Virus Research 169 (2012) 310–315

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Agroinoculation of the cloned infectious cDNAs of *Lettuce chlorosis virus* results in systemic plant infection and production of whitefly transmissible virions

Angel Y.S. Chen, Anita Pavitrin, James C.K. Ng*

Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, United States

ARTICLE INFO

Article history:
Received 1 May 2012
Received in revised form 4 August 2012
Accepted 9 August 2012
Available online 19 August 2012

Keywords:
Crinivirus
Bemisia tabaci
Nicotiana benthamiana
Agroinoculation
Binary plasmids
Transmission

ABSTRACT

Lettuce chlorosis virus (LCV) is a single stranded, positive strand RNA virus that is solely transmitted by specific whitefly vectors (*Bemisia tabaci* biotypes A and B) but not by mechanical leaf-rub inoculation. The roles of viral encoded proteins involved in the infection cycle of LCV have not yet been characterized due to the lack of reverse genetic tools. We present here a report of the successful development of an *Agrobacterium*-mediated inoculation system for the cloned cDNA constructs of LCV. The cDNAs of both LCV RNAs 1 and 2 were engineered into binary vectors in which the expression of LCV RNAs was regulated under a *Cauliflower mosaic virus* (CaMV) 35S promoter. In addition, by engineering the sequence elements of the *Hepatitis delta virus* ribozyme and the nopaline synthase 3′ untranslated region immediately downstream of the last nucleotide of LCV RNAs 1 and 2 in the binary vector constructs, the *in planta* produced LCV transcripts were expected to bear authentic 3′ termini. Both constructs were transformed into *Agrobacterium tumefaciens* cells and infiltrated in *Nicotiana benthamiana* plants. Three to four weeks post-agroinoculation, the *N. benthamiana* plants developed typical interveinal chlorosis and LCV infection was detected in the systemic leaves by reverse transcription-PCR. Virions purified from the LCV-infected *N. benthamiana* plants were flexuous rod-shaped and were transmissible by both *B. tabaci* biotypes A and B following membrane feeding. These results support the conclusion that *Agrobacterium*-mediated inoculation of LCV binary vectors in *N. benthamiana* plants results in LCV infection and the production of biologically active, whitefly transmissible virions. This system represents an important tool for use with reverse genetics designed for the study of LCV gene functions.

Members of the genus *Crinivirus* (family *Closteroviridae*) are damaging to many plant species and are distributed in different parts of the world (Navas-Castillo et al., 2011). In the case of disease caused by *Lettuce chlorosis virus* (LCV), a crinivirus that is endemic throughout South Western USA, susceptibility has been reported for a number of plant species, with most economic losses occurring for lettuce and sugar beet (Duffus et al., 1996; Wisler et al., 1997). The bipartite RNA genome (8591-nucleotide [nt] RNA 1 and 8556-nt RNA 2) of LCV, similar to those of other criniviruses, is among the largest and most complex of the single stranded positive-sense RNA viruses (Martelli et al., 2012). LCV RNA 1 contains two definitive open reading frames (ORFs), 1a and 1b, containing motifs of the viral replicase [papain-like leader (P-PRO), methyltransferase (MTR), helicase (HEL) and RNA-dependent RNA polymerase (RdRP)]. LCV RNA 2 contains up to 10 ORFs that are mostly homologous with ORFs of other criniviruses. ORFs that are unique to LCV, *i.e.* they do not show similarity with any known genes, are also present and they make up approximately 3.8% of its genome (Salem et al., 2009). Immunoblot and immunogold labeling transmission electron microscopy (TEM) analyses of *Lettuce infectious yellows virus* (LIYV), the type member of the genus *Crinivirus*, suggest that its genomic RNAs are encapsidated in filamentous capsids each composed of four LIYV RNA 2 encoded components: the heat shock protein 70 homolog (HSP70h), a 59 kDa protein (P59), the major coat protein (CP), and the minor coat protein (CPm) (Tian et al., 1999). Consistent with this observation are the results of immunoblot analyses of LCV, demonstrating that at least three LCV RNA 2 encoded proteins (CP, CPm and P60 [the P59 homolog of LIYV]) that are homologous to those of LIYV are present on the LCV virion (Ng and Chen, 2011).

