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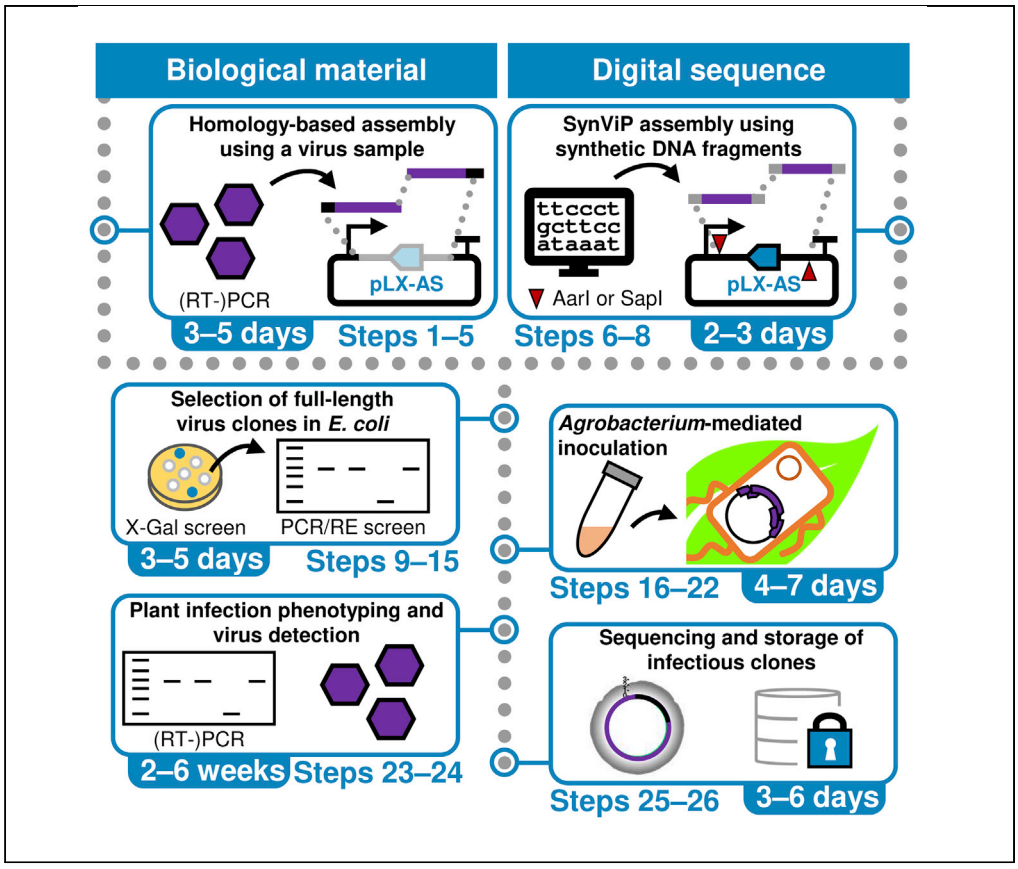
Protocol

Assembly of plant virus agroinfectious clones using biological material or DNA synthesis



Infectious clone technology is universally applied for biological characterization and engineering of viruses. This protocol describes procedures that implement synthetic biology advances for streamlined assembly of virus infectious clones. Here, I detail homology-based cloning using biological material, as well as SynViP assembly using type IIS restriction enzymes and chemically synthesized DNA fragments. The assembled virus clones are based on compact T-DNA binary vectors of the pLX series and are delivered to host plants by *Agrobacterium*-mediated inoculation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

One-step assembly of virus clones using biological material and homology-based cloning

One-step assembly of virus clones using the SynViP synthetic genomics framework

Infectivity assessment of assembled clones by *Agrobacterium*-mediated delivery

One-step verification of infectious clones by high-throughput sequencing

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Assembly of plant virus agroinfectious clones using biological material or DNA synthesis

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SUMMARY

Infectious clone technology is universally applied for biological characterization and engineering of viruses. This protocol describes procedures that implement synthetic biology advances for streamlined assembly of virus infectious clones. Here, I detail homology-based cloning using biological material, as well as SynViP assembly using type IIS restriction enzymes and chemically synthesized DNA fragments. The assembled virus clones are based on compact T-DNA binary vectors of the pLX series and are delivered to host plants by *Agrobacterium*-mediated inoculation.

For complete details on the use and execution of this protocol, please refer to Pasin et al. (2017, 2018) and Pasin (2021).

BEFORE YOU BEGIN

Background information

High-throughput sequencing advances have allowed discovering of an unprecedented number of plant viruses (Dolja et al., 2020; Kutnjak et al., 2021; Moubset et al., 2022). Their biological characterization is now a major bottleneck that limits disease causality studies, evaluation of agronomical and ecological impact of infections, and implementation of virus control strategies (Hou et al., 2020; Massart et al., 2017). Infectious clone assembly is a technology universally applied for virus reverse genetics and functional genomics studies (Carrillo-Tripp et al., 2015; Gao et al., 2019; Li and Zhao, 2021; Pasin et al., 2019; Rihn et al., 2021; Thi Nhu Thao et al., 2020; Xie et al., 2020).

Protocols were reported to develop plant virus infectious clones (Nagata and Inoue-Nagata, 2015; Peremyslov and Dolja, 2007), and whose flexibility and efficiency have been greatly enhanced by improved cloning methods that allow *in vitro* assembly of linear fragments with terminal homology (Blawid and Nagata, 2015; Pasin et al., 2017; Tuo et al., 2015; Wang et al., 2015; Wieczorek et al., 2015; Zhao et al., 2020).

Viruses can be rescued through chemical DNA synthesis of authentic or recoded genomic sequences (Venter et al., 2022). SynViP, a synthetic genomics framework with plant virome capacity, was recently conceived for one-step, seamless virus clone assembly using chemically synthesized DNA. SynViP allowed the use of a digital template to rescue a genuine RNA virus without biological material requirements and sub-cloning steps (Pasin, 2021).

Plant infection is efficiently started by *Agrobacterium*-mediated delivery (agroinfection) of virus clones in T-DNA binary vectors (Pasin et al., 2019). The pLX series consists of novel T-DNA binary vectors that implement synthetic biology principles of minimization and modularity to simplify the

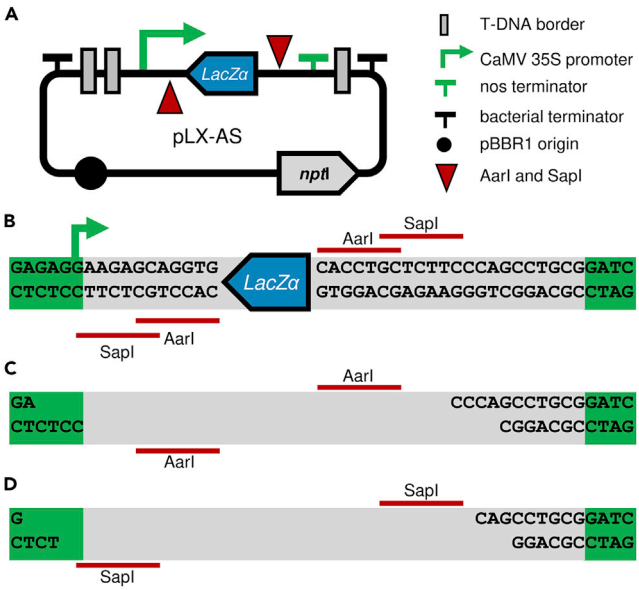

Figure 1. Features of pLX-AS

(A) Diagram of pLX-AS, a mini T-DNA binary vector including divergent AarI and SapI recognition sites. Vector components including the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (nos) for plant expression are indicated (right); *LacZα*, *E. coli* reporter gene; *npt*I, kanamycin resistance gene.

(B) Detail of the pLX-AS cloning cassette with *LacZα* flanked by AarI and SapI sites; the CaMV 35S promoter 3′ end and nos terminator 5′ end are in green; arrow indicates transcription initiation site *in planta*.

(C) Sequences of 4-nt overhangs generated by AarI digestion.

(D) Sequences of 3-nt overhangs generated by SapI digestion.

assembly and engineering of virus infectious clones. The pLX backbone is small, autonomously replicates in *Escherichia coli* and *Agrobacterium*, and includes plasmid stabilizing features (Pasin et al., 2017). pLX outperformed binary vectors based on RK2, the replicon of pCB301 and its derivative pJL-89 (Lindbo, 2007), and was reported for clone assembly and agroinfection of several DNA and RNA viruses (Aragonés et al., 2022; Bao et al., 2020; Klenov and Hudak, 2021; Pasin, 2021; Pasin et al., 2017, 2018; Zhao et al., 2020).

Here, I describe procedures for one-step assembly of virus infectious clones using either a biological sample or a digital sequence as starting materials. Viral sequences are assembled into pLX-AS (Addgene: 188870), a compact 4.5-kb binary vector of the pLX series (Figure 1). Assembled clones are suitable for agroinfection of plants.

Strategic planning

Planning of infectious clone assembly should consider short- and long-term research targets, which may include engineering of expression vectors for biotechnology and synthetic biology applications (Ellison et al., 2021; Pasin et al., 2019, 2022; Torti et al., 2021). Virus propagation in experimental hosts can cause a rapid adaptive evolution and host range alteration (Kurth et al., 2012). A starting material from virus-infected natural hosts should be preferred when infectious clones should be used in disease causality studies or when aiming for development of biotechnological tools functional in a specific crop or plant species.

Biosafety

Bacteria containing recombinant or synthetic nucleic acid molecules need to be handled and disposed according to institutional regulations. Plant viruses pose potential agronomical and environmental risks. Researchers need to comply with governmental and institutional regulations before conducting experiments requiring virus propagation in plants and working with plant infectious

agents. Biosafety considerations and working practices for handling plant viruses and derived infectious clones were reviewed elsewhere (Brewer et al., 2018).

Preparation of competent *Escherichia coli* cells

Timing: 3 days

Prepare chemically-competent *E. coli* TOP10 cells as detailed in Method S1 (see supplemental information).

⚠ CRITICAL: TOP10 is an isolate of the *E. coli* strain DH10B, which was designed for efficient transformation of large insert DNA library clones and maintenance of large plasmids (Durfee et al., 2008), and was shown to outperform the common laboratory strain DH5α in full-length infectious clone assembly (Blawid and Nagata, 2015).

⚠ CRITICAL: Method S1 has been optimized for TOP10 (DH10B; Jessee et al., 2005), and may result in poor transformation efficiencies with other *E. coli* strains.

⚠ CRITICAL: Use cell lots giving a number of colony forming units per μg of plasmid DNA of $\sim 1 \times 10^8$ or above in downstream experiments.

