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Virology

e-Xtra*

Identification of a New Cotton Disease Caused by an Atypical Cotton Leafroll Dwarf Virus in Argentina

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ABSTRACT

An outbreak of a new disease occurred in cotton (*Gossypium hirsutum*) fields in northwest Argentina starting in the 2009–10 growing season and is still spreading steadily. The characteristic symptoms of the disease included slight leaf rolling and a bushy phenotype in the upper part of the plant. In this study, we determined the complete nucleotide sequences of two independent virus genomes isolated from cotton blue disease (CBD)-resistant and -susceptible cotton varieties. This virus genome comprised 5,866 nucleotides with an organization similar to that of the genus *Polerovirus* and was closely related to cotton leafroll dwarf virus, with protein identity ranging from 88 to 98%. The virus was subsequently transmitted to a CBD-resistant cotton variety using *Aphis gossypii* and symptoms were successfully reproduced. To study the persistence of the virus, we analyzed symptomatic plants from CBD-resistant varieties from different cotton-growing fields between 2013 and 2015 and showed the presence of the same virus strain. In addition, a constructed full-length infectious cDNA clone from the virus caused disease symptoms in systemic leaves of CBD-resistant cotton plants. Altogether, the new leafroll disease in CBD-resistant cotton plants is caused by an atypical cotton leafroll dwarf virus.

Cotton (*Gossypium* spp.) is the most important fiber crop worldwide and 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). Cotton is an important crop for northwest Argentina, where the average planted area for the last 5 years has been approximately 600,000 ha. Cotton blue disease (CBD) affects cotton crops in Africa, Asia, and South America (Cauquil 1977), and is transmitted by the aphid vector *Aphis gossypii* Glover (Cauquil and Vaissayre 1971). Cotton leafroll dwarf virus (CLRDV; family *Luteoviridae*, genus *Polerovirus*) is the causal agent of CBD (Corrêa et al. 2005; Distéfano et al. 2010). Cotton plants affected by this disease show a stunted phenotype, with shortening of internodes, leaf rolling, vein yellowing, and intensive green color of the foliage (Cauquil and Vaissayre 1971). The disease is controlled with cotton varieties derived from African cotton germplasm resistant to CBD and with insecticides that can effectively control cotton aphids and, consequently, CBD (Fang et al. 2010). These insecticides, however, are expensive and potentially harmful to the environment, and do not provide season-long protection (Fang et al. 2010). Currently, 99% of the varieties sown in Argentina are resistant to CBD. This resistance, in some *Gossypium hirsutum* cultivars, is conditioned by one dominant gene called *Rghv1* (*resistance to Gossypium hirsutum virus 1*) (Junior et al. 2008).

The complete genomic sequence of an Argentinian isolate of CLRDV has been recently obtained (Distéfano et al. 2010). The CLRDV genome consists of a monopartite, single-stranded, positive-sense RNA of 5,866 kb, and contains seven open reading frames (ORF) in two blocks separated by a noncoding sequence. The 5′ block comprises three ORF—ORF0, ORF1, and ORF2—which are expressed by translation of genomic RNA (gRNA). In poleroviruses, ORF0 encodes the P0 protein that functions as a suppressor of RNA silencing; ORF1 and -2, which overlap one another, encode the replication-related proteins P1 and P1-P2. The 3′ block consists of four ORF—ORF3, ORF3a, ORF4, and ORF5—which are translated from subgenomic RNA (sgRNA). ORF3 encodes the major coat protein (P3); the product of ORF4, P4, is thought to be a movement protein; ORF5 is translated by in-frame readthrough of the P3 stop codon; and P3-P5 is involved in aphid transmission and virus accumulation in plants (Brault et al. 1995; Bruyère et al. 1997; Domier 2012). The small ORF3a was recently described and encodes the P3a protein, which is required for long-distance movement of the virus in the plant (Smirnova et al. 2015). The most variable genomic region within the genus *Polerovirus* is ORF0. Recently, CLRDV P0 protein was characterized as a suppressor of RNA silencing (Delfosse et al. 2014). In accordance, Cascardo et al. (2015) reported similar results for P0 proteins from Brazilian CLRDV isolates, which showed variable degrees of suppression activity.

New polerovirus species have been recently described in several crop plants such as barley, cotton, cucurbits, and maize (Chen et al. 2016; Ellis et al. 2013; Knierim et al. 2014; Zhao et al. 2016). The molecular species demarcation threshold within the genus *Polerovirus* is amino acid sequence identity of any gene product less than 90% (Domier 2012).

In the 2009–10 growing season, an outbreak of a new disease occurred in two cotton-growing regions in the province of Chaco, probably because of a virus infection. The symptoms associated with the disease included slight leaf rolling and, sometimes, crumpled leaves. In general, the disease appeared in the field at the end of the cotton growing season. The plants, which probably were infected later in development, displayed normal morphology in the lower part of the plant but a bushy phenotype in the upper part, with deformations of the apical leaves (Fig. 1A). The plants also presented a reduction in the amount of fiber and seed inside the bolls, with a significant decrease in fiber quality and poor defoliation, which affects harvesting. The disease was associated with the presence of the cotton aphid vector (*A. gossypii*) and affected cotton varieties that are resistant ('NuOpal', 'Deltapine 402', and 'Guazuncho II') or susceptible ('NC33B') to CBD. Since the 2010 growing season, the new disease spread to all cotton-planting areas in the northwest of

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*The *e-Xtra* logo stands for "electronic extra" and indicates that one supplementary table and two supplementary figures are published online.

Argentina, with a high proportion of affected plants (nearly 20% incidence), thus showing that it constitutes a new threat to cotton agriculture.

Infectious full-length cDNA clones have been obtained for several species of the family *Luteoviridae* and, recently, we successfully developed a full-length infectious cDNA clone from CLRDV, which was able to efficiently infect *G. hirsutum* and *Nicotiana benthamiana* via *Agrobacterium tumefaciens* (Delfosse et al. 2013; 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 new pathosystems (Delfosse et al. 2013).

In this study, we report the complete genomic sequence of two independent virus isolates from *G. hirsutum* CBD-resistant and CBD-susceptible cultivars associated with the new disease. The results of this study show that this virus should be classified in the genus *Polerovirus* as an atypical strain of CLRDV (CLRDV-at). Additionally, we successfully developed a full-length infectious cDNA clone from CLRDV-at, which was able to efficiently infect CBD-resistant *G. hirsutum* plants.

MATERIALS AND METHODS

Nucleic acid extraction and cDNA synthesis. Total DNA was isolated from symptomatic leaves as described by Dellaporta et al. (1983) and total RNA extraction was performed using a PureLink RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was done with 3 µg of total RNA using SuperScript III reverse transcription and random primers (Thermo Fisher Scientific) following the manufacturer's recommended conditions.

