IDENTIFICATION OF A *COTTON LEAFROLL DWARF VIRUS-LIKE* POLEROVIRUS INFECTING COTTON IN ALABAMA DURING 2017-2018

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Abstract

Cotton *Gossypium hirsutum* (L.) is one of the most economically important crops in the southeast United States. Cotton blue disease has been associated with two exotic polerovirus variants, *Cotton leafroll dwarf virus* (ACLRDV) and *Cotton leafroll dwarf virus* (CLRDV) known to infect cotton in Argentina and Brazil. The virus is thought to be endemic in Africa or Asia and has spread to South America only recently. During 2017, an aphid-transmitted poleroviruses, was identified for the first time infecting symptomatic cotton plants **i**n Alabama. Provisional identification was based on 1143 bp fragment based on Illumina RNA sequencing, and cloned viral amplicons obtained by RT-PCR amplification. To obtain the full-length viral genome, RNA was isolated from symptomatic cotton leaves and petioles collected in Barbour and Macon counties during 2018 and subjected to Illumina sequencing. Analysis of the assembled contigs revealed a ~5770 nt viral genome that most closely matches *Cotton leafroll dwarf virus* variants from South America. Phylogenetically, it is unique and an outlier to ACLRDV and CLRDV isolates. Based on pairwise distances, the polerovirus isolate from Alabama shares its highest nucleotide (nt) identity with CLRDV from Brazil [HQ827780], at 95.6%. The apparently full-length genome contains six open reading frames (ORFs), ORF0-ORF5. The most divergent coding region among the six available genomes is ORF0, which encodes a viral suppressor of host-gene silencing, at 90.6-92.0% nt identity and 82.4-88.5% amino acid similarity, the latter, which is suggestive of a distinct species (>10% divergence). In 2018, the extent of its distribution in Alabama cotton plantings was investigated using reverse transcriptase-PCR and previously validated primers. The expected size amplicon of 310 bp was detected in 196 of 400 cotton samples from Alabama, and selected amplicons were cloned and sequenced, confirming virus presence in 20 counties.

Introduction

Cotton blue disease (CBD) has been reported affecting cotton in Africa, Asia, and South America. Cotton plants affected by CBD display variable symptoms including stunted phenotype, shortening of internodes, leaf rolling, vein yellowing, and an intense green to blue leaf color (Brown 2001, Cauquil and Vaissayre, 1971). Although the disease was reported in Central African Republic in 1949 (Cauquil 1977), the etiology was not known until more than 50 years later (Corrêa et al. 2005) when it was associated with the presence of *Cotton leafroll dwarf virus* (CLRDV) (genus *Polerovirus,* family, *Luteoviridae*). In 2010 the complete genome of CLRDV was sequenced revealing it was a positive-sense single stranded RNA virus, approximately 5.8kb in length. The virus is transmitted by the cotton aphid *Aphis gossypii* (Glover) in a circulative, persistent manner. It is not known to be seedborne (Mayo and Ziegler-Graff 1996, Distéfano et al. 2010).

After the introduction of CLRDV into Argentina and Brazil, the disease has been managed by growing CBD-resistant varieties and through the application of insecticides to control the aphid vector. During 2006, typical symptoms were observed in CBD-resistant cultivars in Brazil that were not consistent with CBD, despite the detection of CLRDV. Virus-infected plants exhibited mild symptoms including foliar reddening, distortion of the leaf lamina and veins, and vertically-positioned leaves (Silva et al. 2015). Shortly thereafter, in 2009-2010, a virus-like disease outbreak occurred in Argentina, affecting CBD-resistant cultivars. The symptoms consisted of foliar reddening and withering of leaves held vertically, compared to their more upright stature on the uninfected cotton plants, and mild leaf-rolling and crumpling. The latter virus-infected cotton plants produced less fiber and fewer seeds, negatively affecting yields

(Agrofoglio et al 2017, Mukherjee et al. 2012). A comparison of the full length-genome sequence associated with the atypical symptoms, with previously sequenced CLRDV isolates from Argentina and Brazil indicated that atypical symptoms were associated with a previously unknown, apparently resistance-breaking strain of CLRDV, referred to as atypical CLRDV (ACLRDV). The virus isolates shared high amino acid identities with the open reading frames (ORFs) encoded by previously determined CLRDV genomes (Distéfano et al. 2010), with the exception of ORF0, with which it shared only 86-88% amino acid similarity (da Silva et al. 2015), which encodes a protein involved in suppression of host plant gene-silencing (Delfosse et al. 2014).

