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Short communication

A simple, rapid and efficient way to obtain infectious clones of potyviruses

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ABSTRACT

The availability of an infectious cDNA clone is a prerequisite for genetic studies on RNA viruses. However, despite important improvement in molecular biology techniques during the last decades, obtaining such clones often remains tedious, time-consuming and rather unpredictable. In the case of potyviruses, cDNA clones are frequently unstable due to the toxicity of some viral proteins for bacteria. The problem can be overcome by inserting introns into the viral sequence but this requires additional steps in the cloning process and depends on the availability of suitable restriction sites in the viral sequence or adjunction of such sites by mutagenesis. Homologous recombination in yeast rather than *in vitro* restriction and ligation can be used to build infectious clones or other viral constructs. This paper describes how, by using recombination in yeast and fusion PCR, infectious intron-containing clones were obtained within a few weeks for two strains of watermelon mosaic virus (WMV, *Potyvirus*), whereas previous attempts using "classical" cloning techniques had failed repeatedly. Using the same approach, intronless infectious clones of two other potyviruses, zucchini yellow mosaic virus (ZYMV) and papaya ringspot virus (PRSV), were obtained in less than two weeks.

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The molecular tools for manipulating RNA remain scarce. Infectious cDNA clones have therefore been developed in the last 30 years for genetic studies of plant RNA viruses. For potyviruses, that represent about 25% of all known plant viruses, the first infectious clone was obtained in 1989 (Domier et al., 1989). More than 20 years later, about 40 clones belonging to 15 different virus species are now available, and such tools are still missing for many agronomically important viruses. Among the difficulties relevant to the building of potyvirus infectious clones are the length of the viral RNA (about 10 kb, making one-step cloning poorly efficient), the highly deleterious effects of adding any extra nucleotides in the 5′ or 3′ extremity of the genome during the cloning process (Boyer and Haenni, 1994), and the toxicity of some viral proteins in *Escherichia coli*, resulting in spontaneous deletions of some clones in bacterial cells. The most toxic proteins appear to be P3 and CI encoded in the central region of the genome (Jakab et al., 1997; Chikh Ali et al., 2011). In order to overcome these difficulties, several techniques have been used, including cloning the genome in two halves that have to be religated *in vitro* before inoculation (Jakab et al., 1997), adding introns in the cDNA to prevent expression in *E. coli* (Johansen, 1996), or removing cryptic prokaryotic promoter elements in the P3 coding region by site-directed mutagenesis (Chikh Ali et al., 2011). One drawback of these techniques, besides the fact that they are time-consuming, is the risk of adding unwanted mutations in extensively manipulated clones, resulting in non-infectivity or impaired biological properties. To limit theses drawbacks, a simple protocol involving a very limited number of cloning steps and proven to be very efficient was developed.

Purified virions of strains WMV-FMF00-LL2 and WMV-FMF00-LL1 (Desbiez et al., 2007) obtained with standard protocols applied in the laboratory (Lecoq and Pitrat, 1985) immediately before use (in the case of FMF00-LL2) or kept in glycerol (1:1, v:v) at −20 °C for 2 years were used for the production of viral RNA. The virus particles were precipitated by ultracentrifugation for 2 h at 130,000 × *g* in a Beckman LE-80 ultracentrifuge with a 50.2Ti rotor, and resuspended in 0.02 M potassium phosphate buffer at pH 7.4. One microgram of purified virus was subjected to a Proteinase K treatment for 40 min at 37 °C (100 μM Proteinase K in buffer Tris 10 mM, EDTA 5 mM, SDS 0.5% pH 8), then to one phenol extraction followed by a phenol:chloroform:isoamylate (25:24:1) extraction, ethanol precipitation and resuspension in 20 μl RNAse-free water. Viral genomes were amplified in four overlapping fragments of 1.9–3.5 kb using specific primers with at least a 20-base overlap (Fig. 1 and Table 1). The primers corresponding to the 5′ and 3′ extremities of the genome contained an extra 20–30-base tail corresponding to the extremities of the vector (see below). Reverse transcription reactions were performed on 2 μl RNA, using 2.5 U AMV reverse transcriptase (Promega, France) in the manufacturer's buffer, 0.5 mM each dNTP, and 0.5 μM reverse primers WMV-A-RV, C-RV, E-RV and F-RV (Table 1). The reactions were performed for 1 h at 42 °C. Two microliters of each cDNA were used for PCR amplification using 1–2 U Pfu DNA polymerase (Promega, France)

