EPIDEMIOLOGY AND MANAGEMENT OF EMERGING VIRUS DISEASES ON *GOSSYPIUM HIRSUTUM* L

by

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(Under the Direction of SUDEEP BAG)

ABSTRACT

Emerging viral diseases are a growing concern for cotton production in the USA. Cotton leafroll dwarf virus (CLRDV), which causes cotton leafroll dwarf disease (CLRDD), poses a persistent threat to the cotton industry. In this study, we analyzed P0 gene diversity, tissue tropism, and epidemiology of CLRDV, providing critical insights into advancing disease management strategies for Upland cotton (*Gossypium hirsutum* L) production in Georgia (GA). To optimize diagnosis, we examined CLRDV distribution across cotton tissues. We synthesized cDNA clones of CLRDV and assessed their infectivity via agroinoculation and infiltration under greenhouse conditions, along with aphid-mediated transmission, to investigate disease progression and symptom development. To address the grower's concern in GA, we evaluated the role of caulimovirid-like endogenous viral elements (EVE-GA) in terminal abortion and yield loss. Additionally, field monitoring was conducted from 2022 to 2024 in on-farm variety trials across Georgia to evaluate the involvement of CLRDV in symptom expression resembling bronze wilt in specific cultivars and associated yield loss. Our findings revealed that the P0 gene of GA isolates exhibited atypical genotypes. Phylogenetic analysis showed that North American isolates form a distinct clade, with cotton and weed species isolates in the USA segregating into two

clusters. Tissue tropism analysis detected high viral gene copy numbers in newly growing top branches. No association was found between EVE-GA and terminal abortion, and no significant yield loss was observed in symptomatic plants. Greenhouse studies using virulent aphids and cDNA clones did not result in disease progression or symptom development. Field monitoring revealed spatial and temporal variability in CLRDD incidence and yield loss. Specific cultivars infected at early growth stages exhibited severe wilting and bronzing, with higher viral gene copy numbers than their asymptomatic counterparts, suggesting a role of the virus in CLRDV-induced bronze wilt. Symptomatic plants experienced complete yield loss, whereas asymptomatic plants showed no significant yield reduction. These findings provide critical insights into CLRDD epidemiology and symptomatology, highlighting the need for future research on disease development factors. This knowledge will support breeding efforts and inform disease management strategies to sustain cotton production in the USA.

INDEX WORDS: Cotton leafroll dwarf virus-induced bronze wilt, *Gossypium hirsutum* L, Cotton, terminal abortion, endogenous viral elements, tissue tropism

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DEDICATION

To My Family and Friends

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CHAPTER 1

INTRODUCTION

History of Cotton

The word *cotton* originated from the Arabic term *qutn*, which became *algodón* in Spanish and *cotton* in English. Cotton (*Gossypium* sps.) crop belongs to the Malvaceous family and cultivation dates to the 6th millennium B.C. or before (Moulherat et al., 2002). The origins of cotton remain uncertain, but archaeological evidence provides key insights. In Mexico's Tehuacán Valley, cotton bolls, seeds, and cloth remnants were found dating back to approximately 3500 BCE and possibly as early as 5800 BCE, resembling modern American varieties of *Gossypium hirsutum* (MacNeish, 1967; Smith & Stephens, 1971; Georgia Cotton Commission, 2016). In present-day Pakistan's Indus River Valley, cotton fibers from Mehrgarh, traced to 4500 BCE, indicate early use, with widespread cultivation and weaving established by 3000 BCE (Moulherat et al., 2002; Lee & Fang, 2015). Cotton in Egypt's Nile Valley emerged later, likely introduced by around 500 BCE through trade, with broader cultivation developing in subsequent centuries.

Background and importance of cotton in Georgia

There are four domesticated species of cotton, two of which are native to the Old World and two to the New World. *Gossypium arboreum* L. and *Gossypium herbaceum* L. are diploid species that originated in the Old World. *Gossypium arboreum* remains an important crop in India, where it has been grown for centuries. In contrast, *Gossypium herbaceum*, once significant, is now primarily cultivated in drier regions of Africa and Asia for local use. On the other

hand, *Gossypium barbadense* L. and *Gossypium hirsutum* L., both allotetraploids, evolved in the New World. *Gossypium barbadense* is known for its high-quality fibers and is mainly grown in tropical and subtropical regions, while *Gossypium hirsutum* is the most widely cultivated species globally due to its high yield and adaptability, making it the dominant variety in commercial cotton production (Lee and Fang, 2015; Wendel & Cronn, 2003). These species have played a crucial role in the history and global production of cotton. The United States of America (USA) is a world-leading exporter, accounting for 35 percent of global cotton, primarily grown in the southern "Cotton Belt" states ranging from Virginia to California. Texas is the largest cotton producer in the USA, contributing 40% of the total USA production, followed by Georgia, Mississippi, and Arkansas (Economic Research Service, USDA, 2024). Among the two new world tetraploid cotton species, Pima cotton (*Gossypium barbadense*) accounts for 4%, while Upland cotton (*Gossypium hirsutum*) comprises about 90% of the total cotton production in the world (Lu et al., 1997, McCarty et al., 2004). Cotton is an important fiber crop contributing more than $120 billion to the USA economy. Georgia is the second leading cotton producer in the USA, with 1.93 million bales of cotton lint and 548,000 tons of cotton seed produced in 2024 (NASS USDA 2024).

Cotton leaf roll dwarf virus

Among the numerous viruses transmitted by insect pests in crops, the aphid-transmitted *Polerovirus*, cotton leafroll dwarf virus (CLRDV) is a pathogen of concern in cotton in the USA. CLRDV is an emerging pathogen causing CLRDD in the USA and a causal agent of cotton blue disease (CBD) in different parts of the world. CBD was first reported around 1949 in the Central African Republic and later in South America (Edula et al., 2023). In the USA, CLRDV was first observed in Alabama from cotton in 2017. CLRDV is a phloem-limited virus and is transmitted

efficiently by the cotton aphid (*Aphis gossypii* Glover) in a circulative and non-propagative manner (Cauquil & Vaissayre, 1971; Heilsnis et al., 2023; Michelotto & Busoli, 2003). The symptom expression varies due to multiple biotic and abiotic factors, including cotton variety, geographic location, planting date, aphid population density, and environmental conditions (Bag et al. 2021). Plants infected with CLRDV showed symptoms including intense dark green to bluish foliage, reddening of stems and petioles, curling and drooping of leaves, internodal shortening, and moderate to severe stunting. CLRDV has been associated with significant but variable yield losses in cotton production in the USA cotton belt. Field diagnosis is difficult due to symptom overlap with abiotic stress and unexplained physiological disorders, such as bronze wilt, demanding reliable molecular detection for accurate assessment of virus involvement and cultivar-specific responses. Moreover, CLRDV was detected in symptomatic and asymptomatic plants from both commercial fields and research trials (Tabassum et al., 2021; Edula et al., 2023). In some cultivars, plants infected with CLRDV in younger stages could suffer complete yield loss, whereas the losses decrease when plants are infected at the mature stages of plant development (Parkash et al., 2021). Although the incidence of the virus in some commercial cotton fields in Georgia and Alabama is 80%–100%, no significant yield losses have been reported (Mahas et al., 2022). Hence, vigilant monitoring of disease incidence on commercial cotton cultivars in growers' fields and checking host response to CLRDV infections is vital to understand the disease impact.

The isolates present in the USA differ from those causing CBD in South America and other regions. The disease caused by CLRDV in the United States is hence referred to as cotton leafroll dwarf disease (CLRDD; Brown et al., 2019). CLRDV is a non-enveloped, spherical virus having a positive ssRNA genome of approximately 5.7kb in length consisting of seven open

reading frames (ORFs). The first ORF, ORF0 encodes a 29kDA protein, P0 which potentially functions as a silencing suppressor (Delfosse et al., 2013). P0 gene is highly variable and the amino acid replacement from isoleucine (I) to valine (V) (I-V) at the 72nd position in the F-box domain of P0 protein is the demarcation between the 'typical' and 'atypical' genotype of CLRDV (Cascardo et al., 2015; Tabassum et al. 2021). The five unique substitutions identified in the P0 protein of CLRDV isolates from the USA could also contribute to different symptoms associated with this disease. Georgia isolates also had a unique insertion of arginine (R) at the 120th position (Tabassum et al., 2021). The significance of these substitutions in ORF P0 is yet to be investigated to understand their role in disease development. Such variations due to random mutations in the P0 sequence might significantly influence disease development and severity. Therefore, characterization of the P0 sequences from CLRDV-infected plants over a period can help to check the mutations in the P0 sequence.

Bronze wilt or bronze wilt complex

In 1995 and early 2000, cotton crop in the USA had a major concern, an unexplained anomaly called bronze wilt (BW) or bronze wilt complex (BWC) (Creech, 1999) in cotton which is also known as sudden wilt, copper top, red top, bronzing, and early fadeout (Bell et al., 1998, 2002). Predominant symptoms associated with BWC were bronzing of leaves, red stems, stunting, rugose leaves, absence of squares, square and boll abortions, reduction in internode length, and wilting (Phipps, 2000; Albers and Guthrie, 2001). Symptomatic leaves were also warmer than average leaf temperatures (Phipps, 2000). During this period, symptoms similar to BW were also described in Argentina, Bolivia, and Brazil, with certain germplasm and cultivars showing more susceptibility and severity (Creech 2000), suggesting a potential cultivar-specific anomaly. The occurrence and economic impact of BW varied across different states in the USA (Bell et al., 1998; Creech, 1999;

Brown, 2000; Phipps, 2000; Creech and Fieber, 2000; Padget et al., 2004). Susceptibility to the BW was strongly associated with varietal response (El-Zik et al., 2001; Nichols, 2001) and environmental factors, particularly temperature, which may have exacerbated symptom severity (Bell, 2000; El-Zik et al., 2001). A few researchers associated the disease with *Agrobacterium* biovar I as a potential causal agent. However, investigations were inconclusive and not widely accepted, since the bacterium is an endophytic parasite commonly present in cotton seeds, and Koch's postulates could not be fulfilled (Bell 1998, 1999, 2000). Researchers believed that molecular approaches could provide deeper insights into the etiology of BW, but no such methodologies were employed at the time to investigate potential viral involvement, leaving a critical gap in the understanding of its causation. Descriptions of both CLRDD and BW show overlapping symptoms, progression, and recovery of symptomatic plants (Albers and Guthrie, 2001; Tabassum et al., 2019). The phenotypic symptom description of the bronze wilt complex in cotton is nearly identical to the CLRDD symptoms (Edula et al. 2023: Parkash et al. 2021). Since bronzing and wilting can result from both biotic and abiotic stresses, a comprehensive investigation of virus involvement is essential to determine their underlying causes.

Endogenous viral elements and terminal abortion symptoms in Upland cotton (*Gossypium hirsutum* L.) in Georgia, USA

In plants, the frequently observed EVEs, called "plant pararetroviruses", belong to the genus *Caulimovirus*, and their sequences are believed to be embedded into their host genomes due to ancient virus infections dating back thousands to millions of years ago (Gayral & Iskra-Caruana, 2009). Replication-competent endogenous viral elements (EVEs) can be activated by factors such as genome hybridization, tissue culture, abiotic stress, and physical wounding. This phenomenon has been observed in several plant hosts, including banana with endogenous

Banana Streak Viruses (eBSVs) (Harper et al., 1999; Ndowora et al., 1999), tobacco with endogenous Tobacco Vein-Clearing Virus (eTVCV) (Lockhart et al., 2000), and petunia species with endogenous Petunia Vein-Clearing Virus (ePVCV) (Harper et al., 2003; Richert-Pöggeler et al., 2003; Richert-Pöggeler & Shepherd, 1997). In June of 2023, upland cotton in Georgia (GA), US, experienced both abiotic and biotic challenges during cooler-than-average summer temperatures and it was noted that young cotton plants frequently exhibited terminal abortion, resulting in the development of profuse vegetative branching symptoms noted in plants at the 3-leaf stage and a few instances at the 5–6-leaf stage (Edula et al., 2024). Causation was unknown, and research at the time suggested the presence of caulimovirid-like endogenous virus elements embedded in the tetraploid cotton genome (Aboughanem-Sabanadzovic et al., 2023). However, their involvement was unknown, and the appearance of terminal abortion in young cotton plants with no apparent cause raised concerns among industry and academic scientists alike.

Expected outcomes and economic implications

Before our investigations, it was unknown whether P0 sequences in CLRDV isolates from Georgia diverged from their global counterparts. Characterization of P0 sequences in infected plants over time may reveal mutation patterns in this silencing suppressor gene. As CLRDV is phloem-limited, targeted tissue sampling is essential for reliable qualitative and quantitative detection, facilitating accurate assessments of CLRDV incidence in cotton crop. Symptomatically, CLRDV infections are frequently confounded with abiotic stress responses or the poorly understood enigmatic bronze wilt phenomenon, previously reported yet undefined. The role of CLRDV in cultivar-specific responses in commercial cotton fields remains unclear. Moreover, recently identified caulimovirid-like endogenous viral elements (EVEs) may influence terminal abortion in cotton crops, an association needing further study.

To understand these aspects, we have investigated the three objectives below:

1) Diversity of P0 and tissue tropism of CLRDV in cotton host
2) Characterization of caulimovirid-like sequences from upland cotton (*Gossypium hirsutum* L.) exhibiting terminal abortion in Georgia, USA.
3) Cotton leafroll dwarf virus-induced bronze wilt in Upland Cotton: The role of cultivar selection in mitigating yield loss in Georgia USA.

This research has deepened our understanding of CLRDV variability in P0 and virus tropism in different tissues of the host. Role of CLRDV in cultivar-specific bronze wilt symptomatology and associated yield loss. It also helped us to understand the role of caulimovirid-like endogenous viral elements (EVEs) in terminal abortion symptoms and associated yield loss. From an applied perspective, these findings provided critical insights into virus- host interactions and their effect on yield in commercial cotton cultivars. The knowledge generated here is disseminated to growers, stakeholders, and the scientific community, facilitating informed decision-making and contributing to the development of sustainable CLRDV management strategies to mitigate yield impacts.

References

Aboughanem-Sabanadzovic, N., Allen, T. W., Frelichowski, J., Scheffler, J., & Sabanadzovic, S. (2023). Discovery and analyses of Caulimovirid-like sequences in upland cotton (*Gossypium hirsutum*) viruses. *Viruses*, *15*(8), 1643.

Albers, D. W., & Guthrie, D. (2001). Field incidence and description of bronze wilt symptoms. In *Proceedings of the Beltwide Cotton Conference* (Vol. 1, pp. 104–105). National Cotton Council.

Bag, S., Roberts, P. M., & Kemerait, R. C. (2021). Cotton leafroll dwarf disease: An emerging virus disease on cotton in the U.S. *Crops & Soils*, *54*(1), 18–22.

Bell, A. A., Cui, Y., Magill, C., Orta, H., & Hawkins, M. (1998). Agrobacterium wilt and bronzing: The new challenge. In *Proceedings of the Beltwide Cotton Conference* (Vol. 1, pp. 136–137). National Cotton Council.

Bell, A. A. (1999). Agrobacterium bronzing and wilt: Cultivar reactions and effects of temperature. Proceedings of the Beltwide Cotton Conference, 1, 117–120. National Cotton Council.

Bell, A. A. (2000). Role of *Agrobacterium* in bronze wilt of cotton. *Proceedings of the Beltwide Cotton Conference*, 1, 154–160. National Cotton Council.

Bell, A. A., Nichols, R. L., Albers, D., Baird, R., Brown, S., Colyer, P., et al. (2002). *Bronze wilt of cotton*. Texas Cooperative Extension, Agricultural Communications, The Texas A&M University System. Available at: http://cotton.tamu.edu/Nematodes/bronzewilt.pdf [Accessed 27 March 2025].

Brown, S. M. (2000). Bronze wilt field symptoms in Georgia. *Proceedings of the Beltwide Cotton Conference*, 1, 151. National Cotton Council.

Brown, S., Conner, K., Hagan, A., Jacobson, A., Koebernick, J., & Lawrence, K. (2019). *Report of a research review and planning meeting on cotton leafroll dwarf virus*. Cotton Incorporated. https://www.cottoninc.com/cotton-production/ag-research/plant-pathology/cotton-leafroll-dwarf-virus-research/

Cauquil, J., & Vaissayre, M. (1971). La "maladie bleue" du cotonnier en Afrique: Transmission de cotonnier à cotonnier par *Aphis gossypii* Glover. *Coton et Fibres Tropicales*, *26*, 463–466.

Creech, J. B. (1999). Bronze wilt in the 1998 Mississippi cotton variety trials. In *Proceedings of the Beltwide Cotton Conference* (Vol. 1, pp. 472–473). National Cotton Council.

Creech, J. B., & Fieber, J. C. (2000). Bronze wilt complex observations in the 1998 and 1999 Mississippi cotton variety trials. *Proceedings of the Beltwide Cotton Conference*, 1, 151–152. National Cotton Council.

Cascardo, R. S., Arantes, I. L. G., Silva, T. F., Sachetto-Martins, G., Vaslin, M. F. S., & Correa, R. L. (2015). Function and diversity of P0 proteins among cotton leafroll dwarf virus isolates. *Virology Journal, 12*(123).

Delfosse, V. C., Casse, M. F., Agrofoglio, Y. C., Kresic, I. B., Hopp, H. E., Ziegler-Graff, V., & 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 Research, 175*(1), 64–70

Edula, S. R., Bag, S., Milner, H., Kumar, M., Suassuna, N. D., Chee, P. W., Kemerait, R. C., Hand, L. C., Snider, J. L., Srinivasan, R., et al. (2023). Cotton leafroll dwarf disease: An enigmatic viral disease in cotton. *Molecular Plant Pathology, 24*, 513–526.

Georgia Cotton Commission. (2016). *Cotton: The story*.https://georgiacottoncommission.org/wp-content/uploads/2020/09/CottontheStory2016.pdf

Gayral, P., & Iskra-Caruana, M.-L. (2009). Phylogeny of banana streak virus reveals recent and repetitive endogenization in the genome of its banana host (Musa sp.). *Journal of Molecular Evolution, 69*(1), 65–80.

Harper, G., Osuji, J. O., Heslop-Harrison, J. S., & Hull, R. (1999). Integration of banana streak virus into the Musa genome: Molecular and cytogenetic evidence. *Virology, 255*(2), 207–213.

Harper, G., Hart, D., Moult, S., Hull, R., & Geering, A. (2003). Detection and significance of endogenous pararetrovirus sequences in petunia. *Acta Horticulturae, 623*, 235–240.

Heilsnis, B., Mahas, J. B., Conner, K., Pandey, S., Clark, W., Koebernick, J., Srinivasan, R., Martin, K., & Jacobson, A. L. (2023). Characterizing the vector competence of *Aphis gossypii*, *Myzus persicae*, and *Aphis craccivora* (Hemiptera: Aphididae) to transmit cotton leafroll dwarf virus to cotton in the United States. *Journal of Economic Entomology, 116*, 719–725.

Lee, J. A., & Fang, D. D. (2015). Cotton as a world crop: Origin, history, and current status. In D. D. Fang & R. G. Percy (Eds.), *Cotton* (p. 1). American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America.

Lockhart, B. E., Menke, J., Dahal, G., & Olszewski, N. E. (2000). Characterization and genomic analysis of tobacco vein clearing virus, a pararetrovirus representing a new genus. *Phytopathology, 90*(6), S46.

