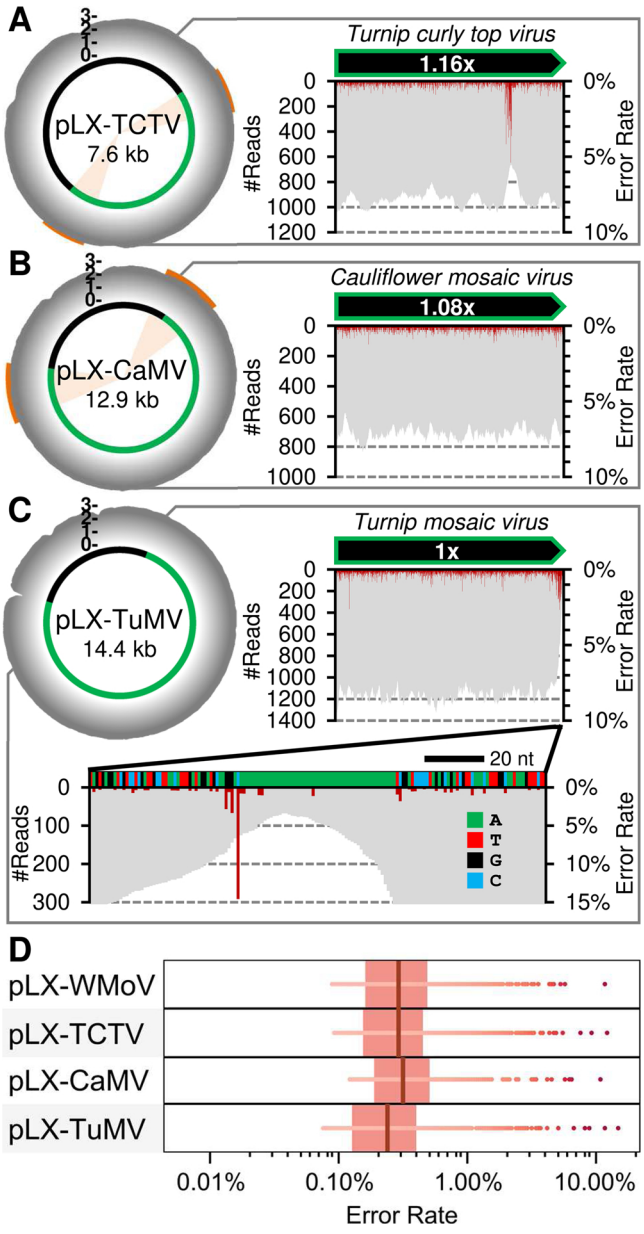

Fig. 2. Use of pLX vectors to generate infectious clones of phylogenetically diverse plant viruses. The pLX-TCTV (A), pLX-CaMV (B), pLX-TuMV (C) binary vectors were generated to deliver genome copies of *Turnip curly top virus*, *Cauliflower mosaic virus* and *Turnip mosaic virus*, respectively (Fig. S1). Full-length plasmid sequences were verified by Illumina; left, read coverage (log10) of plasmid sequences assembled; sequence repeats are labeled in orange. Right, plots of read number (gray) and error rate in percentage (red) of assembled virus genomes (arrows; number of genome repeats are indicated); CaMV 35S promoter sequences from vector backbone are not plotted. Detail of pLX-TuMV poly(A) stretch and its flanking nucleotides are also shown in (C). (D) Error rate statistics in Illumina sequencing of full-length infectious clones. Box plots show the median and upper and lower quartiles of sequencing error rates including both backbone and virus genome sequences; dots indicate values for each plasmid base (log10 scale). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

WMoV sequence in a single reaction without custom-made primers or previous sequence information (Supplementary data). The 6.3-kb WMoV genome sequence obtained had an average coverage of 792 times and a high confidence level (technical error rate below 5%) and was confirmed by partial Sanger sequencing (1870/6297 bp, not shown). This WMoV genome sequence is available at GenBank (accession number MH200607). Phylogenetic analysis of the WMoV genome from the pLX-WMoV clone and representative accessions of virus genomic sequences in the genus *Tobamovirus* showed that the WMoV sequence differed from that of the genus type member *Tobacco mosaic virus* and clustered with other crucifer-infecting tobamoviruses (Fig. 1C) (Adams et al., 2017). In agreement with the origin of the samples used for infectious clone assembly, the WMoV sequence determined was found to be closely related to the WMoV Alishan isolate previously reported in Taiwan (Deng et al., 2016).