⚠ CRITICAL: Keep cell aliquots frozen at −80°C until needed; avoid thawing and refreezing since it dramatically reduces transformation efficiency.

Alternatives: Electrocompetent cells may also be used but procedures should be optimized to avoid salt excess.

Alternatives: Transformation competent cells of DH10B and derivatives are available from commercial providers and can also be used.

Preparation of competent *Agrobacterium* cells

Timing: 3 days

Prepare *Agrobacterium* AGL1 cells suitable for freeze-thaw transformation as detailed in Method S2.

Note: AGL1 is a hypervirulent C58 derivative that carries a disarmed pTiBo542, which confers high transformation efficiencies on numerous plant (and fungal) species (Lazo et al., 1991; Casado-Del Castillo et al., 2021). The strain is a nonfunctional mutant of *recA*, which reduces the occurrence of plasmid recombination without interfering with DNA transfer (Lazo et al., 1991).

Alternatives: This protocol uses T-DNA binary vectors of the pLX series, which have been tested in a variety of *Agrobacterium* strains (Pasin et al., 2018). Strain alternatives to AGL1 include C58C1-313, GV3101::pMP90, and EHA105 (De Saeger et al., 2021; Pasin et al., 2017), as well as any *Agrobacterium* strain capable of plant cell transformation and sensitive to kanamycin, the antibiotic used for binary vector selection.

Alternatives: Transformation competent cells of *Agrobacterium* strains are available from commercial providers and can also be used.

Alternatives: Electrocompetent cells may also be used but procedures should be optimized to avoid salt excess.

Preparation of host plants

Timing: ~3 weeks

Mix an *Arabidopsis thaliana* seed aliquot and tap water in 10 mL tube, and incubate at 4°C (2 d). Fill pots with soil mixture (3 parts of potting substrate and 1 part of vermiculite), place them in JiffyTray-XL trays, and distribute seeds onto pre-watered soil by pipetting. After sowing, cover trays with JiffyDome-XL lids to maintain high humidity level, and place them in a growth chamber at 22°C, with ~65% relative air humidity, a 16 h/8 h day/night cycle and a light intensity of ~200 µmol/m^2/s. Remove lids after 3 days; clear pots from plants in excess after 14 days, and agroinoculate plants after 16–20 days.

⚠ CRITICAL: The protocol uses viruses naturally infecting plants of the family Brassicaceae: *A. thaliana* can be infected by diverse viruses, but it is not a universal virus host and its accessions can display differences in infection susceptibility (Ouibrahim and Caranta, 2013; Shukla et al., 2019).

⚠ CRITICAL: For optimal agroinfection conditions and time saving, ensure to coordinate preparation of plants and *Agrobacterium* strains hosting the virus clones (steps 16–22). Periodically sow new batches of seeds to have a continuous supply of plants.

Alternatives: Alternative plant species, ecotypes or varieties should be used depending on the known host range of the virus of interest. If no host range information is available a panel of plant species can be tested as experimental hosts. *Chenopodium* spp. and *Nicotiana* spp. are the most widely used herbaceous host and indicator plants (Horváth, 2019; Legrand, 2015), and common laboratory strains of *Nicotiana benthamiana* are susceptible to a large number of plant viruses (Goodin et al., 2008).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pLX-AS	Addgene	188870
Bacterial and virus strains		
One Shot™ TOP10 chemically competent *Escherichia coli*	Thermo Fisher Scientific	C404010
Agrobacterium AGL1	(Lazo et al., 1991)	N/A
Experimental models: Organisms/strains		
Arabidopsis thaliana Columbia 0 (Col-0)	ABRC	CS70000
Chemicals, peptides, and recombinant proteins		
Agarose D1 medium EEO	Condalab	8019
Bacteriological agar	Condalab	1800
Tryptone	Condalab	1612
Yeast extract	Condalab	1702
FavorPrep™ MicroElute GEL/PCR purification kit	Favorgen	FAEPK 001
FavorPrep™ plant total RNA mini kit	Favorgen	FAPRK 001
Adenosine 5′-triphosphate (ATP)	New England Biolabs	P0756S
DpnI (20 U/µL)	New England Biolabs	R0176S
NEBuilder® HiFi DNA assembly master mix (2×)	New England Biolabs	E2621S
Q5® high-fidelity 2× master mix	New England Biolabs	M0492S
T4 DNA ligase (400 U/µL)	New England Biolabs	M0202S
Wizard® Plus SV minipreps DNA purification system	Promega	A1330
2-(*N*-Morpholino)ethanesulfonic acid hydrate (MES)	Sigma-Aldrich	M8250
3′,5′-Dimethoxy-4-hydroxyacetophenon (Acetosyringone)	Sigma-Aldrich	D134406

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ampicillin sodium salt	Sigma-Aldrich	A9518
Boric acid	Sigma-Aldrich	1.00165
Calcium chloride dihydrate ($CaCl_2$)	Sigma-Aldrich	C3306
Carbenicillin disodium salt	Sigma-Aldrich	C1389
D(+)-Glucose monohydrate	Sigma-Aldrich	1.04074
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Sigma-Aldrich	E5134
Glycerol (85%)	Sigma-Aldrich	1.04094
Kanamycin	Sigma-Aldrich	K1377
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich	M8266
Magnesium sulfate heptahydrate ($MgSO_4$)	Sigma-Aldrich	1.05886
Manganese(II) chloride tetrahydrate ($MnCl_2$)	Sigma-Aldrich	1.05927
N,*N*-Dimethylformamide	Sigma-Aldrich	D4551
Potassium acetate (KOAc)	Sigma-Aldrich	P1190
Potassium chloride (KCl)	Sigma-Aldrich	1.04933
Rifampicin	Sigma-Aldrich	R3501
Tris(hydroxymethyl)aminomethane (Tris base)	Sigma-Aldrich	T1503
5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal)	Thermo Fisher Scientific	B1690
AarI (2 U/μL)	Thermo Fisher Scientific	ER1581
DreamTaq green PCR master mix (2×)	Thermo Fisher Scientific	K1081
ezDNase™	Thermo Fisher Scientific	11766051
FastDigest BamHI	Thermo Fisher Scientific	FD0054
FastDigest Eco32I (EcoRV)	Thermo Fisher Scientific	FD0303
FastDigest EcoRI	Thermo Fisher Scientific	FD0274
FastDigest SalI	Thermo Fisher Scientific	FD0644
FastDigest SmaI	Thermo Fisher Scientific	FD0663
RiboLock RNase inhibitor (40 U/μL)	Thermo Fisher Scientific	EO0381
SuperScript™ III reverse transcriptase (RT)	Thermo Fisher Scientific	18080044
Sodium chloride (NaCl)	VWR Chemicals	27810.364
Liquid nitrogen	N/A	N/A
Oligonucleotides		
Random hexamers	Thermo Fisher Scientific	N8080127
2050_F GCCATTGTCCGAAATCTCACG	(Pasin et al., 2017)	N/A
2051_R CTGGAAATGCGATTCTCTTAGC	(Pasin et al., 2017)	N/A
Q9_F GACCGTATGAGCAAAGAAATCACAGC	(Pasin, 2021)	N/A
Q10_R AATGTACTGAGGGAAGCAAGAATGGA	(Pasin, 2021)	N/A
W1_R AGCACAGACAGCTCGGATAA	(Pasin et al., 2018)	N/A
W2_R CTTTTGCTTAGGCTGACTCTTGAT	(Pasin et al., 2018)	N/A
W3_R TGGGCCCCTACCCGGGGTTAGGGAGGATTC	(Pasin et al., 2018)	N/A
W4_F AGGAAGTTCATTTCATTTGGAGAGGGTT TAAATTATTGCAACAACAACAACAAATTACAA	(Pasin et al., 2018)	N/A
W5_R CCTTGAACCTCATGGACCGTATTCACA	(Pasin et al., 2018)	N/A
W6_F TGTGAATACGGTCCATGAGGTTCAAG	(Pasin et al., 2018)	N/A
W7_R GGACTCATCAGACCGGAAAGCA CATCCGGTGACTGGGCCCCTACC CGGGGTTAGGGAGGA	(Pasin et al., 2018)	N/A
W8_R CCTCTCCAAATGAAATGAACTTCCT	(Pasin et al., 2018)	N/A
W9_F GCTTTCCGGTCTGATGAGTCCGTG AGGACGAAACTGGGCCTGCGGA TCGTTCAAACATTT	(Pasin et al., 2018)	N/A
W83_F CGCAGGTTGTGAGAGGGAA	(Pasin et al., 2018)	N/A
W154_F GGAGTTAGCCGCTCGAAATAGAT	This protocol	N/A
W155_R GTTTTCCGCCTCTGGTTCTTTG	This protocol	N/A
Other		
JiffyTray-XL plant tray	Jiffy	110041
JiffyDome-XL plant tray lid	Jiffy	110042

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Material and equipment for molecular biology work (table-top centrifuges, dry block heaters, electrophoresis system, nucleic acid staining dyes, blue light transilluminator, water purification system, etc.)	N/A	N/A
Material and equipment for bacterial handling and culture (petri dishes, tubes, autoclave, microbiological cabinet, static and shaking incubators, etc.)	N/A	N/A
Materials and equipment for plant maintenance (soil substrate, pots, greenhouse or plant growth chamber, etc.)	N/A	N/A

MATERIALS AND EQUIPMENT

Virus sample

Timing: as needed

Plant virus samples can be obtained from international resource centers such as the American Type Culture Collection (ATCC, U.S.A., https://www.atcc.org), the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany, https://www.dsmz.de), or the National Agriculture and Food Research Organization Genebank (NARO, Japan, https://www.gene.affrc.go.jp).

⚠ CRITICAL: Depending on research targets, multiple isolates of the same virus species can be cloned in parallel, since they can display large variation in pathogenicity and virulence.

Media and solutions

Timing: 1–2 days

Unless otherwise indicated, standard molecular cloning methods and reagents are used (Sambrook and Russell, 2001); 18.2 MΩ · cm purified water (ultrapure water) is obtained using a Milli-Q® system (Millipore).

- Acetosyringone (0.1 M): Dissolve 98 mg of acetosyringone powder in 5 mL of DMSO, and store in single-use 150-μL aliquots at −20°C in the dark for up to 6 months.
- Ampicillin (100 mg/mL): Dissolve 1 g of ampicillin sodium salt in 10 mL of ultrapure water, sterilize through a 0.2-μm filter, and store 1-mL aliquots at −20°C for up to 1 year.

  Note: Used only in competent cell preparation (Method S1).