Toward the end of the 2017 cotton-growing season in the southeastern cotton-growing states of the U.S., virus-like symptoms were observed in commercial cotton fields. In Alabama, Florida, Georgia, Louisiana, and Mississippi, similar symptoms were observed in cotton that consisted of foliar distortion, curling, rolling, and bluish-green discoloration, vein-clearing, and shortened internodes that resulted in dwarfing of plants, swollen and brittle stems, and reduced boll set. Symptoms were distinct from herbicide damage, and more severe than those characteristically caused by whitefly or aphid feeding alone. In south coastal Alabama, leaf crumpling and chlorosis, vertical stature of leaves, and at times vein-reddening were observed in cotton fields in the six counties initially sampled (Figure 1A-D). Whiteflies were abundant in cotton fields in Alabama coincident with symptom appearance and low-level aphid populations had been noted earlier in the season. To investigate the identity of the suspect causal virus, leaves were collected from symptomatic cotton *Gossypium hirsutum* (L.) plants in Barbour County in late August and mid-September 2017. Because whiteflies were abundant in the cotton fields, the presence of a whitefly-transmitted viral disease was suspected, namely, the New World, bipartite *Cotton leaf crumple virus* (CLCrV) or *Squash leaf curl virus* (SLCV) native to the southwestern US and western Mexico (Brown, 1992), and the Old-World cotton leaf curl virus disease complex (CLCuD) endemic to Pakistan and India, among which the *Cotton leaf curl Multan virus* (CLCuMuV), recently introduced into China and Philippines.

The objective of this study was to determine the identity of the suspect plant virus associated with symptomatic cotton in Alabama during 2017-2018. Initially, DNA was isolated from cotton leaves and petioles and subjected to polymerase chain reaction (PCR) using primers designed for detecting whitefly-transmitted geminiviruses, which facilitate broad- or specific-begomovirus and/or betasatellite detection (Brown et al. 2017). No begomovirus was detected in any of the symptomatic cotton samples. Second, an approach that relies upon Next Generation Sequencing (NGS) of DNA or RNA from symptomatic samples was implemented, as a relatively new approach for pathogen discovery for which no *a priori* knowledge of the nature of the suspect pathogen is needed. The NGS platforms are capable of producing millions of DNA or RNA sequence reads that are *de novo* assembled bioinformatically. Assembled partial or full-length viral genome(s) of suspect DNA or RNA viruses are identified by matching the resultant sequence(s) to the closest relatives for which a partial and/or full-length genome sequence is available in public databases, including NCBI GenBank. The Illumina-DNA sequencing yielded no DNA virus-like genomes or partial genomes, however, the RNAseq produced sequence that allowed for tentative identification of this CLRDV-like polerovirus, an exotic aphid-transmitted virus of cotton not previously identified in the U.S. Here, the results of the Illumina sequencing of 2017 and 2018 isolates are reported, together with those based on reverse-transcriptase PCR (RT-PCR) amplification using primers based on the resultant Illumina-derived viral genome sequence, and cloning and Sanger sequencing of the amplicons. The results confirmed the identification of a CLRDV-like virus, based on preliminary characterization of the full-length genome sequence, and phylogenetic and pairwise distance analyses.