3.2. Application of the improved workflow to assemble pLX-based infectious clones of viruses from the Geminiviridae, Caulimoviridae and Potyviridae families

The pLX vectors and Illumina sequencing verification were applied to generate pLX-based infectious clones of TCTV, CaMV and TuMV, designated pLX-TCTV, pLX-CaMV and pLX-TuMV, respectively (Fig. S1). The sizes of the generated binary vectors ranged from 7.6 to 14.4 kb (Fig. 2), with that of pLX-TuMV being the largest. The presence of a 487-bp repeat (in pLX-TCTV) and a 1-kb repetitive element with up to 759-bp identities (pLX-CaMV) did not interfere with the *de novo* read assembly as one circular high quality contig of each clone was obtained (Fig. 2A, B; Suppl. Data). Like the 3′ termini of potyvirus genomes, the pLX-TuMV clone contained a poly(A) tail, a 52-bp homopolymer stretch that did not hinder the sequencing and read assembly of the complete clone (Fig. 2C; Suppl. Data). Sequencing data of the full-length plasmids showed that the median error rate per base ranged from 0.23% to 0.31% (Fig. 2D), with a few extremes detected at sites with lower relative coverage. Full-length sequences of the pLX-TCTV, pLX-CaMV and pLX-TuMV binary vectors were determined by *de novo* read assembly without the aid of a reference genome. The *de novo*-assembled genome sequences matched those of infectious clone templates used in the cloning procedure (Fig. S3). These results indicate that Illumina sequencing could be a convenient, reliable alternative to Sanger and primer walking strategies for sequence verification of novel infectious clones.

3.3. Stability of an intron-free pLX-based clone of a potyvirus

The viral polyprotein coding sequence of the pLX-TuMV clone does not include any intron and is further enlarged by a reporter gene (Fig. 3). Clone stability during propagation in *E. coli* was evaluated. No instability was detected in the conditions tested, since restriction enzyme digestion profiles of input and output plasmids were identical after multiple growth cycles in *E. coli* (Fig. 3B). *Arabidopsis thaliana* plants were agro-inoculated to confirm infectivity of the pLX-TuMV clone. In the upper uninoculated leaves, the TuMV coat protein was detected by immunoblot analysis and the reporter protein by fluorescence imaging (Fig. 3C). The results supported the stability of the pLX-TuMV clone and the use of pLX vectors for the delivery of intron-free potyvirus genomes into plants.

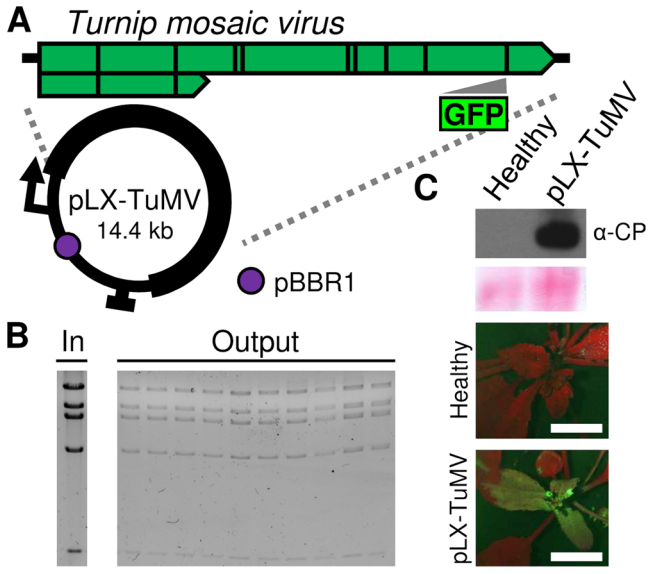

Fig. 3. Stability and *Agrobacterium*-mediated infection of a pLX-based cDNA clone of *Turnip mosaic virus*. (A) The pLX-TuMV vector for delivery of a GFP-tagged *Turnip mosaic virus* cDNA clone. (B) Stability of pLX-TuMV in *E. coli*. Output, the EcoRI-digested plasmid after six growth cycles, each lane is an individual colony sample; In, the EcoRI-digested pLX-TuMV input plasmid is shown as a reference. (C) The pLX-TuMV vector was agro-inoculated into *Arabidopsis thaliana* plants and data were collected after 11 days. Upper uninoculated leaves were imaged: green, GFP fluorescence; red, chlorophyll autofluorescence; scale bar, 1 cm. Virus accumulation was assessed by coat protein (CP) immunoblot analysis; Ponceau red-stained blot is shown as a loading control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Agrobacterium-mediated delivery of pLX-based clones of single-stranded RNA, single-stranded DNA and double-stranded DNA viruses