Lu, Z., Chen, J., Percy, R. G., & Zeiger, E. (1997). Photosynthetic rate, stomatal conductance and leaf area in two cotton species (*Gossypium barbadense* and *Gossypium hirsutum*) and

their relation with heat resistance and yield. *Australian Journal of Plant Physiology, 24*(6), 693–700.

Mahas, J. W., Hamilton, F. B., Roberts, P. M., Ray, C. H., Miller, G. L., Sharman, M., et al. (2022). Investigating the effects of planting date and *Aphis gossypii* management on reducing the final incidence of cotton leafroll dwarf virus. *Crop Protection, 158*, 106005.

MacNeish, R. S. (1967). *The prehistory of the Tehuacán Valley: Vol. 1. Environment and subsistence*. University of Texas Press.

McCarty, J. C., Jenkins, J. N., & Wu, J. (2004). Primitive accession derived germplasm by cultivar crosses as sources for cotton improvement. *Crop Science, 44*(4), 1231–1235.

Michelotto, M. D., & Busoli, A. C. (2003). Eficiência de ninfas e adultos de *Aphis gossypii* Glover na transmissão do vírus do mosaico das nervuras do algodoeiro. *Bragantia, 62*, 255–259.

Moulherat, C., Tengberg, M., Haquet, J.-F., & Mille, B. T. (2002). First evidence of cotton at Neolithic Mehrgarh, Pakistan: Analysis of mineralized fibres from a copper bead. *Journal of Archaeological Science, 29*(12), 1393-1401.

Ndowora, T., Dahal, G., LaFleur, D., Harper, G., Hull, R., Olszewski, N. E., & Lockhart, B. E. (1999). Evidence that Badnavirus infection in Musa can originate from integrated pararetroviral sequences. *Virology*, 255(2), 214–220.

Nichols, R. L. (2001). Introduction to two newly recognized disorders of cotton – bronze wilt and seed rot. *Proceedings of the Beltwide Cotton Conference*, 1, 104. National Cotton Council.

Parkash, V., Sharma, D. B., Snider, J., Bag, S., Roberts, P., Tabassum, A., et al. (2021). Effect of cotton leafroll dwarf virus on physiological processes and yield of individual cotton plants. *Frontiers in Plant Science, 12*, 734386.

Padgett, B., Garber, B. W., Rea, W., & Price, J. (2004). Epidemiology of bronze wilt in Northeast Louisiana. *Proceedings of the Beltwide Cotton Conference*, 105–110. National Cotton Council.

Phipps, B. J. (2000). Bronze wilt symptoms. In *Proceedings of the Beltwide Cotton Conference* (Vol. 1, p. 688). National Cotton Council.

Richert-Pöggeler, K. R., & Shepherd, R. J. (1997). Petunia vein-clearing virus: A plant pararetrovirus with the potential for integrating into the host genome. *BioEssays*, 19(5), 427–434.

Richert-Pöggeler, K. R., Noreen, F., Schwarzacher, T., Harper, G., & Hohn, T. (2003). Induction of infectious Petunia vein clearing (caulimo) virus from endogenous provirus in petunia. *The EMBO Journal*, 22(19), 4836–4845.

Smith, C. E., & Stephens, S. G. (1971). Critical identification of Mexican archaeological cotton remains. *Economic Botany, 25*(2), 160–168.

Tabassum, A., Bag, S., Suassuna, N. D., Conner, K. N., Chee, P., Kemerait, R. C., & Roberts, P. (2021). Genome analysis of cotton leafroll dwarf virus reveals variability in the silencing suppressor protein, genotypes, and genomic recombinants in the USA. *PLoS One*, *16*(7), e0252523.

U.S. Department of Agriculture, National Agricultural Statistics Service. (2024). *2024 State agriculture overview: Georgia*. https://data.nass.usda.gov/

U.S. Department of Agriculture, Economic Research Service. (2024). Cotton sector at a glance. *Economic Research Service*. https://www.ers.usda.gov/topics/crops/cotton-and-wool/cotton-sector-at-a-glance

Wendel, J. F., & Cronn, R. C. (2003). Polyploidy and the evolutionary history of cotton. *Advances in Agronomy*, *78*, 139–186.

CHAPTER 2

COTTON LEAFROLL DWARF DISEASE: AN ENIGMATIC VIRAL DISEASE IN COTTON

[PATHOGEN PROFILE]

Published Article:

Edula, S. R., Bag, S., Milner, H., Kumar, M., Suassuna, N. D., Chee, P. W., Kemerait, R. C., Hand, L. C., Snider, J. L., Srinivasan, R., & Roberts, P. M. (2023). Cotton leafroll dwarf disease: An enigmatic viral disease in cotton. *Molecular Plant Pathology*, *24*(6), 513–526.

Abstract

Taxonomy: *Cotton leafroll dwarf virus* (CLRDV) is a member of the genus *Polerovirus*, family *Solemoviridae*.

Geographical Distribution: CLRDV is present in most cotton-producing regions worldwide, prominently in North and South America.

Physical Properties: The virion is a nonenveloped icosahedron with $T = 3$ icosahedral lattice symmetry that has a diameter of 26–34 nm and comprises 180 molecules of the capsid protein. The CsCl buoyant density of the virion is 1.39–1.42 g/cm^3 and S_{20w} is 115–127S.

Genome: CLRDV shares genomic features with other poleroviruses; its genome consists of monopartite, single-stranded, positive-sense RNA, is approximately 5.7–5.8 kb in length, and is composed of seven open reading frames (ORFs) with an intergenic region between ORF2 and ORF3a.

Transmission: CLRDV is transmitted efficiently by the cotton aphid (*Aphis gossypii* Glover) in a circulative and non-propagative manner.

Host: CLRDV has a limited host range. Cotton is the primary host, and it has also been detected in different weeds in and around commercial cotton fields in Georgia, USA.

Symptoms: Cotton plants infected early in the growth stage exhibit reddening or bronzing of foliage, maroon stems and petioles, and drooping. Plants infected in later growth stages exhibit intense green foliage with leaf rugosity, moderate to severe stunting, shortened internodes, and

increased boll shedding/abortion, resulting in poor boll retention. These symptoms are variable and are probably influenced by the time of infection, plant growth stage, varieties, soil health, and geographical location. CLRDV is also often detected in symptomless plants.

Control: Vector management with the application of chemical insecticides is ineffective. Some host plant varieties grown in South America are resistant, but all varieties grown in the United States are susceptible. Integrated disease management strategies, including weed management and removal of volunteer stalks, could reduce the abundance of virus inoculum in the field.

Keywords: cotton blue disease, cotton leafroll dwarf disease, cotton leafroll dwarf virus, *Gossypium hirsutum*, Polerovirus.

Review of Literature

Cotton (*Gossypium hirsutum*) is a major cash crop in many parts of the world, including the United States. In 2021, 4.1 million hectares of cotton were harvested in the United States, valued at approximately $7.4 billion (USDA, 2021). Cotton blue disease (CBD) is a viral disease capable of causing significant losses in the cotton industry. CBD was first described in the Central African Republic in 1949 and has since then been reported from several regions in Africa, Asia, and the Americas (Cauquil, 1977). However, the nature of the causal agent of CBD was not known until 2005, when the entire capsid gene and a partial RNA-dependent RNA polymerase gene revealed its association with a virus belonging to the genus Polerovirus, family Luteoviridae, and named it cotton leafroll dwarf virus (CLRDV) (Corrêa et al., 2005). Distéfano et al. (2010) sequenced the complete genome of a CLRDV isolate from Argentina. Recently, ICTV reclassified CLRDV as a member of the family *Solemoviridae* (Sõmera et al., 2021). In 2006, a less aggressive resistance-breaking genotype of CLRDV was observed in Brazil on cotton varieties known to be resistant against CBD. This new disease was referred to as "atypical" CBD (Agrofoglio et al., 2017; da Silva et al., 2015).

In the United States, CLRDV was first reported in Alabama from cotton in 2019. Plants infected with CLRDV showed symptoms including intense dark green to bluish foliage, reddening of stems and petioles, curling and drooping of leaves, internodal shortening, and moderate to severe stunting. The genome sequences from Alabama and Georgia isolates were characterized. The isolates present in the United States differ from those causing CBD in South America and other regions. The disease caused by CLRDV in the United States is hence referred to as cotton leafroll dwarf disease (CLRDD; Brown et al., 2019). It has been observed that cotton plants infected in younger stages could suffer complete yield loss, whereas the losses decrease when

plants are infected at the mature stages of plant development (Parkash et al., 2021). Although the incidence of the virus in some commercial cotton fields in Georgia and Alabama is 80%–100%, no significant yield losses have been reported (Mahas et al., 2022).

Geographical Distribution

CBD was first reported around 1949 in the Central African Republic. The disease spread towards Benin (previously known as Dahomey), Chad, Cameroon, Congo, and Ivory Coast (Cauquil, 1977; Cauquil & Follin, 1983; Cauquil & Vaissayre, 1971). Later, CBD was introduced in Brazil and Argentina (Corrêa et al., 2005; Costa & Carvalho, 1962) and Asian countries including India (Mukherjee et al., 2012), Thailand (Sharman et al., 2015), and Timor-Leste (Ray et al., 2016; Figure 2.1a). More recently, CLRDV was reported in China (Feng et al., 2017), South Korea (Igori et al., 2022), and Uzbekistan (Kumari et al., 2020; Figure 2.1a). In North America, symptoms associated with CBD were first observed in the autumn of 2017 on the Gulf Coast of Alabama, and the identity of CLRDV was confirmed in 2019 (Avelar et al., 2019). Currently, CLRDV is widespread in all major cotton-growing regions in the United States (Aboughanem-Sabanadzovic et al., 2019; Alabi et al., 2020; Ali & Mokhtari, 2020; Ali et al., 2020; Avelar et al., 2019; Faske et al., 2020; Ferguson & Ali, 2022; Iriarte et al., 2020; Price et al., 2020; Tabassum et al., 2019; Thiessen et al., 2020; Wang et al., 2020).

The incidence of CLRDV infection in different states in the United States as estimated based on symptomatology ranges from 2% to 100% (Aboughanem-Sabanadzovic et al., 2019; Alabi et al., 2020; Ali & Mokhtari, 2020; Avelar et al., 2019, 2020). In Georgia, a disease incidence of less than 10% was recorded, but CLRDV was detected in many plant tissues with and without symptoms in commercial fields and research plots (Figure 2.1b; Tabassum et al., 2020, 2021).

Symptomatology

Symptoms including leaf rolling, crumpling, and a bushy top were reported in the province of Chaco in north-west Argentina (Agrofoglio et al., 2017). Plants infected in the mature stages had normal morphology in the lower plant parts (stem and internodes) but a bushy phenotype in the top, with deformed apical leaves (Agrofoglio et al., 2017). Symptomless CLRDV infections in cotton are very common in the United States (Bag et al., 2021; Tabassum et al., 2020, 2021). In addition, CLRDV-induced symptoms are highly variable at different locations in the United States. On the Gulf Coast of Alabama, symptoms such as leaf deformation with bluish-green discolouration, vein clearing, leaf curling and rolling, and dwarf, stacked internodes were observed during the autumn of 2017 (Avelar et al., 2019). In Georgia, CLRDV-infected cotton plants exhibit a multitude of symptoms depending on the plant growth stage (Bag et al., 2021; Figure 2.2a,b). Plants infected early (within 50 days after emergence) exhibit symptoms such as reddening of leaves, petioles, and stems, puckering, crinkling, deformation of the leaf lamina, wilting, and downward leaf drooping with V-shaped lamina folding (Figure 2.2c–f). Infected plants often wilt and some may recover but do not produce any bolls as they mature (Parkash et al., 2021). Plants infected later in the season at maturity exhibit more turgidity with a leathery texture, crinkling, and square/leaf dropping along with wilting (Figure 2.2g–j). Often, these plants have more vegetative growth in the upper branches of the stem, exhibiting stacked internodes and ceasing reproductive development (Figure 2.2k,l). Terminal whips or accentuated verticality and a bushy phenotype are also observed, indicating infection during the early flowering or boll setting stage that leads to noticeable yield loss (Figure 2.2m,n). Other structural abnormalities such as parrot beak fruits (Figure 2.2o) with reduced seeds are also observed in infected plants. Some of these symptoms are observed across Georgia irrespective of the variety.

The development of symptoms is influenced by plant age during infection, plant vigour, environmental conditions, and soil health. In plants infected at early stages exhibiting reddening and wilting of foliage (Figure 2.2p), it has also been observed that the symptoms diminish and the plants reappear green but remain dwarf without any harvestable bolls (Figure 2.2q,r). Symptoms described for CLRDD are strikingly similar to the symptoms of bronze wilt (Parkash et al., 2021), another wilting disease with unknown causes. Bronze wilt, synonymous with “copper top, sudden wilt, and phloem wilt", was first identified in Mississippi and Louisiana in 1995, causing significant yield loss (Bell et al., 2002). Outbreaks were later reported in the late 1990s from Missouri without much yield loss (Phipps, 2000). But in 1998, Georgia recorded about $25 million in losses due to severe disease pressure during a long dry spell with temperatures above 35°C (Brown, 2000; McGraw, 2000). Short-season varieties of Upland and Pima cotton have been reported to be more susceptible to bronze wilt, especially if the pedigree involved crosses with Tamcot SP-37 or its progeny Miscot T8-27 (Bell et al., 2002). In other countries, symptoms such as red leaves, red wilt, and anthocyanosis are associated with bronze wilt and are considered a physiological disorder induced by numerous abiotic factors (Gade et al., 2013). Studies that were conducted to understand the aetiology of bronze wilt and its causal biotic or abiotic factors were inconclusive due to the inconsistency of the disease symptoms and severity (Padgett et al., 2004). The combined role of biotic (virus) and abiotic factors in causing bronze wilt disease has never been evaluated. Due to the similarity of CLRDD and bronze wilt symptoms, the cause of symptoms observed in cotton in Georgia and the surroundings is still under discussion.

Physiology of CLRDV-Infected Cotton

Because CLRDV is a relatively new cotton pathogen in the United States, minimal research has been conducted on the physiological response of cotton plants to CLRDV infection. However, leaf reddening in response to other stresses is a result of anthocyanin production, which is speculated to attenuate solar radiation or to reduce reactive oxygen species levels (Close & Beadle, 2003). Similar to plants exposed to other wilt-inducing stresses like drought (Chastain et al., 2014, 2016), recent research has shown that plants with CLRDV symptoms significantly reduce stomatal conductance and the net photosynthetic rate under field conditions (Parkash et al., 2021). Even in the earliest stages of the disease, when severe wilting is not yet observable, stomatal conductance and the net photosynthetic rate were reduced by 94% and 84%, respectively. Because declines in conductance limit transpirational cooling, leaf temperature also significantly increased by 0.5–3.8°C at advanced stages of the disease (Parkash et al., 2021). Using stomatal conductance as a reference indicator of stress, Medrano et al. (2002) and Parkash et al. (2021) were also able to show that the electron transport rate through photosystem II was less sensitive to CLRDV-induced stress than carbon assimilation. The possibility that these differences in sensitivity lead to oxidative stress and subsequent increases in anthocyanin production should be explored further.

Host Range

Crops and weeds are vital components in virus disease epidemiology and spread into new geographical locations. While cotton is the primary host of CLRDV, weeds and other crops that serve as hosts for this virus can influence its spread and aid in reoccurrence in agricultural landscapes. In Georgia, where the winters are mild, CLRDV was detected in a large number of overwintering cotton stalks and regrowth from these stalks (Figure 2.2s,t; Sedhain et al., 2021).

CLRDV was also detected in 23 weed species growing near cotton fields in Georgia (Sedhain et al., 2021). In greenhouse experiments, CLRDV was detected by reverse transcription (RT)-PCR following aphid-mediated transmission in hibiscus (*Hibiscus acetosella*), okra (*Abelmoschus esculentus*), *Nicotiana benthamiana*, Palmer amaranth (*Amaranth palmeri*), and prickly sida (*Sida spinosa*) (Pandey et al., 2022). Aphids feeding on CLRDV-infected malvaceous hosts including hibiscus, prickly sida, and okra were able to acquire CLRDV and back-transmitted it to noninfected cotton seedlings (Pandey et al., 2022). These volunteer cotton stalks and weed hosts could potentially act as a "green bridge" and as a source of primary inoculum for the next growing season (Bag et al., 2021; Sedhain et al., 2021).

In addition to cotton, CLRDV also infects a number of crop species worldwide. For example, chickpea (*Cicer arietinum*) was shown to be a host for CLRDV (Kumari et al., 2020). Symptoms induced by CLRDV on chickpea are similar to those observed on cotton, including chlorosis, stunting, necrosis, yellowing, and reddening (Kumari et al., 2020). In addition, CLRDV capsid protein gene sequences share 89.4% to 100% homology with chickpea stunt disease-associated virus (CpSDaV) isolates from India (Mukherjee et al., 2016). The virus was also transmitted from cotton to chickpea, suggesting that CLRDV and CpSDaV are strains of the same virus (Mukherjee et al., 2016). Natural infection of CLRDV has also been observed on *Hibiscus syriacus* exhibiting vein-clearing symptoms in South Korea upon mixed infection with other viruses (Igori et al., 2022). CLRDV was reported infecting cacao (*Theobroma cacao*) trees in Bahia state in north-eastern Brazil (Ramos-Sobrinho et al., 2022), suggesting its movement to different cultivated crop species.

Morphology and Genome Organization

As a primary confirmation of Koch's postulates, CLRDV was detected in infected plants using electron microscopy. Virion particles were partially purified from young bark tissues from cotton plants with CLRDV symptoms (Figure 2.2c,d). The virion preparation was examined under a transmission electron microscope as described by Al Rwahnih et al. (2021). A minimal number of spherical virion particles with a diameter of 25–35 nm were observed (Figure 2.3a), suggesting the titer of the virus particles in the sample was low. RT-PCR further confirmed the presence of CLRDV in the same bark tissues following the protocol described in Tabassum et al. (2020). Previously Takimoto et al. (2009) conducted anatomical studies on CLRDV-infected cotton petioles and midrib tissues that exhibited more callose depositions and inclusion bodies in phloem cells (Takimoto et al., 2009).

The CLRDV genome organization resembles those of other poleroviruses (Avelar et al., 2020; Corrêa et al., 2005; Distéfano et al., 2010; Tabassum et al., 2020, 2021). The virus genome comprises seven open reading frames (ORFs) with an intergenic region between ORF2 and ORF3a (Figure 2.3b). ORF0 encodes the P0 protein (28.9 kDa), a silencing suppressor (Delfosse et al., 2014). ORF1 encodes the P1 protein (70.1 kDa), predicted to be expressed through leaky scanning. ORF1–2 encodes a fused protein P1–P2 replication-related protein of 118.7 kDa through ribosomal frameshift. ORF3–5 are expressed through subgenomic RNAs. ORF3 encodes the P3 (capsid) protein (22.4 kDa), ORF4 encodes the P4 (movement) protein (19.4 kDa), and P5 is translated through an in-frame read through the P3 stop codon. The P3–P5 protein (77.2 kDa) is essential for aphid transmission and virus accumulation in plants (Agrofoglio et al., 2017; Distéfano et al., 2010; Silva et al., 2008; Figure 2.3b).

