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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*

Verónica C. Delfosse[a], María F. Casse[b], Yamila C. Agrofoglio[a], Iván Bonacic Kresic[b], Horacio E. Hopp[a,c], Véronique Ziegler-Graff[d], Ana J. Distéfano[a,*]

[a] *Instituto de Biotecnología, CICVyA, CNIA, INTA, Buenos Aires, Argentina*
[b] *EEA Saenz Peña, INTA, Chaco, Argentina*
[c] *Facultad de Ciencias Exactas y Naturales, UBA, Buenos Aires, Argentina*
[d] *Institut de Biologie Moléculaire des Plantes, Laboratoire Propre du CNRS Conventionné avec l'Université de Strasbourg, Strasbourg, France*

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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

* Corresponding author at: Instituto de Biotecnología, CICVyA, CNIA, INTA, Los Reseros y Las Cabañas s/n (1686), Hurlingham, Buenos Aires, Argentina. Tel.: +54 11 4621 1447; fax: +54 11 4621 0199.
E-mail addresses: vdelfosse@cnia.inta.gov.ar (V.C. Delfosse), mfcasse@chaco.inta.gov.ar (M.F. Casse), yagrofoglio@cnia.inta.gov.ar (Y.C. Agrofoglio), ibonacic@chaco.inta.gov.ar (I.B. Kresic), ehopp@cnia.inta.gov.ar (H.E. Hopp), Veronique.Ziegler-Graff@ibmp-cnrs.unistra.fr (V. Ziegler-Graff), adistefano@cnia.inta.gov.ar, anajuliadistefano@gmail.com (A.J. Distéfano).

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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

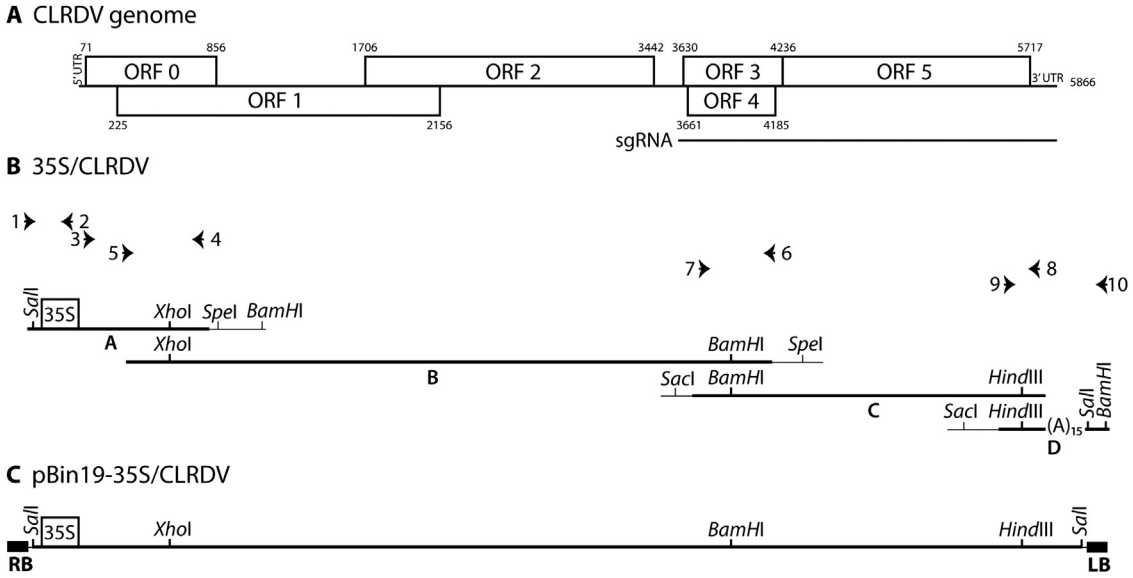

Fig. 1. Construction of the full-length cDNA clone of CLRDV. (A) Genome organization of CLRDV RNA. The rectangles indicate the predicted ORFs and the horizontal line below indicates the subgenomic RNA (B) PCR fragments A, B, C and D involved in the synthesis of the full-length cDNA. The arrows above indicate the primers used for PCR. Relevant restriction sites are *BamH*I, *Hind*III, *Sac*I, *Sal*I, *Spe*I and *Xho*I. The bold lines correspond to CLRDV cDNA sequences and the thin lines (not to scale) to the vector flanking sequences. (C) To construct the infectious full-length cDNA clone, the complete replicon was inserted into pBin19 vector, generating pBin19-35S/CLRDV.