In transient expression assays, the use of the succinamopine-type EHA105 and AGL1 strains of *Agrobacterium* enhanced the accumulation of a reporter protein delivered by a pLX-based vector (Fig. S4); the AGL1 strain was therefore chosen for agro-infections of *A. thaliana*. Ecotype Col-0 plants agro-inoculated with the pLX-based virus clones showed diverse responses. TuMV, CaMV and TCTV infectious clones induced severe symptoms that were not apparent in the corresponding WMoV- and mock-treated plants (Fig. 4A). Measurement of the height of these plants indicated significant growth differences between them ($p < 0.05$; Fig. 4A). Virus genome/transcript accumulation in the upper uninoculated leaves was confirmed by a multiplex RT-PCR assay (Fig. 4C). Virus DNA accumulation was detected by PCR analyses of DNA from the upper leaves of agro-inoculated plants (Fig. S5). The presence of infectious WMoV in the agro-inoculated *A. thaliana* Col-0 plants was confirmed when crude extracts from the upper uninoculated leaves were used to mechanically inoculate *Nicotiana benthamiana* leaves. Necrotic lesions typical of hypersensitive response appeared at inoculation sites (Fig. 4D). Consistent with the *A. thaliana* Col-0 results, *Agrobacterium*-mediated delivery of pLX-TuMV, pLX-CaMV and pLX-TCTV also induced growth defects in *A. thaliana* ecotype Ws-2 plants (Fig. S6) but no apparent symptoms were observed in the corresponding mock-treated and WMoV-inoculated plants (Fig. S6). Mechanical inoculation of crude extracts from the upper uninoculated leaves of the WMoV agro-inoculated Ws-2 plants nonetheless elicited a local hypersensitive response in *N. benthamiana* plants (Fig. S6C).

4. Discussion

Generation of infectious clones is a resource-consuming task and a major bottleneck in plant virology. The technical challenges of infectious clone assembly include the amplification of large virus genomes, plasmid instability issues, and complex cloning designs that might need several intermediate subcloning steps. *Agrobacterium*-mediated inoculation is a simple and efficient procedure, but large backbones of binary vectors can complicate cloning of virus genomes and subsequent reverse genetic studies. Reports showed that a rational cloning design, binary vectors with special features, and adoption of overlap-based cloning methods are key requirements for the rapid, streamlined assembly of stable full-length infectious clones (Peremyslov and Dolja, 2007; Bedoya and Daròs, 2010; Wieczorek et al., 2015; Blawid and Nagata, 2015; Tuo et al., 2015).

In this study, a workflow employing the specially designed pLX vectors, overlap-dependent cloning and Illumina sequencing validation was used successfully for one-step assembly of binary infectious clones of phylogenetically diverse plant viruses from the *Virgaviridae, Geminiviridae, Caulimoviridae and Potyviridae* families. To our knowledge, the first infectious clone of *Wasabi mottle virus*, a crucifer-infecting tobamovirus, is also reported herein. These findings highlight both the broad utility and the flexibility of our assembly workflow, which includes several advanced features that overcome constraints previously encountered in infectious clone assembly. Firstly, enhanced binary vectors were used. The pLX plasmids are mini T-DNA vectors (~3 kb) that replicate autonomously in both *E. coli* and *Agrobacterium*. We previously used pLX binary vectors in a disarmed octopine-type

