Infectious clones of the crinivirus cucurbit chlorotic yellows virus are competent for plant systemic infection and vector transmission

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Cucurbit chlorotic yellows virus (CCYV), a recently identified bipartite crinivirus, causes economic losses in cucurbit plants. CCYV is naturally transmitted only by whitefly *Bemisia tabaci*. Here we constructed full-length cDNA clones of CCYV (RNA1 and RNA2) fused to the T7 RNA polymerase promoter and the cauliflower mosaic virus 35S promoter. CCYV replicated and accumulated efficiently in *Cucumis sativus* protoplasts transfected with *in vitro* transcripts. Without RNA2, RNA1 replicated efficiently in *C. sativus* protoplasts. Agroinoculation with the infectious cDNA clones of CCYV resulted in systemic infection in the host plants of *C. sativus* and *Nicotiana benthamiana*. Virus derived from the infectious clones could be transmitted between cucumber plants by vector whiteflies. This system will greatly enhance the reverse genetic studies of CCYV gene functions.

Received 4 March 2016
Accepted 8 March 2016

Cucurbit chlorotic yellows virus (CCYV) is a recently found cucurbit-infecting crinivirus within the family *Closteroviridae* (Abrahamian et al., 2012; Gu et al., 2011; Hamed et al., 2011; Okuda et al., 2010). The bipartite RNA genome (8607 nt RNA1 and 8041 nt RNA2) of CCYV is among the largest single-stranded positive-sense RNA viruses (Martelli et al., 2012). Among the whitefly species tested, the virus is transmitted solely by the sweet potato whitefly, the MEAM1 (formerly B biotype) and MED (formerly Q biotype) of the *Bemisia tabaci* complex in a semipersistent manner (Gyoutoku et al., 2009).

Recent studies on CCYV (Kubota et al., 2011; Okuda et al., 2013; Wang et al., 2014, 2015) were hampered by the lack of reverse genetic tools. Construction of full-length infectious cDNA clones will facilitate the investigation of viral determinants in virus replication and movement, as well as the interactions between viral proteins and host factors. Two major strategies can be used to construct infectious cDNA clones of plant viruses: (1) obtain infectious viral RNA via *in vitro* transcription of cDNAs fused to SP6, T3 or T7 promoters (Nagyová & Subr, 2007), or (2) produce *in vivo* transcripts under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Both strategies have been applied in studies involving members of the family *Closteroviridae* (Dolja & Koonin, 2013). To date, infectious cDNA clones have been obtained from three members of the genus *Crinivirus*: lettuce infectious yellows virus (LIYV), lettuce chlorotic virus (LCV) and tomato chlorosis virus (ToCV) (Chen et al., 2012; Wang et al., 2009; Orilio et al., 2014; Mongkolsiriwattana et al., 2011; Klaassen et al., 1996).

However, these infectious clones of criniviruses are unable to establish direct infection in their original hosts. Here, we developed full-length infectious cDNA clones for CCYV RNA1 and RNA2. The capped *in vitro* transcripts replicated in *Cucumis sativus* protoplasts. Furthermore, an agroinoculation system for delivering CCYV to host plants supported the systemic infection of CCYV in *Nicotiana benthamiana*, as well as in its natural host *C. sativus*, indicating a wide range of applications.

The strategy used for constructing full-length cDNA clones of RNA1 and RNA2 is outlined in Fig. 1. To generate full-length cDNA clones under the T7 promoter, the pUC18 vector was engineered to insert RNA1 at the *AflII* site and RNA2 at the *EcoRV* site immediately upstream of *XbaI* using primer pairs P1/P2 or P1/P3 (Table S1, available in the online Supplementary Material). Two fragments covering the full-length genomic sequence of RNA1 were amplified using primer pairs P4/P5 and P6/P7 (Table S1) and cloned into modified pUC18 vector to generate the full-length cDNA clone pCCYV13. Two fragments covering the full-length genomic sequence of RNA2 were amplified using primer
primer pairs P8/P9 and P10/P11 (Table S1) and cloned into modified pUC18 vector to generate the full-length cDNA clone pCCYV27 (Fig. 1a). Clones of RNA1 (pCCYV13) and RNA2 (pCCYV27) with the expected insert sizes were sequenced, and the correct insertion was confirmed. Sequence analysis revealed the presence of 28 nt that differed from the Beijing RNA1 (GenBank accession no. JQ904628) and RNA2 (GenBank accession no. JQ904629) isolates. The two clones of RNA1 (pCCYV13) and RNA2 (pCCYV27) were used for in vitro transcription reactions and subsequent protoplast transfection. Capped in vitro transcription reactions were performed according to the Ribomax large-scale RNA production system manual (Promega) using BamHI-linearized pUCCCYVRNA1 and XmaI-linearized pUCCCYVRNA2 as templates. Cucumber protoplasts were prepared and transfected using the methods described by Huang et al. (2013) with minor modifications. A total of 2 × 10^7 cucumber protoplasts were transfected with 20 % polyethylene glycol (PEG) using 5 µg of the in vitro transcripts. Reverse transcription (RT)-PCR were performed using primer sets P22/P23 and P24/P25 (Table S1). P22/P23 was designed to amplify the P22 coding sequence, and P24/ P25 was designed to amplify the coat protein (CP) coding sequence. RT-PCR and Western blot analysis using CP antisem confirmed infection with the cDNA clones (Fig. 2a, b). The results indicated that the nucleotide changes did not abolish the infectivity of pCCYV13- and pCCYV27-derived transcripts. Furthermore, Northern blot analyses were conducted using DIG-labelled specific RNA probes for RNA1 (566 nt within the P22 gene) and RNA2 (698 bp within the CP gene), which were synthesized using DIG RNA labelling mix (Roche) with specific primers P26/P27 and P28/P29 (Table S1). Consistent with LIYV (Klaassen et al., 1996; Wang et al., 2009), LCV (Mongkolsiriwattana et al., 2011) and ToCV (Orilio et al., 2014), CCYV RNA1 showed strong replication at 1-day post-infection (dpi) (Fig. 2c). Furthermore, time course accumulation of RNA1 and RNA2 determined at 0.5 h post-infection (hpi), 1 dpi and 2 dpi showed strong replication of CCYV RNA1 and RNA2 at 1 and 2 dpi (Fig. 2d).