of culture medium (LB media supplemented with 10 mM MES pH 5.7, 100 μg/ml kanamycin, 100 μg/ml rifampicin and 25 μM acetosyringone) were inoculated with 400 μl of the 48-h culture and grown at 28 °C overnight. The cells were collected by centrifugation, resuspended in infiltration buffer (10 mM MES, pH 5.7, 10 mM $MgCl_2$, 150 μM acetosyringone) at a final OD_{600} of 0.8 (*G. hirsutum* and *N. benthamiana*) or 1 (*A. thaliana*) and were incubated at room temperature for at least 3 h. Infiltration was carried out into the abaxial surface of plants leaves (*N. benthamiana* and *A. thaliana*) or cotyledons (*G. hirsutum*) using a 1 ml needleless syringe. Prior to the Agrobacterial infiltration, the underside of cotyledons of cotton plants were punched once or twice with a 25 G needle.

2.3. Aphids transmission test

Non-viruliferous *Aphis gossypii* were transferred to a pBin19-35S/CLRDV agroinfected plant showing typical blue disease symptoms. The aphids were maintained in contact with the agroinfected plants under greenhouse conditions for 15 days in order to acquire the virus. Subsequently, the aphids were transferred to 60 healthy young *G. hirsutum* cv. NC33B (approximately three aphids per plant) for an inoculation period of 10 days and then the aphids were eliminated by insecticide treatment. Finally, the development of symptoms was scored 4–5 weeks postinoculation and virus detection was performed 4 weeks postinoculation by RT-PCR.

2.4. Detection of viral RNA

Total RNA was extracted from 100 mg of frozen leaf material using RNAqueous small scale phenol-free total RNA isolation kit (Ambion) following the protocol provided by the manufacturer. Total RNA concentrations were estimated using spectrophotometer (NanoDropTechnologies) and then the RNA was treated with DNase I (Invitrogen). The synthesis of cDNA from 3 μg of total RNA was carried out using SuperScript III reverse transcriptase and random primers according to manufacturer's instructions (Invitrogen). The PCR assays for virus capsid RNA detection were performed with Platinum Taq DNA Polymerase (Invitrogen) and specific primers CP up (5′-ATGAATACGGTCGTGGGTAG-3′, nt 3630–3649) and CP low (5′-CTATTTTGGATTGTGGAATT-3′, nt 3235–3216). The PCR reaction was carried out with a denaturation step at 94 °C for 4 min followed by 40 cycles of 1 min at 94 °C, 1 min at 52 °C, and 1 min at 72 °C, with a final extension at 72 °C for 10 min. For northern blot analyses, 10 μg of total RNA isolated from *G. hirsutum* leaves at 6 weeks postinfection, from *N. benthamiana* leaves at 2 weeks (IL: infiltrated leaves) or 3 weeks (SL: systemic non-infiltrated leaves) postinfection, and 20 μg of total RNA isolated from *A. thaliana* leaves at 1 week (IL) or 4 weeks (SL) postinfection were resolved by denaturing 1% agarose-gel electrophoresis, transferred onto a Hybond N^+ membrane and finally hybridized with a radiolabelled capsid fragment to use as a probe employing the Prime-a-Gene Labeling System (Promega) in the presence of [α-32P] dCTP. The membrane was incubated for hybridization with ULTRAhyb solution (Ambion) at 42 °C overnight. The 5′ and 3′ termini of progeny viral RNA were analyzed by Rapid Amplification of cDNA Ends (RACE) (Frohman et al., 1988) employing a kit from Invitrogen. Briefly, the RNA was denatured and 3′ termini were polyadenylated using 5 U of poly(A) polymerase I (Ambion) before performing first-strand synthesis and amplification. For 3′ RACE, the specific CLRDV oligonucleotides were primer 19 (5′-AACCAACACGATCAATTTCA-3′ nt 5363–5382) and nested primer 20 (5′-AGAAGCCGTTCCAGTTGGGG-3′ nt 5536–5555) (Distéfano et al., 2010). For 5′RACE, the specific CLRDV internal antisense oligonucleotides were primer II (5′-CCGCGAGTGCAGAGATACT-3′ nt 519–500) and nested-specific primer III (5′-CGACCAAAGAGCGAATAGCA-3′ nt 480–461) (Distéfano et al., 2010). The amplified products were purified using a QIAEX II Gel Extraction Kit (Qiagen), cloned into pGEM®-T Easy Vector (Life Technologies) and sequenced using an ABI 3730 XL automated sequencer.