ORF0 is a highly variable genomic region of CLRDV (Cascardo et al., 2015; Delfosse et al., 2014); the P0 protein is the most diverse protein. The P0 protein contains a conserved F-box (LPxx[L/I]) domain located near the N-terminus and a conserved sequence ([K/R]IYGEDGXXXFWR) at the C-terminus (Delfosse et al., 2021) considered essential for viral silencing suppressor activity (Pazhouhandeh et al., 2006; Zhuo et al., 2014). Silencing suppression activity varies in different species and can be systemic or local, and a few proteins have null activity (Delfosse et al., 2021). In isolates collected from the United States, five unique amino acid substitutions were present; however, their role in disease development and expression is unknown. The amino acid substitution from isoleucine (I) to valine (V) at the 72nd position (I72V), in the F-box domain, differentiates between the typical and atypical genotypes of CLRDV (Delfosse et al., 2014). The significance of this substitution in symptom development and virulence is not yet determined. The sequenced isolates from Georgia and Alabama resemble the South American resistance-breaking atypical genotypes with valine (V) at the 72nd position, whereas isolates from Texas have both typical (I) and atypical (V) residues at the 72nd position (Tabassum et al., 2020, 2021).

Diversity and Evolution of CLRDV

Since the first report of CLRDV in Alabama, isolates from different host species and geographical locations in the United States were sequenced and compared with the isolates of other parts of the world to further understand the diversity and evolution of CLRDV (Avelar et al., 2020; Ramos-Sobrinho et al., 2021; Tabassum et al., 2020, 2021). A study by Tabassum et al. (2021) revealed that the P0 proteins of CLRDV isolates from Texas and Alabama were >90% identical to those of the CLRDV isolates from Georgia. However, Georgia isolates have >10% divergence in their amino acid sequences compared to other South American CLRDV sequences.

These data might be helpful in further understanding the diversity, degree of genetic differentiation, and population dynamics of CLRDV. A recent study suggests that eight newly determined full-length genome sequences of CLRDV isolated from cotton plants in Alabama, Florida, and Texas were phylogenetically related with a CLRDV genotype from Alabama (Ramos-Sobrinho et al., 2021). Furthermore, these isolates have been shown to cluster into a monophyletic group among the CLRDV populations in North America. These data further suggest that horizontal gene flow may have occurred between CLRDV isolates during the course of evolution.

A more in-depth analysis of ORF0 of various CLRDV isolates was performed with the sequence information available as of 2023-01-10 ($n = 76$) using MEGA-XI and iTOL (Table S1; Letunic & Bork, 2021; Tamura et al., 2021). CLRDV isolates were separated into four major clades mostly based on geographical location (Figure 2.4a). The Asian clade consisted of three CLRDV isolates, of which two infect the family Malvaceae (*H. syriacus* and *Malvaviscus arboreus*) in South Korea and one was reported from soybean aphid (*Aphis glycines*) in China. The CLRDV P0 sequences ($n = 20$) from South American cotton isolates diverged into three separate clades: (i) one clade containing "typical" CLRDV genotypes (mostly from Brazil), (ii) one clade containing "atypical" CLRDV genotypes (mostly from Argentina; da Silva et al., 2015), and (iii) one clade containing two "atypical" isolates from cacao (*T. cacao*) trees in Brazil (Ramos-Sobrinho et al., 2022). The most diverse and most extensive clade consisted of 53 sequences from North America. Among the CLRDV isolates reported from the United States, isolates associated with cotton ($n = 43$) and weed ($n = 10$) species were segregated into two distinct clusters. Such segregation suggests genetic recombination, which might help the viruses

to broaden their host range and foster successful establishment in the ecosystem (Vassilakos et al., 2016).

The same set of sequences was also used to study the amino acid substitution rates and evolutionary pattern of CLRDV using BEAST v. 1.10.4 and Tracer v. 1.7.1 (Rambaut et al., 2018; Suchard et al., 2018). BEAST analysis showed that the amino acid substitution rate of P0 protein from different hosts combined was 2.194×10^{-3} substitutions per site per year. However, when P0 from only weeds was considered, the amino acid substitution rate was higher (6.313×10^{-10} substitutions per site per year) (Table 2.1). Genome-wide mutations and recombination in viruses allow them to jump from one host to another (Calvo et al., 2014). The higher amino acid substitution rate in cultivated and non-cultivated host species emphasizes the potential threat to cotton cultivation and other crops in the future.

Based on the CLRDV ORF0 sequences available in GenBank from 2005 to 2022, BEAST analysis estimated the age (root) of the CLRDV population in the year 1945 during the period 1938–1962 when CLRDV symptoms were first observed and documented in the Central African Republic (Cauquil, 1977; Cauquil & Follin, 1983; Costa & Carvalho, 1962). However, the 95% highest posterior density credible interval spans from 1914 to 1971 (Bryant et al., 2007; Figure 2.4b). These data provide insight into virus transmission among cotton and other species and suggest that CLRDV was probably introduced in South America from the African continent (as a CBD).

Insect Vector and Transmission

The cotton or melon aphid, *Aphis gossypii* is a major agricultural pest found worldwide in over 170 countries (CABI Compendium, 2021). The earliest known investigations of CLRDV transmission were conducted in Africa in the early 1970s when CLRDV was commonly known

as CBD. Aphids collected from cotton plants with symptoms in the field were able to transmit the virus to cotton (Cauquil & Vaissayre, 1971). Studies conducted in Brazil determined that susceptible cultivars required fewer viruliferous aphids to develop symptoms while more resistant cultivars required a minimum of 10 aphids to develop symptoms (Takimoto, 2003). *A. gossypii* transmits CLRDV to cotton plants in a circulative and non-propagative manner (Michelotto & Busoli, 2003). Both winged and wingless morphs can transmit the virus after a 48-h inoculation access period for up to 12 days (Michelotto & Busoli, 2006, 2009).

A. gossypii is an annual pest of cotton in the south-eastern United States. Seasonal dynamics of cotton aphid populations and their potential role in the spread of CLRDV were investigated in field plots in Georgia and Alabama. A higher incidence of *A. gossypii* was observed during June and July. *A. gossypii* was detected in all test plots irrespective of the intensity of insecticide use surveyed in 2019. CLRDV was detected in 60% to 100% of the samples tested (Mahas et al., 2022), suggesting a correlation between vector and disease spread in the region.

Soybean aphids (*A. glycines*) collected in 2017 in China were found to harbour CLRDV, although cotton is a nonpreferred host of this aphid (Feng et al., 2017). *Aphis craccivora* and the green peach aphid *Myzus persicae* transmit CpSDaV (Horn et al., 1995), which is suspected to be another strain of CLRDV (Mukherjee et al., 2016). These findings indicate that more than one aphid species could transmit CLRDV and might contribute to the introduction and spread of the virus to different hosts and geographical regions. In the United States, *A. craccivora*, *Aphis fabae*, *Macrosiphum euphorbiae*, *Protaphis middletonii*, and *Rhopalosiphum rufiabdominale* are known to colonize cotton (Blackman & Eastop, 2000; Mahas et al., 2022). However, transmission experiments with these aphids need to be conducted to confirm their ability to transmit CLRDV. Due to the presence of sweet potato whitefly (*Bemisia tabaci*) on cotton in the

south-eastern United States, its ability to transmit CLRDV on cotton was tested. *B. tabaci* could neither acquire nor transmit the virus, suggesting *A. gossypii* is the only efficient vector transmitting CLRDV onto cotton in the United States (Heilsnis et al., 2022).

Diagnosis

The symptoms of CLRDV-infected cotton plants are complex and depend on the plant growth stage at the time of infection, the variety, and the geographical location, making it difficult to diagnose solely on the basis of symptomology. Serological tests performed on CBD samples from north-eastern Argentina suggested its relationship to barley yellow dwarf virus serotypes RPV and PAV members of the family *Luteoviridae* (Lenardon, 1994). Recently, antibodies raised against CLRDV capsid protein antigens were used in double antibody sandwich-ELISA to detect CLRDV in *Commelina* sp. weed samples (Hoffmann et al., 2022).

After the CLRDV genome was sequenced, PCR-based methods were developed for detection. Initially, degenerate primers targeting ORF0 and ORF3 were used to detect the virus (Corrêa et al., 2005). Later, specific primers targeting ORF3 were developed for detection assays (Silva et al., 2008). With the emergence of CLRDV in the United States, PCR assays using primers designed specifically targeting the USA isolates were developed (Tabassum et al., 2020). Another set of degenerate primers targeting ORF3 were designed to detect several polero and luteoviruses from Argentina, Brazil, and India, including CLRDV isolates (Sharman et al., 2015). SYBR Green-based RT-quantitative PCR (RT-qPCR) assays targeting ORF3 were developed for more precise and quick detection (Pandey et al., 2022).

High-throughput sequencing plays a significant role in the early detection of CLRDV on plants with symptoms in commercial fields in Alabama, Florida, and Texas (Avelar et al., 2019, 2020; Ramos-Sobrinho et al., 2021). CLRDV was found as coinfections with other viruses in cotton

plants exhibiting leaf roll and vein yellowing symptoms (Yang et al., 2021) in China and on *H. syriacus* exhibiting vein clearing symptoms (Igori et al., 2022) in South Korea.

Cotton leafroll dwarf virus Tissue Tropism

CLRDV is phloem-limited, and during initial studies conducted in Georgia, detection in whole leaf tissues gave inconsistent results due to the low virus titre (Figure 2.3a). To determine the best tissue to be sampled for accurate detection of CLRDV in cotton, virus titre was estimated in different tissues from plants exhibiting symptoms associated with CLRDV. The copy number of the virus in samples was estimated by the method described by Kavalappara et al. (2022). A partial capsid protein gene amplified and cloned in a Topo-TA PCR cloning vector (Invitrogen) was used as standard. Six 10-fold serial dilutions of the linearized plasmids were prepared, and a standard curve was made. The number of copies of standard was calculated based on the spectrophotometric determination of the plasmid DNA concentration using a previously reported formula (Rotenberg et al., 2009). In general, CLRDV titre was higher in the upper parts of infected cotton plants than in the lower parts. CLRDV titre was highest in the main stem, followed by the branches and petioles. Fruits carried the lowest CLRDV titre among various tissues tested (Table 2,2). Thus, young phloem tissues in the upper canopy would be the ideal candidate for sample collection for accurate and efficient detection.

Economic Importance

In Argentina and Brazil, yield losses of up to 80% were reported due to CLRDV infection in susceptible varieties (Distéfano et al., 2010; Silva et al., 2008). Yield reductions of up to 99% due to a decrease in boll number and boll mass resulting from a lower number of seeds per boll have been recorded on individual plants (Parkash et al., 2021). However, overall yield losses are variable in the United States (Avelar et al., 2019; Mahas et al., 2022). Many commercial cotton

fields in the south-eastern United States, where CLRDV was first detected in 2019, have met their production goals (authors' personal observations). It is unknown whether variation in disease and yield loss among locations is due to varietal, environmental, crop age, or vector-related factors. Most of the cotton varieties cultivated in the United States are tolerant to CLRDV; the virus may be present, but the host plants do not exhibit any symptoms. Furthermore, only a small number of commercial cotton varieties exhibit disease symptoms such as reddening of leaves, wilting, and downward leaf drooping with V-shaped lamina when the plant is infected by the virus early in the season. Consequently, CLRDD seems to be a minor concern at present. However, given the previous virus outbreaks and the yield losses in South American countries, monitoring of the evolving virus species/strains and its epidemics in commercial cotton varieties may be a good preventive measure. A thorough study of virus–host interactions will help in developing management strategies for future outbreaks.

Disease Management

Vector management is one of the major components of integrative pest management strategies for virus management. However, aggressive aphid sprays (weekly application) during 2019–2020 did not reduce the occurrence of CLRDV in an experimental plot in Georgia. It was also observed that insecticide application did not prevent aphid colonization or decrease CLRDV incidence in both Alabama and Georgia. Moreover, it could adversely impact other beneficial insects as well as elevate the risk of insects developing insecticide resistance. In addition, adjusting the planting date was also found to be ineffective in reducing the incidence of CLRDD (Mahas et al., 2022).

CBD was effectively mitigated in South America by deploying varieties possessing a single dominant resistance gene, *Rghv1* (Pupim Junior et al., 2008). Morello et al. (2010)

developed BRS-293, a midseason high-yielding cultivar that is moderately resistant to typical and atypical CLRDV but was found to be susceptible to the prevalent CLRDV in the south-eastern United States (Brown et al., 2019). While assessing resistant populations obtained from cotton cultivar Delta Opal, possessing a single dominant resistance gene, *Cbd*, Fang et al. (2010) identified two tightly linked simple-sequence repeat markers associated with the *Cbd* gene, localized on chromosome 10. However, genetic analyses have indicated that varieties carrying the *Cbd*gene were susceptible to CLRDV (Brown et al., 2019), suggesting that breeding for resistance would necessitate screening to identify new sources of resistance.

Conclusion

CLRDD is an emerging viral disease in cotton-producing regions in the United States. Several weed species and overwintering cotton stalks might act as a potential green bridge and source of virus inoculum. Early and late season symptoms are yield-limiting in the infected plant but not detrimental over a large acreage due to low disease incidence since its first report in 2018. Earlier recorded yield losses, the increase in host range, and its worldwide distribution are still concerning. At present, CLRDD may not be yield-limiting in the United States, but it can cause significant yield loss in individual infected plants, as observed in commercial fields and experimental plots in Georgia (Parkash et al., 2021) and in other cotton-producing countries. Basic research on virus–host interactions using a reverse genetic approach will be advantageous to devise appropriate management strategies and breeding approaches for future crops.

CLRDV is phloem-limited, but little is known about its interactions with phloem proteins and interference with host phloem activities (Ψw, Ψp, Ψs, phloem loading, and unloading), highlighting that further research is needed. Systemic infection with plant viruses can affect the phloem tissue and its translatome (Collum & Culver, 2017). CBD viruses trigger severe

symptoms and yield losses to which resistant germplasm was developed, which was soon overcome by the resistance-breaking atypical strain causing mild symptoms. Reasons for the varied host response to different virus strains are not well known, and could include abiotic factors, virus–host interactions, or a combination of both, resulting in leaf drooping, wilting, reddening, stunting, and stacking internode symptoms. The CLRDV P0 protein is a silencing suppressor; it contains a highly variable region with five amino acid substitutions that are uniquely present in the isolates collected in Georgia, USA. The effects of these amino acid substitutions in P0 on symptom development and virulence need further investigation. The number of aphid vectors is very high from early June to mid-July in Georgia. Aggressive sprays (once weekly) reduced the aphid populations but did not inhibit the occurrence of CLRDV and negatively influenced beneficial insects. The soilborne fungus *Neozygites fresenii* acts as a biological (natural) control and collapses the aphid population in the region, reducing the dependence on chemical control. The development and deployment of resistant varieties remain the most efficacious strategy for CLRDD management. Further investigations of the disease symptomatology, virus interactions with crop and noncrop hosts, vectors, the virus population, and cultural practices to develop and implement integrated disease management strategies are necessary.

References

Aboughanem-Sabanadzovic, N., Allen, T. W., Wilkerson, T. H., Conner, K. N., Sikora, E. J., Nichols, R. L., & Sabanadzovic, S. (2019). First report of cotton leafroll dwarf virus in upland cotton (*Gossypium hirsutum*) in Mississippi. Plant Disease, 103(7), 1798.

Agrofoglio, Y. C., Delfosse, V. C., Casse, M. F., Hopp, H. E., Kresic, I. B., & Distéfano, A. J. (2017). Identification of a new cotton disease caused by an atypical cotton leafroll dwarf virus in Argentina. *Phytopathology*, *107*(3), 369-376.

Al Rwahnih, M., Alabi, O. J., Hwang, M. S., Tian, T., Mollov, D., & Golino, D. (2021). Characterization of a new nepovirus infecting grapevine. *Plant Disease*, *105*(05), 1432-1439.

Alabi, O. J., Isakeit, T., Vaughn, R., Stelly, D., Conner, K. N., Gaytán, B. C., Villegas, C., Hitzelberger, C., De Santiago, L., Monclova-Santana, C., & Brown, J. K. (2020). First report of cotton leafroll dwarf virus infecting upland cotton (*Gossypium hirsutum*) in Texas. *Plant Disease*, *104*(3), 998.

Ali, A., & Mokhtari, S. (2020). First report of cotton leafroll dwarf virus infecting cotton (*Gossypium hirsutum*) in Kansas. *Plant Disease*, *104*(6), 1880.

Ali, A., Mokhtari, S., & Ferguson, C. (2020). First report of cotton leafroll dwarf virus from cotton (*Gossypium hirsutum*) in Oklahoma. *Plant Disease*, *104*(9), 2531.

Avelar, S., Ramos-Sobrinho, R., Conner, K., Nichols, R. L., Lawrence, K., & Brown, J. K. (2020). Characterization of the complete genome and P0 protein for a previously unreported genotype of cotton leafroll dwarf virus, an introduced polerovirus in the United States. *Plant Disease*, *104*(3), 780-786.

Avelar, S., Schrimsher, D. W., Lawrence, K., & Brown, J. K. (2019). First report of cotton leafroll dwarf virus associated with cotton blue disease symptoms in Alabama. *Plant Disease*, *103*(3), 592-592.

Bag, S., Roberts, P. M., & Kemerait, R. C. (2021). Cotton leafroll dwarf disease: an emerging virus disease on cotton in the US. *Crops & Soils*, *54*(2), 18-22.

Bell, A. A., Nichols, R. L., & Lemon, R. G. (2002). Bronze wilt of cotton.

Blackman, R. L., & Eastop, V. F. (2000). Aphids on the World's Crops. *An identification and information guide* (pp. 466). Wiley.

Brown, S., Conner, K., Hagan, A., Jacobson, A., Koebernick, J., Lawrence, K., Brown, J., Nichols, R. L., & Kemerait, R. (2019). *Report of a research review and planning meeting on cotton leafroll dwarf virus*. Cotton Incorporated. https://www.cottoninc.com/cotton-production/ag-research/plant-pathology/cotton-leafroll-dwarf-virus-research/

Brown, S. M. (2000). Bronze wilt field symptoms in Georgia. In C. P. Dugger & D. A. Richter (Eds.), *Proceedings of the 2000 Beltwide Cotton Conferences* (p. 151). Memphis, TN: National Cotton Council.

Bruyère, A., Brault, V., Ziegler-Graff, V., Simonis, M. T., Van den Heuvel, J. F., Richards, K., Guilley, H., Jonard, G., & 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*(2), 323–334.

Bryant, J. E., Holmes, E. C., & Barrett, A. D. (2007). Out of Africa: a molecular perspective on the introduction of yellow fever virus into the Americas. *PLoS pathogens*, *3*(5), e75.

CABI Compendium. (2021) *Aphis gossypii* (cotton aphid). https://doi.org/10.1079/cabicompendium.6204

Calvo, M., Malinowski, T., & García, J. A. (2014). Single amino acid changes in the 6K1-CI region can promote the alternative adaptation of Prunus- and Nicotiana-propagated Plum pox virus C isolates to either host. *Molecular plant-microbe interactions*, *27*(2), 136–149.

Cascardo, R. S., Arantes, I. L., Silva, T. F., Sachetto-Martins, G., Vaslin, M. F., & Corrêa, R. L. (2015). Function and diversity of P0 proteins among cotton leafroll dwarf virus isolates. *Virology journal*, *12*, 123.

Cauquil, J. (1977) Etudes sur une maladie d'origine virale du cotonnier, la maladie bleue. *Coton et Fibres Tropicales*, *32*, 259–278.

Cauquil, J. & Follin, J.C. (1983) Presumed virus and mycoplasma-like organism diseases in sub-Saharan Africa and in the rest of the world. *Cotton et Fibres Tropicales*, *38*, 293–317.