Methods

Cotton samples were collected in Barbour Co., Alabama, during 2017 (Figure 1A-D). Total DNA was extracted from cotton leaves using CTAB (Doyle and Doyle, 1990), and total RNA was extracted using Direct-zol Miniprep Plus kit (Zymo Research). Total DNA and RNA were subjected to DNA and RNAseq Illumina Hi-Seq 2500 (paired-end reads, 150 bp) shotgun sequencing. Reads were *de novo* assembled using DNASTAR NGEN v. 12.0. Contigs were annotated using BLASTn (NCBI database). Among the sequences assembled from the RNAseq run, several small and one large contig of 1143 bp in size was recovered. Sequences shared its closest BLASTn sequence match to the genome sequence for isolates of Atypical *Cotton leafroll dwarf virus* (ACLRDV), Accession number KF359947, corresponding to an isolate from Argentina, at 97% similarity (100% coverage). To verify the identity of the CLRDV-like_AL viral contigs from the 2017 samples, the PCR primer pair, AL674F-5'-CCGTAGCGGTCATCGTCTTT/AL1407R-5'-TAACCCTGACACAGTGGGGA, was designed based on a region conserved in the six available CLRDV genome sequences [GenBank Accession no: GU167940, HQ827780,

KF359946, KF359947, KF906260, KF906261]. The AL674F/AL1407R primers were used in parallel with the previously reported polerovirus-specific primers, CLRDV3675F and Pol3982R (Sharman et al. 2015), for RT-PCR amplification from total RNA, purified from symptomatic cotton plants (n=3). The identity of the virus as CLRDV was confirmed by bi-directional Sanger DNA sequencing of the cloned PCR amplicons. Pairwise comparisons with the six CLRDV reference genomes were carried out using Sequence Demarcation Tool (SDT v.1.2, Muhire et al. 2014). Because the 2017 cotton samples were collected near the end of the 2017 growing season, additional samples could not be obtained for independent verification or to determine the complete genome sequence of the virus until the 2018 growing season.

In contrast to the 2017 season, symptoms were observed in several counties in Alabama by mid-summer during 2018. Symptomatic leaves were collected from plants in the locations where symptoms were observed, and throughout the cotton-growing season from counties as symptoms became evident. Petiole and leaf samples were collected from symptomatic plants across Alabama. Total DNA was isolated from petioles (DNeasy kit, Qiagen) or from cotton leaves (CTAB, per 2018 sample preparation). The DNA from the first 200 samples was tested for the presence of begomoviruses (*Cotton leaf curl Multan virus,* which is endemic to south Asia*,* and *Cotton leaf crumple virus,* which is endemic to the SW-US), using degenerate primers that amplify a conserved region of the coat protein gene of most begomoviruses (Brown et al., 2001).

Total RNA was isolated from 400 samples using RNeasy kits (Qiagen). Synthesis of cDNA from all RNA extracts was performed using the SuperScript™ IV First-Strand Synthesis System (Invitrogen™), according to the manufacturer's instructions, and the virus-primer, Pol3982R. The primers CLRDV3675F and Pol3982R (Sharman et al. 2015) were used for diagnostic RT-PCR of CLRDV.

For Illumina sequencing, total RNA was purified from the petioles collected from symptomatic cotton plants in Barbour, and Macon counties, using a modified silica capture RNA isolation method (adapted from Rott and Jelkmann, 2001), and subjected to RNAseq Illumina shotgun sequencing (NextSeq v2 2x75 High output, paired-end reads, 75 bp). The reads were mapped to CLRDV reference sequences using Bowtie2 (Langmead and Salzberg, 2012). Mapped reads were reference-guided assembled, and viral open reading frames (ORFs) were annotated using the most similar CLRDV reference sequence [GenBank, HQ827780] as template in Geneious Prime v. 2019.0.4 (http://www.geneious.com, Kearse et al., 2012). The assembled contig was aligned with six CLRDV reference genomes using MUSCLE algorithm (Edgar et al. 2004). Pairwise comparisons with the six CLRDV references were performed with SDT v.2.1 (Muhire et al. 2014). The Maximum Likelihood (ML) tree was reconstructed (1000 bootstrap iterations) using MEGA X v.10.0.4 (Kumar et al. 2018).