Virus source, cloning, and sequencing. Two virus isolates were obtained from two cotton cultivars with disease symptoms; the viruses were called CLRDV-at M3 and M5 and came from NuOpal (CBD resistant) and NC33B (CBD susceptible), respectively. These cultivars were from a crop field located in Sáenz Peña, department Comandante Fernández, in the province of Chaco, Argentina. The general polerovirus primers PLF and PLR (primers 8 and 9, respectively) were used to amplify parts of ORF2 and ORF3

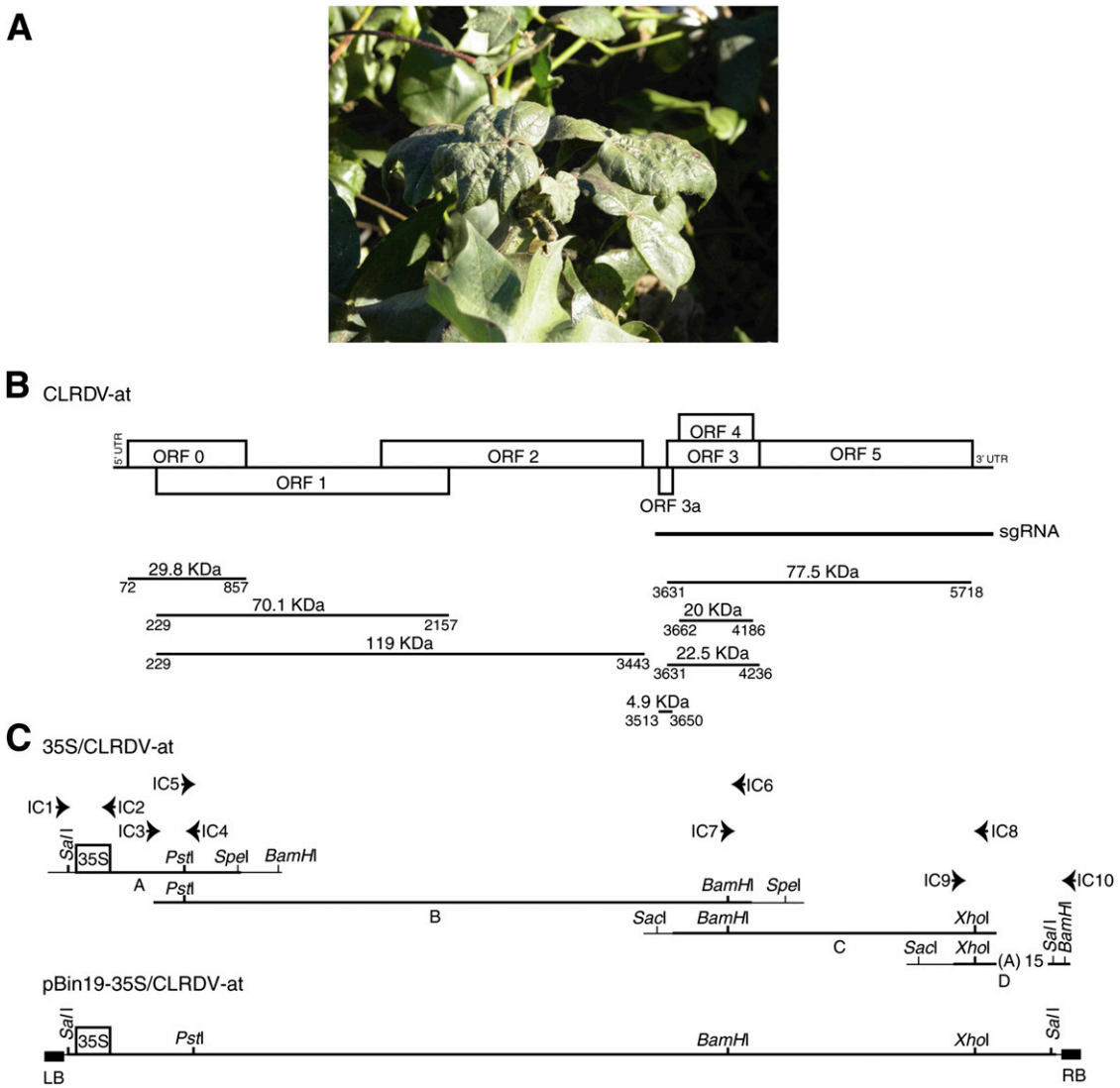

Fig. 1. Symptoms, genome organization, and an infectious clone of an atypical strain of cotton leafroll dwarf virus (CLRDV-at). **A,** Symptoms associated with the new disease. A bushy phenotype, leaf rolling, and crumple symptoms of *Gossypium hirsutum* (variety NuOpal) leaves in the field. **B,** CLRDV-at genome organization. The rectangles correspond to the predicted open reading frames (ORF). Protein products are represented by a thin line, positions are marked at the ends, and the predicted sizes of the proteins are listed above the indicated protein regions. **C,** Construction of the full-length cDNA of CLRDV-at. Fragments A, B, C, and D were amplified by polymerase chain reaction using the primers indicated with arrows and joined with restriction sites *Bam*HI, *Pst*I, *Sac*I, *Spe*I, and *Xho*I. Bold lines correspond to CLRDV-at cDNA sequences and thin lines (not to scale) to the vector-flanking sequences. 35S corresponds to the *Cauliflower mosaic virus* promoter. 35S/CLRDV-at was cloned into pBin19 vector in the *Sal*I restriction site, generating pBin19-35S/CLRDV-at.

(a fragment of 1,065 bp). The primer sequences are listed in Supplementary Table S1. The polymerase chain reaction (PCR) 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 2 min at 72°C; with a final extension at 72°C for 10 min. Subsequent amplifications were performed with similar PCR cycling profiles. ORF0, ORF1, and the missing part of the ORF2 sequence were obtained in three fragments using primers 2 and 3 (fragment of 339 bp), primers 4 and 5 (fragment of 1,129 bp), and primers 6 and 7 (fragment of 818 bp), respectively. Finally, the ORF3 and ORF5 complete sequences were amplified in three fragments using primers 10 and 11 (fragment of 565 bp), primers 12 and 13 (fragment of 834 bp), and primers 14 and 15 (fragment of 829 bp), respectively. To complete the sequence of the 3′ and 5′ ends of the CLRDV-at genome, rapid amplification of cDNA ends (RACE) was performed employing kits from Thermo Fisher Scientific and following the manufacturer's instructions. For 5′ RACE, the CLRDV-at specific internal antisense oligonucleotide was primer 1 and, for 3′ RACE, the CLRDV-at specific oligonucleotide was primer 16.

Two independent PCR with Platinum Pfx DNA polymerase (Thermo Fisher Scientific) were performed and two clones from each amplicon were sequenced in order to confirm the sequence. The PCR products were purified using QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA), cloned into pGEM-T Easy vector (Promega Corp., Madison, WI) and sequenced with an ABI 3500 XL automated sequencer. The complete nucleotide sequence was assembled and analyzed with the software Vector NTI Advance 11.0 (Thermo Fisher Scientific). The complete nucleotide sequences were submitted to GenBank under accession numbers KF359946 (M3 isolate) and KF359947 (M5 isolate).