Cauquil, J. & Vaissayre, M. (1971) La "maladie bleue" du cotonnier en Afrique: transmission de cotonnier à cotonnier par Aphis gossypii Glover. *Coton et Fibres Tropicales*, *26*, 463–466.

Chastain, D. R., Snider, J. L., Choinski, J. S., Collins, G. D., Perry, C. D., Whitaker, J., Grey, T. L., Sorensen, R. B., van Iersel, M., Byrd, S. A., & Porter, W. (2016). Leaf ontogeny strongly influences photosynthetic tolerance to drought and high temperature in *Gossypium hirsutum*. *Journal of plant physiology*, *199*, 18–28.

Chastain, D. R., Snider, J. L., Collins, G. D., Perry, C. D., Whitaker, J., & Byrd, S. A. (2014). Water deficit in field-grown Gossypium hirsutum primarily limits net photosynthesis by decreasing stomatal conductance, increasing photorespiration, and increasing the ratio of dark respiration to gross photosynthesis. *Journal of plant physiology*, *171*(17), 1576–1585.

Close, D. C., & Beadle, C. L. (2003). The ecophysiology of foliar anthocyanin. *The Botanical Review, 69*(2), 149-161.

Collum, T. D., & Culver, J. N. (2017). Tobacco mosaic virus infection disproportionately impacts phloem associated translatomes in Arabidopsis thaliana and *Nicotiana benthamiana. Virology, 510,* 76–89.

Corrêa, R. L., Silva, T. F., Simões-Araújo, J. L., Barroso, P. A., Vidal, M. S., & Vaslin, M. F. (2005). Molecular characterization of a virus from the family Luteoviridae associated with cotton blue disease. *Archives of Virology, 150*(7), 1357–1367.

Costa, A.S. & Carvalho, A. (1962) Virus diseases of the cotton plant in the state of São Paulo. Bragantia, 21, 45–62.

da Silva, A. K., Romanel, E., Silva, T.daF., Castilhos, Y., Schrago, C. G., Galbieri, R., Bélot, J. L., & Vaslin, M. F. (2015). Complete genome sequences of two new virus isolates associated with cotton blue disease resistance breaking in Brazil. *Archives of Virology, 160*(5), 1371–1374.

DeBlasio, S. L., Johnson, R., Mahoney, J., Karasev, A., Gray, S. M., MacCoss, M. J., & Cilia, M. (2015). Insights into the polerovirus-plant interactome revealed by coimmunoprecipitation and mass spectrometry. *Molecular plant-microbe interactions, 28*(4), 467–481.

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

Delfosse, V. C., Barrios Barón, M. P., & Distéfano, A. J. (2021). What we know about poleroviruses: Advances in understanding the functions of polerovirus proteins. *Plant Pathology, 70*(5), 1047-1061.

Distéfano, A. J., Bonacic Kresic, I., & 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. *Archives of Virology, 155*(11), 1849–1854.

Fang, D. D., Xiao, J., Canci, P. C., & Cantrell, R. G. (2010). A new SNP haplotype associated with blue disease resistance gene in cotton (*Gossypium hirsutum* L.). TAG. Theoretical and applied genetics. *Theoretische und angewandte Genetik, 120*(5), 943–953.

Faske, T. R., Stainton, D., Aboughanem-Sabanadzovic, N., & Allen, T. W. (2020). First report of cotton leafroll dwarf virus from upland cotton (*Gossypium hirsutum*) in Arkansas. *Plant Disease, 104*(10), 2742.

Feng, Y., Krueger, E. N., Liu, S., Dorman, K., Bonning, B. C., & Miller, W. A. (2017). Discovery of known and novel viral genomes in soybean aphid by deep sequencing. *Phytobiomes, 1*(1), 36-45.

Ferguson, C., & Ali, A. (2022). Complete genome sequence of cotton leafroll dwarf virus infecting cotton in Oklahoma, USA. *Microbiology resource announcements, 11*(7), e0014722.

Fusaro, A.F., Correa, R.L., Nakasugi, K., Jackson, C., Kawchuk, L., Vaslin, M.F. et al. (2012) The enamovirus P0 protein is a silencing suppressor which inhibits local and systemic RNA silencing through AGO1 degradation. *Virology, 426*, 178–187.

Gade, R. M., Tanwar, R. K., Jeyakumar, P., & Kanwar, V. (2013). *Leaf reddening and its management in cotton* (Report No. 30). ICAR - National Centre for Integrated Pest Management.

Haupt, S., Stroganova, T., Ryabov, E., Kim, S. H., Fraser, G., Duncan, G., Mayo, M. A., Barker, H., & Taliansky, M. (2005). Nucleolar localization of potato leafroll virus capsid proteins. *The Journal of General Virology, 86*(Pt 10), 2891–2896.

Heilsnis, B., McLaughlin, A., Conner, K., Koebernick, J., & Jacobson, A. L. (2022). Vector competency of Aphis gossypii and Bemisia tabaci to transmit cotton leafroll dwarf virus. *Journal of Cotton Science, 26*, 23–30.

Hoffmann, L. V., Branquinho, A. A., Barroso, P. A. V., & Vaslin, M. F. S. (2022). Antibodies for the coat protein of cotton leafroll dwarf virus detect *Commelina sp.* as an intermediary host for cotton blue disease. *Frontiers in Plant Science, 13*, 814119.

Hofius, D., Herbers, K., Melzer, M., Omid, A., Tacke, E., Wolf, S., & Sonnewald, U. (2001). Evidence for expression level-dependent modulation of carbohydrate status and viral resistance by the potato leafroll virus movement protein in transgenic tobacco plants. *The Plant Journal, 28*(5), 529–543.

Horn, N. M., Reddy, S. V., Van den Heuvel, J. F. J. M., & Reddy, D. V. R. (1996). Survey of chickpea (*Cicer arietinum* L.) for chickpea stunt disease and associated viruses in India and Pakistan. *Plant Disease, 80*(3), 286–290.

Igori, D., Shin, A. Y., Kim, S. E., Kwon, S. Y., & Moon, J. S. (2022). First report of cotton leafroll dwarf virus infecting *Hibiscus syriacus* in South Korea. *Plant Disease, 106*(11), 3003.

Iriarte, F. B., Dey, K. K., Small, I. M., Conner, K. N., O'Brien, G. K., Johnson, L., Savery, C., Carter, E., Sprague, D., Nichols, R. L., & Wright, D. L. (2020). First report of cotton leafroll dwarf virus in Florida. *Plant Disease, 104*(10), 2744.

Jaag, H. M., Kawchuk, L., Rohde, W., Fischer, R., Emans, N., & Prüfer, D. (2003). An unusual internal ribosomal entry site of inverted symmetry directs expression of a potato leafroll polerovirus replication-associated protein. *Proceedings of the National Academy of Sciences of the United States of America, 100*(15), 8939–8944.

Kavalappara, S. R., Riley, D. G., Cremonez, P. S. G., Perier, J. D., & Bag, S. (2022). Wild radish (*Raphanus raphanistrum* L.) is a potential reservoir host of cucurbit chlorotic yellows virus. *Viruses, 14*(3), 593.

Kumari, S. G., Sharman, M., Moukahel, A., Ziyaev, Z., & Ahmed, S. (2020). First report of *Cotton leafroll dwarf virus* affecting chickpea (*Cicer arietinum*) in Uzbekistan. *Plant Disease, 104*(9), 2532.

Latourrette, K., Holste, N. M., & Garcia-Ruiz, H. (2021). Polerovirus genomic variation. *Virus Evolution, 7*, veab1021.

Lenardon, S. (1994). Caracterización del agente causal de la enfermedad azul del algodón en el área centro chaqueña. *Informe del PIC, 320*, 536.

Letunic, I., & Bork, P. (2021). Interactive Tree of Life (iTOL) v5: An online tool for phylogenetic tree display and annotation. *Nucleic Acids Research, 49*(W1), W293–W296.

Mahas, J. W., Hamilton, F. B., Roberts, P. M., Ray, C. H., Miller, G. L., Sharman, M., Conner, K., Bag, S., Blythe, E. K., Toews, M. D., & Jacobson, A. L. (2022). Investigating the effects of planting date and *Aphis gossypii* management on reducing the final incidence of cotton leafroll dwarf virus. *Crop Protection, 158*, 106005.

McGraw, L. (2000). The cause of bronze wilt of cotton. *Agricultural Research, 48*(11), 21. https://agresearchmag.ars.usda.gov/ar/archive/2000/nov/wilt1100.pdf

Medrano, H., Escalona, J. M., Bota, J., Gulías, J., & Flexas, J. (2002). Regulation of photosynthesis of C3 plants in response to progressive drought: Stomatal conductance as a reference parameter. *Annals of Botany, 89*(Spec No 7), 895–905.

Michelotto, M. D., & Busoli, A. C. (2003). Eficiência de ninfas e adultos de *Aphis gossypii* Glov. na transmissão do vírus do mosaico das nervuras do algodoeiro. *Bragantia, 62*, 255–259.

Marcos, D. M., & Busoli, A. C. (2006). Efeito da época de inoculação do vírus do mosaico das nervuras por *Aphis gossypii* Glover (Hemiptera: Aphididae) no desenvolvimento e na produção do algodoeiro [Effect of the time of inoculation of the cotton vein mosaic virus by *Aphis gossypii* Glover (Hemiptera: Aphididae) on growth and yield of cotton plants]. *Neotropical Entomology, 35*(2), 251–256.

Michelotto, M. D., & Busoli, A. C. (2009). Biologia de *Aphis gossypii* em plantas infectadas pelo vírus do mosaico das nervuras do algodoeiro. *Bragantia, 68*, 1018–1024.

Morello, C. D. L., Nelson, D. S., Farias, F. J. C., Lamas, F. M., Pedrosa, M. B., Ribeiro, J. L., et al. (2010). BRS 293: A midseason high-yielding upland cotton cultivar for Brazilian savanna. *Crop Breeding and Applied Biotechnology, 10*, 180–182.

Mukherjee, A. K., Chahande, P. R., Meshram, M. K., & Kranthi, K. R. (2012). First report of *Polerovirus* of the family *Luteoviridae* infecting cotton in India. *New Disease Reports, 25*(22), 2044-0588.

Mukherjee, A. K., Mukherjee, P. K., & Kranthi, S. (2016). Genetic similarity between cotton leafroll dwarf virus and chickpea stunt disease associated virus in India. *The Plant Pathology Journal, 32*(6), 580–583.

Opalka, N., Tihova, M., Brugidou, C., Kumar, A., Beachy, R. N., Fauquet, C. M., & Yeager, M. (2000). Structure of native and expanded sobemoviruses by electron cryo-microscopy and image reconstruction. *Journal of molecular biology, 303*(2), 197–211.

Padgett, G. B., Colyer, P. D., & Whitam, H. K. (2004). Bronze wilt in Louisiana cotton. *Louisiana Agriculture, 47*(3), 24-25.

Pandey, S., Bag, S., Roberts, P., Conner, K., Balkcom, K. S., Price, A. J., Jacobson, A. L., & Srinivasan, R. (2022). Prospective alternate hosts of an emerging polerovirus in cotton landscapes in the southeastern United States. *Viruses, 14*(10), 2249.

Parkash, V., Sharma, D. B., Snider, J., Bag, S., Roberts, P., Tabassum, A., West, D., Khanal, S., Suassuna, N., & Chee, P. (2021). Effect of cotton leafroll dwarf virus on physiological processes and yield of individual cotton plants. *Frontiers in plant science, 12*, 734386.

Patton, M. F., Bak, A., Sayre, J. M., Heck, M. L., & Casteel, C. L. (2020). A polerovirus, potato leafroll virus, alters plant-vector interactions using three viral proteins. *Plant, cell & environment, 43*(2), 387–399.

Pazhouhandeh, M., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., Hemmer, O., Kretsch, T., Richards, K. E., Genschik, P., & Ziegler-Graff, V. (2006). F-box-like domain in the polerovirus protein P0 is required for silencing suppressor function. *Proceedings of the National Academy of Sciences of the United States of America, 103*(6), 1994–1999.

Peter, K. A., Gildow, F., Palukaitis, P., & Gray, S. M. (2009). The C terminus of the polerovirus p5 readthrough domain limits virus infection to the phloem. *Journal of virology, 83*(11), 5419–5429.

Phipps, B. J. (2000). Cotton variety response to bronze wilt in Missouri and northern Tennessee. In C. P. Dugger & D. A. Richter (Eds.), *Proceedings of the 2000 Beltwide Cotton Conferences*, 4–8 January 2000 (pp. 152–154). National Cotton Council.

Price, T., Valverde, R., Singh, R., Davis, J., Brown, S., & Jones, H. (2020). First report of cotton leafroll dwarf virus in Louisiana. *Plant Health Progress*, *21*, 142–143.

Pupim Junior, O., Schuster, I., Pinto, R. B., Pires, E., Belot, J. L., Silvie, P., et al. (2008). Herança da resistência do algodoeiro à doença-azul. *Pesquisa Agropecuária Brasileira*, *43*, 661–665.

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, *67*(5), 901–904.

Ramos-Sobrinho, R., Adegbola, R. O., Lawrence, K., Schrimsher, D. W., Isakeit, T., Alabi, O. J., & Brown, J. K. (2021). Cotton leafroll dwarf virus US genomes comprise divergent subpopulations and harbor extensive variability. *Viruses*, *13*(11), 2230.

Ramos-Sobrinho, R., Ferro, M. M. M., Lima, G. S. A., & Nagata, T. (2023). First report of cotton leafroll dwarf virus infecting cacao (Theobroma cacao) trees in Brazil. *Plant Disease*, *107*(4), 1251.

Ray, J. D., Sharman, M., Quintao, V., Rossel, B., Westaway, J., & Gambley, C. (2016). Cotton leafroll dwarf virus detected in Timor-Leste. *Australasian Plant Disease Notes*, *11*, 29.

Rotenberg, D., Krishna Kumar, N. K., Ullman, D. E., Montero-Astúa, M., Willis, D. K., German, T. L., & Whitfield, A. E. (2009). Variation in tomato spotted wilt virus titer in Frankliniella occidentalis and its association with frequency of transmission. *Phytopathology*, *99*(4), 404–410.

Sedhain, N. P., Bag, S., Morgan, K., Carter, R., Triana, P., Whitaker, J., et al. (2021). Natural host range, incidence on overwintering cotton and diversity of cotton leafroll dwarf virus in Georgia, USA. *Crop Protection, 144*, 105604.

Sharman, M., Lapbanjob, S., Sebunruang, P., Belot, J. L., Galbieri, R., Giband, M., et al. (2015). First report of cotton leafroll dwarf virus in Thailand using a species-specific PCR validated with isolates from Brazil. *Australasian Plant Disease Notes, 10*, 24.

Silva, T. F., Correa, R. L., Castilho, Y., Silvie, P., Belot, J. L., & Vaslin, M. F. S. (2008). Widespread distribution and a new recombinant species of Brazilian virus associated with cotton blue disease. *Virology Journal, 5*, 123.

Smirnova, E., Firth, A. E., Miller, W. A., Scheidecker, D., Brault, V., Reinbold, C., Rakotondrafara, A. M., Chung, B. Y., & Ziegler-Graff, V. (2015). Discovery of a small non-AUG-initiated ORF in poleroviruses and luteoviruses that is required for long-distance movement. *PLoS Pathogens, 11*(5), e1004868.

Sõmera, M., Fargette, D., Hébrard, E., Sarmiento, C., & ICTV Report Consortium. (2021). ICTV virus taxonomy profile: Solemoviridae 2021. *The Journal of General Virology, 102*(12), 001707.

Suchard, M.A., Lemey, P., Baele, G., Ayres, D.L., Drummond, A.J. & Rambaut, A. (2018) Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evolution, 4*, vey016.

Sun, Q., Li, Y. Y., Wang, Y., Zhao, H. H., Zhao, T. Y., Zhang, Z. Y., Li, D. W., Yu, J. L., Wang, X. B., Zhang, Y. L., & Han, C. G. (2018). Brassica yellows virus P0 protein impairs the antiviral activity of NbRAF2 in *Nicotiana benthamiana*. *Journal of Experimental Botany, 69*(12), 3127–3139.

Tabassum, A., Bag, S., Roberts, P., Suassuna, N., Chee, P., Whitaker, J. R., Conner, K. N., Brown, J., Nichols, R. L., & Kemerait, R. C. (2019). First report of cotton leafroll dwarf virus infecting cotton in Georgia, USA. *Plant Disease, 103*(7), 1803.

Tabassum, A., Bag, S., Suassuna, N. D., Conner, K. N., Chee, P., Kemerait, R. C., & Roberts, P. (2021). Genome analysis of cotton leafroll dwarf virus reveals variability in the silencing suppressor protein, genotypes, and genomic recombinants in the USA. *PLoS ONE, 16*(7), e0252523.

Tabassum, A., Roberts, P. M., & Bag, S. (2020). Genome sequence of cotton leafroll dwarf virus infecting cotton in Georgia, USA. *Microbiology Resource Announcements, 9*(34), e00812-20.

Takimoto, J. K. (2003). *Estudo da relação vetor-patógeno-hospedeiro para a doença azul do algodoeiro* (Master's thesis). Instituto Agronômico de Campinas, São Paulo, Brazil.

Takimoto, J. K., Benetti Queiroz-Voltan, R., & Alberto, J. (2009). Alterações anatômicas em algodoeiro infectado pelo vírus da doença azul. *Bragantia, 68*, 109–116.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution, 38*, 3022–3027.

Thiessen, L. D., Schappe, T., Zaccaron, M., Conner, K., Koebernick, J., Jacobson, A., & Huseth, A. (2020). First report of cotton leafroll dwarf virus in cotton plants affected by cotton leafroll dwarf disease in North Carolina. *Plant Disease, 104*(12), 3275.

U.S. Department of Agriculture. (2021). *NASSQuickstat, a comprehensive tool for accessing agricultural data published by National Agricultural Statistics Service*. U.S. Department of Agriculture. https://quickstats.nass.usda.gov

Vassilakos, N., Simon, V., Tzima, A., Johansen, E., & Moury, B. (2016). Genetic determinism and evolutionary reconstruction of a host jump in a plant virus. *Molecular Biology and Evolution, 33*(2), 541–553.

Wang, H., Greene, J., Mueller, J., Conner, K., & Jacobson, A. (2020). First report of cotton leafroll dwarf virus in cotton fields of South Carolina. *Plant Disease, 104*(9), 2532.

Yang, X., Du, M., Li, S., & Zhou, X. (2021). Coinfection of Cotton Plants with Watermelon Mosaic Virus and a Novel Polerovirus in China. *Viruses, 13*(11), 2210.

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., & 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. *Molecular Plant-Microbe Interactions, 27*(6), 515–527.

Tables

TABLE 2.1 Analysis of the rate of mutation and the age (root) of the CLRDV P0 protein using the BEAST tool (v. 1.10.4)

Sequence source	**Mean rate**	**95% HPD interval**	**S**	**Variation**	**ESS/no. of samples**	**Age (root)**	**95% HPD interval**
All 76 sequences	2.194×10^{-3}	1.293×10^{-3} to 3.081×10^{-3}	0.1015	0.0103	8,871/9,001	1945	1914 to 1971
Only weed species	6.313×10^{-10}	1.266×10^{-23} to 1.398×10^{-10}	1.129×10^{-12}	1.275×10^{-22}	566/9,001	–	–

Abbreviations: ESS, effective sample size, that is, the number of independent samples or the chain length; HPD, highest posterior density; Stdev, standard deviation of the mean.