Results

For the 2017 samples, none of the suspect ssDNA-containing begomoviruses were detected using diagnostic PCR primers specific for begomoviruses, or by 'discovery' using the Illumina DNA sequencing platform, *de novo*-assembly of contigs, and BLASTn analysis against begomoviral sequences available in the GenBank database. However, one RNA *de novo* assembled contig comprising 103 reads assembled into a 1143 bp fragment [Accession no. MK015625], that was most closely related to a previously characterized, atypical CLRDV isolate from Argentina [KF359947], having a 97.1% similarity score with 98% sequence coverage (BLASTn, GenBank). Tentatively, the CLRDV-like virus belongs to the genus, *Polerovirus* (family, *Luteoviridae*), a group of aphid-transmitted, positive-sense, single-stranded RNA viruses, with a genome size of ~5.8 kb (Distéfano et al. 2010). Virus presence in samples was confirmed by bi-directional Sanger sequencing of cloned PCR amplicons obtained using AL674F/1407R and 3675F/Pol3982R. The amplicons had BLASTn similarity scores of 94-99%, with closest matches to two poleroviruses from Argentina and Brazil, CLRDV and ACLRDV. Pairwise distance analysis (Muhire et al. 2014) of the amplicons for the Alabama isolate with analogous genome fragments for the six available leaf roll isolates indicated they shared 91.5-99.3% nt sequence identity. This indicates that the isolate is closely related to, but divergent from, previously reported leaf roll disease isolates from Brazil and Argentina, based on the available partial genome sequence.

For 2018 samples, the viral sequences determined from the 2018 samples provided a nearly complete genome sequence of 5771 bp in size (minus the predicted 94 nt located at the non-coding 3'end). Validation of the sequence by cloning, and primer walking has been completed for most of the genome, and the remainder, in progress, is expected to be completed very soon. Based on comparative analysis of the apparently complete genome sequence, it shared 95.6% similarity at 100% coverage (BLASTn, GenBank) to an isolate from Brazil [HQ827780], indicating the U.S.

isolate virus is closely related, but distinct from to isolates from Brazil and Argentina, available in GenBank. The sequence encodes six predicted coding regions, or open reading frames (ORFs), ORF0 to ORF5. The ORF0 encodes for a putative viral suppressor of host gene silencing (Delfosse et al. 2014), and it was found to be the most divergent among ORFs, at 90.6 - 92.0% shared nt identity, and 82.4-88.5% amino acid similarity in relation to the six full-length reference genome sequences for two CLRDV-like isolates from South America.

Using six degenerate and virus-specific primers and PCR amplification, begomovirus was not detected from symptomatic cotton samples collected in AL during 2018, a result that is consistent with that obtained for 2017 samples. Also consistent with a recent report (Avelar et al., 2019), the expected size amplicon of 310 bp in size was obtained for 196 of 400 samples by PCR amplification using primers, CLRD_3675F/Pol3982R, which is the expected size for CLRDV and closely related polerovirus isolates from Argentina and Brazil. Cloned amplicons for representative, positive cotton samples were subjected to DNA sequencing (Sanger), confirming that the amplicons shared high (97-99%) identity to the analogous region of CLRDV isolates (GenBank). Based on PCR amplification, the exotic polerovirus was detected in 20 counties in Alabama, including the far northern, southern, eastern, and western regions of the state.