2.5. Protein extraction and immunoblot analysis

Total proteins were extracted from 0.1 g of CLRDV-aphid inoculated or pBin19-35/CLRDV-agroinfected *G. hirsutum* leaves after grinding to a powder in liquid nitrogen with one volume of 8 M urea/0.1 M Tris/0.2 M borate buffer (Schmidt and Wells, 1986).

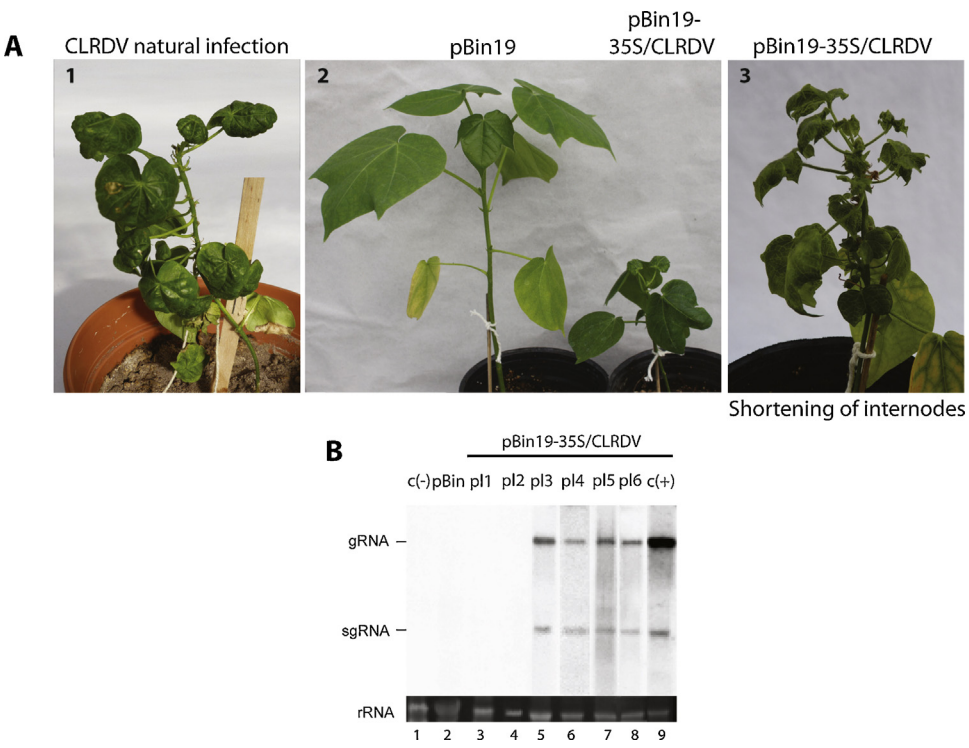

Fig. 2. Infectivity of pBin19-35S/CLRDV on *Gossypium hirsutum* mediated by agroinfiltration. (A) *G. hirsutum* NC33B plants infiltrated with *A. tumefaciens* culture containing the CLRDV genome in pBin19 vector at 6 weeks (panel 2) or 8 weeks (panel 3) postinfection. The empty vector pBin19 was used as a negative control (panel 2, left). *G. hirsutum* NC33B plants were infected by aphids that were fed on virus-infected cotton leaves (CLRDV natural infection) (panel 1). (B) Northern blot analysis of CLRDV RNA accumulation from systemic cotton leaves 6 weeks after infection. Total RNA extracted from pBin19-35S/CLRDV-agroinfected cotton plants (lanes 3–8) and from pBin19-inoculated plant (lane 2). Lane 1 contains total RNA from healthy cotton plant (negative control) and lane 9 contains total RNA from aphid-inoculated cotton plant (positive control). Viral genomic RNA (gRNA) and subgenomic RNA (sgRNA) were detected with ^{32}P-labeled DNA probe specific for the capsid gene. Ribosomal RNAs were stained with ethidium bromide and used as loading control (lower panel).

