SURVEILLANCE OF *COTTON LEAFROLL DWARF VIRUS* (CLRDV) IN THE US BY MOLECULAR DIAGNOSTICS AND PHYLOGENY OF ORF0 AND ORF3 FRAGMENTS

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Abstract

Cotton leafroll dwarf virus (CLRDV) is an aphid-transmitted *Polerovirus* (*Solemoviridae*). The virus has been associated with symptomatic cotton plants in the Asian-Pacific region, in South America, and most recently in the US (Avelar et al., 2019). Since the initial 2017 U.S. outbreak, CLRDV has been identified in all southern US states where cotton is grown. In 2020, the distribution and spread of CLRDV has been monitored in sentinel plots planted with a standardized group of cotton genotypes. Asymptomatic leaves and leaves showing diverse symptoms reminiscent of CLRDV were collected from sentinel plots from seven states. Initial CLRDV detection was carried out in the Plant Diagnostic Laboratory, Auburn University, by RT-PCR amplification of a fragment of the viral coat protein (*CP*) (Sharman et al., 2015). Frozen leaf samples and/or total RNA from CLRDV-positive and negative samples were shipped to the Plant Virus Diagnostic Laboratory, The University of Arizona. Samples were subjected to RT-PCR

amplification with CLRDV ORF0-specific primers and amplicons (868 bp) cloned, and sequenced, revealing CLRDV presence in 67% of samples. The CLRDV-negative samples (33%) were subjected to RT-PCR amplification with CLRDV ORF4-specific primers, and amplicons (310 bp), were cloned and sequenced. The CLRDV was detected in 10% of cotton samples. Pairwise nucleotide (nt) analysis of ORF0 and ORF3 sequences shared 93.4 - 98.7% and 93.8 - 99.4% nt identity, respectively. Phylogenetic analyses (Maximum Likelihood) of the partial sequences revealed phylogeographic distribution of isolates from the US and South America, with the US isolates grouping as sister clades within one monophyletic clade. Among the CLRDV sequences available in GenBank their closet relative was CLRDV AL_US (MN071395) from a sample collected in Alabama. Based on this study, the ORF0 variation observed among US isolates suggested that several different CLRDV variants have been introduced into one or multiple locales in the US, and that post-introduction, at least some of these variants may have diverged possibly following aphid-mediated spread from the primary site(s) of introduction.

Introduction

Cotton leafroll dwarf virus (CLRDV) is a non-native plant virus species that has recently been reported infecting cotton plants in several cotton-growing states in the United States (US) (Aboughanem-Sabanadzovic et al., 2019; Avelar et al., 2019; Alabi et al., 2020; Faske et al., 2020; Price et al., 2020; Iriarte et al., 2020; Tabassum et al., 2019). CLRDV has a positive sense, single-strand RNA (ssRNA) genome and encodes seven proteins (Cascardo et al., 2015; Miller et al., 1997). The virus belongs to the genus *Polerovirus*, family *Solemoviridae* (ICTV, 2021). It is transmitted from plant to plant by cotton aphid (*Aphis gossypii*) in a persistent, circulative, non-propagative manner, and is phloem-limited in infected plants (Gray et al., 2003; Wang et al., 1995). The P0 protein of CLRDV is highly variable, added to being the suppressor of the host's post-transcription gene (PTGS) silencing mechanism (Cascardo et al., 2015; Agrofoglio et al., 2019). In addition, the P0 protein has been identified as an avirulence (Avr) determinant in cotton (Agrofoglio et al., 2019). As a result of the rapid spread of the CLRDV across several cotton growing US states, epidemiological studies aimed at early detection of new, emerging, and newly introduced strain/variant(s) of CLRDV and other plant viruses infecting cotton has become essential to shed new light on the recognition of the recent CLRDV outbreak in the US. This is because new strains of the virus may pose an existential threat to the sustainable production of cotton in the US because there is very little information available about sources of genetic resistance in cotton. In addition, improved molecular epidemiological tools are needed to facilitate accurate detection and monitoring the diversity and the potentially variable co-evolutionary signals of CLRDV populations associated with different cotton genotypes.