The presence of viruses from the family *Geminiviridae* and the genus *Ilarvirus* were analyzed using GeV and GeC, universal geminivirus primers for dicotiledoneus (Briddon and Markham 1994), and ILAR 2F5 and ILAR 2R, universal ilarvirus primers (Untiveros et al. 2010). The presence of viruses from the family *Potyviridae* was analyzed using a universal primer (Sprimer) to detect the 3′-terminal genome region, as described by Chen et al. (2001).

The CBD-resistant plants with atypical disease symptoms were randomly collected from foci of infected plants present in different fields in 2013 (M10/13 isolate, Sáenz Peña and M12/13 and M13/13 isolates, Gancedo), 2014 (M5/14 and M14/14, Sáenz Peña), and 2015 (M13/15 isolate, Sáenz Peña and M8/15 isolate, la Chiquita). RNA was purified and reverse-transcription (RT)-PCR of ORF0 was performed using P0up and P0low primers (amplified fragment of 786 bp). ORF0 was directly sequenced or cloned in pGEM-T Easy vector and sequenced. The nucleotide sequences were submitted to GenBank under accession numbers KU934226 (M10/13 isolate), KU934227 (M12/13 isolate), KU934228 (M13/13 isolate), KU934229 (M14/14 isolate), KU934230 (M15/14 isolate), KU934233 (M8/15 isolate), and KU934234 (M9/15 isolate).

Sequence and phylogenetic analysis. Nucleotide and amino acid sequences of the newly isolated viruses were compared with GenBank databases using the BLAST programs (Altschul et al. 1990). The sequences of selected viruses were aligned using ClustalX (Thompson et al. 1997) and distance trees were constructed using MEGA6 (Tamura et al. 2013). A maximum-likelihood method based on the JTT matrix-based model was used and the statistical validity of the clusters obtained was verified with a bootstrap value calculated on 1,000 trials. The following sequences from viruses belonging to the *Luteoviridae* family were used in the phylogenetic analyses: from *Luteovirus* genus: *Barley yellow dwarf virus*-GAV (BYDV-GAV, AY220739), BYDV-MAV (D11028), and BYDV-PAS (AF218798); from *Polerovirus* genus: *Beet chlorosis virus* isolate-2a (AF352024), *Beet mild yellowing virus* (KC121026), *Beet western yellows virus* (AF473561), *Carrot red leaf virus* strain UK-1 (AY695933), *Cereal yellow dwarf virus* isolate 010 (EF521830), *Chickpea chlorotic stunt virus* isolate Et-fb-am1 (AY956384), CLRDV-at M3 (KF359946), CLRDV-at M5 (KF359947), CLRDV-Acr3 (KF906260), CLRDV-ARG (GU167940), CLRDV-Br (HQ827780), CLRDV-IMA2 (FK906261), *Cucurbit aphid-borne yellows virus* (X76931), *Melon aphid-borne yellows virus* (EU000534), *Potato leafroll virus* (PLRV, NC001747), *Sugarcane yellow leaf virus* (AY236971), *Tobacco vein-distorting virus* (EF529624), and *Turnip yellow virus* (TuYV, X13063); from *Enamovirus* genus: *Pea enation mosaic virus-1* (NC003629); and from unassigned genus: *Cotton bunchy top virus* (CBTV, JF803842).

Aphid transmission tests. An *A. gossypii* colony was obtained from a CBD-resistant cotton plant with symptoms of the new disease from a field located in Sáenz Peña (growing season 2014). The viruliferous aphids were removed with a brush and transferred to 10 healthy 3-week-old stage NuOpal cotton seedlings with one expanded leaf. Ten aphids per plant were placed on the true leaf for an inoculation feeding period of 15 days. The plants were placed into separate aphid-proof cages under controlled conditions (22°C with a 16-h photoperiod). Finally, the aphids were eliminated by spraying the plants with the insecticide imidacloprid. The same experimental transmission was achieved using laboratory-reared nonviruliferous *A. gossypii*. The development of the symptoms was scored 3 to 4 weeks postinoculation and virus detection was performed in systemic leaves 6 weeks postinoculation by RT-PCR using P0up and P0low primers (see above). The nucleotide sequences were submitted to GenBank under accession numbers KU934231 (Mtr2/14 isolate) and KU934232 (Mtr10/14 isolate).

Synthesis of full-length CLRDV-at cDNA transcription vector. Total RNA from infected plant tissue with CLRDV-at isolate M3 was used as a template for the synthesis of cDNA, as described above. PCR amplifications were performed with specific primers using FideliTaq (Affymetrix, Santa Clara, CA). The final construct containing the full-length CLRDV-at cDNA under the control of the *Cauliflower mosaic virus* 35S promoter (hereafter referred to as 35S promoter) was prepared similarly to that described by Delfosse et al. (2013). Briefly, to construct this vector, we followed a PCR megaprimer approach. A first amplicon containing the 35S promoter sequence (401 bp) with the first 11 CLRDV-at nucleotides was produced with oligonucleotides IC1 and IC2 as primers. The nonviral *Sal*I restriction site was incorporated into the oligonucleotide IC1 to allow the cloning step. The second amplicon corresponded to the 5′ CLRDV-at sequence (nucleotides 1 to 519) flanked with the last 6 nucleotides of the 35S promoter and was amplified with oligonucleotides IC3 and IC4. The two purified PCR fragments were mixed in equal amounts and used as templates to obtain PCR fragment A (Fig. 1C) using primers IC1 and IC4. PCR amplicons were synthesized using primers IC5 and IC6 (fragment of 4,019 bp), IC7 and IC8 (fragment of 1,813 bp), and IC9 and IC10 (fragment of 331 bp) to produce fragments B, C, and D, respectively. *Bam*HI and *Sal*I restriction sites were incorporated into the oligonucleotide IC10 to allow the cloning step and, finally, 15 adenine residues (poly(A) tail) were added at the 3′ end of the cDNA to increase the stability of the transcript. No transcription termination signal was added in the construction to minimize the nonviral residue. Then, the amplicons were cloned into pGEM-T vector (Promega Corp.) and sequenced. Digestion of pGEM-A, -B, -C, and -D with *Pst*I, *Sac*I, *Xho*I, and *Bam*HI enzymes was done to produce the pGEM-ABCD fragment, which contains the full-length copy of CLRDV-at cDNA under the control of the 35S promoter (35S/CLRDV-at). The pGEMT-35S/CLRDV-at was completely sequenced using an ABI 3500 XL automated sequencer. For the agroinfection experiments, the 35S/CLRDV-at fragment was digested with *Sal*I endonuclease and subcloned into the *Sal*I site of the binary vector pBin19 to produce pBin19-35S/CLRDV-at (Fig. 1C). Cotton seedlings at approximately the 2-week-old stage with two fully expanded cotyledons were inoculated with *A. tumefaciens* LBA 4404 carrying the binary vector pBin19-35S/CLRDV-at, as described by Delfosse et al. (2013).