- $CaCl_2$ (2.5 M): Dissolve 73.51 g of $CaCl_2$ in ultrapure water to a final volume of 200 mL, sterilize by autoclaving, and store at room temperature indefinitely.

  Note: Used only in competent cell preparation (Method S1).

- Carbenicillin (100 mg/mL): Dissolve 0.5 g of carbenicillin disodium salt in 5 mL of ultrapure water, sterilize through a 0.2-μm filter, and store 1-mL aliquots at −20°C for up to 1 year.

  Note: Used only in competent cell preparation (Method S2).

- EDTA (0.5 M): Mix 93.05 g of EDTA and 0.4 L of distilled water on a magnetic stirrer, adjust pH to 8.0 with ~10 g of NaOH pellets (EDTA will not go into solution until the solution reach pH ~8.0), take the volume up to 0.5 L with distilled water, sterilize by autoclaving, and store at room temperature indefinitely.

- Glucose (1 M): Dissolve 9.91 g of *D*(+)-glucose monohydrate in ultrapure water to a final volume of 50 mL, sterilize through a 0.2-μm filter, and store at room temperature indefinitely.
- Glycerol for bacterial stocks: Aliquot 0.2 mL of 85% glycerol into 2-mL screw cap tubes, sterilize by autoclaving, and store at room temperature indefinitely.

Note: Glycerol is a viscous liquid; use slow aspiration and dispensing speeds to reduce pipetting errors.

- Kanamycin (50 mg/mL): Dissolve 0.5 g of kanamycin powder in 10 mL of ultrapure water, sterilize through a 0.2-μm filter, and store 1-mL aliquots at −20°C for up to 1 year.
- KOAc (1 M): Dissolve 4.91 g of KOAc in ultrapure water to a final volume of 50 mL, sterilize through a 0.2-μm filter and store at room temperature for up to 1 year.

Note: Used only in competent cell preparation (Method S1).

- MES pH 5.5 (1 M): Dissolve 9.762 g of MES powder in 40 mL of ultrapure water, adjust pH to 5.5 with 1 N KOH, take the volume up to 50 mL with ultrapure water, sterilize through a 0.2-μm filter, and store at room temperature in the dark for up to 6 months.
- $MgCl_2$ (2 M): Dissolve 9.52 g of $MgCl_2$ in ultrapure water to a final volume of 50 mL, sterilize through a 0.2-μm filter and store at room temperature indefinitely.
- $MnCl_2$ (2.5 M): Dissolve 9.90 g of $MnCl_2$ in ultrapure water to a final volume of 50 mL, sterilize through a 0.2-μm filter and store at room temperature indefinitely.

Note: Used only in competent cell preparation (Method S1).

- Rifampicin (50 mg/mL): Dissolve 0.5 g of rifampicin powder in 10 mL of DMSO, and store 1-mL aliquots at −20°C in the dark for up to 6 months.
- Tris-HCl pH 8.0 (0.1 M): Mix 6.06 g of Tris base and 0.4 L of distilled water on a magnetic stirrer, adjust pH to 8.0 with HCl, take the volume up to 0.5 L with distilled water, sterilize by autoclaving and store at room temperature for up to 1 year.

Note: Used only in competent cell preparation (Method S2).

- X-Gal (40 mg/mL): Dissolve 0.4 g of X-Gal powder in 10 mL of *N*,*N*-dimethylformamide, and store 1-mL aliquots at −20°C in the dark for up to 6 months.
- Media.

Lysogeny broth (LB)

Reagent	Final concentration	Amount
Tryptone	1.0%	10 g
Yeast extract	0.5%	5 g
NaCl	1.0%	10 g
Ultrapure water	–	Up to 1 L
Total	–	**1 L**

Note: Sterilize by autoclaving and store at room temperature indefinitely. Aliquot and supplement antibiotics as needed before use; handle in a microbiological cabinet to prevent contaminations.

Note: Add 15 g/L of bacteriological agar for solid medium preparation. Sterilize by autoclaving, cool the medium down to ~50–60°C and, in a microbiological cabinet, supplement

antibiotic solutions and mix vigorously. Aliquot into petri dishes, and once solidified store at 4°C in the dark.

Note: X-Gal solution can be spread onto LB agar plates before use.

Super optimal broth (SOB)		
Reagent	Final concentration	Amount
Tryptone	2.0%	20 g
Yeast Extract	0.5%	5 g
NaCl	10 mM	0.584 g
KCl	2.5 mM	0.186 g
$MgSO_4$	20 mM	2.4 g
Ultrapure water	–	Up to 1 L
Total	–	**1 L**

Note: Sterilize by autoclaving, and store at room temperature indefinitely.

Note: Handle in a microbiological cabinet to prevent contaminations.

SOC		
Reagent	Final concentration	Amount
SOB	–	9.8 mL
Glucose (1 M)	20 mM	0.2 mL
Total	–	**10 mL**

Note: Sterilize through a 0.2-μm filter and store at room temperature indefinitely.

Note: Handle in a microbiological cabinet to prevent contaminations.

SOB-Glycerol		
Reagent	Final concentration	Amount
SOB	–	164.7 mL
Glycerol (85%)	15%	35.3 mL
Total	–	**200 mL**

Note: Used only in competent cell preparation (Method S1).

Note: Glycerol is a viscous liquid; use slow aspiration and dispensing speeds to reduce pipetting errors.

Note: Sterilize by autoclaving, and store at room temperature indefinitely.

- Buffers.

5× TBE (Tris-borate-EDTA) buffer		
Reagent	Final concentration	Amount
Tris base	450 mM	54 g
Boric acid	450 mM	27.5 g
EDTA pH 8.0 (0.5 M)	10 mM	20 mL
Distilled water	–	Up to 1 L
Total	–	**1 L**

Note: Store at room temperature indefinitely. For agarose gel preparation and electrophoresis running, dilute 100 mL of the 5× TBE stock with distillate water up to 1 L.

Note: Premade 5× TBE solutions are commercially available.

Induction buffer		
Reagent	Final concentration	Amount
MES pH 5.5 (1 M)	10 mM	1 mL
$MgCl_2$ (2 M)	10 mM	0.5 mL
Acetosyringone (0.1 M)	150 μM	0.15 mL
Ultrapure water	–	Up to 100 mL
Total	–	**100 mL**

Note: Freshly prepared before use.

CCMB80 buffer		
Reagent	Final concentration	Amount
KOAc (1 M)	10 mM	5 mL
$CaCl_2$ (2.5 M)	80 mM	16 mL
$MgCl_2$ (2 M)	10 mM	2.5 mL
$MnCl_2$ (1 M)	20 mM	10 mL
Glycerol (85%)	10%	58.8 mL
Ultrapure water	–	Up to 0.5 L
Total	–	**0.5 L**

Note: Used only in competent cell preparation (Method S1).

Note: Glycerol is a viscous liquid; use slow aspiration and dispensing speeds to reduce pipetting errors.

Note: Adjust the pH of the final solution to 6.4 with 0.1 M HCl.

Note: Filter sterilize and store at room temperature indefinitely.

- Oligonucleotides: Custom DNA oligonucleotides are purchased desalted after synthesis as 100 μM solutions. Prepare working stocks at 10 μM (for PCR) and 2 μM (for cDNA synthesis) in autoclaved ultrapure water, and store at −20°C indefinitely.

⚠ CRITICAL: Ensure to avoid contaminations during the handling and preparation of oligonucleotide stocks.

STEP-BY-STEP METHOD DETAILS

Procedures of this protocol are applied to a scenario in which the researcher has access to biological samples as starting materials, as well as to an alternative one in which only a digital sequence of the virus genome is available (e.g., ancient or environmental samples, impossibility to obtain a physical sample from other researchers or countries, etc.).

Start from step 1 if a sample is available, or step 6 if the virus clone will be assembled using the SynViP synthetic genomics framework. The mini binary vector pLX-AS is used in both approaches (Figure 1). Steps common for the two above-mentioned scenarios are then described, which include full-length virus clone recovery from *E. coli* (steps 9–15), clone transformation into *Agrobacterium* cells and

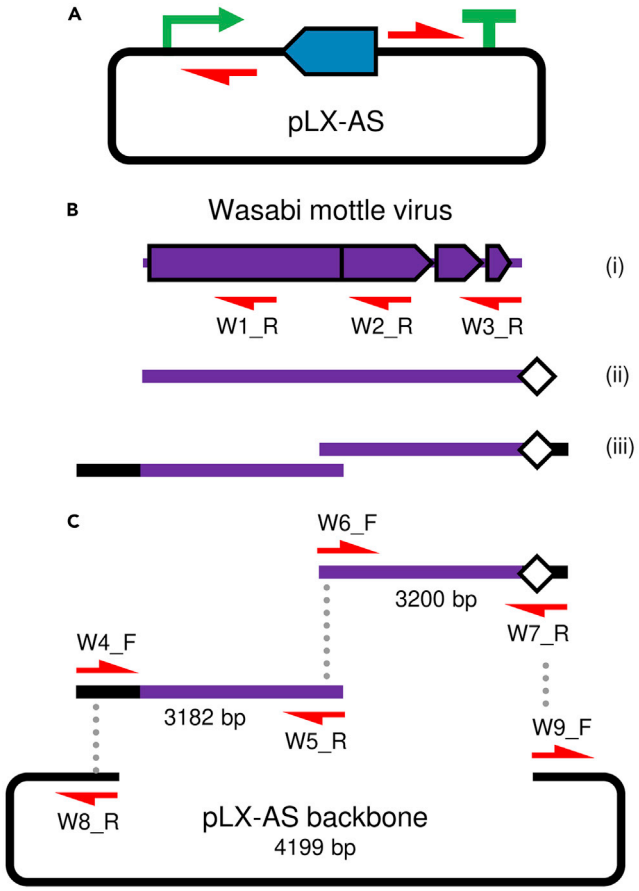

Figure 2. Homology-based clone assembly using biological material

(A) PCR primers for pLX-AS linearization are designed to allow functional linkage of the vector CaMV 35S promoter and nos terminator (green) with viral sequences.

(B) The RNA genomic sequence of wasabi mottle virus (WMoV) is retrieved and cDNA synthesis primers are designed (i), a ribozyme sequence is appended (diamond; ii), and RT-PCR products are designed to have terminal homology to each other and with the PCR-linearized pLX-AS.

(C) Amplicons are assembled *in vitro* by homology-based cloning; cloning primers are depicted and fragment sizes are indicated.

delivery to plant by agroinfection (steps 16–22), infectivity evaluation (steps 23 and 24), and, finally, complete sequencing of the assembled infectious clones and long-term storage (steps 25 and 26).