Methods

Symptomatic and asymptomatic leaf samples with leaf petiole attached were collected from cotton plants maintained in Sentinel plots in Alabama (AL), Florida (FL), Louisianan (LA), North Carolina (NC), Oklahoma (OK), South Carolina (SC), and Texas (TX). The samples were immediately flash-frozen in liquid nitrogen and shipped on dry ice under a domestic USDA-APHIS permit to the Brown lab, Plant Sciences, The University of Arizona. Samples were immediately removed from the dry ice and stored in a -80C freezer.

Total RNA was isolated using the modified silica extraction method according to Rott and Jelkman (2001). The amount and quality of total RNAs were estimated using Qubit fluorometer (ThermoFisher Scientific, Waltham, MA).

First strand cDNA synthesis was carried using SuperScript IV reverse transcription system (Invitrogen, Carlsbad, CA). PCR amplification was carried out using oligonucleotide primers designed to amplify the CLRDV open reading frame zero (ORF0) (P0_51F/P0_916R) and ORF3 (CLRDV3675F/Pol3982) viral fragments (Avelar et al., 2020; Sharman et al., 2015). The primer pairs were designed to amplify a fragment of the highly variable ORF0/P0 region of 868 base pairs (bp) and of the more conserved ORF3 region (310 bp) of the viral genome, respectively. Polymerase chain reaction (PCR) reaction amplification was carried out using REDTaq® ReadyMix™ PCR reaction mix (Sigma-Aldrich, St. Louis, MO) with the addition 0.2 µM of each primer and 2.0 µl of the cDNA template. The cycling conditions consisted of an initial denaturation of 95°C for 2 min, followed by 35 cycles of denaturation: 95°C for 30 sec, annealing: 56-62°C for 30 sec, and extension: 72°C for 40 sec-1 min, and final extension of 72°C for 10 min. Five microliters of each PCR product was analyzed by agarose gel (1%) electrophoresis containing GelRed (Biotium, Fremont, CA) to identify samples that yielded the expected size amplicon, respectively.

Amplicons of the expected size were ligated to pGEM®-T Easy vector plasmid vector (Promega Corp., Madison, WI), and transformed into chemically competent *Escherichia coli* cells. The presence of an expected size of the DNA insert was confirmed by colony PCR. Two independent clones were sequenced in both orientations using Sanger sequencing (chain-termination method). All steps were carried out using standard molecular biology protocols.

The plasmid vector sequences were moved from raw sequence reads and the forward and reverse reads were assembled in Geneious Prime (Biomatters, Inc., San Diego, CA), and a consensus sequence of each isolate was derived from two independent clones. Gene fragments of ORF0 and ORF3 of CLRDV isolates were obtained from the GenBank database (Benson et al., 2013).

Multiple sequence alignments were carried out using Muscle (Edgar, 2004), pairwise nucleotide and amino acid (aa) sequence comparisons of the ORF0 and ORF3 of CLRDV isolates from this study and global CLRDV isolates was carried out using Sequence Demarcation Tool (SDT) v.1.2 (Muhire et al., 2014).

Phylogenetic trees of CLRDV ORF0 and ORF3 were inferred using randomized axelerated maximum likelihood (RAxML) program (Stamatakis, 2014) with a bootstrap support of 1,000 replicates. The rate of evolutionary changes were modeled using general time reversible (GTR) model plus a gamma (G) distribution, and the best tree was selected. Phylogenetic analyses was carried out on the CIPRES web portal (Miller et al., 2010). Phylogenetic trees were displayed using the interactive tree of life tool (iTOL) v6.4 (Letunic and Bork, 2021).

Results and Discussion

During the 2020 growing season, two hundred and eighty-one (281) virus-symptomatic and virus-asymptomatic leaf samples were received from Alabama (216), Florida (31), Louisiana (10), North Carolina (5), Oklahoma (2), South Carolina (15), and Texas (2) (Figure 1).