Detection of viral RNA. For RT-PCR analysis, RNA was treated with DNaseI (Thermo Fisher Scientific) and cDNA was

synthesized as described above. For detection of the viral capsid, Platinum Taq Polymerase (Thermo Fisher Scientific) and specific CPup and CPLow primers were used. Primers specific for *G. hirsutum* ubiquitin mRNA (GhUbiUp and GhUbiLow) were used to ensure that the lack of viral detection was not due to a failure in the cDNA reaction (Artico et al. 2010). A total of 10 µg of RNA from healthy and symptomatic leaves (PCR positive) was fractionated on a denaturing 1% agarose gel and transferred onto a Hybond N+ membrane (GE Healthcare, Little Chalfont, UK). The probe was produced with CPup and CPlow primers and radiolabeled with [α-^{32}P] dCTP with the Prime-a-Gene Labeling System kit (Promega Corp.).

RESULTS

Genome sequencing of two virus isolates. Samples were obtained from symptomatic leaves (with leaf rolling and bushy phenotypes) (Fig. 1A) from CBD-resistant (isolate M3) and CBD-susceptible (isolate M5) cotton varieties collected in the province of Chaco, the most important cotton-producing area of Argentina. In order to identify the etiological agent, the samples were screened by RT-PCR with the polerovirus primers PLF and PLR (Corrêa et al. 2005), universal geminivirus primers GeV and GeC (Briddon and Markham 1994), ilarvirus primers ILAR 2F5 and ILAR 2R9 (Untiveros et al. 2010), and a universal potyvirus primer (Chen et al. 2001). The two samples were positive only for the presence of a polerovirus. The 1,065-kb fragment of the virus genome was sequenced, which included part of ORF2-RNA-dependent RNA polymerase (RdRp), the intergenic region, and part of ORF3-coat protein (CP) and ORF4-movement protein (MP) genes (Fig. 1B). The sequence identities of the deduced amino acid sequences for partial RdRp, CP, and MP ranged from 95.4 to 98.5% for isolate M3 and 97.7 to 98.5% for isolate M5 to CLRDV-ARG (Argentinian isolate), a recently sequenced and characterized polerovirus (Distéfano et al. 2010). Subsequently, the complete nucleotide sequence of virus isolates M3 and M5 was obtained.

TABLE 1. Percent amino acid sequence identity between cotton leafroll dwarf virus (CLRDV)-ARG and CLRDV-at isolates M3 and M5

CLRDV-ARG	CLRDV-at M3	CLRDV-at M5
P0	88.2	87.0
P1	93.6	93.8
P1-P2	95.6	96.0
P3	98.0	98.0
P3a	100.0	100.0
P4	95.4	97.1
P3-P5	96.6	96.4

CLRDV-at genome structure, phylogeny, and persistence. The viral genome was 5,866 nucleotides long for both isolates M3 and M5, with six predicted ORF typical for *Polerovirus* genus members (Mayo and Ziegler-Graff 1996). Both isolates had the recently described a novel short ORF, termed ORF3a, that is positioned upstream of ORF3 and whose translation initiates at a non-AUG codon (Smirnova et al. 2015). The features of the sequences and the sizes of the ORF are shown in Figure 1B.

For individual ORF, the isolates M3 and M5 have a high level of identity in their nucleotide (98 to 100%; for example, 98% in P1P2 and 100% in P3a) and amino acid (98.1 to100%; for example, 98.1% in P0) sequences. This finding indicates that the strains isolated from both cotton varieties correspond to the same virus species. The comparison between the seven putative encoded proteins of M3 and M5 isolates and the encoded protein from CLRDV-ARG displayed significant divergence in P0 protein (88.2% in M3 and 87% in M5) while keeping a high identity in the other proteins (93 to 100%) (Table 1). This result reveals that CLRDV-at is very closely related to CLRDV-ARG. We also compared isolate M3 with two cotton poleroviruses: CLRDV-Acr3, a new CLRDV genotype present in Brazil which was recently sequenced (da Silva et al. 2015), and CBTV, a recombinant virus present in Australia that was partially sequenced (Ellis et al. 2013). When P0, P3, and P4 proteins from the M3 isolate were analyzed, a high identity of 95, 99, and 97%, respectively, was observed with the corresponding CLRDV-Acr3 proteins. On the other hand, P2, P3, and P4 proteins from M3 isolate showed identity of 92.1, 68, and 54%, respectively, with the corresponding CBTV proteins. In contrast, comparison of M3 isolate with noncotton polerovirus sequences revealed a lower identity for P0 protein (26 to 29%) and somewhat lower for P3 protein (60 to 80%).

According to the criteria established by the International Committee on Taxonomy of Viruses (ICTV) (Domier 2012), the M3 and M5 isolates could be proposed as members of a new species within the genus *Polerovirus*, because differences greater than 10% were observed in the P0 amino acid sequences with regard to CLRDV-ARG. However, because the amino acid sequence differences in the other gene products were less than 10%, we propose that the two isolates represent an atypical strain of CLRDV (CLRDV-at). The phylogenetic analysis between CLRDV-at M3 and M5 isolates and members of the family *Luteoviridae*, based on P0 and CP amino acid sequences, is displayed in Figure 2. The topology of CP and P0 trees showed that CLRDV-at isolates share a cluster with CLRDV isolates, as expected by their high sequence identities. Nevertheless, CLRDV-at M3 and M5 form a branch separated from the Brazilian isolates of CLRDV-IMA2, -Acr3, and -BR and CLRDV-ARG in the

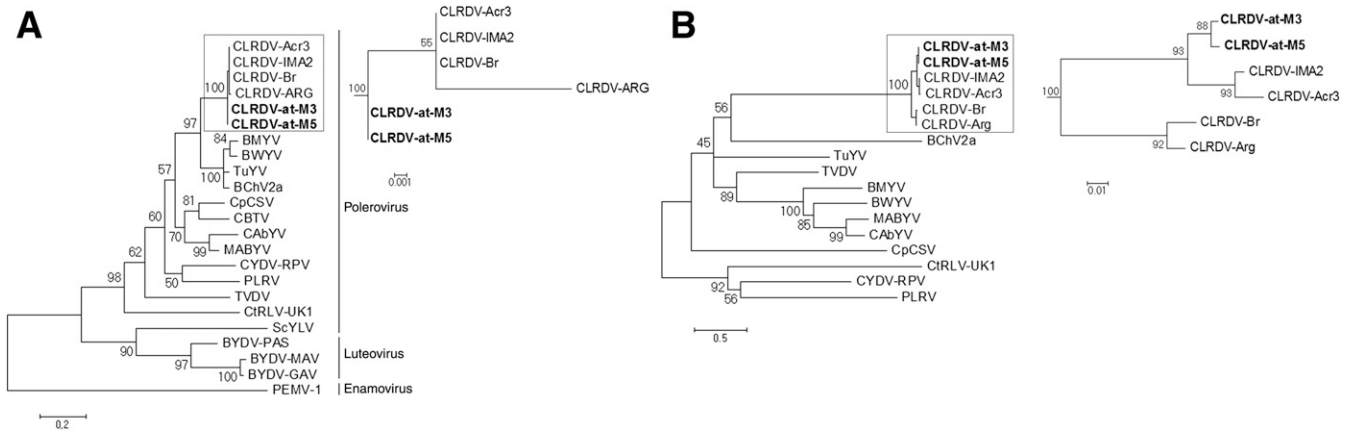

Fig. 2. Phylogeny of an atypical strain of cotton leafroll dwarf virus (CLRDV-at). Phylogenetic trees constructed using the maximum likelihood method based on **A,** the predicted sequence of the P3 (coat protein) with a zoom in of the boxed cluster and **B,** the P0 coding region with a zoom in of the boxed cluster. Numbers in the nodes correspond to bootstrap values from 1,000 replicates. Virus abbreviations and sequences used are listed in Materials and Methods.