TABLE 2.2 Estimated copy number of cotton leafroll dwarf virus (CLRDV) in different parts of infected cotton plants.

Part of the plant		**Estimated copy number per ng of total RNA**
Underground	Roots	1.78×10^5
Lower plant parts	Main stem	5.01×10^5
	Branches	4.47×10^5
	Petioles	2.63×10^3
	Fruit bracts and skin	2.00×10^3
Upper plant parts	Main stem	8.91×10^5
	Branches	6.15×10^6
	Petioles	1.58×10^5
	Fruit bracts and skin	1.26×10^4

Note: Values are averages from eight CLRDV-infected plants as determined by reverse transcription-PCR. Samples from each plant were run in triplicate.

Supplementary information

Table 2.3 (S1): Cotton leafroll dwarf virus (CLRDV) P0 protein sequences generated and analyzed in this study

Accession number	Protein ID-P0	Collection date	Isolate	Host	Country	Reference
ON954058	UXR10333	2022	TCBR01	*Theobroma cacao*	Brazil	Ramos-Sobrinho et al., 2022
ON954059	UXR10334	2022	TCBR02	*T. cacao*	Brazil	Ramos-Sobrinho et al., 2022
OQ054191	Not available	2021	Terrell	*Gossypium hirsutum*	USA	This study
OQ054192	Not available	2021	Tifton	*G. hirsutum*	USA	This study
OQ054193	Not available	2021	Dooly	*G. hirsutum*	USA	This study
OQ054194	Not available	2021	Sumter	*G. hirsutum*	USA	This study
OQ054195	Not available	2021	Crisp	*G. hirsutum*	USA	This study
OQ054196	Not available	2021	Ben Hill	*G. hirsutum*	USA	This study
OQ054197	Not available	2021	Bulloch	*G. hirsutum*	USA	This study
OQ054183	Not available	2020	Ben Hill	*G. hirsutum*	USA	This study
OQ054184	Not available	2020	Lauren	*G. hirsutum*	USA	This study
OQ054185	Not available	2020	Montgomery	*G. hirsutum*	USA	This study
OQ054186	Not available	2020	Evans	*G. hirsutum*	USA	This study
OQ054187	Not available	2020	Jeff Davis	*G. hirsutum*	USA	This study
OQ054188	Not available	2020	Plains	*G. hirsutum*	USA	This study
OQ054189	Not available	2020	Worth	*G. hirsutum*	USA	This study
OQ054190	Not available	2020	Dawson	*G. hirsutum*	USA	This study
OQ054178	Not available	2019	Screven	*G. hirsutum*	USA	This study
OQ054179	Not available	2019	Early	*G. hirsutum*	USA	This study
OQ054180	Not available	2019	Wayne	*G. hirsutum*	USA	This study
OQ054181	Not available	2019	Coffee	*G. hirsutum*	USA	This study
OQ054182	Not available	2019	Brooks	*G. hirsutum*	USA	This study
MN506243	QKV49422	2019	128.01a	*G. hirsutum*	USA	Alabi et al., 2020
MN506244	QKV49423	2019	128.01b	*G. hirsutum*	USA	Alabi et al., 2020

MN506245	QKV49424	2019	1808001.06a	*G. hirsutum*	USA	Alabi et al., 2020
MN506246	QKV49425	2019	1808001.06b	*G. hirsutum*	USA	Alabi et al., 2020
MN872302	QHB18532	2019	Texas CS4	*G. hirsutum*	USA	Ali & Mokhtari 2020
OK073299	UID85580	2019	SK	*Hibiscus syriacus*	South Korea	Igori et al., 2022
OK185939	UGY70907	2019	CLRDV_USA_AL_MC2	*G. hirsutum*	USA	Ramos-Sobrinho et al., 2021
OK185940	UGY70914	2019	CLRDV_USA_FL_SC4	*G. hirsutum*	USA	Ramos-Sobrinho et al., 2021
OK185941	UGY70921	2019	CLRDV_USA_TX_CT2	*G. hirsutum*	USA	Ramos-Sobrinho et al., 2021
OK185942	UGY70928	2019	CLRDV_USA_TX_CT3	*G. hirsutum*	USA	Ramos-Sobrinho et al., 2021
OK185943	UGY70935	2019	CLRDV_USA_TXa	*G. hirsutum*	USA	Ramos-Sobrinho et al., 2021
OK185944	UGY70942	2019	CLRDV_USA_TXb	*G. hirsutum*	USA	Ramos-Sobrinho et al., 2021
OK185945	UGY70949	2019	CLRDV_USA_TXc	*G. hirsutum*	USA	Ramos-Sobrinho et al., 2021
OK185946	UGY70956	2019	CLRDV_USA_TXd	*G. hirsutum*	USA	Ramos-Sobrinho et al., 2021
MW629382	QXI66601	2019	W_08	*Arachis glabrata*	USA	Sedhain et al., 2021
MW629383	QXI66602	2019	W_34	*Lamium amplexicaule*	USA	Sedhain et al., 2021
MW629384	QXI66603	2019	S_55	*L. amplexicaule*	USA	Sedhain et al., 2021
MW629385	QXI66604	2019	S_69	*Oenothera laciniata*	USA	Sedhain et al., 2021
MW629386	QXI66605	2019	W_332	*O. laciniata*	USA	Sedhain et al., 2021
MW629387	QXI66606	2019	W_382	*Amaranthus palmeri*	USA	Sedhain et al., 2021
MW629388	QXI66607	2019	W_348	*Physalis minima*	USA	Sedhain et al., 2021
MW629389	QXI66608	2019	W_350	*Portulaca pilosa*	USA	Sedhain et al., 2021
MW629390	QXI66609	2019	W_384	*Mollugo verticillata*	USA	Sedhain et al., 2021
MW629391	QXI66610	2019	W_402	*Richardia scabra*	USA	Sedhain et al., 2021
MT633122	QKV28728	2018	GA_53	*G. hirsutum*	USA	Tabassum et al., 2020
MT800932	QWJ75369	2018	GA_40	*G. hirsutum*	USA	Tabassum et al., 2021
MT800933	QWJ75377	2018	GA_72	*G. hirsutum*	USA	Tabassum et al., 2021
MT814774	QWJ75382	2018	GA_67	*G. hirsutum*	USA	Tabassum et al., 2021
MT814775	QWJ75388	2018	GA_77	*G. hirsutum*	USA	Tabassum et al., 2021
MT814776	QWJ75394	2018	GA_58	*G. hirsutum*	USA	Tabassum et al., 2021
MT814777	QWJ75400	2018	AL	*G. hirsutum*	USA	Tabassum et al., 2021

MN071395	QEU57990	2018	AL_US	*G. hirsutum*	USA	Avelar et al., 2020
MN046206	QEU57984	2018	AL_US	*G. hirsutum*	USA	Avelar et al., 2020
MN057665	QLJ58259	2017	YN	*Malvaviscus arboreus*	China	Unpublished
KU934233	APZ88682	2015	M8/15	*G. hirsutum*	Argentina	Agrofoglio et al., 2017
KU934234	APZ88683	2015	M9/15	*G. hirsutum*	Argentina	Agrofoglio et al., 2017
KU934229	APZ88678	2014	M14/14	*G. hirsutum*	Argentina	Agrofoglio et al., 2017
KU934230	APZ88679	2014	M15/14	*G. hirsutum*	Argentina	Agrofoglio et al., 2017
KU934231	APZ88680	2014	Mtr2/14	*G. hirsutum*	Argentina	Agrofoglio et al., 2017
KU934232	APZ88681	2014	Mtr10/14	*G. hirsutum*	Argentina	Agrofoglio et al., 2017
KF906260	AIU96343	2011	IAC25RMD	*G. hirsutum*	Brazil	da Silva et al., 2015
KF359946	AID69208	2010	M3 atypical	*G. hirsutum*	Argentina	Agrofoglio et al., 2017
KF359947	AID69214	2010	M5 atypical	*G. hirsutum*	Argentina	Agrofoglio et al., 2017
KX588248	ARU09826	2009	CN-S5	*Aphis glycines*	China	Feng et al., 2017
HQ827780	AEK06218	2007	Transcriptomic data	*G. hirsutum*	Brazil	Silva et al., 2011
KR185733	AKZ17159	2007	CLRDV-Acr9	*G. hirsutum*	Brazil	Cascardo et al., 2015
KR185734	AKZ17160	2007	CLRDV-Pm1	*G. hirsutum*	Brazil	Cascardo et al., 2015
KR185735	AKZ17161	2007	CLRDV-Hol1	*G. hirsutum*	Brazil	Cascardo et al., 2015
KR185736	AKZ17162	2007	CLRDV-Ipa4	*G. hirsutum*	Brazil	Cascardo et al., 2015
KR185737	AKZ17163	2007	CLRDV-Pal3	*G. hirsutum*	Brazil	Cascardo et al., 2015
KF906261	AIU96349	2006	Delta Opal	*G. hirsutum*	Brazil	da Silva et al., 2015
GU167940	ADN83833	2006	ARG	*G. hirsutum*	Argentina	Distefano et al., 2010
NC_014545	YP_003915147	2006	ARG	*G. hirsutum*	Argentina	Distefano et al., 2010
OM687235	UPA61483	2021	EC4	*G. hirsutum*	USA	Ferguson & Ali 2022

Figures:

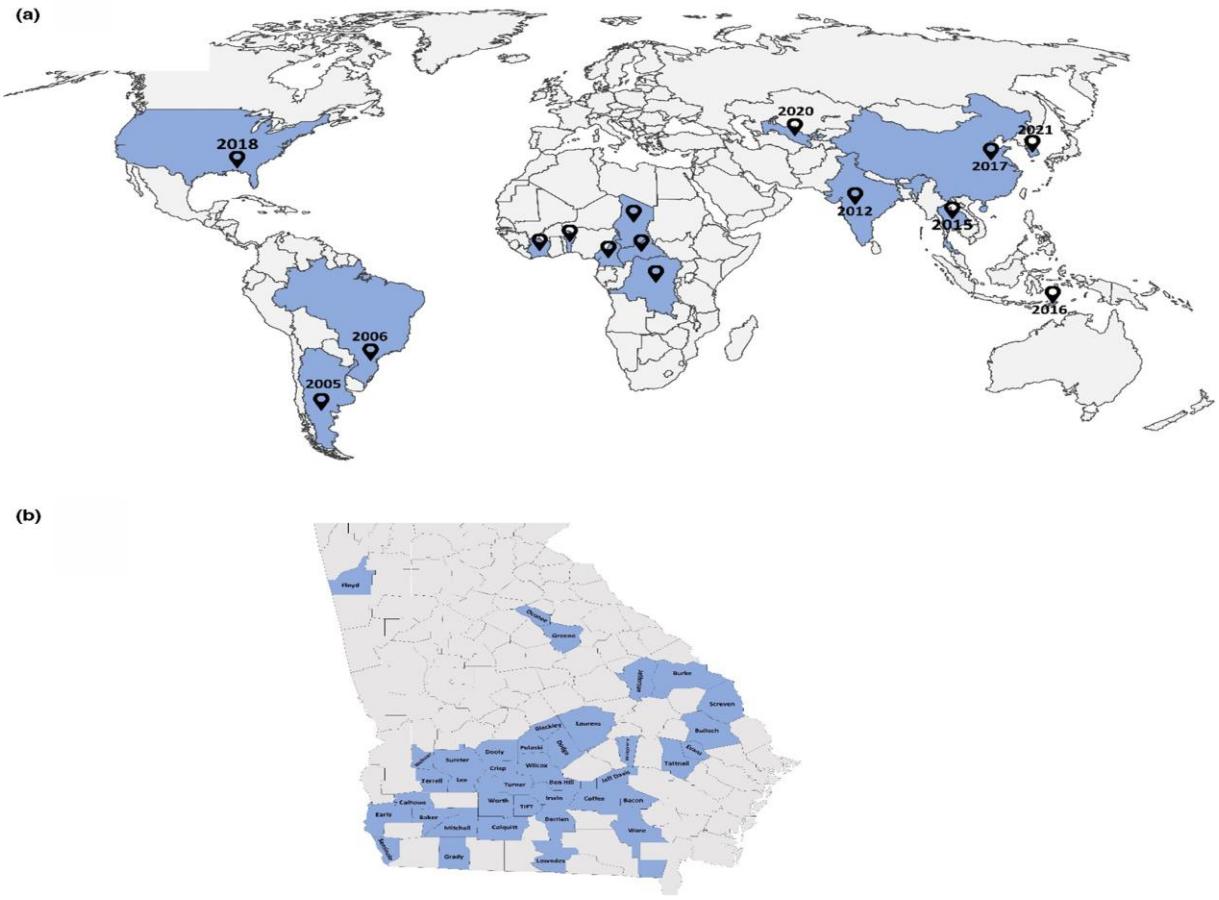


Figure 2.1

(a) Worldwide distribution of cotton blue disease and cotton leafroll dwarf disease. (b) Counties where cotton leafroll dwarf virus (CLRDV) was detected on cotton fields in Georgia, USA, are highlighted in blue. Pointers in (a) indicate where CLRDV was discovered, and the year of the first report in the respective country. For the African continent, virus discovery dates are not known; hence, only disease presence is shown.

Figure 2.2

Symptoms observed on cotton plants infected with cotton leafroll dwarf virus (CLRDV) grown in commercial cotton fields in Georgia, USA. (a) Leaf deformation with bluish-green discolouration. (b) Vein clearing, leaf curling, rolling, and deformation. (c) Reddening of leaf, petioles, and stem. (d) Puckering and crinkling. (e) Wilting and downward leaf drooping (V-shaped lamina folding). (f) Deformation of the leaf lamina. (g) Leaf turgidity with a leathery texture. (h) Crinkling. (i) Square/leaf dropping. (j) Wilting.

(k,l) Vegetative growth in the upper branches of the stem and stacked internodes and ceased reproductive growth. (m) Bushy phenotype. (n) Terminal whips or accentuated verticality. (o) Parrot beak fruits. (p) Plant infected in the early stage. (q,r) Symptoms diminish in early infected plants but plants remain dwarf without any harvestable bolls. (s,t) Volunteer cotton stalks.

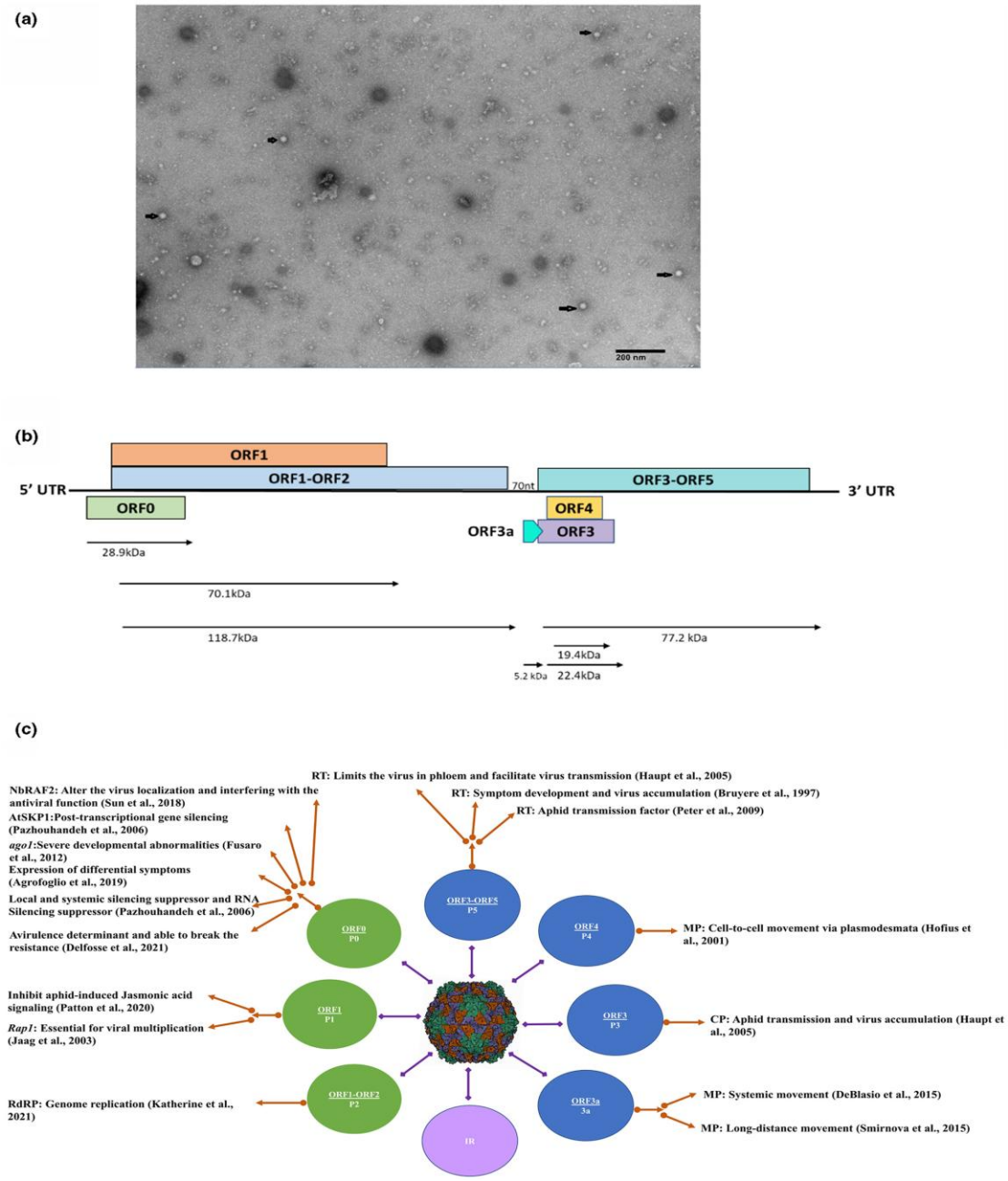


Figure 2.3

(a)Transmission electron micrograph of virion particles of cotton leafroll dwarf virus (CLRDV) from bark tissues of an infected cotton plant with symptoms. The presence of CLRDV in these bark tissues was further confirmed by reverse transcription-PCR. (b) Schematic representation of the genome of CLRDV and open reading frames

(ORFs). (c) Functional overview of proteins associated with CLRDV, including a schematic representation and their functions. An artificially coloured cryo-electron microscopy image of rice yellow mottle virus (family *Solemoviridae*) is shown in the centre (ICTV; https://ictv.global/report/chapter/solemoviridae/solemoviridae and Opalka et al., 2000). The functional role played by each viral protein (shown in green, blue, and lavender-coloured circles) encoded by CLRDV and other members of the genus Polerovirus are depicted at the periphery corresponding to their functional characterization in that virus species.