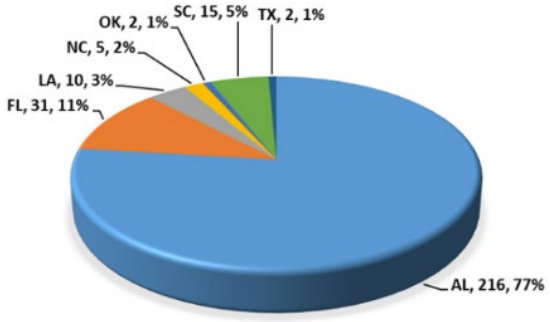

Figure 1. Proportion of cotton samples received from seven cotton growing US states

Leaf samples were analyzed for 20 cotton cultivars, with PHY 480 W3FE comprising 25.6% (72) of the samples received (Table 1), followed by DP 1646 B2XF (24.2%, 68); DP359Pima (12.5%, 35); EXP1 B3XF (11.7%, 33); and DG 3615 B3XF (8.5%, 24). Additional cultivars from AL represented 0.4% to 1.7% of the samples (Table 1).

Table 1. Cultivar composition of cotton samples received from different states.

	States								
Cultivar name	AL	FL	LA	NC	OK	SC	TX	Total	Percent
DG 3615 B3XF	15	5	3	0	0	1	0	24	8.5
PHY 480 W3FE	54	8	1	3	0	5	1	72	25.6
EXP1 B3XF	14	12	4	2	0	1	0	33	11.7
DP 1646 B2XF	52	6	1	0	0	8	1	68	24.2
PHY 360	0	0	1	0	0	0	0	1	0.4
DP 359 Pima	35	0	0	0	0	0	0	35	12.5
CG 3885 B2XF	3	0	0	0	0	0	0	3	1.1
CG 9608 B3XF	3	0	0	0	0	0	0	3	1.1
CG 1546 B2XF	4	0	0	0	0	0	0	4	1.4
CG 2038 B3XF	4	0	0	0	0	0	0	4	1.4
CG 2055 B3XF	3	0	0	0	0	0	0	3	1.1
PHY 400 W3FE	3	0	0	0	0	0	0	3	1.1
PHY 500 W3FE	3	0	0	0	0	0	0	3	1.1
PHY 580 W3FE	4	0	0	0	0	0	0	4	1.4
NG 4969 B3XF	4	0	0	0	0	0	0	4	1.4
NG 4068 B3XF	3	0	0	0	0	0	0	3	1.1
NG 5711 B3XF	3	0	0	0	0	0	0	3	1.1
ST 4990 B3XF	4	0	0	0	0	0	0	4	1.4
ST 5600 B3XF	2	0	0	0	0	0	0	2	0.7
ST 5610 B3XF	3	0	0	0	0	0	0	3	1.1
NIL	0	0	0	0	2	0	0	2	0.7
Sum	216	31	10	5	2	15	2	281	100.0

*Alabama (AL), Florida (FL), Louisiana (LA), North Carolina (NC), Oklahoma (OK), South Carolina (SC), Texas (TX)

Two hundred and forty-four samples were analyzed by RT-PCR for CLRDV presence using primers specific for ORF0 and ORF3. Of the 244 samples tested with the ORF0 primers, one hundred and sixty-three were positive (Fig. 2a) and eighty-one (81) samples were negative for CLRDV.