CP protein tree (Fig. 2A). The same topology was observed when an MP phylogenetic tree was constructed (data not shown). Finally, the phylogenetic tree of the P0 protein showed that CLRDV-at M3 and M5 form a group with CLRDV-IMA1 and -Acr3, and separated from CLRDV-BR and -ARG (Fig. 2B).

To study the persistence of CLRDV-at, we analyzed symptomatic plants from CBD-resistant cotton varieties from different fields in Chaco from 2013 to 2015. We amplified ORF0, which codes for the P0 protein, the most variable protein in poleroviruses. The amino acid sequence alignments between position 24 and 174 of P0 protein from field isolates, which contained the polerovirus-conserved F-Box-like motif (68-LPxxL/Ix$_{(10)}$P-84), were performed (Supplementary Fig. S1). Interestingly, CLRDV-ARG and -BR have an isoleucine (I) at position 72, while CLRDV-at M3 and M5 and isolates from 2013 to 2015 had a valine (V), as previously described for CLRDV-Acr3 and -IMA2 (da Silva et al. 2015). On the other hand, a comparison of field isolates with CLRDV-at M3 and M5 isolates revealed a high identity for P0 protein (from 98.7 to 99.6% at the nucleotide level and 98 to 99% at the amino acid level). Furthermore, almost all the amino acid changes in CLRDV-at M3 and M5 with respect to CLRDV-ARG were conserved in field isolates collected from 2013 to 2015.

Aphid transmission analysis. We evaluated the ability of CLRDV-at to be transmitted by *Aphis gossypii*. The aphids were transferred to healthy young *G. hirsutum* NuOpal and 2 of 10 plants (Mtr2/14 and Mtr10/14) developed leaf rolling 4 to 5 weeks postinoculation (Fig. 3A and B). The ORF0 was amplified in both plants by RT-PCR, sequenced, and analyzed. P0 from Mtr2/14 and Mtr10/14 revealed a high identity with CLRDV-at M3 and M5 (99% at the nucleotide level and from 98 to 99% at the amino acid level) and that almost all the positions that changed in CLRDV-at M3 and M5 with respect to CLRDV-ARG were conserved.

Construction of a full-length cDNA clone of CLRDV-at and agroinfection of cotton plants. To demonstrate that the new cotton disease is produced by CLRDV-at, we constructed a full-length cDNA clone. The overall strategy employed for the construction of the CLRDV-at infectious clone was similar to that for CLRDV (Delfosse et al. 2013) and is outlined in Figure 1C. The 35S/CLRDV-at containing the full-length copy of CLRDV-at cDNA was then successfully cloned into the pBin19 vector (Fig. 1C).

We tested the ability of pBin19-35S/CLRDV-at to infect *G. hirsutum* NC33B, a cotton variety susceptible to both CLRDV and CLRDV-at, and NuOpal, a variety resistant to CLRDV but susceptible to CLRDV-at. Cotyledons of both varieties were agroinoculated with *Agrobacterium tumefaciens* carrying the binary vector pBin19-35S/CLRBV. After 6 weeks, symptoms such as slight leaf rolling began to appear in *G. hirsutum* NC33B (Fig. 3C) and NuOpal systemic leaves (Supplementary Fig. S2A). Also, small leaves emerged in the lateral buds of symptomatic plants but not in uninfected plants, and no dwarfing phenotype was apparent in infected plants (Fig. 3C). In contrast, *G. hirsutum* NuOpal infected with pBin19-35S/CLRDV remained uninfected (data not shown) and *G. hirsutum* NC33B developed typical CBD symptoms such as pronounced leaf rolling, intensive dark-green color of the foliage, vein yellowing in the systemic leaves, shortening of internodes, and stunted phenotype (Fig. 3C). After 6 weeks of inoculation, 38% of *G. hirsutum* NuOpal and 37% of *G. hirsutum* NC33B inoculated with pBin19-35S/CLRDV-at contained viral RNA at their systemic leaves (Fig. 4A, lanes 6 to 10), which was demonstrated by RT-PCR. Viral gRNA and sgRNA were detected by Northern blot hybridization of total RNA extracted from systemic leaves of pBin19-35S/CLRDV-at-infected plants that displayed symptoms (Fig. 4B, lanes 4 to 6) but not in the control plants (inoculated with pBin19; Fig. 4B, lane 1).