To establish a CCYV agroinoculation system to deliver CCYV to host plants, the full-length cDNA sequences of CCYV RNA1 and RNA2 were inserted separately into the binary vector pCB301M to construct pCBCCYVRNA1 and pCBCCYVRNA2, respectively. The binary vector pCB301-2×35S-MCS-HDVaz-NOS (Yao et al., 2011) was engineered to insert the ApaLI restriction enzyme site using the primer pair P12/P13 to generate pCB301M. To create a binary construct for RNA1 agroinoculation, two fragments covering the full-length genomic sequence of RNA1 were amplified using primer pairs P14/P15 and P16/P17 (Table S1) and cloned into pCB301M to generate the full-length clone pCBCCYVRNA1. The full-length clone pCBCCYVRNA2 was constructed using the primer pairs P18/P19 and P20/ P21. pCBCCYVRNA1 and pCBCCYVRNA2 was transformed into Agrobacterium tumefaciens strain GV3101 by freeze-thaw transformation. In the constructed binary plasmids, the CaMV 35S promoter was positioned immediately

Fig. 1. Schematic representation of the construction of full-length cDNA clones of CCYV. (a) and (b) construction of full-length cDNA clones of RNA1 and RNA2 under the control of the T7 promoter and 35S promoter (spotted box). Rz, Ribozyme (black box); NOS, nopaline synthase polyadenylation signal (grey box).
upstream of the first nucleotides of CCYV RNA1 and RNA2 to direct the expression of the CCYV genome. In addition, the hepatitis delta virus (HDV) ribozyme sequence and the nopaline synthase (NOS) polyadenylation signal were positioned immediately downstream of the last nucleotides of CCYV RNA1 and RNA2 to generate authentic 3′ termini (Fig. 1b).

To test infectivity, A. tumefaciens strain GV3101 containing pCBCCYVRNA1 and pCBCCYVRNA2 were prepared and co-inoculated with A. tumefaciens transformed with pCBP1/HC-Pro [a silencing suppressor from sugarcane mosaic virus (SCMV)] at a ratio of 1 : 1 : 0.7, into the leaves of N. benthamiana at the four/five true leaf-stage and C. sativus at the cotyledon stage. At 30 days post-infiltration, leaves of C. sativus plants agroinfiltrated with these strains developed yellowing symptoms on older leaves, typical of CCYV infection in plants (Fig. 3a), whereas no symptoms were observed on N. benthamiana leaves, which was consistent with a previous report (Okuda et al., 2010). Total RNA was extracted from the systemic leaves of both N. benthamiana and C. sativus plants and analysed by RT-PCR using the primer pair P24/P25, which is specific to the CP coding sequence (Table S1). All samples displayed amplification products of the expected sizes (Fig. 3b). The amplification products were purified and sequenced, which verified CCYV infection. Western blot analysis confirmed the detection of CCYV CP protein in the systemic leaves of C. sativus (Fig. 3c). The infection rates for C. sativus and N. benthamiana varied in different experiments [67–100 % (3/3, 8/8, 2/3) for C. sativus; 82–83 % (5/6, 9/11) for N. benthamiana].

To test the transmissibility of the infectious clone-derived CCYV, cucumber plants were infiltrated with the infectious clones and maintained for 30 days for the infection to build up. The non-viruliferous adult MED whiteflies (formerly biotype Q) were fed on the infected plants for 24 h to acquire the virus. Subsequently, approximately 200 of the whiteflies were transferred to healthy cucumber plants for 24 h, after which they were killed by the pesticide imidacloprid at a concentration of 50 mg l⁻¹. Two weeks after this, CCYV infection was confirmed by RT-PCR (infection rate: 67–100 % (3/3, 8/8, 2/3) for C. sativus; 82–83 % (5/6, 9/11) for N. benthamiana).
8/8, 5/7) whereas control plants challenged with non-virulent whiteflies remained uninfected (0/8, 0/7). At 30 dpi, the whitefly-inoculated cucumber plants developed typical CCYV interveinal yellowing symptoms on older leaves (data not shown).

Unlike previously reported crinivirus infectious clones of LIYY, LCV and ToCV, which are fully active in the host plants after grafting or virion transmission by whiteflies from agroinfected N. benthamiana plants (Wang et al., 2009; Chen et al., 2012; Orílio et al., 2014), our CCYV infectious clone successfully established direct infection in the host plant C. sativus, as well as N. benthamiana, indicating a potentially wide range of host applications. To our knowledge, our infectious clone of CCYV represents the first report of successful infection of a crinivirus in the natural host plant and in N. benthamiana.

We developed two sets of full-length CCYV cDNA clones under the control of the T7 RNA polymerase and 35S promoters. The infectious clone of CCYV will greatly facilitate future studies investigating the infection process of CCYV in host plants and offers the potential for further defining viral gene functions.

Acknowledgements

Financial support was provided by Foundation for University Key Teacher by the Henan Province, China (2014GGJS-037) and Henan Key Laboratory of Fruit and Cucurbit Biotechnology (HNS-201508-9). We would like to thank Dr Xiaorong Tao for providing the binary vector.

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