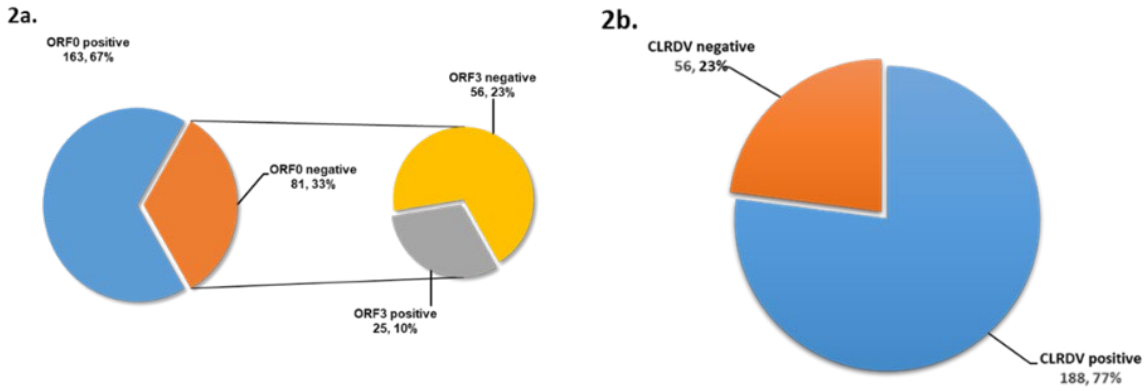

Figure 2a-b. Proportion of cotton samples positive for CLRDV using oligonucleotide primers that amplify ORF0 and ORF3 from cotton growing states in the US.

The eighty-one (81) samples negative for CLRDV ORF0 (Figure 2a) were subjected to a second round of PCR assays using primers that amplify a fragment of CLRDV ORF3. Of these samples that were initially negative for CLRDV using ORF0 specific primers, twenty-five (25) representing 10% of the original total number of samples were positive using primers specific to ORF3 (Figure 2a) indicating that primers designed to amplify ORF0 were not able to detect all CLRDV isolates. Overall, one hundred and eighty-eight (188) samples representing 77% of the total number of samples were positive for CLRDV using either ORF0 or ORF3 primers (Figure 2b) whereas, fifty-six (56) samples

representing 23% of the total number of samples were negative using either ORF0 or ORF3 primers. This observation shows a high incidence of CLRDV in cotton fields especially in Alabama from where most of the samples originated. Inability of primers P0_51F/P0_916R designed to amplify the ORF0 gene of the virus genome shows that variability within the virus populations precludes the amplification of some isolates, which could lead to false negatives in a disease diagnostics situation. Notably, none of the North Carolina isolates were amplified by the P0_51F/P0_916R primers. Consequently, sequencing additional complete genome sequences for CLRDV isolates occurring in other world regions, particularly Africa and Asia, could aid in better understanding global CLRDV diversity and paths of present and future spread, and provide more comprehensive database for designing PCR primers that permit molecular detection of diverse CLRDV isolates.

The ORF0 fragment of the CLRDV isolates sequenced in this study shared 94.5% - 100% nt identify with each other. Sequence comparisons of the isolates with those available in the GenBank database indicated nt identities ranging from 93.4% - 98.7%. The isolates shared the lower percent nt identities with GenBank isolates from Argentina or Brazil in South America, while they shared the higher percent nt identities with GenBank isolates from GA.

At the aa level, the P0 (ORF0) proteins of isolates from this study shared 91.6% - 100% aa similarity, compared to the other isolates available in GenBank, at 89.7% - 97.7% similarity. Similar to the nt sequence comparisons, the P0 proteins of the isolates shared the lowest aa similarity with the P0 proteins of isolates from South America and shared a higher aa similarity with the P0 proteins of GA isolates.

The ORF3 fragment of the US isolates shared 97.4% - 100% nt identity among themselves, and 93.8% - 99.4% identity compared to isolates from GenBank. At the aa level, the P3 protein of the US isolates were 96.1% - 100% similar, whereas a comparison of P3 among US and GenBank isolates indicated 92.2% - 100% similarity.

Among all known CLRDV isolates, ORF3 has been shown to exhibit greater aa sequence conservation than ORF0, observations that were corroborated by the results reported here. These results indicate that CLRDV isolates infecting cotton in the US are more like each other than they are to the ‘typical’ and ‘atypical’ CLRDV isolates described from South America. These observations do not support the hypothesis that CLRDV isolates extant in the US were recently introduced from either Argentina or Brazil, despite the evidence that they are the closest known CLRDV relatives to US isolates.