Homology-based clone assembly using biological material

Timing: 3–5 days

This section describes the use of homology-based assembly to generate a full-length clone of wasabi mottle virus (WMoV), a plant RNA virus of the family *Virgaviridae* (Figure 2). Genomic sequences and plant samples infected with WMoV were available. For additional details on the WMoV infectious clone assembly using biological material, please refer to Pasin et al. (2018).

⚠ CRITICAL: This procedure has been optimized for pLX-AS; use of alternative binary vectors is not recommended.

Note: NEBuilder® HiFi DNA assembly is used in this protocol for homology-based cloning. Gibson assembly can also be used (Gibson et al., 2009); the required enzymatic mix can be in-house prepared as detailed (Zhao et al., 2020), or purchased from commercial providers (e.g., Gibson Assembly® Master Mix, cat. no. E2611S, New England Biolabs). If Gibson assembly is used, no protocol changes are required and steps 1–5 should be followed as detailed herein.

Note: pLX-B2-TagRFP-T (GenBank: KY825142) was originally used for WMoV cloning (Pasin et al., 2018). pLX-AS is used herein since it includes the *E. coli LacZα* reporter for white-blue screen of recombinant clones (Figure 1).

1. Primer and cloning design (Figure 2).
   a. Retrieve the WMoV genomic sequence from NCBI (GenBank: AB017504, Wasabi mottle virus genomic RNA, complete genome, isolate: Tochigi).
   b. Import the sequence into a bioinformatics software suitable for nucleic acid sequence editing and cloning design.

   Note: Multiple bioinformatics tools for *in silico* cloning design are available (Baek et al., 2014).

   c. Primer design for cDNA synthesis (Figure 2B).
      i. Generate a WMoV genomic consensus by using GenBank: AB017504 as a query in a nucleotide BLAST (blastn) search against the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed August 2022), and running a multiple sequence alignment with the identified genomic sequences (i.e., AB017503, KJ207375, MK431779).

   ⚠ CRITICAL: Ensure that ~30 nt of AB017504 genomic termini are conserved and can be used for primer design.

      ii. Design the primer W3_R hybridizing to the very 3′ end of AB017504 (6268–6297 nt).
      iii. Design the primers W1_R and W2_R hybridizing to conserved genomic regions at internal positions (1740–1759 nt, 3927–3950 nt, respectively).

   Note: The virus-specific primers are designed to enhance efficiency of viral cDNA synthesis.

   d. *In silico* assembly of the WMoV clone in a T-DNA binary vector.
      i. In a bioinformatics software, append to the 3′ end of AB017504 the 46-nt ribozyme sequence from tobacco ringspot virus satellite RNA (GenBank: M14879): GTCACCG GATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAAC.

   Note: Ribozyme is included to remove non-viral nucleotides from the WMoV 3′ end after *in planta* transcription.

      ii. Append downstream the ribozyme sequence the 3-nt reverse complement of the WMoV 3′ terminus: TGG.

   Note: The sequence is included to promote ribozyme folding and processing.

      iii. Retrieve the sequence of the binary vector pLX-AS from NCBI (GenBank: MW281334, Cloning vector pLX-AS, complete sequence).
      iv. Import the pLX-AS sequence into a bioinformatics software suitable for *in silico* cloning, and replace the cloning cassette at positions 1204–1565 with the sequence designed in the step d.ii.
   e. Cloning primer design (Figure 2C).

i. For vector linearization, design the primer pair W8_R and W9_F, hybridizing to the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (nos) terminator of pLX-AS, respectively.

Note: The predicted amplicon size is 4199 bp.

ii. Design a first primer pair W4_F and W5_R for RT-PCR amplification of the WMoV genomic 5′ half.

Note: The predicted amplicon size is 3182 bp and includes a 25-bp overlap with the linearized pLX-AS.

iii. Design a second primer pair W6_F and W7_R for RT-PCR amplification of the WMoV genomic 3′ half.

Note: The predicted amplicon size is 3200 bp and includes a 27-bp overlap with the product of the previous step, and 21-bp overlap with the linearized pLX-AS.

Note: The ribozyme sequence is included in the cloning primers W7_R and W9_F, and reconstituted by correct clone assembly.

⚠ CRITICAL: The WMoV genome is ~6 kb and can be amplified by a single RT-PCR. It is however preferred to amplify it in two ~3-kb fragments (i) to limit DNA damage introduced during temperature cycling and increase amplification fidelity (Potapov and Ong, 2017), and (ii) to avoid amplification of defective viral molecules with correct termini but an incomplete genomic sequence.

f. Verify that the cloning design is correct by *in silico* simulation of a homology-based assembly reaction including the linearized pLX-AS and the RT-PCR fragments.

Note: Assembly can be simulated online using NEBuilder® assembly tool (https://nebuilder.neb.com, accessed August 2022).

Note: Correct assembly will result in a sequence equivalent to pLX-WMoV (GenBank: MH200603) with WMoV genomic variation specific to the Taiwanese isolate used as the starting material (6213/6297 identities to AB017504).

2. Viral cDNA fragment preparation.
   a. Collect material samples from a virus-infected plant, and grind them in liquid nitrogen in a pre-chilled mortal to a fine powder.

⚠ CRITICAL: In sample collection consider that some viruses may cause no clear infection symptoms, and be limited to specific plant organs or tissues.

⚠ CRITICAL: Handle liquid nitrogen in well-ventilated areas using protective clothing.

Pause point: Plant material powder can be stored for weeks at −80°C.

   b. Proceed with total RNA purification from ~100 mg of plant material powder using FavorPrep™ plant total RNA mini kit (FAVORGEN) per manufacturer's instructions, except that
      i. after washing, centrifuge columns to remove the residual liquid and then dry them by incubating at 60°C (5 min) with the tube cap open, and
      ii. use autoclaved ultrapure water preheated at 60°C for the final elution.

⚠ CRITICAL: Avoid contaminating nucleases that may compromise RNA integrity and yield of the subsequent cDNA synthesis reaction.

Note: Other commercial plant RNA purification kits can also be used.

Pause point: RNA samples can be stored for weeks at −80°C.

c. Determine RNA yield and purity using a spectrophotometer (e.g., NanoDrop™, Thermo Fisher Scientific).
d. Synthesis of viral genome cDNA.
   i. Prepare the cDNA synthesis mix.

cDNA synthesis mix

Reagent	Final concentration	Amount
First-Strand Buffer (5×) [a]	1×	2.0
dNTP Mix (10 mM each) [a]	0.5 mM each	0.5
DTT (0.1 M) [a]	0.05 M	0.5
Random hexamers (50 μM)	2.5 μM	0.5
W1_R (2 μM)	0.1 μM	0.5
W2_R (2 μM)	0.1 μM	0.5
W3_R (2 μM)	0.1 μM	0.5
RiboLock RNase inhibitor (40 U/μL)	2 U/μL	0.5
SuperScript™ III RT (200 U/μL)	1 U/μL	0.5
Total RNA	~100 ng/μL	~1 μg
Autoclaved ultrapure water	–	Up to 10 μL
Total	–	**10 μL**

[a]Supplied with the SuperScript™ III RT enzyme.

Note: Other commercial cDNA synthesis enzymes can also be used.

Note: A mix of random hexamers and the virus-specific primers are used to enhance recovery of the complete genomic cDNA.

   ii. Sequentially incubate the mix at 25°C (20 min), 55°C (2 h), 70°C (15 min), and on ice (2 min).
   iii. Dilute the cDNA synthesis reaction by adding 50 μL of autoclaved ultrapure water.

Pause point: Reactions can be stored for several days at −20°C.

e. RT-PCR amplification of viral cDNA fragments.
   i. Prepare the RT-PCR mixes to amplify the two WMoV genomic halves.

RT-PCR mix

Reagent	Final concentration	Amount
Q5® high-fidelity 2× master mix	1×	10 μL
Diluted cDNA	–	4 μL
Forward primer (10 μM)	0.75 μM	1.5 μL
Reverse primer (10 μM)	0.75 μM	1.5 μL
Autoclaved ultrapure water	–	Up to 20 μL
Total	–	**20 μL**

⚠ CRITICAL: Q5® high-fidelity DNA polymerase has an error rate ~280-fold lower than that of Taq DNA polymerase. Alternative PCR enzymes may work, but their use may affect efforts required to recover error-free clones and total costs.

Note: Use the primer pair W4_F/W5_R for WMoV genomic 5′ half.

Note: Use the primer pair W6_F/W7_R for WMoV genomic 3′ half.

ii. Incubate the mixes according to the RT-PCR cycling conditions.

RT-PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	98°C	5 min	1
Denaturation	98°C	10 s	30
Ramp	from 72°C to 56°C	−0.2°C/s	
Annealing	56°C	10 s	
Extension	72°C	3 min + 1 s/cycle	
Final extension	72°C	5 min	1
Final incubation	4°C	Hold	

Note: A temperature ramp is included to increase primer annealing specificity and secondary structure melting; alternative cycling conditions may work.

Pause point: Reactions can be stored for several days at −20°C.

3. pLX vector backbone preparation.
   a. Linearize the binary vector pLX-AS (Addgene cat. no. 188870).
      i. Prepare the inverse PCR reaction mix.

Inverse PCR mix

Reagent	Final concentration	Amount
Q5® high-fidelity 2× master mix	1×	10 μL
W9_F (10 μM)	0.75 μM	1.5 μL
W8_R (10 μM)	0.75 μM	1.5 μL
pLX-AS	–	~10 ng
Autoclaved ultrapure water	–	Up to 20 μL
Total	–	**20 μL**

⚠ CRITICAL: Q5® high-fidelity DNA polymerase has an error rate ~280-fold lower than that of Taq DNA polymerase. Alternative PCR enzymes may work, but their use may affect efforts required to recover error-free clones and total costs.

ii. Incubate the PCR mix tube according to the inverse PCR cycling conditions.

Inverse PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	98°C	5 min	1
Denaturation	98°C	10 s	28
Ramp	from 72°C to 58°C	−0.2°C/s	
Annealing	58°C	10 s	
Extension	72°C	3 min 40 s + 1 s/cycle	
Final extension	72°C	5 min	1
Final incubation	4°C	Hold	

Note: A temperature ramp is included to increase primer annealing specificity and secondary structure melting; alternative cycling conditions may work.

b. Remove the template plasmid of the PCR reaction.
   i. Prepared a DpnI digestion mix.

DpnI digestion mix		
Reagent	Final concentration	Amount
rCutSmart™ Buffer (10×) [a]	1×	0.5 μL
DpnI (20 U/μL)	0.6 U/μL	0.7 μL
Autoclaved ultrapure water	–	3.8 μL
PCR reaction	–	18 μL
Total	–	**23 μL**

[a]Supplied with the enzyme.

   ii. Incubate at 37°C (1 h), and on ice (2 min).

