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Agroinoculation of a full-length cDNA clone of cotton leafroll dwarf virus (CLRDV) results in systemic infection in cotton and the model plant *Nicotiana benthamiana*

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ABSTRACT

Cotton blue disease is the most important viral disease of cotton in the southern part of South America. Its etiological agent, cotton leafroll dwarf virus (CLRDV), is specifically transmitted to host plants by the aphid vector (*Aphis gossypii*) and any attempt to perform mechanical inoculations of this virus into its host has failed. This limitation has held back the study of this virus and the disease it causes. In this study, a full-length cDNA of CLRDV was constructed and expressed *in vivo* under the control of cauliflower mosaic virus 35S promoter. An agrobacterium-mediated inoculation system for the cloned cDNA construct of CLRDV was developed. Northern and immunoblot analyses showed that after several weeks the replicon of CLRDV delivered by *Agrobacterium tumefaciens* in *Gossypium hirsutum* plants gave rise to a systemic infection and typical blue disease symptoms correlated to the presence of viral RNA and P3 capsid protein. We also demonstrated that the virus that accumulated in the agroinfected plants was transmissible by the vector *A. gossypii*. This result confirms the production of biologically active transmissible virions. In addition, the clone was infectious in *Nicotiana benthamiana* plants which developed interveinal chlorosis three weeks postinoculation and CLRDV was detected both in the inoculated and systemic leaves. Attempts to agroinfect *Arabidopsis thaliana* plants were irregularly successful. Although no symptoms were observed, the P3 capsid protein as well as the genomic and subgenomic RNAs were irregularly detected in systemic leaves of some agroinfiltrated plants. The inefficient infection rate infers that *A. thaliana* is a poor host for CLRDV. This is the first report on the construction of a biologically-active infectious full-length clone of a cotton RNA virus showing successful agroinfection of host and non-host plants. The system herein developed will be useful to study CLRDV viral functions and plant–virus interactions using a reverse genetic approach.

1. Introduction

Cotton (*Gossypium* spp.) is the most important fiber crop worldwide that sustains one of the world's largest industries (textiles) and also serves as a source of feed, foodstuff, oil and biofuel production (Sunilkumar et al., 2006). This crop is grown in more than 80 countries and its production worldwide was reported to be around 123 million bales during the 2011/2012 growing season (United States Department of Agriculture, 2012).

Cotton blue disease (CBD) is an important disease present in cotton crops in South America, Africa and Asia (Cauquil, 1977). CBD is a serious problem for cotton production in Argentina; importantly, its incidence is steadily increasing every year and this disease can reduce yield potential by up to 20%. Cotton leafroll dwarf virus (CLRDV) is the causal agent of CBD and is transmitted by the *Aphis gossypii* Glover (Cauquil and Vaissayre, 1971; Corrêa et al., 2005; Distéfano et al., 2010). The complete genomic sequence of an Argentinian isolate of CLRDV has been recently obtained. In addition, the difference in amino acid sequence identity levels of all CLRDV gene products with the species in the genus *Polerovirus* (greater than 10%) and the phylogenetic analysis strongly suggests that it should be classified as a member of a new species of the *Luteoviridae* family within the genus *Polerovirus* (Distéfano et al., 2010). The cotton plants affected by this disease show a moderate to

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severe stunting phenotype due to shortening of internodes, leaf rolling, vein yellowing and intensive dark green color of the foliage (Cauquil and Vaissayre, 1971). Viruses in the genus *Polerovirus* contain monopartite, single-stranded, positive-sense RNA genomes and are transmitted in a circulative, non-propagative manner by several aphid species (Harrison, 1984; King et al., 2011; Mayo and Ziegler-Graff, 1996). These viruses cannot be transmitted by mechanical inoculation (Mayo and D'Arcy, 1999) which represents a major problem in the study of these viruses. Poleroviruses exhibit a vascular tissue tropism limiting virus replication and movement to companion cells, phloem parenchyma cells and sieve tubes of specific host plants (Mutterer et al., 1999). Like other members of the genus *Polerovirus*, CLRDV is restricted to the phloem tissues in cotton (Takimoto et al., 2009) and additionally mechanical transmission to cotton plants has not been possible so far.

Infectious full-length cDNA clones have been obtained for several species of the *Luteoviridae* family, such as *Turnip yellows virus* (formerly known as beet western yellows virus-FL1), *Potato leafroll virus*, *Cucurbit aphid-borne yellow virus*, *Barley yellow dwarf virus-PAV* and *Cereal yellow dwarf virus-RPV* and agroinfection has been used as an alternative to aphids for introducing virus into plants *via* inoculation with *Agrobacterium* (Franco-Lara et al., 1999; Lee et al., 2005; Leiser et al., 1992; Prüfer et al., 1995; Veidt et al., 1992; Yoon et al., 2011). The use of full-length infectious clones, coupled with site-directed mutagenesis, facilitates reverse genetic studies to assay viral gene expression and their functions, virus replication and virus–host interactions; which will be helpful in better understanding the pathosystems. The study of CLRDV–cotton interaction remained so far extremely difficult without infectious clones.