The CLRDV is aphid-transmitted, as is known for other poleroviruses. The mode of transmission is persistent and circulative, with transmission by viruliferous aphids continuing for up to 12 days, post-acquisition-access (Michelotto and Busoli 2007). In the southern U.S., cotton aphid infestations occur annually in the cotton belt (Abney et al. 2008, Gore et al. 2013, Kerns et al. 2015). During 2018, the cotton aphid infested cotton across central and southern Alabama during the latter part of June (authors, personal observations). The widespread abundance of aphids, and relatively high predicted rates of transmission, combined with the propensity for aphids to disperse long distances on wind, appears to have facilitated the spread of CLRDV-like isolate throughout much of Alabama (and in other coastal cotton belt states). The capacity of aphids to migrate across the southern U.S. has been demonstrated by the recent invasion of the sugarcane aphid in sorghum crops, spreading across the southern U.S. over a two-year period (Bowling et al. 2016), and into the southwestern U.S. by 2017 (J.K. Brown, personal observation). These phenomena underscore the potential for CLRDV to spread to other cotton-producing states in the southern U.S., and into cotton-growing areas of Texas and several midwestern states, as well as to Arizona and California. Cotton production and aphid management practices may require a serious re-evaluation to protect the U.S. cotton crop from potentially disastrous losses, if virus outbreaks occur in subsequent years.

Summary

Virus-like symptoms were observed in cotton plantings in Alabama during 2017 and again during 2018. Illumina-RNAseq discovery and verification by RT-PCR amplification, the presence of the exotic ssRNA virus, CLRDV, was identified in symptomatic cotton samples. Despite heavy infestations by the B mitotype of whitefly *Bemisia tabaci* (Genn.), the insect vector of the genus, *Begomovirus* (*Geminiviridae)*, begomoviruses known to infect cotton in the U.S., including *Cotton leaf crumple virus*, or elsewhere, worldwide, such as the *Cotton leaf curl virus* complex, were not detected by Illumina DNA sequencing or PCR amplification. CLRDV is endemic to Africa (Brown, 1990) from where it has apparently spread to the Asian-Pacific region (Ray et al. 2016). Two closely-related polerovirus variants, CLRDV and ACLRDV, have been identified as exotic introductions in Brazil and Argentina (Corrêa et al. 2005, Distéfano et al. 2010). Until now, CLRDV-like polerovirus have not been reported in Central or North America or in the Caribbean region. The route by which the exotic CLRDV-like virus has been introduced in commercial cotton, in Alabama, Florida, Georgina, Louisiana, and Mississippi (authors, personal observation) has not been determined. Studies are needed to track the dispersal of the cotton aphid and associated spread of this exotic polerovirus in the U.S. Very little is known about the host range of CLRDV/ACRDV in South America. Whether alternate hosts of the U.S. isolate of the CLRDV-like virus exist that may serve as over-wintering reservoirs is not known. Also, whether the virus will become established persistently in the southern U.S. cotton belt states, and/or spread to western U.S. cotton producing areas is not known. To combat the spread of this exotic virus, aphid management practices require a critical re-evaluation, and evaluation and identification of virus resistant-germplasm are now essential to protect the U.S. cotton crop from losses due to virus infection. To our knowledge, this is the first report of the complete genome sequence determined for the exotic polerovirus found infecting cotton in Alabama during 2017-2018, and of its' introduction into the southern U.S. The virus isolate reported here is genomically distinct from the cotton-infecting polerovirus variants occurring in South America and will likely be considered a strain of CLRDV by the International Committee on Taxonomy of Viruses.