The extracts were centrifuged at 10,000 × *g* for 10 min at 10 °C to remove insoluble materials. Total proteins were extracted from 0.1 g of pBin19-35/CLRDV-agroinfected *N. benthamiana* and *A. thaliana* leaves after grinding to a powder in liquid nitrogen with one volume of 1× Laemmli buffer (Laemmli, 1970). Equalized loadings of protein extracts were electrophoresed on a 12% SDS-polyacrylamide gel and blotted onto Hybond ECL nitrocellulose membranes (Amersham Bioscience). The broad-range luteovirus monoclonal antibody 5G4 (AS-0227/1; DSMZ; an anti-BLRV CP antibody) was used to detect CLRDV CP at a dilution of 1/250. An anti-Xpress™ antibody (Invitrogen) against the recombinant CLRDV CP protein (pRSET-CLRDV P3) was also used at a dilution of 1/4000. An anti-mouse antibody conjugated to alkaline phosphatase (Sigma) was used as secondary antibody at a dilution of 1/5000.

3. Results

3.1. Construction of a full-length cDNA clone of cotton leafroll dwarf virus

The overall strategy employed for the construction of a full-length cDNA clone of CLRDV is described in detail under Section 2.1 and is also outlined in Fig. 1. Briefly, four large overlapping cDNA clones of an Argentinian isolate of CLRDV were constructed. To obtain an infectious clone suitable for the agroinfection technology, the full-length CLRDV cDNA was placed under the control of the CaMV 35S promoter and a poly(A) tail was added at the end of the cDNA to increase the stability of the transcript. No transcription termination signal was added in the construction. As the non-viral extensions in the 5′ terminus were reported to potentially interfere with transcript infectivity (Leiser et al., 1992; Veidt et al., 1992; Ziegler-Graff et al., 1988), the cloning strategy was chosen to minimize the non-viral residue, and therefore a single G was added at this extremity. So far the poly(A) tail at the transcript 3′ terminus has not been reported to be deleterious for the infectivity of a polerovirus transcript. To test the biological activity of the full-length cDNA, we analyzed the ability of 35S/CLRDV to replicate in tobacco *BY2* protoplasts. The construction proved to be infectious as revealed by the time course appearance of genomic and subgenomic viral RNAs detected in extracts of protoplasts by Northern blot (supplementary figure 1). The 35S/CLRDV containing the full-length copy of CLRDV cDNA was then successfully cloned into pBin19 vector (Fig. 1C).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.04.007.

3.2. Agroinfection with pBin19-35S/CLRDV in cotton plants

To analyze the biological activity of the full-length cDNA *in planta*, we tested the ability of pBin19-35S/CLRDV to agroinfect cotton plants. *G. hirsutum* NC33B plants were agroinoculated in the cotyledons with *A. tumefaciens* carrying the binary vector pBin19-35S/CLRDV, as described in Section 2.2. After 3–4 weeks, typical cotton blue disease symptoms such as leaf rolling and intensive dark green color of the foliage began to appear in the systemic leaves. These and other symptoms (shortening of internodes, stunting phenotype and vein yellowing) became more pronounced 6–8 weeks after infection (Fig. 2A and supplementary figure 2). In four different experiments, CLRDV infection was confirmed by the