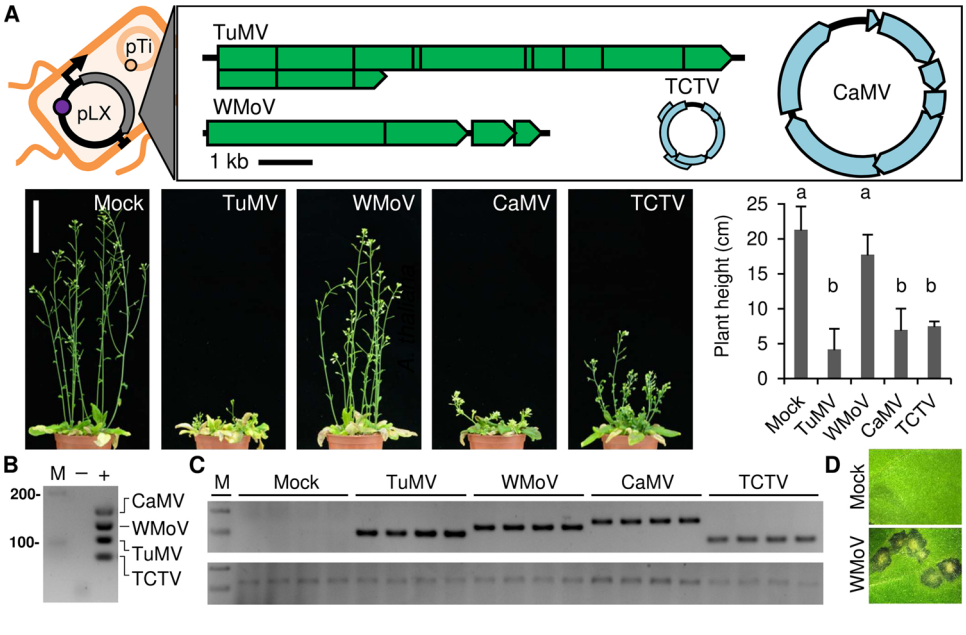

Fig. 4. Agro-infection of *Arabidopsis thaliana* plants with viruses of diverse families. (A) The pLX-TuMV for delivery of turnip mosaic virus (TuMV), pLX-WMoV for wasabi mottle virus (WMoV), pLX-CaMV for cauliflower mosaic virus (CaMV) and pLX-TCTV for turnip curly top virus (TCTV) were transformed into *Agrobacterium* cells; pTi, tumor-inducing plasmid (not to scale); organization of virus genomes is depicted; green and cyan, RNA and DNA viruses, respectively. Plants (ecotype Col-0) were mock treated (Mock) or agro-inoculated using the binary vectors indicated and data were collected after one month. Photographs of plants are shown; scale bar, 5 cm. Plant heights are plotted ($n = 8$); letters indicate $p < 0.05$. (B) Optimization of a multiplex RT-PCR assay for simultaneous detection of viral targets. PCR reactions were resolved by electrophoresis: −, no DNA template reaction; +, reaction included a mixture of pLX-CaMV, pLX-WMoV, pLX-TuMV and pLX-TCTV binary vectors. Viral specific amplicons are indicated (right); M, DNA size marker, 100- and 200-bp fragment bands are indicated (left). (C) Virus detection in agro-inoculated plants. RT-PCR reactions using viral specific primers (top panel) or actin primers (bottom), each sample is a pool of two plants; M, DNA size marker. (D) Photographs of *Nicotiana benthamiana* leaves at 6 days post-inoculation with extracts from mock-treated (Mock) or pLX-WMoV agro-inoculated (WMoV) *A. thaliana* Col-0 plants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Agrobacterium strain; this study showed that the nopaline- and succinamopine-type strains are also suitable for pLX vector use. Despite their small size and broad host range, the pLX vectors showed no instability. Plasmids that host large cDNA copies of potyvirus genomes can degenerate during propagation in bacteria (Bedoya and Daròs, 2010; Tuo et al., 2015; Sun et al., 2017). Clone stability also depends on plasmid features and was linked to the toxicity of undesired viral gene expression in bacterial hosts (Nagyová and Subr, 2007; Bedoya and Daròs, 2010). Interruption of viral cistrons with eukaryotic introns avoids unwanted viral protein accumulation and toxicity in bacteria (Nagyová and Subr, 2007). Insertion of introns into virus genomes helps increase plasmid stability but complicates cloning. In contrast, the pLX-TuMV clone containing the full-length copy of an intron-free potyvirus genome showed no instability. The medium-copy origin of pLX vectors can promote clone stability in bacteria by reducing the burden associated with propagation of large inserts. In addition, the bacterial terminators of the pLX backbones might enable the cloning of intron-free genomes of plant viruses by avoiding transcriptional read-through into the T-DNA cassettes and toxicity in bacteria.