Of the 163 isolates for which an ORF0 amplicon was obtained, the nt sequences of 142 isolates were determined by Sanger sequencing. The maximum likelihood (ML) analysis of the CLRDV ORF0 fragment determined here with sequences available in Genbank indicated that most of the US isolates are closely related to US_AL CLRDV variant (GenBank accession no: MN071395) reported by Avelar et al (2019; 2020). The ML tree for all known CLRDV ORF0 sequences grouped the isolates into two monophyletic clades, with the US isolates forming two subclades, with the basal clade representing the most divergent US isolates, which were all from GA (Figure 3a).

Figure 3a. Phylogenetic analysis of the CLRDV ORF0 sequence determined for each isolate from Alabama (AL), Florida (FL), Louisiana (LA), Oklahoma (OK), South Carolina (SC), and Texas (TX). The evolutionary tree was reconstructed using random axelerated maximum likelihood (RAxML) method and 1,000 bootstrap replicates. Evolutionary distances were calculated using the General Time Reversible (GTR) model with the rate variation among sites modeled with a gamma distribution. Evolutionary analyses was carried out in CIPRES. The phylogenetic tree was drawn using iTOL. Color code: red=AL, blue=FL, brown=LA, yellow=OK, green=SC, black=TX, GA=Georgia, ARG=Argentina, BRZ=Brazil, SA=South America, US=United States.

Within clade 1a, a divergent cluster of isolates from Texas was resolved (arrowed). The closest relative to the latter isolates were isolates from GA (clade 1b). Collectively, they diverge from the most commonly occurring isolates that grouped with the 2017 isolate from Alabama (CLRDV US_AL MN071395). The second monophyletic clade (clade 2) harbored all CLRDV isolates known from Brazil and Argentina, indicating that two divergent phylogeographic groups of CLRDV occur in North America and South America, respectively. Thus, based on phylogenetic analysis of ORF3 sequences for the 25 CLRDV isolates amplified by ORF3-specific primers, the isolates grouped as two sister groups in the same clade, and are clearly divergent from the atypical and typical isolates known from South America (Figure 3b).

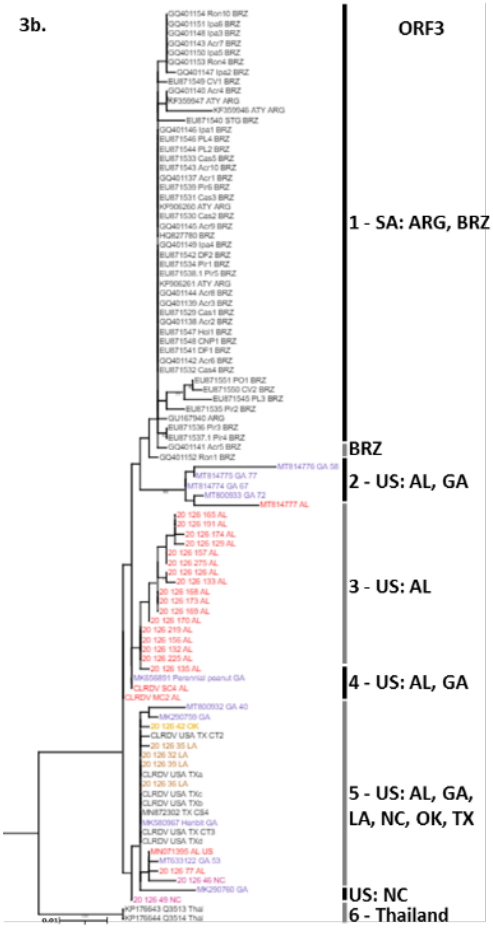

Figure 3b. Phylogenetic analysis of the CLRDV ORF0 sequence determined for each isolate from Alabama (AL), Louisiana (LA), Oklahoma (OK), North Carolina (NC), and Texas (TX) in the US (Clades 2-5), and for isolates available in the GenBank database from Argentina and Brazil (Clade 1), and Thailand (Clade 6). The evolutionary tree was inferred in CIPRES using random axelerated maximum likelihood (RAxML) method with 1,000 bootstrap replicates. The distances were calculated using the General Time Reversible (GTR) model with the rate variation among sites model and gamma distribution. The phylogenetic tree was drawn using iTOL with the following color code: red=AL, brown=LA, yellow=OK, pink=NC, black=TX. GA=Georgia, ARG=Argentina, BRZ=Brazil.