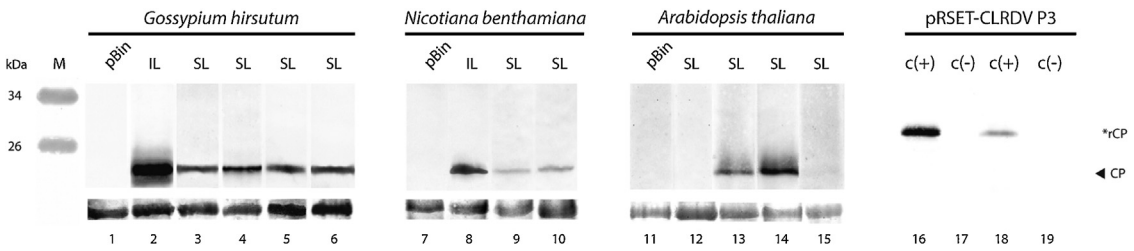

Fig. 3. Immunoblot analysis of P3 capsid protein from *G. hirsutum* NC33B, *N. benthamiana* and *A. thaliana* plants infected with CLRDV by agroinoculation. Total protein extracts from pBin19-inoculated plants (lane 1: cotton systemic leaves, lane 7: *N. benthamiana* systemic leaves and lane 11: *A. thaliana* systemic leaves), from pBin19-35S/CLRDV-agroinfected plants (lane 2: cotton cotyledon at 1 wpi, lanes 3–5: cotton systemic non-infiltrated leaves (SL) at 6 wpi, lane 8: *N. benthamiana* infiltrated leaves (IL) at 2 wpi, lanes 9 and 10: *N. benthamiana* systemic non-infiltrated leaves (SL) at 3 wpi and lanes 12–15: *A. thaliana* systemic leaves at 3 wpi) and from aphid-infected cotton plants (lane 6) were analyzed using an anti-BLRV CP monoclonal antibody (1:250 dilution). The CLRDV P3 capsid protein was identified as a 22.5 kDa protein (CP) (indicated with an arrow). IPTG-induced *E. coli* BL21 pLys cells lysates expressing pRSET-CLRDV P3 were used as a positive control (rCP, expected size: 28 kDa, indicated with an asterisk) (lanes 16 and 18) and IPTG-induced *E. coli* BL21 pLys cells lysates as a negative control (lanes 17 and 19) and visualized with BLRV CP monoclonal antibody (lanes 16 and 17) or anti-Xpress antibody (1/4000 dilution) (lanes 18 and 19). Total proteins stained with Ponceau S after electroblotting are shown below. M: the numbers indicate the molecular mass markers (in kDa).

detection of viral RNA by RT-PCR 3 weeks after inoculation in the systemic leaves of 53–92% of the plants inoculated with pBin19-35S/CLRDV (Table 1).

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Viral genomic RNA (gRNA) and subgenomic RNA (sgRNA) were detected by Northern blot hybridization of total RNA extracted from systemic leaves of pBin19-35S/CLRDV-infected plants that displayed symptoms (Fig. 2B, lanes 5–8) but not in plants without symptoms (Fig. 2B, lanes 3 and 4) or control plants (inoculated with pBin19) (Fig. 2B, lane 2). Next, total protein extracts from agroinfected cotyledon and systemic leaves were subjected to immunoblot analysis using a broad-range luteovirus monoclonal antibody produced against the bean leafroll virus (BLRV) capsid protein. A protein corresponding to the size of CLRDV P3 capsid protein (approximately 22.5 kDa) was detected in agroinfected cotyledons and symptomatic systemic leaves of agroinoculated *G. hirsutum* plants (Fig. 3, lanes 2–5) and in aphid-infected plants (Fig. 3, lane 6). Together these results demonstrate that the CLRDV cDNA clone was fully functional to produce a systemic infection in its host plant.

The CLRDV RNAs produced in agroinoculated *G. hirsutum* plants were expected to contain the 5′ termini from CLRDV (with only one extra G residue added at the beginning of the viral RNA) and a poly(A) tail. To confirm this, total RNA of systemically-infected leaves of two agroinoculated cotton plants was extracted and the 5′ and 3′ termini of progeny viral RNA were analyzed by Rapid Amplification of cDNA Ends (RACE). All the clones analyzed contained the expected 5′ and 3′ terminal sequences without any additional sequence from the 35S promoter at the 5′ termini or from the binary vector at the 3′ termini (data not shown).

Table 1
CLRDV infection efficiency mediated by agroinfiltration in *Gossypium hirsutum*, *Nicotiana benthamiana* and *Arabidopsis thaliana* plants.

Host	Experiment	Systemically infected plants[a]/infiltrated	Percent of infection
G. hirsutum	1	12/14	86%
	2	9/15	60%
	3	12/13	92%
	4	8/15	53%
N. benthamiana	1	2/7	28.6%
	2	4/6	66%
	3	3/6	59%
A. thaliana	1	3/4	74%
	2	1/4	25%

[a] Plants were analyzed by RT-PCR detecting viral capsid region.