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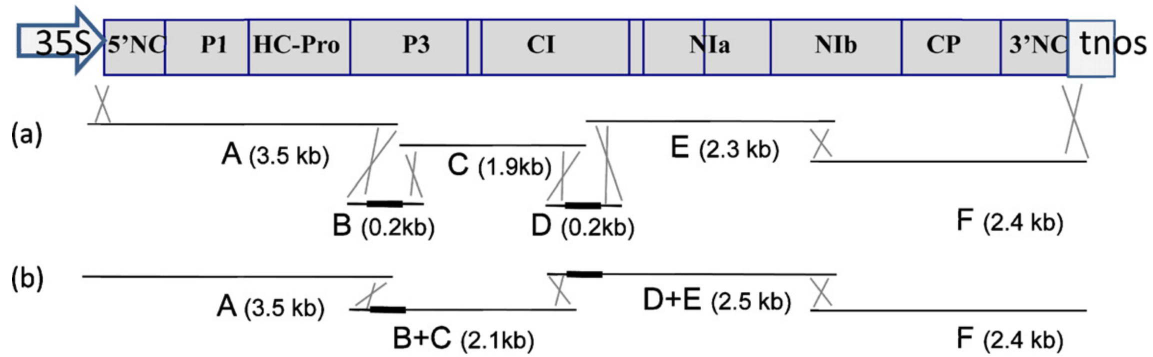

Fig. 1. Cloning strategy for WMV. (a) Cloning of WMV genome using homologous recombination in yeast, with 4 genomic fragments overlapping the complete genome (fragments A, C, E, F) and 2 introns (fragments B and D), in a shuttle vector containing a 35S promoter and nos terminator. (b) Introns B and D were fused to fragments C and E, respectively, by fusion PCR before cloning.

with 0.2 mM of each dNTP and 0.5 μM of each primer. PCR reactions were performed as follows: 5 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at 50 °C and 5 min at 72 °C. PCR fragments of the expected sizes were excised from 1% agarose gel under UV light and purified using standard protocols.

For WMV, numerous attempts had shown that viral clones were highly unstable in *E. coli* (Desbiez, unpublished), and the insertion of introns was chosen as a way to improve the stability of bacterial clones as used by Johansen (1996) for pea seedborne mosaic virus. Introns IV (189 bp) of the ST-LS1 gene from *Solanum tuberosum* ("Intron 1" in this work) and Intron 2 (221 bp) of the NiR gene from *Phaseolus vulgaris* (Johansen, 1996) were amplified from an intron-containing infectious clone of potato virus Y (PVY) SON41p (Moury et al., 2004), using primers "WMV-B-FW + WMV-B-RV" and "WMV-D-FW + WMV-D-RV" containing a 30-base tail complementary to the genome of WMV at positions 3541 and 5397 in the P3 and CI coding regions, respectively (Table 1). Amplified fragments B and D (corresponding to introns 1 and 2, respectively) were fused to fragments C and E, respectively, by fusion PCR (Fig. 1): 0.2 μl of fragments "B and C" and "D and E", respectively, were used in a mix containing 1–2 U Pfu in the manufacturer's buffer, 0.2 mM of each dNTP, 0.5 μM each of primers "WMV-B-FW + WMV-C-RV" and "WMV-D-FW + WMV-E-RV". The amplification conditions were as above with elongation times of 4 min. Fragments of the expected size were excised from agarose gels and purified.