⚠ CRITICAL: DpnI cleaves only when its recognition site is methylated and specifically targets template plasmids purified from an *E. coli dam*+ strain but not PCR products, which significantly reduces the number of vector-only background colonies after of *E. coli* transformation (step 9).

Pause point: Reaction can be stored for several days at −20°C.

4. Gel purification of DNA fragments.
   a. Prepare agarose gel in 0.5× TBE buffer, and run electrophoresis of reactions from steps 2 and 3.

   Note: Agarose D1 medium EEO is used for gel preparation; 0.5% agarose gels provide good electrophoresis resolution of the fragments generated in this protocol and facilitate DNA purification from gel bands.

   b. Cut bands on a blue light transilluminator using single-use disposable scalpels.

   Alternatives: If a blue light transilluminator is not available a UV light transilluminator can used but ensure to avoid prolonged exposure to UV light as it damages DNA samples and dramatically reduces assembly efficiency. If UV light transilluminator is used, wear suitable protection personal protective equipment to avoid UV associated hazards.

   Alternatives: Gel cutting tips and other scalpel surrogates can be used.

   Pause point: Gel bands can be stored for several days at −20°C.

   c. Purify DNA from gel bands using FavorPrep™ MicroElute GEL/PCR purification kit (FAVORGEN). Follow kit manufacturer's instructions, except that
      i. wash columns twice with Wash Buffer,
      ii. after washing, centrifuge columns to remove the residual liquid and then dry them by incubating at 60°C (5 min) with the tube cap open, and
      iii. use autoclaved ultrapure water preheated at 60°C for the final elution.

   Note: Other commercial gel purification kits can also be used.

   d. Check 1 μL of the purified DNA fragments by agarose gel electrophoresis alongside DNA standards and estimate DNA fragment concentration.

Note: Spectrophotometer quantification may also be used.

Pause point: Gel-purified DNA can be stored for several days at −20°C.

5. *In vitro* homology-based assembly.
   a. Prepare a reaction mix including equimolar amounts of the gel-purified (RT-)PCR fragments from step 4 and NEBuilder® HiFi DNA assembly master mix according to manufacturer's instructions, except that a final reaction volume of 3–5 μL is used.

Note: The manufacturer's recommended total DNA amount is 0.2–0.5 pmol in a 20 μL reaction volume; scale-down the DNA amount according to the volume used.

Note: The NEBiocalculator® tool (https://nebiocalculator.neb.com, accessed August 2022) can be used for molarity calculations.

Note: If concentration of the gel-purified DNA fragments is below the recommended by the manufacturer, pool in a PCR tube equimolar amounts of all fragments needed for the assembly reaction in a final volume of 10–20 μL. To concentrate the sample, incubate the tube with the cap open at 65°C until ~2 μL are left (30–60 min; avoid tube drying). Take the tube to ice, add an identical volume (~2 μL) of NEBuilder® HiFi DNA assembly master mix, mix by pipetting and proceed with the next step.

   b. Incubate the mix at 50°C (1 h). At the reaction end, place tubes on ice and proceed with step 9.

Note: To save time, start preparing materials and equipment required for step 9 (Transformation of *E. coli* cells) before the reaction end.

SynViP clone assembly using chemically synthesized DNA fragments

Timing: 2–3 days (plus DNA synthesis time)

This section describes the use of the SynViP synthetic genomics framework to generate a full-length clone of turnip rosette virus (TuRV), a plant RNA virus of the family *Solemoviridae* (Figure 3). A revised, high-confidence sequence of the TuRV genome was reported (Ling et al., 2013; Sõmera and Truve, 2013). For additional details on the TuRV infectious clone assembly using SynViP and chemically synthesized DNA fragments, please refer to Pasin (2021).

⚠ CRITICAL: pLX-AS, the T-DNA binary vector used in SynViP, is compatible with digestion-ligation using AarI, its isoschizomer PaqCI® (cat. no. R0745S, New England Biolabs), or SapI (cat. no. R0569S, New England Biolabs) (Figure 1).

Note: Correct assemblies using up to 24 and 13 fragments have been reported by digestion-ligation with PaqCI® (AarI) and SapI, respectively (New England Biolabs, 2021a, 2021b).

Note: SynViP design and assembly using AarI is detailed herein; use of SapI is present for illustrative purposes in Method S3.

6. Synthetic DNA fragment design for seamless cloning (Figure 3A).
   a. Retrieve the TuRV genomic sequence from NCBI (GenBank: KC778720, Turnip rosette virus isolate TRoV-1, complete genome).
   b. Import the sequence into a bioinformatics software suitable for nucleic acid sequence editing and cloning design.

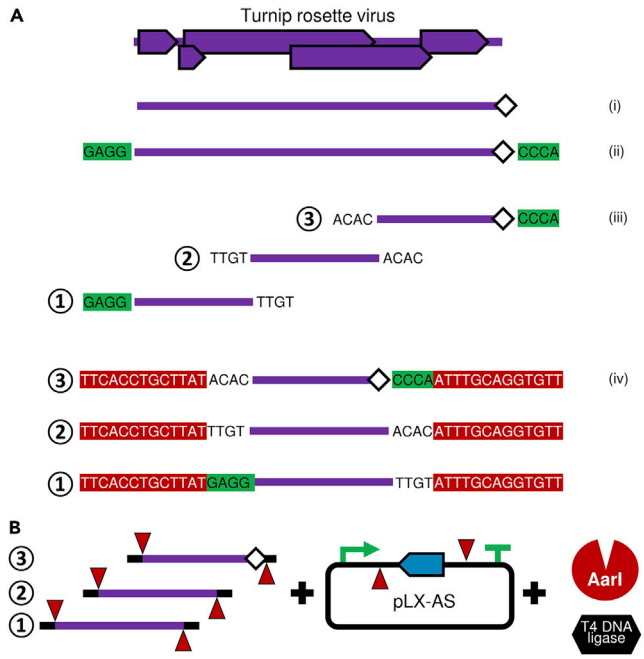

Figure 3. SynViP clone assembly using chemically synthesized DNA fragments

(A) The RNA genomic sequence of turnip rosette virus (TuRV) is retrieved and ribozyme sequence is appended (diamond; i); linkers (green) are added to allow directional cloning in AarI-digested pLX-AS and functional linkage of vector CaMV 35S promoter and nos terminator with viral sequences (ii); the obtained sequence is split in fragments with size < 1.8 kb and internal linkers for seamless cloning are added (iii); finally, each fragment is flanked with convergent AarI recognition sites (iv). Numbers indicate TuRV genomic fragments.

(B) The designed DNA fragments are chemically synthesized and assembled by a digestion-ligation reaction including pLX-AS, AarI and T4 DNA ligase; red triangles indicate AarI recognition sites.

Note: Multiple bioinformatics tools for in silico cloning design are available (Baek et al., 2014).

c. Append to the TuRV genome 3′ end the 46-nt ribozyme sequence from tobacco ringspot virus satellite RNA (GenBank: M14879): GTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGA GGACGAAAC.

Note: Ribozyme is included to remove non-viral nucleotides from the TuRV 3′ end after *in planta* transcription.

d. Downstream the ribozyme sequence, append the 9-nt reverse complement of the TuRV 3′ terminus: CAAAGACAG.

Note: The sequence is included to promote ribozyme folding and processing.

e. Inspect the obtained sequence for the presence of AarI and SapI sites. Consider the enzyme with no site or the lowest abundance for subsequent steps.

Note: AarI recognition site is CACCTGC(4/8).

Note: SapI recognition site is GCTCTTC(1/4).

Note: AarI and SapI are not palindromic; forward and reverse complement sequences should be inspected.

Note: If AarI and SapI show the same abundance, AarI is preferred given its higher efficiency in multiple fragment assembly.

f. Fragment design for AarI seamless cloning (Figure 3A).

⚠ CRITICAL: Use AarI since no recognition sites are present in the TuRV genomic sequence.

i. Append the sequence GAGG to the 5′ terminus, and CCCA to the 3′ terminus of the sequence from step 6.d.

Note: The appended sequences will allow assembly in AarI-digested pLX-AS and *in planta* generation of the correct TuRV 5′ end from the CaMV 35S promoter (Figure 1).

ii. Split the obtained sequence in fragments with a size < 1.8 kb (i.e., compatible with low-cost chemical DNA synthesis). To the 5′ end of each fragment, append 4 bp of the 3′ end of the preceding fragment (internal cloning linker).

Note: In SynViP assembly of TuRV, three fragments ranging from 1.2 to 1.6 kb are designed.

Note: To ensure efficient SynViP assembly, sequences of internal cloning linkers must be non-palindromic and have ≤ 50% identity to each other, and to GAGG and CCCA.

iii. Flank each of the obtained fragments with convergent AarI sites by appending the sequence TTCACCTGCTTAT to 5′ termini, and ATTTGCAGGTGTT to 3′ termini.
iv. Retrieve the sequence of the T-DNA binary vector pLX-AS from NCBI (GenBank: MW281334, Cloning vector pLX-AS, complete sequence).
v. Verify that the cloning design is correct by *in silico* simulation of an AarI-based digestion-ligation including the designed fragments and pLX-AS.

Note: Assembly can be simulated online using NEBridge™ Golden Gate assembly tool (https://goldengate.neb.com, accessed August 2022).

Note: Correct assembly will result in a sequence identical to pLX-TuRV (GenBank: MW281335).

7. Purchase and preparation of synthetic DNA fragments.
   a. Purchase Gene Fragments with adapters with sequences designed in step 6 from Twist Bioscience (U.S.A., https://www.twistbioscience.com, accessed August 2022).

⚠ CRITICAL: Twist Bioscience provides synthetic gene fragments at an industry leading price and error rate. Alternative products may work (e.g., gBlocks™ Gene Fragments, Integrated DNA Technologies; GeneArt™ Strings DNA Fragments, Thermo Fisher Scientific; etc.), but their use may affect efforts required to recover error-free clones and total costs.

⚠ CRITICAL: Ensure to purchase linear, uncloned DNA fragments; if cloned inserts are preferred, ensure to have them in vectors with ampicillin resistance to avoid interference with the SynViP assembly.

   b. Resuspend in the lyophilized synthetic DNA fragments according to manufacturer's instructions in autoclaved ultrapure water preheated at 60°C.
   c. Check 1 μL of each DNA fragments by agarose gel electrophoresis alongside DNA standards and estimate DNA fragment concentration.

⚠ CRITICAL: Supplied amount of synthetic DNA fragments is sufficient for a SynViP assembly reaction. Twist Gene Fragments are flanked by synthesis adapters with standard sequences, which can be used to design a primer pair for their PCR amplification if a larger amount is need.