To complete the characterization of the biological activity of CLRDV, we tested the aphid transmissibility of the viral progeny from agroinoculated *G. hirsutum* plants. Non-viruliferous *A. gossypii* were allowed to feed on agroinfected cotton plants (displaying CLRDV symptoms) and were then transferred to uninfected *G. hirsutum* NC33B plants. Virus transmission occurred at a rate of 12/60 (20%) and typical blue disease symptoms were observed. This transmission efficiency was consistent with those of the greenhouse aphid-maintained CLRDV isolate (Bonacic Kresic, unpublished results). We concluded that the full-length clone of CLRDV produces virions which are fully competent for aphid transmission and symptom development.

3.3. Agroinfection with pBin19-35S/CLRDV in model plants

In order to study whether the CLRDV replicon could infect model plant species, *N. benthamiana* and *A. thaliana* plants were inoculated with *A. tumefaciens* carrying the binary vector pBin19-35S/CLRDV. Of the agroinoculated *N. benthamiana* plants, 2/7 showed interveinal leaf yellowing symptoms in systemic non-infiltrated leaves (plants 2 and 3) 2 weeks after infection (Table 1 and Fig. 4A). CLRDV RNA was specifically detected by RT-PCR in non-infiltrated tissue of the same plants (plants 2 and 3) 3 weeks post-infiltration (data not shown). Northern blot analysis of the RNA from the infiltrated leaves (at 2 weeks p.i.) revealed the presence of viral genomic and subgenomic RNA (gRNA and sgRNA) in plants 2 and 3 and in another tested plant (plant 1) from experiment 2 (Table 1 and Fig. 4B). However, when systemic non-infiltrated leaves were analyzed (at 2 and 3 weeks p.i.) only one of the three PCR-positive plants showed viral RNA (plant 2) (Fig. 4B). In addition, the CLRDV P3 capsid protein was detected in inoculated (plant 2 was shown) and systemic leaves (plants 2 and 3) by immunoblot analysis (Fig. 3, lanes 8–10). In two other experiments, 4/6 and 3/6 of the agroinoculated *Nicotiana* plants became systemically infected as tested by RT-PCR on the upper non-infiltrated leaves (Table 1).

When *A. thaliana* plants were challenged with *A. tumefaciens* carrying pBin19-35S/CLRDV, no viral symptoms were observed 2 weeks after infection and all the plants remained symptomless even after 6 weeks p.i. Systemic non-infiltrated leaves were analyzed by RT-PCR after 3 weeks post-infiltration and CLRDV RNA was detected in systemic leaves of three *A. thaliana* plants out of four tested and in 1/4 plants in a second experiment (Table 1 and supplementary figure 3). Northern blot analysis of the RNA from the infiltrated leaves of three PCR-positive plants (at 1 week p.i.) revealed the presence of viral genomic and subgenomic RNA (Fig. 4C, lanes 2–4). When systemic non-infiltrated leaves were analyzed, no viral RNA could be detected at 3 weeks p.i. (data not