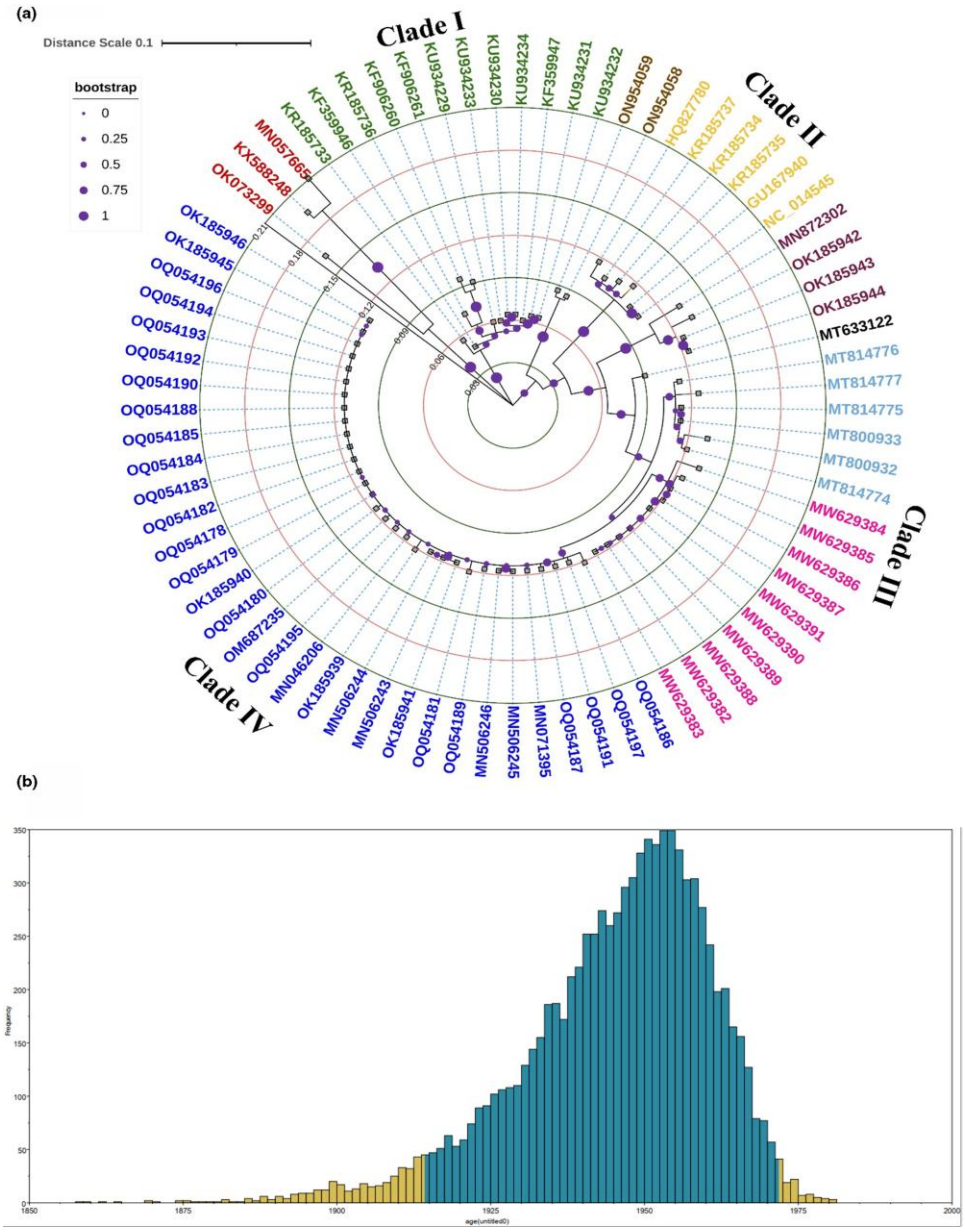


Figure 2.4

(a) The evolutionary history was inferred by using the maximum-likelihood method and a JTT matrix-based model with 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 76 nucleotide sequences. All positions with less than 95% site coverage were eliminated. The blue colour indicates cotton leafroll dwarf virus P0 (CLRDV-P0) sequences obtained from

cotton plants in Alabama, Florida, Georgia, Oklahoma, and Texas, in 2018–2022. The light pink colour indicates CLRDV-P0 sequences from weed species in Georgia. The CLRDV-P0 sequences obtained from cotton in Georgia/Alabama in 2018 are represented in teal. The typical CLRDV-P0 sequences from Brazil are shown in mustard yellow, whereas atypical CLRDV-P0 sequences from cotton are shown in green. Four sequences from Texas forming a subclade are shown in purple. Two sequences (accessions ON954058 and ON954059) shown in golden brown were isolated from cacao (*Theobroma cacao*) trees in Brazil. Three CLRDVs (accessions UID85580, QLJ58259, and ARU09826), isolated from *Hibiscus* sp., *Malvaviscus* sp., and aphid species, are shown in red. The phylogenetic tree was visualized using iTOL v. 4 (Letunic & Bork, 2021). (b) Graph representing the evolution of CLRDV. The virus epidemic may have started around 1945. Analysis was conducted in BEAST v. 1.10.4 and the results were further visualized using Tracer v. 1.7.1.

CHAPTER 3

CHARACTERIZATION OF CAULIMOVIRID-LIKE SEQUENCES FROM UPLAND COTTON (*GOSSYPIUM HIRSUTUM* L.) EXHIBITING TERMINAL ABORTION IN GEORGIA, USA

Published Article:

Edula, S. R., Hand, L. C., Roberts, P. M., Beasley, E., Snider, J. L., Kemerait, R. C., Chee, P. W., & Bag, S. (2024). Characterization of Caulimovirid-like sequences from upland cotton (*Gossypium hirsutum* L.) exhibiting terminal abortion in Georgia, USA. *Viruses*, *16*(7), 1111.

Abstract

In this study, we investigated the potential involvement of endogenous viral elements (EVEs) in the development of apical tissue necrosis, resulting in the terminal abortion of upland cotton (*Gossypium hirsutum* L.) in Georgia. The high-throughput sequence analysis of symptomatic and asymptomatic plant tissue samples revealed near-complete EVE-Georgia (EVE-GA) sequences closely related to caulimoviruses. The analysis of EVE-GA's putative open reading frames (ORFs) compared to cotton virus A and endogenous cotton pararetroviral elements (eCPRVE) revealed their similarity in putative ORFs 1–4. However, in the ORF 5 and ORF 6 encoding putative coat protein and reverse transcriptase, respectively, the sequences from EVE-GA have stop codons similar to eCPRVE sequences from Mississippi. In silico mining of the cotton genome database using EVE-GA as a query uncovered near-complete viral sequence insertions in the genomes of G. hirsutum species (~7 kb) but partial in *G. tomentosum* (~5.3 kb) and *G. mustelinum* (~5.1 kb) species. Furthermore, cotton EVEs' episomal forms and messenger RNA (mRNA) transcripts were detected in both symptomatic and asymptomatic plants collected from cotton fields. No significant yield difference was observed between symptomatic and asymptomatic plants of the two varieties evaluated in the experimental plot. Additionally, EVEs were also detected in cotton seeds and seedlings. This study emphasizes the need for future research on EVE sequences, their coding capacity, and any potential role in host immunity or pathogenicity.

Keywords: endogenous viral elements; terminal abortion; upland cotton; lncRNA; small RNA

Introduction

Endogenous viral elements (EVEs) were discovered within the genome of numerous protists (Bellas et al.; Koslová et al., 2024; Veglia et al., 2023) fungi (Zhao et al., 2023), insects (Flynn & Moreau, 2019; Huang et al., 2023; Liu et al., 2020; Suzuki et al., 2020), fish (Naville & Volff, 2016), birds(Cui et al., 2014; Mason et al., 2020), plants(Mason et al., 2020), animals(Belyi et al., 2010; Katzourakis & Gifford, 2010), humans(Katoh & Honda, 2023; Stein & DePaola, 2023; Weiss, 1967), and other mammals (Skirmuntt et al., 2020). These are considered relics of ancient infections, occasionally referred to as genomic fossils. The first EVEs were discovered in the late 1960s much before the discovery of reverse transcriptase were endogenous retroviruses, otherwise called endogenous retrovirus elements (Weiss, 2006). They integrate into the host nuclear genome with the help of 'integrase' a virally encoded recombinase which catalyzes the viral DNA insertion into the host cell's DNA (Andrake & Skalka, 2015; Weiss, 2006). Among plants, EVEs were first discovered in the tobacco (*Nicotiana tabacum*) genome having geminivirus-related DNA (GRD) (Ashby et al., 1997; Bejarano et al., 1996) followed by the caulimovirid (members of the family *Caulimoviridae*) like DNA in the tobacco (*N. tabacum*), and banana (*Musa balbisiana*) genomes (Diop et al., 2018; Jakowitsch et al., 1999). The GRD found in tobacco originated from an ancient New World geminivirus integration believed to be an illegitimate recombination event in a New World *N. tabacum* (Bejarano et al., 1996). Such similar phenomenon (non-homologous DNA end-joining) must have endogenized the partial or near complete sequences of the members belonging to the family *Caulimoviridae* during the host meiotic (crossovers) or mitotic recombination (somatic DNA repair) during exogenous

ancient infections (Aboughanem-Sabanadzovic et al., 2023; Geering et al., 2014). Such integrations in host germline cell chromosomes can lead to vertical transmission and are further inherited as host alleles (Holmes, 2011; Katzourakis & Gifford, 2010). Endogenous viral elements represent the DNA sequences of viruses that integrate into the host genome and are capable of vertical transmission. Viral DNA integration into bacterial and animal host chromosomes is a common phenomenon. Such integrations in plant genomes are still under study (Pahalawatta et al., 2008). The most described EVEs are from viruses with DNA genomes (ssDNA, genus: *Geminivirus* and dsDNA, genus: *Caulimovirus*) or viruses that can exhibit DNA phase in their replication.

In plants the frequently observed EVEs called “plant pararetroviruses”, belong to the genus *Caulimovirus* and their sequences are believed to be embedded into their host genomes due to ancient virus infections dating back thousands to millions of years ago (Gayral & Iskra-Caruana, 2009). The family *Caulimoviridae* (order *Ortervirales*) (Krupovic et al., 2018), comprises 11 genera: *Badnavirus, Caulimovirus, Cavemovirus, Dioscovirus, Petuvirus, Rosadnavirus, Ruflodivirus, Solendovirus, Soymovirus, Tungrovirus*, and *Vaccinivirus* (Teycheney et al., 2020). Members of this family consist of either isometric or bacilliform shaped non-enveloped virions and comprise reverse-transcribing non-covalently closed circular dsDNA genomes of 7.1–9.8 kbp in size, which replicate through RNA intermediates. Depending on the genus, members of the family *Caulimoviridae* may comprise one to nine open reading frames (ORFs) in their genome. Despite their diversity, all members code for various common proteins, including a movement protein (MP), a coat protein (CP), a pepsin-like aspartic protease (AP), reverse transcriptase (RTase) with a bound RNase H1 (RH1) and several genera

also code for TAV-transactivator/viroplasm protein (Hull, 2014; Teycheney et al., 2020). Genome replication of the *Caulimoviridae* family members is done by reverse transcription, similar to retroviruses, but without integration into the host DNA. Further, transcription of terminally redundant RNA of caulimovirus is done by episomal DNA mini chromosome using host RNA polymerase II in the infected nuclei (Pfeiffer & Hohn, 1983; Schoelz, 2008). Caulimoviruses encode a polycistronic mRNA by reinitiating the ribosomes to provide translational coupling of individual genes (Schoelz, 2008; Scholthof et al., 1992).

Among the existing 11 members of the family *Caulimoviridae*, EVEs were reported from *Badnavirus, Cavemovirus, Caulimovirus, Petuvirus*, and *Tungrovirus* (Chabannes et al., 2013; Harper et al., 1999; Jakowitsch et al., 1999; Ndowora et al., 1999; Saito et al., 2023). Caulimovirus EVEs occurrence was discovered in many vascular monocots, dicots, and even ferns (Chen et al., 2018; de Tomás & Vicient, 2022; Diop et al., 2018; Gong & Han, 2018; Muller et al., 2021; Schmidt et al., 2021; Yu et al., 2019). Most of the integrated EVEs are replication-defective and distributed in varied sizes and numbers among their host genome, ranging from shorter fragments to near-complete virus sequences (Aboughanem-Sabanadzovic et al., 2023). Until recently, such EVEs were not discovered in the cotton genome.

Upland cotton (*Gossypium hirsutum* L.) is extensively cultivated worldwide, primarily for its fiber. However, it is susceptible to a range of pathogens and pests, including plant viruses (geminiviruses, ilarviruses, and poleroviruses) and their insect vectors (whiteflies, thrips, and aphids). In early June of 2023, upland cotton in Georgia, USA experienced both abiotic and biotic challenges. This period was marked by cooler-

than-average summer temperatures alongside above-average infestations of tarnished plant bugs (TPB; *Lygus lineolaris* Palisot de Beauvois) as reported in the UGA extension newsletter, June 2023 (http://www.ugacotton.com/newsletter/). Along with these issues, it was noted that young cotton plants frequently exhibited TA, resulting in the development of profuse vegetative branching. The cause of these symptoms could not be attributed to the other issues occurring in the 2023 growing season. Affected young cotton plants exhibiting TA developed two or more vegetative branches, with major occurrences noted in plants at the 3-leaf stage and a few instances at the 5-6 leaf stage.

Various thrips species serve as vectors for transmitting plant viruses including *Orthotospovirus tomatomaculae* (formally known as tomato spotted wilt virus-TSWV) in crops like peanut, pepper, and tomato (Culbreath et al., 1992; Gitaitis et al., 1998) and tobacco streak virus (TSV) in cotton (Rageshwari et al., 2023). TSV is widespread in cotton-growing countries worldwide (Rageshwari et al., 2023) and has a broad host range (Bag et al., 2019; Daliyamol et al., 2019; Hosseini et al., 2012; McDaniel, 1992; Sharman et al., 2009; Zambrana-Echevarría et al., 2021). While TSV has not been documented in cotton in the USA, its presence has been reported in yellow summer squash (*Cucurbita pepo* L.) in GA, USA (Bag et al., 2019).

Initially, the early infestation of thrips on emerging cotton led to the hypothesis that thrips mediated TSV could be a potential pathogen causing the symptoms observed, including TA. Additionally, another suspected viral pathogen was cotton leafroll dwarf virus (CLRDV), which is prevalent in Georgia and other cotton-growing regions in the USA (Edula et al., 2023). Recent discoveries of endogenous cotton pararetroviral elements (eCPRVE) sequences (Aboughanem-Sabanadzovic et al., 2023) and a novel

cotton virus A (CotVA) (West-Ortiz et al., 2023) in cotton from Mississippi prompted us to investigate the presence of such viruses and viral elements in the field samples in GA. With the advancement of sequencing technologies, high-throughput sequencing (HTS) of small RNA (Kavalappara et al., 2023; Silva et al., 2011) and lncRNA (Aboughanem-Sabanadzovic et al., 2023) is widely used for the detection of known and novel viruses without any prior knowledge (Pecman et al., 2017). Further, this study aims (i) to characterize EVEs within the cotton genome using HTS and to conduct *in-silico* assessment of cotton (*Gossypium* species) genomes for endogenous caulimovirid-like sequences using cotton genomic databases, namely Phytozome (https://phytozome-next.jgi.doe.gov) (Goodstein et al., 2012) and Cottongen (https://www.cottongen.org/) (Yu et al., 2021), (ii) evaluate the presence of their episomal forms and mRNA transcripts of movement protein gene, in addition to investigating for unknown or cryptic viruses in the small RNA sequences (Adeleke et al., 2022; Xin et al., 2017) extracted from field samples.

Materials and Methods

Sample Collection

In early June of 2023, during the vegetative stage of the crop, cotton plants exhibiting terminal abortion (symptomatic) and plants devoid of such symptoms (asymptomatic) (n = 54) were collected from commercial cotton fields in two locations: the Sunbelt Agricultural Expo (n = 28) in Colquitt County and Hopeful (n = 26) in Mitchell County, GA. The tissue sample (petiole, leaf, and tissue near the vegetative branching) from each plant was combined and processed to diagnose the presence of potential viruses impacting cotton at the Virology lab, UGA Tifton, GA. Samples from Colquitt County

were pooled into three subsamples: symptomatic S1 (*n* = 12), S2 (*n* = 12), and asymptomatic S3 (*n* = 4). Similarly, samples from Hopeful were pooled into three subsamples: symptomatic S4 (*n* = 11), S5 (*n* = 11), and asymptomatic S6 (*n* = 4), resulting in six composite samples.

An experimental plot was established at the UGA Bowen research farm in Tifton, GA, to evaluate the incidence of terminal abortion and its potential impact on yield. Two varieties of cotton, 'Dyna-Gro 3615 B3XF' and 'Dyna-Gro H959 B3XF' (DG3615 and DGH959, Loveland Products, Inc., Loveland, CO, USA), were planted in individual plots, which were 16 rows wide and 750 feet in length. The four middle rows were selected for each variety to evaluate the association of EVEs with the terminal abortion observed in the commercial fields, excluding the four border rows to avoid border effects. During the vegetative growth phase for both varieties, DG3615 and DGH959, a symptomatic plant was selected in each row and marked along with an adjacent asymptomatic plant. This was replicated five times in each row, and there were twenty replications for each variety. For DGH959 only, one adjacent asymptomatic plant was additionally selected, and the apical bud was manually terminated to induce terminal abortion. Twenty symptomatic and twenty asymptomatic plants were selected for each variety, and an additional twenty induced terminal abortion plants were selected in DGH959 only. A total of 100 individual plant samples were labeled/tagged and monitored for symptom development and progress. Yield data were hand-harvested from individual plants.

Statistical Analysis

For DG3615, symptomatic and asymptomatic plants are considered two treatments, and a paired student's *t*-test was performed to determine the effect of treatments on the seedcotton yield plant^{-1} and the boll density plant^{-1}. There were three treatments (symptomatic, asymptomatic, and induced terminal abortion plants) in DGH959, and a one-way mixed effects analysis of variance (ANOVA) was used to determine the effects of treatments on the same response variables. Significant effects of treatments were considered at $p < 0.05$. Statistical analysis was performed using JMP Pro version 16 (SAS Institute; Cary, NC, USA).

Seed and Seedling Assessment

Seeds and seedlings from four different commercial cotton varieties, Deltapine 1646 B2XF (DP1646, Bayer Crop Science, St. Louis, MO, USA), Stoneville 4595 B3XF (ST 4595, BASF Corporation, Research Triangle Park, NC, USA), DG 3615 B3XF, and DG H959 B3XF, were evaluated in both laboratory and greenhouse settings. This evaluation was aimed at ascertaining whether the EVE's expression was induced in field conditions due to abiotic factors or was also evident in controlled environments. Ten seeds from each variety were sown directly in soil (Pro-Mix Premier HP, peat-based growing medium) using 6-well trays of 1.5″ square by 2.25″ depth and were maintained inside the insect-free cages (BugDorm, 160 µm aperture, MegaView Science Co., Ltd. Taichung, Taiwan, China) in the greenhouse facility at the University of Georgia, Tifton, GA. The greenhouse was maintained at a temperature of 28 ± 3 °C and 50 ± 20% relative humidity throughout the experiment. Simultaneously, the other set of ten seeds from each variety were kept on water-soaked filter paper in a petri dish and incubated at room temperature

under dark conditions to induce seed germination. All varieties except DP1646 were available pre-treated with fungicide. After 96 h, sprouted seeds and seedlings were divided into three parts for EVEs testing. The seed coat (testa) was separated, and the sprouted seed was divided into the upper shoot with plumule and epicotyl (P + E) and lower root parts, including the hypocotyl, and root (H + R) (Figure 3.1A). Meanwhile, the seedlings grown under greenhouse conditions were collected, and each seedling was separated into three different parts for EVE analysis (cotyledon leaves, an inch of stem including the meristem, and root parts) (Figure 3.1B).

Nucleic Acid Extraction (DNA, RNA, and TNA)

The total RNA was extracted from the six commercial composite field samples from Colquitt and Mitchell County using Spectrum^{TM} Plant Total RNA extraction kit (Sigma–Aldrich, St Louis, MO, USA) following the manufacturer's protocol. The total DNA was extracted from the same set of samples using the DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). Extracted RNA and DNA were used for PCR assays, and the total RNA was used for library preparation and high-throughput sequencing.