To prepare the vector, a construct containing the full-length clone of PVY SON41p in pAGUS1 (Johansen, 1996; Moury et al., 2004), containing the 2 μ yeast replication origin and Trp-1 promoter and gene as a selectable marker (Sikorski and Hieter, 1989) was digested with PflFI and NcoI in order to excise almost completely the PVY insert. The 5.5 kb fragment corresponding to the vector was excised from an agarose gel and purified.

The diploid *Saccharomyces cerevisiae* strain YPH501 (YPH500xYPH499) (MATa/MATα ura3-52/ura3-52 lys2-801_amber/lys2-801_amber ade2-101_ochre/ade2-101_ochre trp1-Δ63/trp1-Δ63 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1),

Table 1
Primers used for PCR amplification of WMV, ZYMV and PRSV.

Primer	Position	Sequence
WMV-A-FW	1	*ATATAAGGAAGTTCATTTCATTTGGAGAGG*AAATTAAAACAACTCATAAAGAC
WMV-A-RV	3522	CTCGCACTGATGCAGTCCAAGC
WMV-B-FW	3514	GTGACCAAGCTTGGACTGCATCAGTGCGAGGTAAGTTTCTGCTTCTACC
WMV-B-RV	3544	GTAGCGTTGCTATGATAAATCTGACAAAACCTGCATATCAACAAATTTTG
WMV-C-FW	3541	CTCGCACTGATGCAGTCCAAGC
WMV-C-RV	5403	CTGATGTGGTATAGCCAACTTGTG
WMV-D-FW	5397	ATGCTACACAAGTTGGCTATACCACATCAGGTAAGTATGCACTTAAAGA
WMV-D-RV	5427	TCTTTCACTGATATCCATTGCCCGACAAACTGCATAATTTCAAAGATTG
WMV-E-FW	5428	TTTGTCGGGCAATGGATATC
WMV-E-RV	7763	ATTGAGCACCGACAGCAGC
WMV-F-FW	7659	TTCGAGAAGGCAGTCGAGGG
WMV-F-RV	10,017	*GCGAATCTAGA*TTTTTTTTTTTTTTTTTTTTAGGACAACAAACATTACCG
ZYMV-A-FW	1	*TATAAGGAAGTTCATTTCATTTGGAGAGG* AAATTGAAACAAATCACAAAG
ZY-3100-RV	3124	ACAAGTTCCGACGAGAGCC
ZY-3060-FW	3058	ATCACGACCCGAGTACATG
ZY-5370-RV	5370	GARTCAGCAATTCCAGCRAG
ZY-5080-FW	5081	GGAGTGCACGTTCAATGCCA
ZY-7550-RV	7531	CGTGTTTTGTTAGCCCTGAC
ZY-7500-FW	7481	AAGGGTCTGTGGAATGGATC
ZY-3NC-RV	9582	*GCGAATCTAGA*TTTTTTTTTTTTTTTTTTTTAGGCTTGCAAACGGAGTC
PRSV-A-FW	1	*ATAAGGAAGTTCATTTCATTTGGAGAGG* AAATAAAACATCTCAACACAAC
PRSV-2670-RV	2670	CTTRGGATCACCAGAATTACC
PRSV-2550-FW	2550	GGACCAATYACTCGTGAGTG
PRSV-5230-RV	5230	TCAAGTGTGACGCCATTCTC
PRSV-5210-FW	5210	AAGAAGCATTTCATAGTTGCCAC
PRSV-8020-RV	8020	GCTCCRACWGCTGCATCCAT
PRSV-7820-FW	7280	CCACTTATGGGTCACTAYATG
PRSV-3NC-RV	10,311	*GCGAATCTAGA*TTTTTTTTTTTTTTTTTTTTCTCTCATTCTAAGAGG

Underlined sequences correspond to the extremities of the introns. Sequences in italics correspond to the extremities of the vector.

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