As with all criniviruses, LCV is strictly restricted to the phloem of infected plants and requires specific phloem-feeding whiteflies to facilitate its transmission. These whiteflies belong specifically to the A and the B biotypes of the *Bemisia tabaci* species complex, being listed as one of the world's 100 worst invasive species (Brown et al., 1995; De Barro, 2008; De Barro et al., 2011; Duffus et al., 1996; Ng and Chen, 2011; Wisler et al., 1998). When either of the two *B. tabaci* biotypes is allowed to membrane-feed on

* Corresponding author at: Department of Plant Pathology and Microbiology, 900 University Ave., University of California, Riverside, CA 92521, United States. Tel.: +1 951 827 4223; fax: +1 951 827 4294.
E-mail address: jamesng@ucr.edu (J.C.K. Ng).

0168-1702/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2012.08.010

artificial diet augmented with virions purified from LCV infected plants, they readily transmit the virus upon being transferred to uninfected target plants, suggesting that plant-purified LCV virions are biologically active and contain all the essential components required for transmission by both *B. tabaci* biotypes (Ng and Chen, 2011).

We have recently constructed the cloned complementary (c)DNAs of LCV RNAs 1 and 2, whose *in vitro* produced transcripts were demonstrated to be biologically active when inoculated to tobacco protoplasts (Mongkolsiriwattana et al., 2011). Although protoplast inoculation is useful, it can be tedious to perform. In addition, it cannot address questions on viral processes that occur at the inter-cellular level, including inter-cellular trafficking and systemic virus movement. These viral processes are best addressed by using a plant inoculation system. However, the phloem-restriction of LCV and criniviruses poses a challenge to the use of reverse genetic approaches to studying viral gene functions directly in plants as mechanical leaf-rub inoculation of susceptible plants using the *in vitro* produced transcripts of the cloned cDNAs of these viruses do not result in successful plant infection. This limitation can be circumvented by the use of *Agrobacterium*-mediated transient expression (agroinoculation) to facilitate the direct inoculation of viral constructs to plants. Agroinoculation has been successfully developed for a number of phloem-limited plant viruses, including viruses in the families *Luteoviridae*, *Geminiviridae* and *Closteroviridae* (Ambros et al., 2011; Kheyr-Pour et al., 1994; Leiser et al., 1992; Nurkiyanova et al., 2000; Prokhnevsky et al., 2002; Stephan and Maiss, 2006; Yoon et al., 2011). So far, LIYV is the only crinivirus within the *Closteroviridae* for which an agroinoculation system has also been established (Wang et al., 2009). The work by Wang et al. (2009) reported that agroinoculation of LIYV WT constructs in *Nicotiana benthamiana* plants consistently resulted in systemic plant infection. Virions purified from the infected *N. benthamiana* plants have typical LIYV virion morphology and are transmissible to lettuce by *B. tabaci* biotype A (Wang et al., 2009). This system has also been used successfully in a number of follow-up studies to identify viral determinants involved in cytopathology *in planta* and in the whitefly transmission of LIYV (Chen et al., 2011; Stewart et al., 2010; Wang et al., 2009).

The purpose of this paper is to report the development of an LCV agroinoculation system, which represents only the second of such systems to be established for a crinivirus (following that of LIYV). We determined the feasibility of using this system to infect three different plant hosts: *N. benthamiana*, *Arabidopsis thaliana*, and *Lactuca sativa*. We also determined the biological activity of virions derived from agroinoculated *N. benthamiana* plants by studying their transmission by *B. tabaci* biotypes A and B.

To determine whether the cloned cDNAs corresponding to full-length LCV genomic RNAs 1 and 2 (Mongkolsiriwattana et al., 2011) were infectious at the whole plant level, these cDNAs were engineered into binary plasmids for *Agrobacterium*-mediated inoculation. The binary plasmid p35SLCVRNA1 was constructed by replacing the cDNA of LIYV genomic RNA 1 in the binary plasmid pJW100 (Wang et al., 2009) with that of LCV RNA 1, while p35SLCVRNA2 was constructed by replacing the cDNA of LIYV genomic RNA 2 in the binary plasmid pJW168 (Wang et al., 2009) with that of LCV RNA 2 (see Supplementary Figs. S1, S2 and Materials and Methods for the detailed construction procedures of p35SLCVRNA 1 and p35SLCVRNA2). In these constructs, the *Cauliflower mosaic virus* (CaMV) 35S promoter was positioned immediately upstream of the first nucleotide of LCV RNAs 1 and 2 to regulate the *in planta* expression of the LCV genome (Fig. 1). In addition, sequence elements of the *Hepatitis delta virus* (HDV) ribozyme and the nopaline synthase (NOS) 3′ untranslated region (3′ UTR) required for the self-cleaving activity and 3′ polyadenylation of the transcript, respectively, were juxtaposed immediately downstream of the last nucleotide of LCV RNAs 1 and 2 to ensure that the transcripts generated *in planta* bore authentic 3′ termini (Fig. 1).