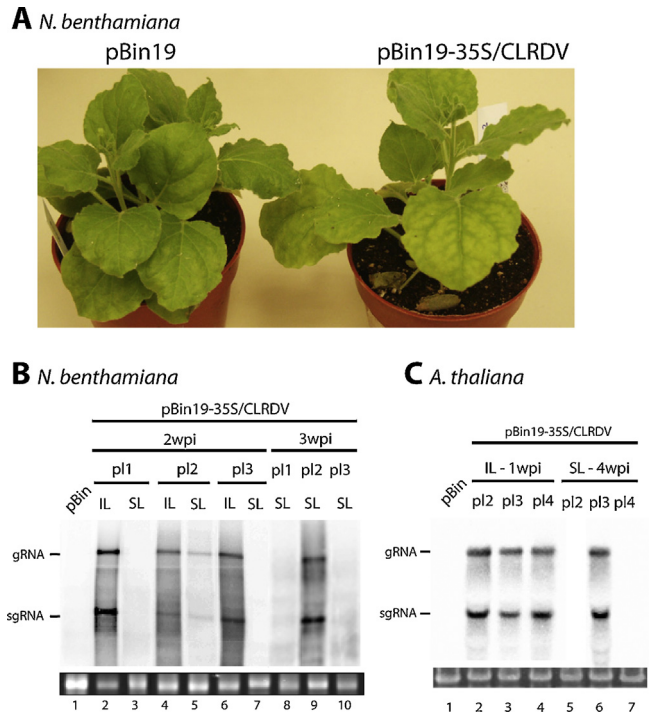

Fig. 4. Infectivity of pBin19-35S/CLRDV on *N. benthamiana* and *A. thaliana*. (A) *N. benthamiana* plants infiltrated with *A. tumefaciens* culture containing pBin19-35S/CLRDV (right) or an empty vector pBin19 (left) at 3 weeks postinfection. (B) Northern blot analysis of CLRDV RNA accumulation from infiltrated (IL) and systemic non-infiltrated *N. benthamiana* leaves (SL), 2 and 3 weeks post infection (wpi). Total RNA extracted from pBin19-35S/CLRDV-agroinfected plants (lanes 2–10) and from pBin19-inoculated plant (negative control, lane 1). Viral genomic RNA (gRNA) and subgenomic RNA (sgRNA) were detected with ^{32}P-labeled DNA probe specific for the viral capsid. Ribosomal RNAs were stained with ethidium bromide and used as loading control (lower panel). (C) Northern blot analysis of CLRDV RNA accumulation from infiltrated (IL) and systemic non-infiltrated (SL) *A. thaliana* leaves, 1 and 4 weeks post infection (wpi). Total RNA extracted from pBin19-35S/CLRDV-agroinfected plants (lanes 2–7) and from pBin19-inoculated plant (negative control, lane 1). Viral genomic RNA (gRNA) and subgenomic RNA (sgRNA) were detected with ^{32}P-labeled DNA probe specific for the capsid gene. Ribosomal RNAs were stained with ethidium bromide and used as loading control (lower panel).

shown) but both genomic and subgenomic RNAs were detected in a single plant at 4 weeks p.i. (Fig. 4C, lane 6). In addition, the CLRDV P3 capsid protein was detected in systemic leaves of some positive RT-PCR plants at 3 weeks p.i. by immunoblot analysis (Fig. 3, lanes 13 and 14).

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These results show that the full-length cDNA clone of CLRDV could be delivered by agroinoculation to the non-natural host *N. benthamiana* where it was able to replicate and move systemically. Although CLRDV cDNA clone was able to multiply in inoculated *A. thaliana* leaves, the infection of systemic leaves was inefficient.

4. Discussion

The construction of a full-length infectious clone of CLRDV associated to an efficient inoculation method provide an excellent tool to investigate virus–host interactions through mutated or recombinant versions of the virus. Our data support that CLRDV infection, including viral replication, cell-to-cell and systemic movement, can be achieved by the use of agrobacterium-mediated inoculation of the full-length CLRDV cDNA controlled by the 35S promoter. In this work, the host range of CLRDV has been examined by means of CLRDV-agroinfection. This system led to systemic infections in its natural host *G. hirsutum*. Interestingly, it also produced systemic infection in the model plant *Nicotiana benthamiana*. This result extends the knowledge of the plant species that could be systemic CLRDV hosts.

The biological activity of the progeny virus derived from the infectious cDNA clone of CLRDV appeared to be similar to that caused by the wild type virus. The particles of CLRDV assembled in the leaf tissue of agroinfected cotton plants were acquired and transmitted by the aphid *A. gossypi* to new cotton plants where typical CBD symptoms developed. These results highlight the potential use of CLRDV-agroinfection instead of the aphid transmission, which is the current method for CLRDV inoculation (Casse et al., 2011; Distéfano et al., 2010). The maintenance of healthy aphids colonies is not only labor intensive; the transmission rates are generally lower than the infection efficiency obtained with agroinfection of infectious clones.