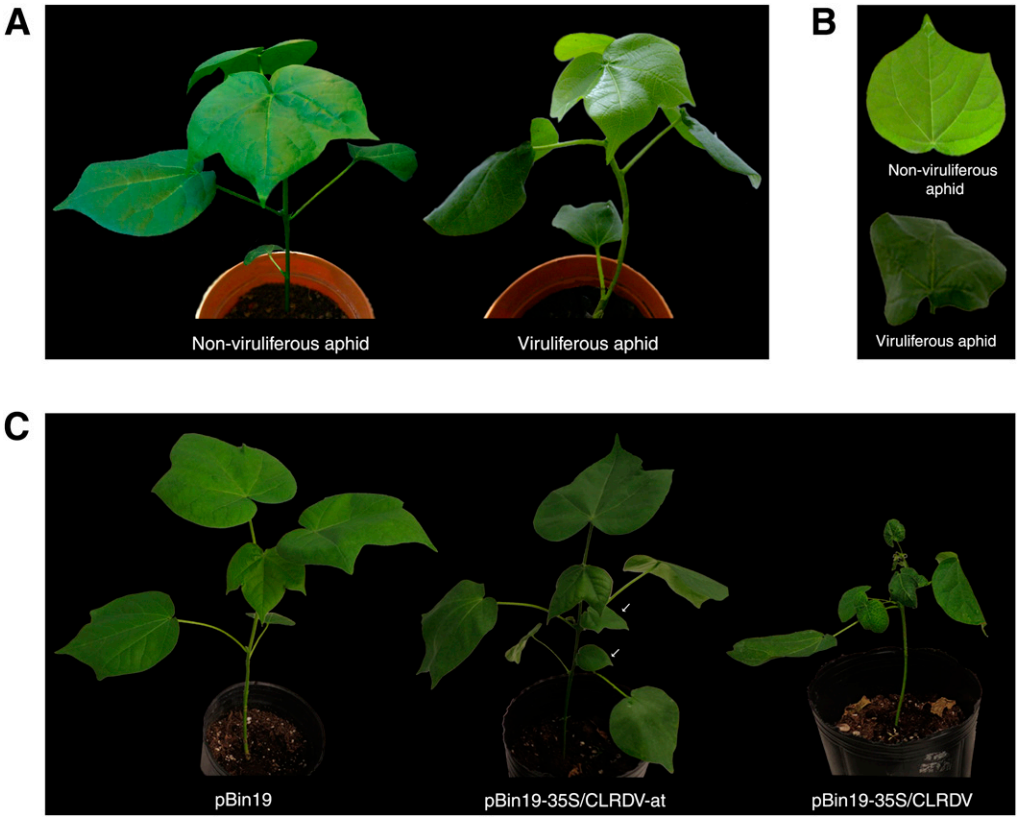

Fig. 3. Aphid transmission and agroinfection of an atypical strain of cotton leafroll dwarf virus (CLRDV-at). **A,** *Gossypium hirsutum* NuOpal plant inoculated with nonviruliferous aphids (left) and with viruliferous aphid collected from a symptomatic plants (right) 3 weeks postinoculation. **B,** Symptoms of *G. hirsutum* (NuOpal) leaf infected with viruliferous aphids (lower) and nonviruliferous aphids (upper). **C,** *G. hirsutum* NC33B plant inoculated with control pBin19 (left), pBin19-35S/CLRDV-at (middle), and pBin19-35S/CLRDV (right) 6 weeks postinoculation.

DISCUSSION

Poleroviruses represent an important threat to agriculture worldwide because they are transmitted by aphids and can infect a wide range of plants. In the last decade, new cotton poleroviruses besides CLRDV have been described in Australia and Brazil; in particular, CBTV and Brazilian isolates PL3, CV2, and PO1, which were all recombinants between CLRDV and an unidentified virus from the family *Luteoviridae* (Ellis et al. 2013; Silva et al. 2008). Here, we describe a new cotton disease in Argentina caused by CLRDV-at. Sequence information and phylogenetic analysis showed that CLRDV-at was closely related to CLRDV-ARG, although the symptoms produced by these two viruses in cotton plants are quite different. The ICTV virus species demarcation criteria for the genus *Polerovirus* states that differences in amino acid sequence identity of any gene product greater than 10% is considered a new species (Domier 2012). CLRDV-at P0 protein has less than 90% identity with CLRDV-ARG P0 protein and, therefore, CLRDV-at might represent a new species within the genus *Polerovirus.* However, because the other proteins share a high identity in amino acid sequence (more than 94%) and for consistency with recent publication on new poleroviruses (da Silva et al. 2015; Knierim et al. 2014), we consider this isolate to be a new strain of CLRDV. The new disease was observed on the field at the end of the cotton growth cycle, at flowering and fruiting stage (bolls development); the plants had normal morphology in the lower part but a bushy phenotype in the upper part, with slight leaf rolling in apical leaves. These symptoms were evident on CBD-resistant and -susceptible cultivars. Currently, 99% of the cotton cultivars sown in Argentina are resistant to CLRDV and to species of Lepidoptera (due to the presence of a *cry1A* transgene) and, for these reasons, the management practices for cotton dramatically changed approximately 10 years ago. By the end of the cotton growth cycle, there is a reduced use of insecticide and, as a consequence, a higher density of aphid populations able to transmit CLRDV-at. The development of a full-length infectious clone of CLRDV-at allows us to investigate the symptom development of CLRDV-at compared with CLRDV-ARG in young cotton plants under controlled conditions (Fig. 3C). In this assay, CLRDV-ARG developed pronounced leaf rolling, intensive dark-green color of the foliage, vein yellowing in the systemic leaves, shortening of internodes, and stunting phenotype on susceptible cultivars. In contrast, CLRDV-at displayed slight leaf rolling without vein yellowing and small leaves emerged in the lateral buds but not in the uninfected or CLRDV-infected plants. No obvious stunting phenotype was detected on the CLRDV-at infected plants.

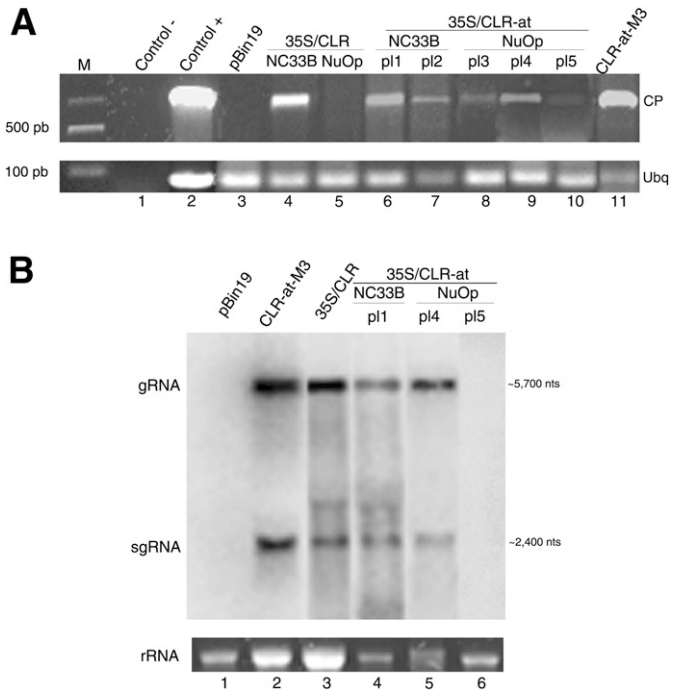

Fig. 4. Detection of an atypical strain of cotton leafroll dwarf virus (CLRDV-at). **A,** Agarose gel of reverse-transcription polymerase chain reaction (PCR) products produced using primers specific for open reading frame ORF3 (capsid protein) (upper panel). *Gossypium hirsutum* NC33B inoculated with pBin19 (lane 3), pBin19-35S/CLRDV (lane 4), or pBin19-35S/CLRDV-at (lanes 6 and 7) and NuOpal inoculated with pBin19-35S/CLRDV (lane 5) or pBin19-35S/CLRDV-at (lanes 8, 9, and 10) 6 weeks after infection. *G. hirsutum* NuOpal CLRDV-at M3 (lane11) was used as a positive control. Lanes 1 and 2 correspond to negative and positive PCR controls, respectively. Primers specific for *G. hirsutum* ubiquitin were used in the positive control reactions (lower panel). M = DNA size markers. **B,** Northern blot analysis of CLRDV-at genomic RNA (gRNA) and subgenomic RNA (sgRNA) accumulation from systemic cotton leaves 6 weeks after infection. *G. hirsutum* NC33B inoculated with pBin19 (lane 1), pBin19-35S/CLRDV (lane 3), or pBin19-35S/CLDBV-at (lane 4) and NuOpal inoculated with pBin19-35S/CLRDV-at (lanes 5 and 6). *G. hirsutum* NuOpal CLRDV-at M3 (lane 2) was used as a positive control. As a loading control, ribosomal RNA (rRNA) were stained with ethidium bromide (lower panel).