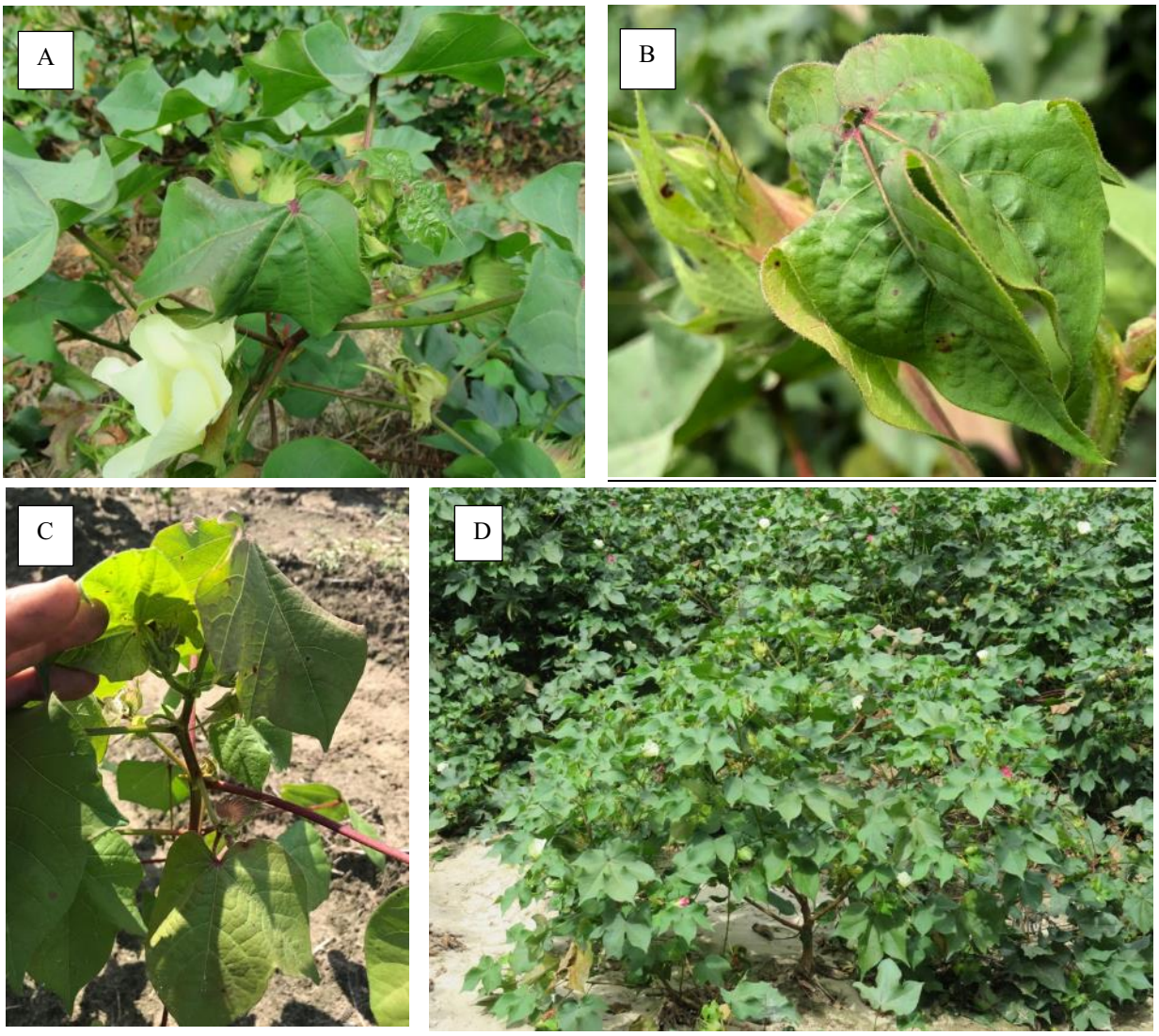

Figure 1A-D. Virus-like symptoms observed in cotton in Alabama during 2017. (A) Downward foliar cupping, reminiscent of a roof-like structure; (B) downward foliar cupping, vein-reddening, puckering, and leathery texture; (C) foliar cupping and vein-thickening on undersides of leaves, and vein-clearing; and (D) overall stunting of the plant, resulting from shortened internodes.

Acknowledgements

The authors gratefully acknowledge the funding provided by several research grants to KL and from Cotton Incorporated grants to JKB and KC. Scouting support and sample collection was supported in 2017 and 2018 by DS Agri-AFC, LLC. Authors also appreciate the additional sample collections in 2018 by Marina Rondon, David Dyer, Bisho Lawaju, Edward Sikora, and Austin Hagan. Partial support for a Graduate Research Assistantship for SA was provided by Cotton Incorporated.

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