CLRDV agrobacterium-mediated inoculation was also tested on the non-natural hosts *N. benthamiana* and *A. thaliana*. The level of accumulation of viral RNA and P3 capsid protein was higher in the infiltrated *N. benthamiana* leaves than in the systemic leaves (Figs. 3 and 4B). As previously described, the difference in viral RNA accumulation levels is presumably because the agroinfiltration is more efficient in transferring the DNA to almost any cells in the infiltrated zone (Llave et al., 2000; Pfeffer et al., 2002), whereas virus infection in the upper leaves is confined to cells of the phloem compartment. *N. benthamiana* has been extensively used for infection of *Potato leafroll virus*, *Turnip yellows virus* and more recently *Barley yellow dwarf virus-PAV* and *Cereal yellow dwarf virus-RPV*, although the last two species do not normally infect dicotyledon plants (Franco-Lara et al., 1999; Leiser et al., 1992; Yoon et al., 2011). In this study, we determined that this species is also a host for CLRDV showing a quite high rate of infection.

It has been previously reported that heterologous virus gene silencing suppressors can enhance the infectivity of infectious clones of several viruses in *Nicotiana* species (Chiba et al., 2006; Liu and Kearney, 2010; Yoon et al., 2011). We studied whether the agroinfection efficiency of CLRDV could be increased by co-infiltration of *A. tumefaciens* harboring the CLRDV replicon and one of the three viral RNA silencing suppressors (HC-Pro of *Potato virus Y*, P19 of *Tomato bushy stunt virus* and 2b of *Cucumber mosaic virus*) to *N. benthamiana* but no infection increase was detected (data not shown). This could be explained by an efficient silencing suppressor activity of CLRDV P0 protein, like that reported for other polerovirus encoding P0s such as the following species: turnip yellow virus, cucurbit aphid-borne yellow virus, sugarcane yellow leaf virus and potato leafroll virus (Fusaro et al., 2012; Mangwende et al., 2009; Pfeffer et al., 2002).

Unlike *N. benthamiana*, *A. thaliana* is not susceptible to a wide range of pathogens. Even though turnip yellows virus, beet mild yellowing virus and beet western yellows virus-USA (BWYV-US) infections of *A. thaliana* plants were reported to remain symptomless, virus accumulation in systemic leaves of these infected plants was easily detected by ELISA 3–4 weeks after infection (Reinbold et al., 2013). The CLRDV infectious clone could be readily infectious in infiltrated *A. thaliana* leaves (Fig. 4C) but the virus apparently did not move efficiently to infect systemic leaves (gRNA and sgRNA were only detecte in one plant) despites the fact that the CLRDV P3 capsid protein detected in systemic leaves of some plants. We concluded that *A. thaliana*, would not be a suitable model host for CLRDV because the infectious clone produced an inefficient systemic infection.

In all three tested plant species, systemic infections could be established, although the success of infection varied considerably between hosts and replicates. Whereas 53–92% of the agroinoculated *G. hirsutum* plants became infected, only 28.6–66% and 25–74% of the *N. benthamiana* and *A. thaliana* plants gave a positive

result for CLRDV, respectively. Even though the cause for this variability is still unknown, there are some features that could affect the level of transient gene expression. For instance, minimal variations in plants age and growth conditions, as well as inoculum amount, could account for this observation as it has been reported for transient gene expression in *A. thaliana* (Kim et al., 2009). The variability in the success of infection may also result from the absence of a transcriptional termination signal in the expression cassette.

The complete genomic sequence of CLRDV present in Argentina was recently obtained; however, the function of most CLRDV encoded proteins is still unknown. With the construction of the CLRDV infectious clone, we now can use reverse genetics to study CLRDV genes involved in virus replication, virion formation, cell-to-cell movement, symptom development in the natural host and virus–aphid interactions. Furthermore, this approach allows screening for resistance genes in cotton germplasm banks. In addition *N. benthamiana* is widely used as a model for plant–microbe interaction studies and for transient expression of foreign genes and *A. thaliana* is a model plant whose genome are fully characterized and that has large collections of mutant (Chory et al., 2000; Pfeffer et al., 2002). Efficient infection of the *N. benthamiana* model plant by the CLRDV infectious clone provides additional tools for further gene-function and virus–host interaction experiments.

To our knowledge, this study represents the first report on the construction of a full-length infectious cDNA clone of CLRDV that is able to infect cotton efficiently and the model plant *N. benthamiana* by an agrobacterium-mediated technology.

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