P0 is the least conserved protein between CLRDV and CLRDV-at, with a sequence identity of 87%. Before polerovirus P0 function were characterized, the P0 protein from PLRV was proposed to be involved in viral symptom expression because potato transgenic plants expressing the P0 protein displayed an altered phenotype resembling virus-infected plants (van der Wilk et al. 1997). Later, P0 protein from TuYV was described as an RNA-silencing suppressor (Pfeffer et al. 2002). In addition, transgenic *Arabidopsis thaliana* plants expressing P0 from TuYV exhibited developmental aberrations with enhanced levels of several miRNA-target transcripts (Bortolamiol et al. 2007), as previously described for other viral suppressors of RNA silencing (Chapman et al. 2004). Although several polerovirus P0 proteins have been described as silencing suppressors, the levels of suppression activity differed among polerovirus species (Delfosse et al. 2014; Zhuo et al. 2014). Also, P0 proteins of isolates of the same species can display different levels of suppression activity or lack of activity (Almasi et al. 2015; Cascardo et al. 2015; Klein et al. 2014; Kozlowska-Makulska et al. 2010). Recently, many studies have linked symptom development with specific interactions between viral and host components involved in phytohormone pathways. These interactions provide the first mechanistic explanation of how viruses modulate phytohormone regulatory systems within their hosts and of how those modulations lead to symptom development (Collum and Culver 2016). CLRDV and CLRDV-at exhibited different symptoms during infection, and the different symptoms could be due to the sequence divergence in P0 proteins. This finding suggests that P0 protein could interact, in a distinct manner, with proteins involved in different pathways of the host plant during infection, thus leading to the development of differential symptoms.

Cotton varieties resistant to CLRDV such as NuOpal or Guazuncho II were susceptible to CLRDV-at, which suggests that the resistance mechanisms broke down or that the genes do not confer cross resistance. Moreover, the negative PCR amplification results for viruses from the family *Geminiviridae*, family *Potyviridae*, and genus *Ilarvirus*, along with the successful transmission of CLRDV-at with *Aphis gossypii* to virus-free plants as well as the capability of the infectious clone to infect CBD-resistant cotton varieties, confirmed that the new disease is associated with this CLRDV-at strain and not with a combination with other viruses. Furthermore, the results on persistence of CLRDV-at from independent symptomatic plants from CBD-resistant cotton varieties which were analyzed during 3 years demonstrated that this new CLRDV variant was always present in plants with symptoms of the new disease.

Recently, a new CLRDV genotype (Acr3) that seems to have acquired the ability to overcome CBD resistance was sequenced in Brazil. Indeed, field-infected plants show mild CBD symptoms associated with reddish, withered leaves with accentuated verticality (da Silva et al. 2015). The CLRDV-at P0 protein displays 95% identity to CLRDV-Acr3 P0 protein, even though symptomatology

between both viruses was clearly different. Thus, further studies are necessary to understand whether CLRDV-at and CLRDV-Acr3 are just geographic variants of the same species that cause different disease symptoms.

Breeding of cotton varieties resistant against CLRDV-at is needed to reduce the incidence and consequent economic losses due to this new disease. The CLRDV-at agroinfection instead of aphid transmission assays will facilitate large-scale screening for resistance genes against this new virus in cotton germplasm banks. Furthermore, the CLRDV-at infectious full-length clone will be useful to study cotton–CLRDV-at interactions using a reverse-genetic approach, which will be helpful in better understanding this new pathosystem.

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LITERATURE CITED

Almasi, R., Miller, W. A., and Ziegler-Graff, V. 2015. Mild and severe cereal yellow dwarf viruses differ in silencing suppressor efficiency of the P0 protein. Virus Res. 208:199-206.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Artico, S., Nardeli, S. M., Brilhante, O., Grossi-de-Sa, M. F., and Alves-Ferreira, M. 2010. Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data. BMC Plant Biol. 10:49.

Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P., and Ziegler-Graff, V. 2007. The polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. Curr. Biol. 17:1615-1621.

Brault, V., van den Heuvel, J. F., Verbeek, M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Garaud, J. C., Guilley, H., Richards, K., and Jonard, G. 1995. Aphid transmission of beet western yellows luteovirus requires the minor capsid read-through protein P74. EMBO J. 14:650-659.

Briddon, R. W., and Markham, P. G. 1994. Universal primers for the PCR amplification of dicot-infecting geminiviruses. Mol. Biotechnol. 1:202-205.

Bruyère, A., Brault, V., Ziegler-Graff, V., Simonis, M. T., van den Heuvel, J. F., Richards, K., Guilley, H., Jonard, G., and Herrbach, E. 1997. Effects of mutations in the beet western yellows virus readthrough protein on its expression and packaging and on virus accumulation, symptoms, and aphid transmission. Virology 230:323-334.

Cascardo, R. S., Arantes, I. L. G., Silva, T. F., Sachetto-Martins, G., Vaslin, M. F. S., and Corrêa, R. L. 2015. Function and diversity of P0 proteins among cotton leafroll dwarf virus isolates. Virol. J. 12:123.

Cauquil, J. 1977. Etudes sur une maladie d'origine virale du cotonnier: La maladie bleue. Cot. Fib. Trop. 32:259-278.

Cauquil, J., and Vaissayre, M. 1971. La "maladie bleue" du cotonnier en Afrique: Transmission de cotonnier a cotonnier par *Aphis gossypii* glover. Cot. Fib. Trop. 26:463-466.

Chapman, E. J., Prokhnevsky, A. I., Gopinath, K., Dolja, V. V., and Carrington, J. C. 2004. Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes Dev. 18:1179-1186.

Chen, J., Chen, J., and Adams, M. J. 2001. A universal PCR primer to detect members of the *Potyviridae* and its use to examine the taxonomic status of several members of the family. Arch. Virol. 146:757-766.

Chen, S., Jiang, G., Wu, J., Liu, Y., Qian, Y., and Zhou, X. 2016. Characterization of a novel *Polerovirus* infecting maize in China. Viruses 8:120.

Collum, T. D., and Culver, J. N. 2016. The impact of phytohormones on virus infection and disease. Curr. Opin. Virol. 17:25-31.

Corrêa, R. L., Silva, T. F., Simões-Araújo, J. L., Barroso, P. A., Vidal, M. S., and Vaslin, M. F. S. 2005. Molecular characterization of a virus from the family *Luteoviridae* associated with cotton blue disease. Arch. Virol. 150: 1357-1367.

da Silva, A. K. F., Romanel, E., Silva, T. F., Castilhos, Y., Schrago, C. G., Galbieri, R., Bélot, J. L., and Vaslin, M. F. S. 2015. Complete genome sequence of two new virus isolates associated with cotton blue disease resistance breaking in Brazil. Arch. Virol. 160:1371-1374.

Delfosse, V. C., Agrofoglio, Y. C., Casse, M. F., Kresic, I. B., Hopp, H. E., Ziegler-Graff, V., and Distéfano, A. J. 2014. The P0 protein encoded by cotton leafroll dwarf virus (CLRDV) inhibits local but not systemic RNA silencing. Virus Res. 180:70-75.