Additionally, total nucleic acid (TNA) was extracted using magnetic bead technology following the protocol as described in Adeleke et al. [63] from individual field samples collected from Colquitt (n = 28) and Mitchell (n = 26) counties, leaf petiole samples (n = 100) collected from UGA Bowen farm experimental plot, and seeds (n = 50) and seedlings (n = 50) from the greenhouse and laboratory.

The quality and quantity of the total nucleic acids were determined using a NanoDrop One UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA,

USA). DNA, RNA, and TNA, with 260/280 absorbance of ≥1.8, were aliquoted for further analysis and stored at −80 °C.

Nucleic Acid Treatment

To detect mRNA transcripts of EVEs and to eliminate erroneous detection of the integrated cotton EVE sequences in the plant genomic DNA, we treated four commercial cotton field composite RNA (S2: symptomatic; S3: asymptomatic; S5: symptomatic; and S6: asymptomatic) and TNA extracted samples with DnaseI (Thermofisher, Waltham, Boston, MA, USA) before cDNA preparation. DNase treatment was performed using <1 µg RNA/TNA and incubated at 37 °C for 30 min. The enzymatic reaction was stopped with 0.5M EDTA (pH 8.0) and incubated at 65 °C for an additional ten minutes.

Before conducting the PCR assay, aliquots of four commercial cotton field composite DNA samples were treated with exonuclease V. This enzyme cleaves linear double-stranded DNA in both 5′ and 3′ directions, enabling caulimovirus circular episomal DNA detection [30]. Similarly, TNA from the seeds of two varieties, DG3615 (n = 10) and ST4595 (n = 10), was treated with exonuclease V to detect the presence of episomal forms of DNA. This treatment was crucial to distinguishing the target episomal DNA from integrated EVE sequences in *G. hirsutum* species. In this treatment, DNA < 1 µg was mixed with Exonuclease V (RecBCD) (NEB, Ipswich, MA, USA) along with ATP and buffers provided by the manufacturer and incubated at 37 °C for 30 min for linear DNA digestion. Later, 0.5 M EDTA (pH 8.0) was added and incubated at 37 °C for an additional 30 min to stop the enzymatic reaction. Linearized DNA digestion of the commercial cotton field samples (S2, S3, S5, S6) was confirmed by analyzing in 0.8%

horizontal agarose gel electrophoresis along with untreated DNA and visualized using the gel documentation system (Analytik Jena UVP UVsolo Touch, Upland, CA, USA).

Virus Detection

cDNA was synthesized using superscript III (Invitrogen) using reverse primers for CLRDV capsid protein and P0 gene, TSV capsid, and movement protein gene, followed by PCR using the gene-specific primer pairs (Table 3.1). An end-point PCR assay was performed targeting the movement protein gene using a primer pair caulimo movement protein primer pair (Caulimo MP-F & Caulimo MP-R) (Table 3.1) on Exonuclease V-treated DNA samples to detect the presence of episomal DNA. A DNase-RT-PCR assay was performed on the cDNA of the same samples to detect the mRNA transcripts of EVEs. All samples (n = 100) collected from the experimental plot were screened for CLRDV-targeting partial capsid protein gene primers in RT-qPCR and for caulimovirus movement protein genes in DNase-RT-PCR using the caulimo movement protein primer pair (Table 3.1).

High-Throughput Sequencing and Analysis

RNA from four commercial field samples S2-symptomatic, S3-asymptomatic, S4-symptomatic, S5-symptomatic was sent to Novogene, Sacramento, CA, USA, for ribodepletion, cDNA library preparation for single-end reads sRNA (1x50bp) and paired-end reads (2 X 150bp) sequencing of long non-coding RNA using the Illumina platform.

Small RNA (sRNA's) sequence analysis was carried out using CLC Genomics Workbench (V.23.0.4) (Qiagen, Redwood City, CA, USA). Sequence reads were *de novo* assembled to create contigs using default parameters. These generated contigs were aligned against the suspected viral sequences using NCBI BLAST. A local virus

nucleotide database and a phytoplasma nucleotide database were downloaded on 05th July 2023 from the National Center for Biotechnology Information (NCBI) using the Create Database feature of the CLC Genomics Workbench 23. These contigs were further compared for similarity using the BLASTn (Altschul et al., 1990) tool against all sequences in the database with default parameters set in the CLC Genomics Workbench 23. Sequence reads were mapped with the individual reference sequences of the suspected viruses like CLRDV (NC_014545.1), TSV (KP256522.1), eCPRVE (OR269951) and CotV-A (OR184923). Similarly, in long non-coding RNAs (lncRNA), sequences were trimmed for the adapter and low-quality sequence reads and mapped against CLRDV (NC_014545.1), TSV (KP256522.1), CotV-A (OR184923) and eCPRVE (OR269951) sequences.

Near-complete caulimovirid-like consensus sequences obtained from field samples were compared with the available sequences in NCBI of CotV-A, eCPRVE, and other members of the family *Caulimoviridae*. Multiple sequence alignments were performed using a maximum likelihood algorithm using multiple sequence alignment software, MEGA 11(Tamura et al., 2021). Among the four EVE sequences obtained, sample PP943202 was used as a reference to mine the plant database, Phytozome (https://phytozome-next.jgi.doe.gov/), a Plant Comparative Genomics portal of the Department of Energy's Joint Genome Institute that consists of updated sequenced genomes of cotton species. The query sequence was compared against the available cotton genome database using the BLAST (BLASTN 2.11.0+) option against the following *Gossypium* species: *G.raimondii*; *G.hirsutum*; *G.mustelinum*_v1_1; *G.tomentosum*_v1_1; *G.barbadense*_v1.1; *G.hirsutum*_v2.1; *G.darwinii*_v1.1;

*G.hirsutum*UGA230; *G.hirsutum*UA48; *G.hirsutum*CSX8308; *G.hirsutum*; *G.hirsutum*DeltaPearl; *G.hirsutum*FM958; *G.hirsutum* Coker genome. In addition, the query sequence was also compared with G. stephensii (AD7) 'AD701', a genome sequence available in the Cottongen database (https://www.cottongen.org) that was not reported earlier.

Results

Symptomatology

During the growing season of 2023, cotton seedlings (2–4 leaf stage) exhibited terminal abortion (Figure 3.2A,B), leading to profuse vegetative branching (Figure 3.2C–E). Samples were collected in two commercial fields and the experimental plot from the plants exhibiting deformed leaf lamina, longer petioles, and stunted plants exhibiting profuse vegetative branching due to terminal abortion (Figure 3.2D,E) and from the asymptomatic plants devoid of such symptoms (Figure 3.2F).

Virus Detection

PCR analysis of commercial field samples (S2: symptomatic; S3: asymptomatic; S5: symptomatic; and S6: asymptomatic) using primer pairs specific for CLRDV and TSV did not amplify any target genes (Figures S1 and S2). The total DNA extracted from samples (Figure 3.3A) was treated with exonuclease (Figure 3.3B) and analyzed in horizontal gel electrophoresis. The treated DNA was further subjected to PCR amplification using the caulimovirus movement protein gene, resulting in an amplicon size of approximately 470 bp from Mitchell County (S5: symptomatic; S6: asymptomatic) (Figure 3.3C) but not in the samples from Colquitt County (S2: symptomatic; S3: asymptomatic). However, a similar amplicon was obtained from both

locations except S3 targeting the caulimovirus movement protein gene in DNase-treated RNA RT-PCR (Figure 3.3C,D), suggesting non-detectable titers of episomal forms along with low RNA transcripts in S2 but none in S3. CLRDV was detected only in two samples (one asymptomatic and the other symptomatic for terminal abortion) among the total collected samples (n = 100) tested from the experimental plot. In the same plot, a total of n = 96 samples, 93% (37/40) of the symptomatic, 98% (39/40) of the asymptomatic, and 95% (19/20) induced terminal abortion samples were positive for EVE detection. Amplicons were further gel-purified, and the sequence was confirmed through Sanger sequencing, matching 98–100% with eCPRVE (OR269951) and CotV-A (OR184923) partial movement protein gene sequence.

Small and Long Non-Coding RNA Analysis

Four cDNA libraries were prepared and sequenced from four commercial field samples (S2: symptomatic; S3: asymptomatic; S4: symptomatic; and S5: symptomatic). Small RNA reads between 19 and 26 million and long non-coding RNA reads between 57 and 83 million were generated from the samples (Table 3.2). Mapped small RNA contigs (18–40 bp) were nonspecific and showed no significant matching to the sequences of CLRDV (NC_014545.1), TSV (KP256522.1), eCPRVE (OR269951), and CotV-A (OR184923) (Table 3.2).

When the paired sequence reads (2 × 150 bp) of long non-coding RNA (lncRNA) of samples (S2: PP943202; S3: PP943203; S4: PP943204; and S5: PP943205) were mapped with near-complete sequences of CotV-A (OR184923) and eCPRVE (OR269951), we obtained 99–100% read coverage (Table 3.2A). Read maps were graphically visualized by creating read tracks showing maximum, minimum, and average

coverage values (Figure 3.4A,B). Among the 80 million clean reads of S2–S5, 0.02–0.06% were matched with eCPRVE (OR269951) and 0.04–0.09% with CotV-A (OR184923) sequences (Table 3.2A). However, the lncRNA sequences did not match the reference sequences of CLRDV (NC_014545.1) and TSV (KP256522.1) (Table 3.2A). The genome organization was represented after comparing the EVE-Georgia (EVE-GA) contigs (PP943202, PP943203, PP943204, and PP943205) with eCPRVE (OR269951) and CotV-A (OR184923). Even though near-complete EVE-GA sequences are 99–100% identical with both eCPRVE and CotV-A (Table 3.2B), ORF-wise EVE-GA sequences are more similar (99–100%) to the eCPRVE sequence with interruptions due to stopping codons in open reading frames (ORFs) that code for putative viral coat proteins and reverse transcriptase (Figure 3.4C).

Validation of HTS Results

In commercial field samples (n = 54) testing, the caulimovirus movement protein gene was detected in both symptomatic and asymptomatic plants from both locations. The presence of the movement protein gene was confirmed in 13 of the 24 symptomatic samples in Colquitt and 15 of the 22 symptomatic samples from Mitchell County. In contrast, it was detected in all the asymptomatic plants in both locations. (Table 3.3).

BLAST, Phylogenetic Analysis, and In Silico Mining

Consensus sequences from the lncRNA sequence of the symptomatic samples collected from growers' fields did not exhibit any matches with CLRDV (NC_014545.1) and Ilarviruses, TSV (KP256522.1) when analyzed with CLC workbench. The sequence matched (98%) with CotV-A and eCPRVE sequences when mapped against the sequences available with NCBI GenBank. In the phylogenetic analysis, the nucleotide

sequences of the near complete sequence from commercial field isolates from GA were 90-98% identical with eCPRVE sequences (OR269936 to OR269951) and DNA virus CotV-A (OR184923) reported earlier from Mississippi, USA. Further, the EVE sequences from GA are 88 % identical to caulimovirus members like plant-associated caulimovirus (OL472131) and grapevine para retrovirus (OP886324). These sequences form a distinct clade from the members of the Family *Caulimoviridae* (Figure 3.5). In our data mining using EVE query (PP943202) sequence obtained from HTS, we found near identical integrated sequences in tetraploid species of *G.hirsutum* cultivars having triplets of high-scoring segment pair (HSPs) of EVEs in A04 chromosome (+/-) with ~7 kb, ~6 kb, and 394bp length showing 97-100% identity (Supplementary Table S1). In addition, we have also observed the integrated near identical EVE sequences in other *G. hirsutum* chromosomes with various matching lengths and percentage identity (Chromosome-D03 (+/+): ~4 kb with 84% identity, A05 (+/+): ~3 kb with 73% identity, D07 (+/−): 1059 bp with 80% identity, A13 (+/−): 1254 bp with 76% identity).

Other than *G. hirsutum* species, we also observed EVE sequences in *G. stephensii* chromosomes A04 with two HSPs in + strands, one with ~7 kb, and the other ~6.2 kb with 99% identity, D03 (+/+): ~3.6 kb with 84% identity, D07 (+/+): ~1 kb with 80% identity, *G. barbadense*, D04 chromosome (+/+): ~1 kb, *G. tomentosum* in A03 (+/+) ~5.3 kb with 79% identity, D04, A13 chromosomes, and *G. mustelinum* in D07 (+/+): ~5.1 kb with 70% identity, and D05, A07 chromosome consisting various lengths of EVE sequence. In the in-silico mining of the cotton genome database, the sequences obtained via HTS data appear to be from a negative-sense copy of EVEs, as described in Aboughanem-Sabanadzovic et al. (2023).

Cotton Yield Components

In DG3615, the yield components, including seedcotton yield and boll density, demonstrated no significant difference between symptomatic and asymptomatic plants (Table 3.4). Similarly, in DGH959, seedcotton yield and boll density demonstrated no significant difference when compared between different treatments (symptomatic, asymptomatic, and induced terminal abortion) (Table 3.4). These results suggest that the terminal abortion symptom observed in the growing season of 2023 did not result in a yield reduction with respect to the varieties tested.

Seed and Seedling Assay

The Caulimovirus movement protein gene was detected in different tissues of the DG3615, DGH959, DP1646, and ST4595 varieties in both germinated seed and seedling stages. In seed and seedling testing, 95% of seeds (38/40) and 75% (30/40) of seedlings were positive for EVE detection (Table 3.3). We detected the caulimovirus movement protein gene in a higher percentage, 85% (34/40) in plumule and epicotyl, followed by 65% (26/40) in seed coat, and 53% (21/40) in hypocotyl and root tissue. However, in the seedling analysis, cotyledon leaves were 65% (26/40), stem tissues were 63% (25/40), and root tissues were 58% (23/40) positive for caulimovirus movement protein gene detection. Among the assessed varieties, DG3615 and DGH959 had comparatively higher percentages (66%) of positives for EVE detection in both seeds and seedlings compared to ST4595 and DP1646 (31%). In the exonuclease V-treated samples of DG3615 and ST4595 (n = 20), 100% (10/10) of samples were positive for the caulimovirus movement protein gene detection in cotyledon leaves.

Discussion

In this study, we evaluated the terminal abortion-symptomatic plants that appeared sporadically in the growing season of 2023 in Tift, Mitchell, and Colquitt County, GA. Concurrently, the identification of EVEs in cotton (Aboughanem-Sabanadzovic et al., 2023; West-Ortiz et al., 2023) solicited the question of whether these elements could play a role in terminal abortion leading to profuse vegetative branching. To enhance our understanding of EVE presence in Georgia-grown cotton, we investigated and detected near-complete sequences (~7.4 kb) in both symptomatic (terminal abortion) and asymptomatic samples. Typically, the terminal abortion of cotton arises from abiotic factors such as wind and hail damage. On occasion, it can also be triggered by biotic elements, including insect feeding, such as tarnished plant bugs commonly found in weed hosts like Palmer amaranth (*Amaranthus palmeri* S. Watson) (Randell et al., 2021). Additionally, sucking pests like thrips (*Frankliniella fusca* Hinds) can induce terminal abortion by feeding on slow-growing cotton seedlings at cold temperatures (Camp Hand, 2023; Shirley, 2023). However, our observations in the fields and thrips infestation predictors indicated that the population of thrips was significantly low in the fields during the early weeks of June 2023 (Kennedy, 2017; Shirley, 2023), potentially due to recurrent rainfalls and routine prophylactic management practices (Cook et al., 2011; Roberts, 2023) implemented at the onset of each crop season.

Based on previous studies, there are instances where replication-competent EVEs are induced by various factors such as genome hybridization, tissue culture, abiotic stress, and wounding. This occurrence has been discovered in hosts like banana (endogenous banana streak viruses, eBSVs) (Harper et al., 1999; Ndowora et al., 1999), tobacco

(endogenous tobacco vein-clearing virus, eTVCV) (Lockhart et al., 2000) and petunia sps. (endogenous petunia vein-clearing virus, ePVCV) (Harper et al., 2003; Richert-Pöggeler et al., 2003; Richert-Pöggeler & Shepherd, 1997). In some cases, EVEs are incapable of autonomous replication due to deficiencies in structural domains, but sometimes their replication is supported by co-infecting viruses from the same family (Eid & Pappu, 2014). Thus, the samples were tested for the presence of viruses, including CLRDV, TSV, or EVEs (CotV-A and eCPRVE), which were suspected to be the potential causal agents of terminal abortion. The absence of suspected viruses like CLRDV and TSV in the RT-PCR assays of samples collected from commercial cotton fields suggests their non-involvement during terminal abortion. The absence of CLRDV in the UGA-Bowen farm experimental field, except for one symptomatic (terminal abortion) and one asymptomatic sample, indicates that CLRDV was not prevalent and unlikely to be a causal agent for terminal abortion. Further, in the Bowen farm experimental plot, the EVE detection rate was over 90% across the treatments (symptomatic, asymptomatic, and induced terminal abortion), despite showing differences among them. This supports the hypothesis that EVEs are unlikely to be the causal agent for terminal abortion.

To comprehend the functional status of recently identified cotton EVEs, we analyzed their episomal forms and mRNA transcripts using the protocol of caulimo movement protein gene primers for CotV-A, as described by Ortiz et al. (2023). Our analysis revealed the presence of EVEs in the episomal forms in Mitchell County samples but not in Colquitt County. Messenger RNA transcripts of the caulimo movement protein gene were detected in both symptomatic and asymptomatic samples

from Mitchell County but only in symptomatic samples from Colquitt County, and non-detectable in the asymptomatic sample from Colquitt County. These results indicate that the formation of episomal DNA from host-integrated sequences may be inconsistent and/or likely a redundant expression and cannot be directly linked to the cause of terminal abortion. As investigated by Squires et al. (2011), the episomal DNA expression of the cauliflower mosaic virus (CaMV) infectious clone in Arabidopsis was not temperature dependent. Similarly, our results corroborate the detection of EVEs' episomal DNA in both symptomatic and asymptomatic field samples exposed to environmental stress (temperature differences) and seeds independent of environmental stress.

In HTS analysis of sRNA and lncRNA sequences, CLRDV and TSV were not detected. However, near-complete, caulimovirid-like sequences were detected for eCPRVE and CotV-A in lncRNAs but not in sRNAs. Commonly, lncRNAs are moderately abundant fractions of eukaryotic transcriptomes (>200 nt) that are lacking coding capacity but are involved in plant gene regulation, and some act as positive or negative regulators of plant immunity (Mattick et al., 2023; Wang & Folimonova, 2023). In comparison, sRNAs are microRNAs (18–40 nt), usually non-coding, and involved in antiviral immunity by guiding argonaut proteins to target viral RNA cleavage (Kavalappara et al., 2023). HTS assays are widely used for the comprehensive assessment of pathogen profiles. They play a crucial role in the discovery of emerging, reemerging, and mixed viral infections in both cultivated crops and wild plant species (González-Pérez et al., 2024; Nizamani et al., 2023). Many plants respond to exogenous virus infections via transcriptional (TGS) and post-transcriptional gene silencing (PTGS),

with PTGS occurring in the cytoplasm and targeting dsRNA intermediates. Although DNA viruses replicate in the nucleus, mRNAs are transported to the cytoplasm for translation or for reverse transcription (pararetorviruses), which makes them potential PTGS targets (Ghoshal & Sanfaçon, 2015). Despite this, the absence of such sRNAs of EVE sequences related to eCPRVEs, CotV-A, or any persistent viruses (Adeleke et al., 2022; Xin et al., 2017) in high-throughput-based sRNA sequences disproves their involvement in terminal abortion. The nonexistence of viral sRNA sequences in the symptomatic and asymptomatic samples, even in the more sensitive HTS, confirms the absence of an active host defense response against the viruses. This inactivity could be attributed to EVE sequences losing infectivity due to various aspects such as insertions and deletions (indels), mutations, and fragmentation during host genome replication (Aboughanem-Sabanadzovic et al., 2023).