Note: Spectrophotometer quantification may also be used.

Pause point: DNA fragments can be stored for several days at −20°C.

8. One-step SynViP assembly by AarI digestion-ligation (Figure 3B).
   a. Prepare the AarI digestion-ligation mix including pLX-AS (Addgene cat. no. 188870) and synthetic DNA fragments.

AarI digestion-ligation mix

Reagent	Final concentration	Amount
pLX-AS	–	~70 fmol [a]
Synthetic DNA fragments	–	~70 fmol/each
ATP (10 mM)	1 mM	1.5 μL
T4 DNA ligase (400 U/μL)	~20 U/μL	0.8 μL
AarI buffer (10×)	1×	1.5 μL
AarI primer (50×) [b]	1×	0.3 μL
AarI (2 U/μL)	~0.07 U/μL	0.5 μL
Autoclaved ultrapure water	–	Up to 15 μL
Total	–	**15 μL**

[a]~70 fmol pLX-AS is equivalent to 100 ng.
[b]Supplied with the enzyme.

Note: The NEBiocalculator® tool (https://nebiocalculator.neb.com, accessed August 2022) can be used for molarity calculations.

Alternatives: AarI can be replaced with its isoschizomer PaqCI® (cat. no. R0745S, New England Biolabs).

Alternatives: A master mix for AarI digestion-ligation is commercially sold as GeneArt™ Type IIs Assembly Kit Aar I (cat. no. A15916, Thermo Fisher Scientific).

   b. Incubate the mix according to digestion-ligation conditions.

Digestion-ligation conditions

Steps	Temperature	Time	Cycles
Initial digestion	37°C	15 min	1
Digestion	37°C	2 min	45
Ligation	16°C	5 min	
Final digestion	37°C	5 min	1
Final ligation	16°C	Hold	

Note: Digestion-ligation conditions are based on those reported by van Dolleweerd et al. (2018); alternative cycling conditions may work.

Assembly reaction transformation and clone selection in *Escherichia coli*

Timing: 3–5 days

In this section, *in vitro* assembly reactions from steps 5 or 8 are transformed into chemically-competent *E. coli* cells for high-efficiency transformation. Selected *E. coli* cells harboring the binary vectors with full-length virus genome copies are identified by white-blue selection and colony PCR, and verified by restriction enzyme digestion.

9. Transformation of *E. coli* cells.
   a. Thaw on ice aliquots of competent *E. coli* TOP10 cells (see Method S1).

   Note: Before starting ensure to have all the required materials and equipment at hand (a water bath at 42°C, LB agar plates with the appropriate antibiotics, etc.).

   Alternatives: A block heater with holds for 1.5–2.0 mL tubes can be used; add to each hold 1 mL of water to promote uniform and rapid heat transfer to the cell tubes.

   b. Mix a 100-μL cell aliquot with 2.5 μL of the assembly reactions from steps 5 or 8.

   Note: Prechill assembly reactions on ice prior to transformation.

   c. Sequentially incubate the mix on ice (30 min), at 42°C (1 min 10 s), and on ice (5 min).
   d. Add 0.7 mL SOC and incubate at 30°C, shaking at 250 r.p.m. (1.5 h).
   e. Harvest bacteria by centrifugation at 3000 × *g* (10 min) and resuspend in 50 μL of SOC.
   f. Plate the bacterial suspension onto LB agar plates supplemented with 50 mg/L kanamycin and 40 μL of X-Gal (40 mg/mL stock), and incubate at 30°C (20–48 h).

   ⚠ CRITICAL: Plate the X-Gal solution on agar plates before use for white-blue screen of recombinant clones.

   Note: Addition of isopropyl β-d-1-thiogalactopyranoside (IPTG), a common reagent for white-blue screens, is not required for this protocol.

   ⚠ CRITICAL: Reduce *E. coli* culturing temperatures from 37°C to 30°C to improve recovery and propagation of full-length virus clones.

   ⚠ CRITICAL: Bacterial cells harboring binary vectors with full-length virus clones generally display low growth rates; extend plate incubation time as needed.

   ⏸ **Pause point:** After colony appearance, plates can be stored for 1–3 days at 4°C.

10. Identification of recombinant binary vectors in *E. coli* transformants by colony PCR.
    a. Prepare 2 mL tubes with 0.3 mL of liquid LB supplemented with 50 mg/L kanamycin.
    b. Prepare a PCR master mix including virus-specific primers.

Colony PCR mix

Reagent	Final concentration	Amount
DreamTaq green PCR master mix (2×)	1×	5 μL
Forward primer (10 μM)	0.8 μM	0.8 μL
Reverse primer (10 μM)	0.8 μM	0.8 μL
Autoclaved ultrapure water	–	Up to 10 μL
Total	–	**10 μL**

Note: Use the primer pair W83_F/W2_R for pLX-WMoV clone identification; the 935-bp amplicon spans the assembly junction of the two viral RT-PCR inserts.

Note: Use the primer pair W154_F/W155_R for pLX-TuRV clone identification; the 538-bp amplicon spans the assembly junction of the two synthetic DNA fragments.

Alternatives: Other commercial PCR reagents can also be used; master mixes including dyes for direct gel loading are recommended to streamline the analysis.

c. Dispense 10-μL mix aliquots into sterile PCR tubes.
d. Screen white colonies selected in step 9.f.
   i. Pick an individual colony from the plate with a sterile P10 micropipette tip.
   ii. Soak the tip in the PCR mix tube for 5–30 s.
   iii. Directly place the tip in a 2 mL tube with LB.

⚠ CRITICAL: Avoid blue colonies.

⚠ CRITICAL: Pick small colonies if a mixture of small and large colonies appears during agar plate selection of transformed bacteria.

Note: It is recommended to screen 4–10 colonies per construct.

e. Incubate the PCR mix tubes according to the colony PCR cycling conditions.

Colony PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial denaturation	95°C	10 min	1
Denaturation	95°C	20 s	30 cycles
Annealing	58°C	25 s	
Extension	72°C	1 min 30 s	
Final extension	72°C	5 min	1
Final incubation	4°C	Hold	

⚠ CRITICAL: An extended initial denaturation step is used to ensure bacterial lysis.

Note: Alternative cycling conditions may work.

f. Incubate the LB tubes at 30°C, shaking at 250 r.p.m. until PCR results are available.
g. Resolve 5 μL of PCR reactions by agarose gel electrophoresis.

11. Culturing of positive transformants identified by colony PCR.
   a. Use LB cultures from step 10.f to individually inoculate 10 mL liquid LB with 50 mg/L kanamycin in 50 mL tubes.
   b. Incubate the tubes at 30°C, shaking at 250 r.p.m. (14–24 h).

Note: Extended culturing time may be necessary.

Note: It is recommended to purify plasmid DNA samples from 2–4 colonies per construct.

12. Glycerol stock preparation of *E. coli* transformants.
   a. Aliquot 650 μL of the culture from the previous step to 2-mL screw cap tubes containing ~200 μL of autoclaved 85% glycerol.

Note: The final glycerol amount is ~20%.

b. Mix by pipetting, freeze the tubes in liquid nitrogen, and store at −80°C indefinitely.

⚠ CRITICAL: Tightly close caps to prevent that liquid nitrogen leaks inside the tubes, which can lead to tube explosion while thawing due to nitrogen expansion.

13. Harvest bacteria from the culture remainder by centrifugation at 3000 × *g* (10 min); discard the supernatant, centrifuge again for 30 s and remove medium residues by pipetting.

Note: Harvested bacterial pellets can be frozen and stored at −80°C for weeks before to proceed with the next step.

14. Purify plasmid DNA from the harvested bacterial pellets using Wizard® Plus SV minipreps DNA purification system (Promega). Follow kit manufacturer's instructions, except that
    a. Use double volumes of resuspension, lysis and neutralization solutions supplied within the kit to improve clearing of bacterial lysates.

Note: After addition of the lysis solution incubate until the cell suspension clears (up to 10 min).

Note: Resuspension (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 μg/mL RNase A), lysis (0.2 M sodium hydroxide, 1% sodium dodecyl sulfate), and neutralization (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid) solutions can be prepared in house in case of run out of those supplied within the kit.

    b. Transfer ~800 μL of the cleared lysate to the column, centrifuge, discard the flowthrough and load again the same column with the remainder of the lysate.

Note: The volume of the cleared bacterial lysate will exceed the spin column capacity.

    c. Use autoclaved ultrapure water preheated at 60°C for the final elution.

Note: Other commercial plasmid DNA purification kits can also be used.

Pause point: Purified plasmids can be stored at −20°C indefinitely.

15. Identification of full-length virus clones by restriction enzyme digestion of plasmids.
    a. Prepare a digestion mix including enzymes and individual plasmid samples.

Digestion mix

Reagent	Final concentration	Amount
FastDigest green buffer (10×) [a]	1×	2 μL
FastDigest enzymes	–	0.5 μL/each
Plasmid DNA	–	~1 μg
Autoclaved ultrapure water	–	Up to 20 μL
Total	–	**20 μL**

[a]Supplied with the enzymes.

Note: The sequence of clones obtained from biological materials can differ from the theoretical one. Digest plasmids with at least two restriction enzymes to enhance the confidence of digestion results.

Note: Simultaneously use FastDigest BamHI, EcoRI, SalI, and SmaI for pLX-WMoV verification.

Note: Simultaneously use FastDigest EcoRI and Eco32I (EcoRV) for pLX-TuRV verification.

b. Incubate reactions at 37°C (30 min).
c. Resolve reactions by agarose gel electrophoresis.

Pause point: Plasmid DNA samples with the correct digestion profiles can be stored at −20°C indefinitely.

Agrobacterium-mediated inoculation

Timing: 4–7 days

In this section, binary vectors with the correct digestion profiles (or the expected virus genome size) are transformed into *Agrobacterium*. Selected bacterial cells harboring the binary vectors are then cultured and prepared for agroinfection, and used to inoculate *A. thaliana* plants.

⚠ CRITICAL: Ensure to coordinate preparation of host plants and *Agrobacterium* strains hosting the virus clones.

16. Transformation of *Agrobacterium* cells.
    a. Thaw on ice aliquots of competent *Agrobacterium* AGL1 cells (see Method S2).

⚠ CRITICAL: Before starting ensure to have all the required materials and equipment at hand (liquid nitrogen, a water bath at 37°C, LB agar plates with the appropriate antibiotics, etc.).

Alternatives: A block heater with holds for 1.5–2.0 mL tubes can be used; add to each hold 1 mL of water to promote uniform and rapid heat transfer to the cell tubes.

    b. Mix a 50-μL cell aliquot with ~0.5 μg of plasmid DNA.