Secondly, use of overlap-based cloning bypassed restriction enzyme constraints and allowed infectious clone assembly of linear and circular viruses without precluding customization. The presence of non-viral sequences at the virus genome termini can reduce infectivity (Turpen et al., 1993; Wang et al., 2015). Vector backbones have been specifically engineered to include the hepatitis delta virus ribozyme and were used in several infectious clones to release the exact 3′ end of the virus RNAs (Lindbo, 2007; Wang et al., 2015; Wrzesińska et al., 2016). For this purpose, a hammerhead ribozyme was incorporated directly during the one-step WMoV clone assembly without intermediate plasmids and cloning steps. With the same approach, it would be possible to generate infectious clones with specific regulatory elements and customized components (*e.g.*, reporter genes).

Thirdly, Illumina sequencing was implemented for convenient verification of the infectious clones assembled. Clone verification *via* Sanger sequencing is a tedious, time-consuming task when dealing with large inserts and/or vectors with low-copy origins. To overcome Sanger limitations, Shapland et al. (2015) developed an Illumina strategy suitable for automated, high-throughput characterization of synthetic constructs. Tandem repeats, long homopolymers, low and high-GC regions can cause coverage and sequencing error bias that interferes with Illumina short read assembly (Ross et al., 2013; Dierckxsens et al., 2017). Despite the presence of tandem repeats in pLX-TCTV and pLX-CaMV as well as a long homopolymer stretch in pLX-TuMV, high quality contigs of complete infectious clones were obtained by Illumina sequencing. Moreover, the Illumina technique provided accurate sequencing of the virus clones with a median error rate per base < 0.5%. Given that clonal plasmids should present no sequence variation, the minor analytical mismatches detected had not weakened the sequencing confidence, as the *de novo* consensus sequences of the viruses cloned into the pLX vectors were identical to the respective genomes of the template plasmids used in the cloning procedures. The Illumina sequencing pipelines thus circumvent customized primer design and data analysis, and they allow accurate, efficient sequence verification of virus infectious clones.

Finally, the infectious clones generated were suitable for *Agrobacterium*-mediated delivery and were used to agro-inoculate the model plant *A. thaliana*. The natural WMoV host is the condiment crop wasabi (*Eutrema japonicum*) that belongs to the *Brassicaceae* family, which includes *Arabidopsis* species. These are natural and experimental hosts of diverse virus species (Pagán et al., 2010; Ouibrahim and Caranta, 2013; Kamitani et al., 2016) and relatives of the plant hosts of the other viruses cloned (Fig. S2). The described binary vector collection of *A. thaliana*-infecting viruses and the agro-infection robustness would allow systematic studies of virus biology and of the physiology of plant infections.

5. Conclusions

Advanced molecular techniques and the pLX binary vectors were applied to easily assemble and validate infectious clones of RNA, as well as ss- and dsDNA plant viruses. Our workflow generated binary infectious clones with (i) reduced sizes, which can facilitate subsequent cloning and reverse genetic studies, (ii) good stability, which would avoid extensive manipulation of virus genome sequences, and (iii) flexibility, since clones replicate autonomously in both *E. coli* and *Agrobacterium* and can be delivered to plants by agro-inoculation. Decreases in DNA synthesis costs enabled the manufacture of infectious clones without virus template requirements (Wimmer et al., 2009;

Cooper, 2014; Lovato et al., 2014; Bouton et al., 2018). The workflow described could also be used to assemble chemically synthesized virus genomes whose sequences are based on metagenomic data or are computationally designed. Rapid generation of infectious clones of human pathogens already paved the way for novel medical applications that overcome the limitations of conventional processes (Dormitzer et al., 2013; Abbink et al., 2018). Similarly, viral vectors and superinfection strategies were recently used to express recombinant polypeptide libraries (Gleba et al., 2014; Julve Parreño et al., 2018), to rewire plant metabolic pathways (Majer et al., 2017), for transient flowering induction (McGarry et al., 2017) as well as for targeted genome editing (Zaidi and Mansoor, 2017), to name a few. The knowledge derived from the use of this technology for in-depth study of virus infections in plants will be valuable for applied research studies and the development of novel biotechnological applications.

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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.2018.09.007.

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