We posit that binary plasmids p35SLCVRNA1 and p35SLCVRNA2 contained all the essential information required for biological activity *in planta*. To test this hypothesis, these plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 for the infiltration of *N. benthamiana* plants (Supplementary Materials and Methods). LCV RNAs 1- and 2-mediated induction of RNA silencing in agroinoculated *N. benthamiana* plants was a legitimate concern in light of results from a recent study aimed at identifying suppressors of RNA silencing in LCV (Kubota and Ng, unpublished). In that study, we found only one LCV protein, the LCV RNA 1 encoded P23 (among seven candidates evaluated), that showed RNA silencing suppressor activity. Namely, P23 was found to be a potent suppressor of systemic silencing but a weak suppressor of local silencing (approx. seven fold lower than that of the gene silencing suppressor [P1/HC-Pro] of *Turnip mosaic virus* [TuMV]). Thus, to overcome local gene silencing triggered by viral infection in agroinoculated *N. benthamiana* plants, p35SLCVRNA1- and p35SLCVRNA2-transformed *A. tumefaciens* were co-inoculated with *A. tumefaciens* transformed with pCB-P1/HC-Pro, a binary vector expressing P1/HC-Pro. Agroinoculation was performed at an ambient temperature of 25 °C using plants that had developed to the four/five true leaf-stage. Three to four weeks post-infiltration, the systemic (non-infiltrated) leaves of agroinoculated *N. benthamiana* plants developed interveinal chlorosis that became increasingly pronounced as the plants aged, typical of LCV infection in plants (Fig. 2A and B). Total RNA was extracted from the non-infiltrated leaves of all agroinoculated *N. benthamiana* plants and tested by RT-PCR using oligonucleotide primers, LCV70-PW and LCV71-PW, specific to a 964 bp region within the 3′ UTR of LCV RNA 2 (Supplementary Materials and Methods). Our results indicated that an RT-PCR amplified product of the expected size was obtained using the total RNA of non-infiltrated leaves of plants that showed chlorotic symptoms (Fig. 2C, lanes 1–3), but not from the total RNA of those that were asymptomatic (Fig. 2C, lane 4). These results suggested that interveinal chlorosis in the non-infiltrated leaves of agroinoculated plants were caused by systemic LCV infection. The infection rate varied; among four experiments performed, approximately 28–90% (29/32, 25/30, 9/32, and 12/32) of infiltrated *N. benthamiana* showed systemic LCV infection.

Virions produced in *N. benthamiana* plants inoculated with p35SLCVRNA1- and p35SLCVRNA2-transformed *A. tumefaciens* were prepared from systemic (non-infiltrated) leaves of infected plants approx. seven to 11 weeks post inoculation. When the resulting purified LCV virions were analyzed by SDS-PAGE and Coomassie blue staining, we observed that their staining properties were consistent with those of LCV virions maintained in *Chenopodium murale* plants by whitefly transmission (a.k.a. greenhouse maintained LCV) (Ng and Chen, 2011; data not shown). The concentration of purified virions ranged from 0.7 to 2.2 μg/μl, which corresponded to a yield of 0.8–2.2 μg of virions per gram of tissues. The yields were slightly lower compared to the 2–20 μg per gram of tissues typically seen in preparations performed using greenhouse maintained LCV (Ng and Chen, 2011). The lower virion yields of LCV was likely due to the fact that virion preparations were performed approx. 7–11 weeks after the agroinoculation of *N. benthamiana* plants. In contrast, greenhouse maintained LCV-infected *C. murale* plants used for virion preparations are typically harvested between two to three months after the whitefly transmission of LCV (Ng and Chen, 2011). LCV purification optimization studies have shown that virion preparations performed using two to three months old *N. benthamiana* plants infected with LCV by whitefly transmission typically resulted in virion yields that are comparable to those performed using similarly treated/infected *C. murale* plants