Note: For each construct, use 2–4 plasmid samples obtained from individual *E. coli* colonies.

    c. Sequentially incubate the mix on ice (5 min), in liquid nitrogen (5 min), at 37°C (5 min), and on ice (5 min).

⚠ CRITICAL: Handle liquid nitrogen in well-ventilated areas using protective clothing.

    d. Add 0.7 mL of SOC and incubate at 28°C, shaking at 250 r.p.m. (2–4 h).
    e. Harvest bacteria by centrifugation at 3000 × *g* (10 min) and resuspend in 50 μL of SOC.
    f. Plate the bacterial suspension onto LB agar plates supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin, and incubate at 28°C (48–72 h).

Note: Bacterial cells harboring binary vectors with full-length virus clones generally display low growth rates; extended plate incubation time may be necessary.

Pause point: After colony appearance, plates can be stored for 1–3 days at 4°C.

17. (Optional) Confirm the presence of the desired binary vectors in *Agrobacterium* transformants by colony PCR as detailed in step 10, except that LB is supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin, and LB tubes are incubated at 28°C.
18. Pick individual colonies harboring the corresponding binary vectors and use them to inoculate 1 mL of liquid LB with 50 mg/L kanamycin and 50 mg/L rifampicin in 10 mL tubes; incubate tubes at 28°C, shaking at 250 r.p.m. (24–48 h).

⚠ CRITICAL: Pick small colonies if a mixture of small and large colonies appears during agar plate selection of bacteria.

Note: If *Agrobacterium* transformants were screened by colony PCR use LB tubes of positive colonies from the previous step as the inoculum source.

19. Prepare 20% glycerol stocks of *Agrobacterium* transformants as detailed in step 12.
20. Use 0.1-mL culture aliquots from step 18 to inoculate 5 mL of liquid LB with 50 mg/L kanamycin and 50 mg/L rifampicin in 50 mL tubes. Incubate tubes at 28°C, shaking at 250 r.p.m. (12–18 h).
21. Preparation of bacterial suspensions for agroinfection.
    a. Harvest bacteria by centrifugation at 3000 × *g* (10 min).
    b. Discard the supernatant, centrifuge again for 30 s and remove medium residues by pipetting.
    c. Resuspend the cell pellet in 2 mL of Induction Buffer (see media and solutions).
    d. Incubate at room temperature in the dark (3 h) for induction of bacterial genes required for T-DNA delivery to plant cells.
22. Plant agroinfection.
    a. Organize, label and water *A. thaliana* plants.

⚠ CRITICAL: Include untreated or mock-treated plants as infection controls.

    b. Measure in a spectrophotometer the optical density at 600 nm (OD_{600}) of the bacterial suspensions, and adjust OD_{600} to 2 with Induction Buffer.
    c. Dip the tips of dissecting forceps into the bacterial suspensions and then pierce young leaves of soil-grown *A. thaliana* plants.

Note: Flame sterilize forceps between suspensions of different bacterial clones.

    d. To avoid fast drying of bacterial suspension on the inoculated leaves, cover pot trays with transparent plastic lids overnight (~14 h).

Note: To avoid cross-contaminations, ensure that plants infected with different constructs do not touch each other and do not share the same watering tray.

Plant infection phenotyping and virus detection

Timing: 2–8 weeks

In this section, plants agroinoculated with full-length virus clones are monitored for appearance of infection symptoms. Biochemical assays are used to confirm virus accumulation.

23. Daily inspect agroinoculated plants and record appearance of infection symptoms.

Note: *A. thaliana* plants inoculated with pLX-TuRV will display severe symptoms characterized by a drastically reduced shoot and inflorescence growth starting after ~2 weeks (Figure 4A).

Note: Virus infection can be very mild or symptomless; *A. thaliana* plants inoculated with pLX-WMoV are almost indistinguishable from the mock control (Figure 4A).

24. Detection of viral nucleic acids in agroinoculated plants.
    a. Collect material samples from agroinoculated and control plants, and proceed with total RNA purification as detailed in steps 2.a–c.

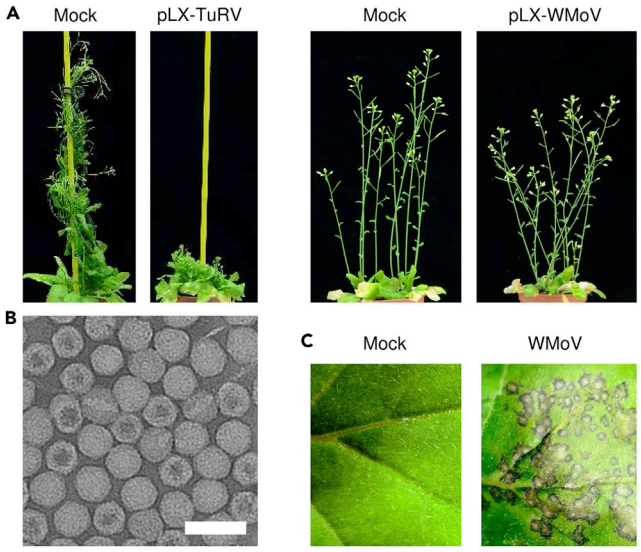

Figure 4. Plant agroinfection and virus recovery

(A) pLX-TuRV and pLX-WMoV were transformed into *Agrobacterium* and delivered to *Arabidopsis thaliana* by agroinfection, and images were taken 3–4 weeks post inoculation; mock condition is shown as a control.

(B) Transmission electron micrograph of virions purified from *A. thaliana* plants agroinoculated with pLX-TuRV; scale bar, 50 nm.

(C) Necrotic lesions in *Nicotiana benthamiana* leaves treated with crude extract of *A. thaliana* plants agroinoculated with pLX-WMoV; mock condition is shown as a control.

⚠ CRITICAL: Agrobacteria are present in inoculated leaves, which host plasmid DNA of the assembled virus clones. Viral RNA molecules and proteins are generated in inoculated cells from T-DNA transcription and translation in absence of viral replication. To prevent false positives, only upper uninoculated plant samples should be analyzed and agrobacteria contamination avoided.

b. Prepare a reaction mix to remove DNA contaminants from total RNA samples, and incubate it at 37°C (10 min), and on ice (2 min).

DNAse mix		
Reagent	Final concentration	Amount
ezDNase Buffer (10×) [a]	1×	0.5 µL
ezDNase	–	0.5 µL
Total RNA	–	~1 µg
Autoclaved ultrapure water	–	Up to 5 µL
Total	–	**5 µL**

[a]Supplied with ezDNase.

c. cDNA synthesis.

i. Prepare a cDNA synthesis mix including virus- and actin-specific primers.

cDNA synthesis mix		
Reagent	Final concentration	Amount
First-Strand Buffer (5×) [a]	1×	2.0 µL
dNTP Mix (10 mM each) [a]	0.5 mM each	0.5 µL
DTT (0.1 M) [a]	0.05 M	0.5 µL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
W2_R (2 μM)	0.1 μM	0.5 μL
W155_R (2 μM)	0.1 μM	0.5 μL
Q10_R (2 μM)	0.1 μM	0.5 μL
RiboLock RNase inhibitor (40 U/μL)	2 U/μL	0.5 μL
SuperScript™ III RT (200 U/μL)	1 U/μL	0.5 μL
DNAse-treated RNA	–	4.5 μL
Total	–	**10 μL**

[a]Supplied with the SuperScript™ III RT enzyme.

Note: The plant actin transcript is used as a template control.

Note: Other commercial cDNA synthesis enzymes can also be used.

ii. Sequentially incubate the mix at 55°C (1 h), 70°C (15 min), and on ice (2 min).
iii. Dilute the cDNA synthesis reaction by adding 50 μL of autoclaved ultrapure water.

Pause point: Reactions can be stored for several days at −20°C.

d. RT-PCR amplification.
i. Prepare RT-PCR mixes for virus- or actin-specific amplification.

RT-PCR mix		
Reagent	Final concentration	Amount
DreamTaq green PCR master mix (2×)	1×	5 μL
Forward primer (10 μM)	0.8 μM	0.8 μL
Reverse primer (10 μM)	0.8 μM	0.8 μL
Diluted cDNA	–	2 μL
Autoclaved ultrapure water	–	Up to 10 μL
Total	–	**10 μL**

⚠ CRITICAL: Carefully run (RT-)PCR assays to prevent false positive results due to contaminations with plasmid DNA of the assembled cloned.

Note: Use the primer pair W83_F/W2_R for WMoV detection.

Note: Use the primer pair W154_F/W155_R for TuRV detection.

Note: Use the primer pair Q9_F/Q10_R for the plant actin transcript detection.

Alternatives: Other commercial PCR reagents can also be used; master mixes including dyes for direct gel loading are recommended to streamline the analysis.

ii. Incubate the mixes according to the RT-PCR cycling conditions.

RT-PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	1
Denaturation	95°C	20 s	25
Annealing	58°C	25 s	

(*Continued on next page*)

Continued

Steps	Temperature	Time	Cycles
Extension	72°C	1 min 30 s	
Final extension	72°C	5 min	1
Final incubation	4°C	Hold	

Note: A low number of total cycles is used to prevent contaminant amplification; alternative cycling conditions may work.

iii. Resolve 5 μL of the reactions by agarose gel electrophoresis.

Note: 935-bp and 538-bp amplicons are respectively detected in WMoV- and TuRV-infected samples; the 125-bp actin amplicon is detected in all plant samples.

Optional: To complement phenotypic inspection and nucleic acid analyses of agroinoculated plants, additional assay can be used to detect virus proteins or particles (Figure 4B). Recovery of infectious virus particles from inoculated plants can be confirmed by mechanical back-inoculation to a suitable host or by leaf lesion assays in indicator plants (Figure 4C).

Verification by high-throughput sequencing and long-term storage of infectious clones

Timing: 3–6 days (plus plasmid shipping time)

In this section, complete sequence of plasmid DNA samples from step 14 and whose infectivity was confirmed (steps 23 and 24) is determined by high-throughput sequencing.

Some protocols adopt laborious multi-step cloning approaches that involve fully sequencing of cloning intermediates and final assembly of virus clones with a consensus genomic sequence. Since (i) the infectivity of consensus sequences or the impact of individual mutations on virus infectivity are difficult to know *a priori*, (ii) agroinfection is easily scalable to simultaneously evaluate multiple clones in parallel, and (iii) agroinfection is usually very efficient and allows to confidently discard clones with deleterious mutations that preclude virus infectivity, I recommend first to test infectivity of assembled full-length clones (steps 23 and 24) and then to fully characterize those with confirmed infectivity, i.e., only those useful in downstream applications.