Clade 1 comprised CLRDV isolates from only Argentina and Brazil. Clades 2 and 4 contained divergent isolates from GA and AL, while isolates in Clade 3 were from AL only. Clade 5 contained isolates found in all US states for which samples were collected, AL, GA, LA, NC, OK, and TX except SC, with one NC isolate, in the basal position. Clade 6 was represented by two isolates from Thailand (Asia) that were overall the most divergent CLRDV isolates. The CLRDV isolates from the US are most closely related to all other US isolates than they are to those from South America or Thailand. Based on the tree structure, at least three CLRDV introductions are hypothesized to have occurred in the US.

Summary and Conclusion

Two hundred and eighty-eight (288) samples were received from the cotton sentinel plots from the following seven states: AL, FL, LA, NC, OK, SC, and TX, of which 244 were analyzed for CLRDV presence by RT-PCR. One hundred and eighty-eight (77%) of samples received from the different states were positive for CLRDV presence based on RT-PCR detection with either or both primer pairs, which were designed to amplify viral ORF0 and ORF3. Finally, 56 cotton samples were found negative for CLRDV presence by RT-PCR using both primer pairs, despite suspect CLRDV-infection. The results indicate that CLRDV was prevalent in all US cotton sentinel plots for which samples were analyzed in this collaborative effort. The inability of P0_51F/P0_916R to detect CLRDV in some of the sentinel

plot samples, despite evidence of virus-like symptoms, may possibly suggest the occurrence of additional ORF0 variants that are undetectable by the available primers (used in this study), and/or that other viruses are responsible for suspect virus-like symptoms or that symptoms are caused by other than a plant virus pathogen. To address these unanswered questions, sequencing of additional CLRDV genomes is underway to provide a more complete inventory of genomic variability within US CLRDV isolates. Additional divergent CLRDV genomes will aid breeding efforts to select/develop CLRDV-resistance genotypes, and potentially inform the design of more robust RT-PCR primers for universal detection of CLRDV globally.

Based on the results of pairwise nt distance analysis and aa sequence comparisons, the CLRDV populations in the US are most closely related to the reference sequence for the first variant reported in the US from Alabama (GenBank accession: MN071395) (Avelar et al., 2019; 2020), compared to typical and atypical CLRDV strains known from Brazil and Argentina (Ramos-Sobrinho et al., 2021). The phylogenetic analyses resolved at least two or three phylogeographic groups among known CLRDV isolates globally, centered in Asia (Thailand), South America, and the US, and offer no reliable clues about the immediate origin(s) of CLRDV introductions into the US. The phylogenetic tree based on the ORF0 fragment showed that CLRDV US_AL (MN071395) is the most common genotype and that it is distributed in all of the US cotton-growing areas. The variability evident in ORF0 sequences, however, suggest more than one introduction has occurred and/or that a single introduction occurred followed by potential rapid divergence based on geographic isolation and/or cotton genotype-isolation associated with geographically favored cotton genotypes. Finally, the potential failure of the ORF0 specific primers (this study) to detect some suspect CLRDV isolates, together with the phylogenetic partitioning of isolates into distinct clades and sister clades based on ORF3 analysis, favors the hypothesis that multiple sources and/or one or multiple CLRDV introductions occurred in the US. Regardless of which hypothesis is born out, it is likely that CLRDV has been established in the US for some period of time prior to its detection in 2017, which may have been abetted by the occurrence of unusual or rare weather or other unsuspected conditions that supported abrupt and/or widespread aphid dispersal of CLRDV throughout the cotton belt.

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