In this study we successfully developed a full-length infectious cDNA clone from CLRDV, which was able to efficiently infect *G. hirsutum* and *N. benthamiana via Agrobacterium* without using aphids. Also, *A. thaliana* supported replication of the virus irregularly. We further determined the biological activity of virions derived from agroinoculated *G. hirsutum* plants by studying their transmission by *A. gossypii.*

2. Materials and methods

2.1. Synthesis of full-length CLRDV cDNA transcription vector

Isolated viral genomic RNA of the Argentinian isolate cotton leafroll dwarf virus (CLRDV) (accession number GU167940) was used as a template for the synthesis of cDNA as previously described (Distéfano et al., 2010). PCR amplifications were performed with specific primers using Platinum Pfx long DNA Polymerase (Invitrogen). The amplified products were purified using a QIAEX II Gel Extraction Kit (Qiagen), subsequently cloned into pGEM®-T Easy Vector (Life Technologies) or pCR® 2.1-Topo (Promega) and finally sequenced using an ABI 3730 XL automated sequencer. The final construct containing the full-length CLRDV cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter (hereafter referred to as 35S promoter) was prepared as follows. To construct a vector containing the 5′CLRDV proximal sequence flanked with the 35S promoter, we followed a PCR megaprimer approach. A first amplicon containing the 35S promoter sequence (401 bp) with the first 11th CLRDV nucleotides was produced using pBin61 as a template and oligonucleotides 1 (5′-CGTCGAC*CCCCTACTCCAAAAATGTCAAAG*-3′) and 2 (5′-**CGTTCTTTTGT***CCCTCTCCAAATGAAATGAAC*-3′) as primers. The non-viral *Sal*I restriction site (see underlined sequence) was incorporated into the oligonucleotide 1 to allow the cloning step. Viral genomic sequences within oligonucleotides are denoted in bold, whereas the 35S promoter sequence is indicated with italicized nucleotides. The second amplicon corresponded to the 5′CLRDV sequence (nucleotides 1–519) flanked with the last 6 nts of the 35S promoter and was produced using pGemt 5′CLRDV clone as a template (Distéfano et al., 2010) and oligonucleotides 3 (5′-*GAGAGGG***ACAAAAGAACGATAGAGGGG**-3′) and 4 (5′-CCGCGAGTGCAGAGATACTC-3′, nt 519–499) as primers. The two purified PCR fragments were mixed in equal amounts and used as templates to amplify PCR fragment A (Fig. 1) with primers 1 and 4. Fragment A, which contains the 35S promoter fused to the 5′CLRDV, was cloned into pCR® 2.1-Topo vector (Topo-A) and sequenced. To produce fragment B, a PCR amplicon was synthesized from CLRDV cDNA using primers 5 (5′-**GGCCGAGCGACCCGCGAAAG**-3′, nt 437–456) and 6 (5′-**CCCATTCTTGGTGATTCCGA**-3′, nt 4063–4082). Then, the amplicon was cloned into pCR® 2.1-Topo vector and sequenced. To produce AB fragment, the fragment B was released by digestion with *Xho*I and *Spe*I enzymes and was then subcloned into Topo-A (also previously digested with the same restriction endonucleases) resulting in Topo-AB. Another PCR fragment (fragment C) was synthesized from CLRDV cDNA using primers 7 (5′-**GGTCTTATTGGAGTTCAT**-3′, nt 3953–3972) and 8 (5′-**TGTTTAACGTCACAGGCTCG**-3′, nt 5779–5758). The purified fragment was cloned into pCR® 2.1-Topo and sequenced. To produce the last fragment (called D), a PCR was performed using pGemt 3′CLRDV clone as a template (Distéfano et al., 2010) and oligonucleotides 9 (5′-T**CTTCCTCTGACAAAGCTGA**-3′, nt 5586–5605) and 10 (5′-GTGGATCCTGTCGAC$(T)_{15}$**ACACCGAAACCCCA**-3′, nt 5866–5853) as primers. Non-viral *BamH*I and *Sal*I restriction sites were incorporated into the oligonucleotide 10 to allow the cloning step and, finally, 15 adenine residues (poly(A) tail) were added at the 3′ end of the cDNA to stabilize the messenger. No further transcription termination signal was added. Amplicon D was cloned into pGEM®-T vector (pGEM-D) and sequenced. To produce CD fragment, the released fragment C, after digestion with *Sac*I and *Hind*III enzymes, was subcloned into pGEM-D previously digested with the same restriction endonucleases; which resulted in pGEM-CD. Finally, to produce ABCD fragment, the released fragment CD, after digestion with *BamH*I enzyme, was subcloned into Topo-AB previously digested with the same restriction endonuclease, giving rise to Topo-ABCD; which contains the full-length copy of CLRDV cDNA under the control of 35S promoter (35S/CLRDV). The Topo-35S/CLRDV was completely sequenced using an ABI 3730 XL automated sequencer. For the agroinfection experiments, the 35S/CLRDV fragment was digested with *Sal*I endonuclease and subcloned into the *Sal*I site of the binary vector pBin19 to produce pBin19-35S/CLRDV (Fig. 1B).

2.2. Protoplast inoculation and Agrobacterium-mediated infection

BY2 N. benthamiana protoplasts were prepared from 4-days-old culture cells. A total of 10^6 protoplasts were electroporated with circular plasmid DNA Topo-35S/CLRDV (5 μg) (subjected to 100 Ω, 125 μF, 180 V) and incubated at 26 °C for 24–48 h as previously described (Gaire et al., 1999). Mock-inoculated protoplasts were used as a control. The recombinant vectors pBin19-35S/CLRDV and pBin19 were introduced into *A. tumefaciens* LBA4404 by electroporation (Mozo and Hooykaas, 1991). Agroinoculation experiments were carried out on *G. hirsutum* cultivar NC33B (two fully-expanded cotyledons and two emerging small true leaves stage), *N. benthamiana* (five to six leaves stage) and *A. thaliana* (Col-0 ecotype, six leaves stage). Plants were grown under 16 h/8 h light/dark conditions at 24 °C. Plant agroinfection was performed as described previously with minor modifications (Voinnet et al., 1998). A single *A. tumefaciens* LBA4404 colony containing a binary construct was grown in LB media supplemented with the antibiotics 100 μg/ml kanamycin and 100 μg/ml rifampicin for 48 h at 28 °C. Then, 7 ml