25. Full-length sequencing of infectious clones.
    a. Place a service order for complete plasmid sequencing at MGH CCIB DNA core facility (U.S.A., https://dnacore.mgh.harvard.edu, accessed August 2022) and send a plasmid aliquot of identified infectious clones according to service provider's instructions.

⚠ CRITICAL: Complete sequencing of the cloned viral genomic sequence is critical for clone verification and subsequent viral vector engineering.

⚠ CRITICAL: Compared to Sanger sequencing, high-throughput plasmid sequencing by Illumina and other platforms requires no reference sequences, no custom primers or data analysis pipelines, avoids time-consuming primer walking strategies, and requires limited DNA amount that facilitates verification of plasmids with low/medium copy origin (which are used herein to enhance stability of the assembled virus clones).

Alternatives: Alternative facilities and companies commercially providing complete plasmid sequencing include seqWell™ (U.S.A., https://seqwell.com, accessed August 2022), and Plasmidsaurus (U.S.A., https://www.plasmidsaurus.com, accessed August 2022).

Note: Sequencing technologies and commercial services suitable for complete plasmid sequencing are rapidly evolving (Shapland et al., 2015; Emiliani et al., 2022); it is recommended to periodically evaluate options available in the market.

b. Record the sequences experimentally obtained in in-house and public databases (e.g., NCBI GenBank).

⚠ CRITICAL: Most commercial services return assembled full-length plasmid sequences as well as assembly statistics, and no bioinformatics skills are needed. Inspect and record any variation of the cloned viral genomic sequence compared to those assembled by *in silico* cloning.

26. Save for long-term storage and prepare backup copies of the *E. coli* glycerol stocks (step 12) and plasmid aliquots (step 14) of infectious clones.

EXPECTED OUTCOMES

The procedures described in this protocol have been successfully used to generate infectious clones of plant viruses using as starting materials either a biological sample or genomic sequences retrieved from public databases. Assembled clones are based on T-DNA binary vectors, and are suitable for *Agrobacterium*-mediated delivery to plants.

Besides its application for WMoV and TuRV clone assembly (Pasin et al., 2018; Pasin, 2021), this protocol was used to generate the first infectious clone of a member of the *Ipomovirus* genus, Ugandan cassava brown streak virus (Pasin et al., 2017). Agroinfectious clones were also obtained for the *Potyvirus* members pokeweed mosaic virus, tobacco vein mottling virus and soybean mosaic virus (Bao et al., 2020; Klenov and Hudak, 2021; Zhao et al., 2020), and for single- and double-stranded DNA viruses of *Geminiviridae* and *Caulimoviridae* (Pasin et al., 2018). SynViP-mediated assembly and rescue of cardamine chlorotic fleck virus, an RNA virus of the family *Tombusviridae* (Skotnicki et al., 1993), was achieved using as a genomic sequence reported in the 1990s and chemically synthesized DNA fragments (author's unpublished results).

The assembled infectious clones were used for biological characterization of plant virus genetics and virus-host interactions (Del Toro et al., 2022; González de Prádena et al., 2020; Hervás et al., 2020; Peng et al., 2022; Rodamilans et al., 2021; Valli et al., 2022), as well as for biotechnology applications including host gene silencing, recombinant protein overexpression and heritable CRISPR/Cas editing of plant genomes (Aragonés et al., 2022).

Finally, *Agrobacterium*-mediated transformation has been implemented for heterologous sequence delivery to many fungal species, and approaches described herein could be adapted for mycovirus characterization (Idnurm et al., 2017; Kondo et al., 2022).

LIMITATIONS

SynViP assembly requires the complete and correct sequence of the full-length virus genome, however sequences determined *de novo* or available in public databases may contain errors that compromise clone infectivity (Cooper, 2014). The homology-based clone assembly detailed here requires limited information of the virus genome sequence. In viruses with linear genomes, it is critical to know the correct sequence of ~ 30-nt terminal regions to recover full-length infectious clones. When dealing with novel viruses with divergent features not conserved in known viruses, it can be difficult to estimate the correctness and completeness of genome termini (Choi et al., 2022; Cobbin et al., 2021; Rose et al., 2019). In novel multipartite viruses, it can be difficult to know *a priori* the total number of genomic segments (Grigoras et al., 2009).

Agrobacterium-mediated delivery of plant virus infectious clones was successfully reported in monocots (Grimsley et al., 1987; Lu et al., 2012; Tzean et al., 2019), but the virus clones assembled according to this protocol have been only tested in dicots.

The protocol has not been tested for negative-stranded or double-stranded RNA viruses, for instance those of the families *Rhabdoviridae*, *Tospoviridae*, *Phenuiviridae*, *Fimoviridae* and *Reoviridae* (Kormelink et al., 2021; Li and Zhao, 2021; Zhang et al., 2021).

TROUBLESHOOTING

Problem 1

High background of vector-only colonies precludes identification of positive clones (steps 9 and 10), or no clone is recovered with the correct digestion profile (step 15).

Potential solution

Get familiar with DNA assembly tools and strategies for plasmid construct generation (Baek et al., 2014; Casini et al., 2015; Chao et al., 2015), and specifically with principles of *in vitro* homology-based assembly (Gibson et al., 2009), and seamless directional cloning using type IIS restriction enzymes (Cockrell et al., 2017; Marillonnet and Grützner, 2020).

Verify that the cloning strategy and materials (primers, synthetic DNA fragments, etc.) are correct.

Work in sterile conditions and avoid plasmid or bacterial contaminations.

Screen more colonies.

In homology-based clone assembly, reduce the amount of template vector used the inverse PCR (step 3.a), and extend DpnI digestion time (step 3.b).

In SynViP assembly, reduce total DNA amount, use more restriction enzyme units and/or extend incubation time at 37°C of the digestion-ligation (step 8).

After *E. coli* cell transformation incubate selection plates at 25°C to improve recovery of full-length virus clones (step 9.f); extended plate incubation time may be necessary.

Biological sequences can differ from those of public databases. In homology-based clone assembly using biological material, proceed with the infectivity evaluation (steps 16–24) if the insert size of the recovered clones is consistent with the expected full-length sequence of the virus genome.

Problem 2

None of the clones is infectious (steps 23 and 24).

Potential solution

Identify additional clones with full-length virus genomic copies (steps 10–15) and test their infectivity (steps 16–24).

Test clone infectivity using additional plant species as experimental hosts (step 22).

Co-express RNA silencing suppressors, and/or proteins implicated in virus replication and movement to enhance agroinfection efficiency (Chiba et al., 2006; Feng et al., 2020; Wang et al., 2015).

Confirm that sequences used in cloning design (steps 1 and 6) are correct and complete.

Problem 3
Virus clones are infectious in model plants but not in the natural host (steps 23 and 24).

Potential solution
Ensure to use a biological sample (step 2) or a reference sequence (step 6) obtained from virus-infected natural hosts and not an experimental one.

Test alternative *Agrobacterium* strains; use of hypervirulent strains can result in an ~1000-fold increase in agroinfection efficiency (Roemer et al., 2018).

Test alternative inoculation methods; purified plasmid DNA of virus clones can be mechanically inoculated to plants.

Problem 4
Sequencing of an infectious clone returns a sequence that is not identical to the theoretical one.

Potential solution
Biological sequences may differ from those of public databases. A varying number of mutations can be anticipated in homology-based clone assembly using biological material (steps 1–5). In SynViP assembly (steps 6–8), efforts required to recover error-free clones would depend on the DNA synthesis error rate of the chosen commercial supplier. For most research targets and applications, once the infectivity of the assembled clone is confirmed the presence of sequence variation has nonetheless minor or no impact. Record the experimentally retrieved sequence of the infectious clone in in-house and public databases.

Problem 5
Low yield of plasmid DNA from *E. coli* cultures.

Potential solution
Plasmid origins with a reduced copy number lessen foreign DNA loads, potential bacterial toxicity of the viral sequences and thus increase the stability of virus infectious clones (Pasin et al., 2019). The binary vectors used herein include by design a medium copy origin to enhance stability; higher culture volumes (steps 11, 13, and 14) should be used to reach yields obtained using with high-copy plasmids. The plasmid DNA amount recovered from step 14 is nonetheless sufficient for the procedures detailed in this protocol, that is restriction enzyme digestion verification (step 15), transformation of *Agrobacterium* cells (step 16), and verification by high-throughput sequencing (step 25).

Problem 6
Slow growth rates of bacteria harboring binary vectors with full-length virus clones.

Potential solution
For most applications, extended bacterial culturing time may solve the problem.

Expression in bacteria of genes encoding enzymatically active products with bacterial toxicity can cause a fitness cost (Rugbjerg and Sommer, 2019). To prevent undesired viral protein accumulation in bacteria, viral genes can be disrupted by insertion of eukaryotic introns that contain multiple stop codons; viral sequence will be reconstituted *in planta* by intron splicing (Johansen and Lund, 2008). Cryptic bacterial promoters can also be removed from viral sequences by targeted mutagenesis (Klenov and Hudak, 2021). Effectiveness of these approaches should be determined empirically.

Problem 7
Sequence alteration and degeneration of full-length infectious clones after propagation in bacteria.

Potential solution

Start experiments using a controlled plasmid aliquot from a backup stock prepared in step 26.

Bacterial cells harboring binary vectors with full-length virus clones generally display low growth rates; avoid contaminations with bacterial strains with fast growth rates. If a mixture of small and large colonies appears during agar plate selection pick small colonies for subsequent analysis.

Reduce of *E. coli* culturing temperatures from 37°C to 25°C–30°C to improve recovery and propagation of full-length virus clones.

Use *E. coli* strains suitable for cloning unstable DNA can be considered (Al-Allaf et al., 2013; Singh and Singh, 1995); commercially available strains include Stbl2™, Stbl3™ and Stbl4™ (Thermo Fisher Scientific), and SURE® (Agilent Technologies).

If the problem persists, engineer viral sequences by intron insertion or mutagenesis of cryptic bacterial promoters as to prevent accumulation of toxic viral products in bacteria that may lead to clone instability (Johansen and Lund, 2008; Klenov and Hudak, 2021; Tran et al., 2019). Effectiveness of these approaches should be determined empirically.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fabio Pasin (f.pasin@csic.es).

Materials availability

pLX-AS is available at Addgene with product no. 188870 (https://www.addgene.org/188870).

Data and code availability

This protocol does not disclose new data or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101716.

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AUTHOR CONTRIBUTIONS

The author conceived and wrote the manuscript.

DECLARATION OF INTERESTS

The author declares no competing interests.

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