Delfosse, V. C., Casse, M. F., Agrofoglio, Y. C., Bonacic Kresic, I., Hopp, H. E., Ziegler-Graff, V., and Distéfano, A. J. 2013. 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*. Virus Res. 175:64-70.

Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA mini-preparation: Version II. Plant Mol. Biol. Rep. 1:19-21.

Distéfano, A. J., Bonacic Kresic, I., and Hopp, H. E. 2010. The complete genome sequence of a virus associated with cotton blue disease, cotton leafroll dwarf virus, confirms that it is a new member of the genus *Polerovirus*. Arch. Virol. 155:1849-1854.

Domier, L. L. 2012. Family *Luteoviridae*. Pages 1045-1053 in: Virus Taxonomy, Ninth Report of the International Committee on Taxonomy of Viruses. A. M. Q. King, M. J. Adams, E. B. Carstens, and E. J. Lefkowitz, eds. Elsevier/Academic Press, London.

Ellis, M. H., Silva, T. F., Stiller, W. N., Wilson, L. J., Vaslin, M. F. S., Sharman, M., and Llewellyn, D. J. 2013. Identification of a new *Polerovirus* (family *Luteoviridae*) associated with cotton bunchy top disease in Australia. 2013. Australas. Plant Pathol. 42:261-269.

Fang, D. D., Xiao, J. H., Canci, P. C., and Cantrell, R. G. 2010. A new SNP haplotype associated with blue disease resistance gene in cotton (*Gossypium hirsutum* L.). Theor. Appl. Genet. 120:943-953.

Franco-Lara, L. F., McGeachy, K. D., Commandeur, U., Martin, R. R., Mayo, M. A., and Barker, H. 1999. Transformation of tobacco and potato with cDNA encoding the full-length genome of potato leafroll virus: Evidence for a novel virus distribution and host effects on virus multiplication. J. Gen. Virol. 80:2813-2822.

Junior, O. P., Schuster, I., Pinto, R. B., Pires, E., Belot, J. L., Silvie, P., Chitarra, L. G., Hoffmann, L. V., and Barroso, P. 2008. Inheritance of resistance to cotton blue disease. Pesqui. Agropecu. Bras. 43:661-665.

Klein, E., Brault, V., Klein, D., Weyens, G., Lefèbvre, M., Ziegler-Graff, V., and Gilmer, D. 2014. Divergence of host range and biological properties between natural isolate and full-length infectious cDNA clone of the beet mild yellowing virus 2ITB. Mol. Plant Pathol. 15:22-30.

Knierim, D., Tsai, W. S., Maiss, E., and Kenyon, L. 2014. Molecular diversity of poleroviruses infecting cucurbit crops in four countries reveals the presence of members of six distinct species. Arch. Virol. 159:1459-1465.

Kozlowska-Makulska, A., Guilley, H., Szyndel, M. S., Beuve, M., Lemaire, O., Herrbach, E., and Bouzoubaa, S. 2010. P0 proteins of European beet-infecting poleroviruses display variable RNA silencing suppression activity. J. Gen. Virol. 91:1082-1091.

Lee, L., Kaplan, I. B., Ripoll, D. R., Liang, D., Palukaitis, P., and Gray, S. M. 2005. A surface loop of the potato leafroll virus coat protein is involved in virion assembly, systemic movement, and aphid transmission. J. Virol. 79:1207-1214.

Leiser, R. M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Lemaire, H., Guilley, H., Richards, K. E., and Jonard, G. 1992. Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus. Proc. Natl. Acad. Sci. USA 89:9136-9140.

Mayo, M. A., and Ziegler-Graff, V. 1996. Molecular biology of luteoviruses. Adv. Virus Res. 46:413-460.

Pfeffer, S., Dunoyer, P., Heim, F., Richards, K. E., Jonard, G., and Ziegler-Graff, V. 2002. P0 of beet western yellows virus is a suppressor of posttranscriptional gene silencing. J. Virol. 76:6815-6824.

Prüfer, D., Wipf-Scheibel, C., Richards, K., Guilley, H., Lecoq, H., and Jonard, G. 1995. Synthesis of a full-length infectious cDNA clone of cucurbit aphid-borne yellows virus and its use in gene exchange experiments with structural proteins from other luteoviruses. Virology 214:150-158.

Silva, T. F., Corrêa, R. L., Castilho, Y., Silvie, P., Bélot, J. L., and Vaslin, M. F. 2008. Widespread distribution and a new recombinant species of Brazilian virus associated with cotton blue disease. Virol. J. 5:123.

Smirnova, E., Firth, A. E., Miller, W. A., Scheidecker, D., Brault, V., Reinbold, C., Rakotondrafara, A. M., Chung, B. Y., and Ziegler-Graff, V. 2015. Discovery of a small non-AUG-initiated ORF in poleroviruses and luteoviruses that is required for long-distance movement. PLoS Pathog. 11:e1004868.

Sunilkumar, G., Campbell, L. M., Puckhaber, L., Stipanovic, R. D., and Rathore, K. S. 2006. Engineering cotton seed for use in human nutrition by tissue-specific reduction of toxic gossypol. Proc. Natl. Acad. Sci. USA 103:18054-18059.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725-2729.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTALX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

Untiveros, M., Perez-Egusquiza, Z., and Clover, G. 2010. PCR assays for the detection of members of the genus *Ilarvirus* and family *Bromoviridae*. J. Virol. Methods 165:97-104.

van der Wilk, F., Houterman, P., Molthoff, J., Hans, F., Dekker, B., van den Heuvel, J., Huttinga, H., and Goldbach, R. 1997. Expression of the

potato leafroll virus ORF0 induces viral-disease-like symptoms. Mol. Plant-Microbe Interact. 10:153-159.
Veidt, I., Bouzoubaa, S. E., Leiser, R.-M., Ziegler-Graff, V. A., Guilley, H., Richards, K., and Jonard, G. 1992. Synthesis of full-length transcripts of beet western yellow virus RNA: Messenger properties and biological activity in protoplasts. Virology 186:192-200.
Yoon, J. Y., Choi, S. K., Palukaitis, P., and Gray, S. M. 2011. Agrobacterium-mediated infection of whole plants by yellow dwarf viruses. Virus Res. 160: 428-434.
Zhao, F., Lim, S., Yoo, R. H., Igori, D., Kim, S.-M., Kwak, D. Y., Kim, S. L., Lee, B. C., and Moon, J. S. 2016. The complete genomic sequence of a tentative new polerovirus identified in barley in South Korea. Arch. Virol. 161:2047-2050.
Zhuo, T., Li, Y. Y., Xiang, H. Y., Wu, Z. Y., Wang, X. B., Wang, Y., Zhang, Y. L., Li, D. W., Yu, J. L., and Han, C. G. 2014. Amino acid sequence motifs essential for P0-mediated suppression of RNA silencing in an isolate of potato leafroll virus from Inner Mongolia. Mol. Plant-Microbe Interact. 27:515-527.