The detection of near full-length EVE sequences in the cotton samples (symptomatic and asymptomatic) collected from growers' fields in Colquitt and Mitchell counties in GA implies its presence in the varieties of *Gossypium hirsutum* L. genetic background (Aboughanem-Sabanadzovic et al., 2023). Moreover, the sequences we obtained consisted of multiple ORFs similar to those submitted by Aboughanem-Sabanadzovic et al. (2023), which are capable of encoding various proteins. However, the sequences from GA had stop codons in the open reading frames 5 and 6 (ORFs) coding for putative viral coat protein and reverse transcriptases similar to eCPRVEs as described in Aboughanem-Sabanadzovic et al. (2023), making them non-functional. Usually, in caulimovirus, mRNA is polycistronic and translated via ribosomal reinitiation (Schepetilnikov & Ryabova, 2014). Interestingly, no such interruptions due to stop codons were observed in

the CotV-A sequence, which requires further evaluation to confirm the functional status of these ORFs (West-Ortiz et al., 2023).

In silico mining of the EVE sequences in the cotton genome database strongly manifests the presence of endogenous viral sequences in various species of *Gossypium* L., which is not limited to the hirsutum species prominent in North and South Americas but also to the species present in Australia (*G.hirsutum* CSX8308). Caulimovirus-like near-complete EVE sequences were also found in *G. stephensii* A04 and D03 chromosomes, showing similar matches to other hirsutum species. This supports the hypothesis of integration predating the speciation events of *G. hirsutum*, estimated at 0.75 mya, as speculated in Aboughanem-Sabanadzovic et al. (2023). However, EVE integrations of approximately ~5 kb with 79% identity were found in Chr A03 in *G. tomentosum* and about ~5 kb with 70% identity in Chr D07 in *G. mustelinum*. These integrations were also present in two other species of tetraploids (*G.barbadense* and *G. darwinii*) in very minimal length ~1 kb sequences with 70% identity (Table S1). This shows a high degree of sequence degradation, raising a query about whether the integration event was much earlier (1.80 mya) (Figure 3.6) than the hirsutum speciation event in the tetraploid "AADD" ancestors or a recent (0.75 mya) multiple independent integrations that needs further investigation. Identifying active mRNA transcripts of ORF3 (movement protein) (Aboughanem-Sabanadzovic et al., 2023) in EVEs only partially evaluates the entire polycistronic mRNA. A deeper insight into the genomic annotations and functions of other ORFs can significantly enhance our understanding of EVEs in cotton. However, additional investigation is essential to ascertain if episomal DNAs are involved in virion formation and infectivity. Although eCPRVE sequences

were discovered in CLRDV-infected cotton plants (Aboughanem-Sabanadzovic et al., 2023), any correlation (synergistic or antagonistic) between these two viruses has not been established yet. Such clarification on their synergism or antagonism is vital in understanding their role in infection and symptom development within the host plant.

In a study of *Dahlia variabilis* endogenous pararetrovirus sequence (DvEPRS), integrated into the host dahlia (*D. variabilis*) genome, it was detected in various tissues, including leaves, roots, seeds, flower petals, and pollen, and was capable of 100% seed transmission (Eid & Pappu, 2014; Pahalawatta et al., 2008; Pahalawatta et al., 2007). Similarly, our results indicate the presence of EVEs in seeds and seedlings, expressed as episomal forms and mRNA transcripts, although their presence in other tissues was not tested. However, the transmission of DvEPRS by mechanical inoculation and through aphids (*Myzus persicae*) was unsuccessful (Eid & Pappu, 2014). Further research is imperative to understand such prospects with EVEs in cotton.

To address the concern about the emerging issue of terminal abortion resulting in profuse vegetative branching, yield impact was assessed in two varieties (DG3615 and DGH959) at an experimental plot at the UGA Bowen research farm in Tifton, GA. The results showed no significant difference in the yield response variables: seedcotton yield (DG3615: $p = 0.4139$, DGH959: $p = 0.8866$) and boll density (DG3615: $p = 0.4933$, DGH959: $p = 0.7028$) between the treatments of two varieties. These findings imply that the terminal abortion leading to profuse vegetative branching observed in the growing season of 2023 did not adversely affect the yield in the varieties tested, which further supports the speculation that terminal abortions may not be concerning at present, and the plants are likely to recover to sustain yields. Despite the inconclusive findings of the

exact causal agent for terminal abortion, it is worthwhile to explore the cotton genotype response to climate change and increasing temperatures. Consequently, our study provides valuable insights for cotton growers and researchers into the significance of caulimovirid-like EVEs in the cotton genome, paving the way for future research on EVEs to assess their activity and involvement in host interactions. In some cases, putative EVEs may confer host resistance to associated viral infections (Aswad & Katzourakis, 2012; Bertsch et al., 2009; Mette et al., 2002; Valli et al., 2023). The discovery of EVE sequences in lncRNAs, but not in small RNAs, prompts the intriguing question of whether they have a role in conferring host immunity, which will be an interesting aspect that needs to be substantiated in future research.

Conclusions

During the 2023 growing season in Georgia, USA, the intermittent appearance of terminal abortion in young cotton plants with no apparent cause raised concerns among industry and academic scientists alike. Therefore, samples were evaluated for multiple aspects to address these concerns. The impact of terminal abortion on cotton yield was mainly assessed, and there was no significant difference between symptomatic and asymptomatic plants. The association between tarnished plant bugs and thrips was also non-significant at the time of symptom appearance. This study further evaluated the presence of EVEs in the cotton genome, finding no correlation between their presence and the occurrence of terminal abortion. Further research and evaluation of cotton EVEs is needed to understand their true functionality and role in pathogenicity or immunity.

Author Contributions

Conceptualization, S.R.E., L.C.H., P.M.R. and S.B.; methodology, S.R.E. and L.C.H.; software, S.R.E.; validation, S.R.E., L.C.H., P.M.R. and S.B.; formal analysis, S.R.E.; investigation, S.R.E., L.C.H., P.M.R., E.B. and S.B.; resources, P.M.R. and S.B.; data curation, S.R.E., L.C.H., P.M.R. and S.B.; writing—original draft preparation, S.R.E. and S.B.; writing—review and editing, S.R.E., L.C.H., P.M.R. and S.B.; supervision, L.C.H., P.M.R., J.L.S., R.C.K., P.W.C. and S.B.; project administration, S.B.; funding acquisition, S.B., L.C.H. and P.M.R. All authors have read and agreed to the published version of the manuscript.

References

Aboughanem-Sabanadzovic, N., Allen, T. W., Frelichowski, J., Scheffler, J., & Sabanadzovic, S. (2023). Discovery and Analyses of Caulimovirid-like Sequences in Upland Cotton (*Gossypium hirsutum*). *Viruses, 15*(8), 1643.

Adeleke, I. A., Kavalappara, S. R., McGregor, C., Srinivasan, R., & Bag, S. (2022). Persistent, and Asymptomatic Viral Infections and Whitefly-Transmitted Viruses Impacting Cantaloupe and Watermelon in Georgia, USA. *Viruses, 14*(6), 1310.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology, 215*(3), 403-410.

Andrake, M. D., & Skalka, A. M. (2015). Retroviral Integrase: Then and Now. *Annual Review of Virology, 2*(1), 241-264.

Ashby, M. K., Warry, A., Bejarano, E. R., Khashoggi, A., Burrell, M., & Lichtenstein, C. P. (1997). Analysis of multiple copies of geminiviral DNA in the genome of four closely related *Nicotiana* species suggest a unique integration event. *Plant Molecular Biology, 35*(3), 313-321.

Aswad, A., & Katzourakis, A. (2012). Paleovirology and virally derived immunity. *Trends in Ecology & Evolution, 27*(11), 627-636

Bag, S., Tabassum, A., Brock, J., & Dutta, B. (2019). First report of Tobacco streak virus infecting summer squash in Georgia, U.S.A. *Plant Disease, 103*(6), 1442.

Bejarano, E. R., Khashoggi, A., Witty, M., & Lichtenstein, C. (1996). Integration of multiple repeats of geminiviral DNA into the nuclear genome of tobacco during evolution. *Proceedings of the National Academy of Sciences, 93*(2), 759-764.

Bellas, C., Hackl, T., Plakolb, M.-S., Koslová, A., Fischer, M. G., & Sommaruga, R. (2023). Large-scale invasion of unicellular eukaryotic genomes by integrating DNA viruses. *Proceedings of the National Academy of Sciences, 120*(16), e2300465120.

Belyi, V. A., Levine, A. J., & Skalka, A. M. (2010). Sequences from ancestral single-stranded DNA viruses in vertebrate genomes: The Parvoviridae and Circoviridae are more than 40 to 50 million years old. *Journal of Virology, 84*(23), 12458-12462.

Bertsch, C., Beuve, M., Dolja, V. V., Wirth, M., Pelsy, F., Herrbach, E., & Lemaire, O. (2009). Retention of the virus-derived sequences in the nuclear genome of grapevine as a potential pathway to virus resistance. *Biology Direct, 4*, 21.

Camp Hand, G. H., Kemerait, B., Liu, Y., Perry, C., Hall, D., Mallard, J., Porter, W., Roberts, P., Smith, A., Virk, S., Bag, S., & Sintim, H. (2023). *Georgia cotton production guide 2023*. University of Georgia Extension.

Chabannes, M., Baurens, F. C., Duroy, P. O., Bocs, S., Vernerey, M. S., Rodier-Goud, M., Barbe, V., Gayral, P., & Iskra-Caruana, M. L. (2013). Three infectious viral species lying in wait in the banana genome. *Journal of Virology, 87*(15), 8624-8637.

Chen, S., Saito, N., Encabo, J. R., Yamada, K., Choi, I. R., & Kishima, Y. (2018). Ancient endogenous pararetroviruses in *Oryza* genomes provide insights into the heterogeneity of viral gene macroevolution. *Genome Biology and Evolution, 10*(10), 2686-2696

Cook, D., Herbert, A., Akin, D. S., & Reed, J. (2011). Biology, crop injury, and management of thrips (Thysanoptera: Thripidae) infesting cotton seedlings in the United States. *Journal of Integrated Pest Management, 2*(2), B1-B9.

Cui, J., Zhao, W., Huang, Z., Jarvis, E. D., Gilbert, M. T. P., Walker, P. J., Holmes, E. C., & Zhang, G. (2014). Low frequency of paleoviral infiltration across the avian phylogeny. *Genome Biology, 15*(12), 539.

Culbreath, A. K., Todd, J. W., & Demski, J. W. (1992). Productivity of Florunner peanut infected with tomato spotted wilt virus. *Peanut Science, 19*(1), 11-14.

Daliyamol, J., Jailani, A. A. K., Vemana, K., Roy, A., Krishnareddy, M., Kobayashi, K., & Mandal, B. (2019). Complete genome sequence and phylogenetic relationships of tobacco streak virus causing groundnut stem necrosis disease in India. *Virus Disease, 30*(2), 227-236.

de Tomás, C., & Vicient, C. M. (2022). Genome-wide identification of reverse transcriptase domains of recently inserted endogenous plant pararetrovirus (Caulimoviridae). *Frontiers in Plant Science, 13*, 1011565.

Diop, S. I., Geering, A. D. W., Alfama-Depauw, F., Loaec, M., Teycheney, P.-Y., & Maumus, F. (2018). Tracheophyte genomes keep track of the deep evolution of the Caulimoviridae. *Scientific Reports, 8*(1), 572.

Edula, S. R., Bag, S., Milner, H., Kumar, M., Suassuna, N. D., Chee, P. W., Kemerait, R. C., Hand, L. C., Snider, J. L., Srinivasan, R., & Roberts, P. M. (2023). Cotton leafroll dwarf disease: An enigmatic viral disease in cotton. *Molecular Plant Pathology, 24*(6), 513-526.

Eid, S., & Pappu, H. R. (2014). Expression of endogenous para-retroviral genes and molecular analysis of the integration events in its plant host *Dahlia variabilis*. *Virus Genes, 48*(1), 153-159.

Flynn, P. J., & Moreau, C. S. (2019). Assessing the diversity of endogenous viruses throughout ant genomes. *Frontiers in Microbiology, 10*, 1139.

Gayral, P., & Iskra-Caruana, M.-L. (2009). Phylogeny of banana streak virus reveals recent and repetitive endogenization in the genome of its banana host (*Musa sp.*). *Journal of Molecular Evolution, 69*(1), 65-80.

Geering, A. D. W., Maumus, F., Copetti, D., Choisne, N., Zwickl, D. J., Zytnicki, M., McTaggart, A. R., Scalabrin, S., Vezzulli, S., Wing, R. A., Quesneville, H., & Teycheney, P.-Y. (2014). Endogenous florendoviruses are major components of plant genomes and hallmarks of virus evolution. *Nature Communications, 5*(1), 5269.

Ghoshal, B., & Sanfaçon, H. (2015). Symptom recovery in virus-infected plants: Revisiting the role of RNA silencing mechanisms. *Virology, 479-480*, 167-179.

Gitaitis, R. D., Dowler, C. C., & Chalfant, R. B. (1998). Epidemiology of tomato spotted wilt in pepper and tomato in southern Georgia. *Plant Disease, 82*(7), 752-756.

Gong, Z., & Han, G. Z. (2018). Euphyllophyte paleoviruses illuminate hidden diversity and macroevolutionary mode of Caulimoviridae. *Journal of Virology, 92*(10).

González-Pérez, E., Chiquito-Almanza, E., Villalobos-Reyes, S., Canul-Ku, J., & Anaya-López, J. L. (2024). Diagnosis and characterization of plant viruses using HTS to support virus management and tomato breeding. *Viruses, 16*(6), 888.

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., & Rokhsar, D. S. (2012). Phytozome: A comparative platform for green plant genomics. *Nucleic Acids Research, 40*(Database issue), D1178-D1186.

Harper, G., Osuji, J. O., Heslop-Harrison, J. S., & Hull, R. (1999). Integration of Banana Streak Badnavirus into the Musa genome: Molecular and cytogenetic evidence. *Virology, 255*(2), 207–213.

Harper, G., Richert-Pöggeler, K. R., Hohn, T., & Hull, R. (2003). Detection of Petunia vein-clearing virus: Model for the detection of DNA viruses in plants with homologous endogenous pararetrovirus sequences. *Journal of Virological Methods, 107*(2), 177–184.

Holmes, E. C. (2011). The evolution of endogenous viral elements. *Cell Host & Microbe, 10*(4), 368–377.

Hosseini, S., Habibi, M. K., Mosahebi, G., Motamedi, M., & Winter, S. (2012). First report on the occurrence of tobacco streak virus in sunflower in Iran. *Plant Pathology Journal, 94*(3), 585–589.

Huang, H.-J., Li, Y.-Y., Ye, Z.-X., Li, L.-L., Hu, Q.-L., He, Y.-J., Qi, Y.-H., Zhang, Y., Li, T., Lu, G., Mao, Q.-Z., Zhuo, J.-C., Lu, J.-B., Xu, Z.-T., Sun, Z.-T., Yan, F., Chen, J.-P., Zhang, C.-X., & Li, J.-M. (2023). Co-option of a non-retroviral endogenous viral element in planthoppers. *Nature Communications, 14*(1), 7264.

Hull, R. (2014). *Profiles of families and genera of plant viruses*. In *Plant virology* (5th ed., p. 981). Elsevier.

Jakowitsch, J., Mette, M. F., van der Winden, J., Matzke, M. A., & Matzke, A. J. M. (1999). Integrated pararetroviral sequences define a unique class of dispersed repetitive DNA in plants. *Proceedings of the National Academy of Sciences, 96*(23), 13241–13246.

Katoh, H., & Honda, T. (2023). Roles of human endogenous retroviruses and endogenous virus-like elements in cancer development and innate immunity. *Biomolecules, 13*(12), 1706.

Katzourakis, A., & Gifford, R. J. (2010). Endogenous viral elements in animal genomes. *PLoS Genetics, 6*(11), e1001191.

Kavalappara, S. R., Bag, S., Luckew, A., & McGregor, C. E. (2023). Small RNA profiling of Cucurbit yellow stunting disorder virus from susceptible and tolerant squash (*Cucurbita pepo*) lines. *Viruses, 15*(3), 788.

Kennedy, G. G. (2017). Thrips infestation predictor for cotton: An online tool for informed thrips management. *APS*. Retrieved June 19 from https://www.planthealthexchange.org/cotton/Pages/GROW-COT-04-17-103.aspx

Koslová, A., Hackl, T., Bade, F., Sanchez Kasikovic, A., Barenhoff, K., Schimm, F., Mersdorf, U., & Fischer, M. G. (2024). Endogenous virophages are active and mitigate giant virus infection in the marine protist *Cafeteria burkhardae*. *Proceedings of the National Academy of Sciences, 121*(11), e2314606121.

Krupovic, M., Blomberg, J., Coffin, J. M., Dasgupta, I., Fan, H., Geering, A. D., Gifford, R., Harrach, B., Hull, R., & Johnson, W. (2018). Ortervirales: New virus order unifying five families of reverse-transcribing viruses. *Virology Journal, 92*(12), 00515-18.

Liu, S., Coates, B. S., & Bonning, B. C. (2020). Endogenous viral elements integrated into the genome of the soybean aphid, *Aphis glycines*. *Insect Biochemistry and Molecular Biology, 123*, 103405.

Lockhart, B. E., Menke, J., Dahal, G., & Olszewski, N. E. (2000). Characterization and genomic analysis of tobacco vein clearing virus, a plant pararetrovirus that is transmitted vertically and related to sequences integrated in the host genome. *Journal of General Virology, 81*(Pt 6), 1579-1585.

Mahmood, M. A., Ahmed, N., Hussain, A., Naqvi, R. Z., Amin, I., & Mansoor, S. (2024). Dominance of Cotton leaf curl Multan virus-Rajasthan strain associated with third epidemic of cotton leaf curl disease in Pakistan. *Scientific Reports, 14*(1), 13532.

Mason, A. S., Lund, A. R., Hocking, P. M., Fulton, J. E., & Burt, D. W. (2020). Identification and characterisation of endogenous avian leukosis virus subgroup E (ALVE) insertions in chicken whole genome sequencing data. *Mobile DNA, 11*(1), 22.

Mattick, J. S., Amaral, P. P., Carninci, P., Carpenter, S., Chang, H. Y., Chen, L.-L., Chen, R., Dean, C., Dinger, M. E., Fitzgerald, K. A., Gingeras, T. R., Guttman, M., Hirose, T., Huarte, M., Johnson, R., Kanduri, C., Kapranov, P., Lawrence, J. B., Lee, J. T., Mendell, J. T., Mercer, T. R., Moore, K. J., Nakagawa, S., Rinn, J. L., Spector, D. L., Ulitsky, I., Wan, Y., Wilusz, J. E., & Wu, M. (2023). Long non-coding RNAs: Definitions, functions, challenges and recommendations. *Nature Reviews Molecular Cell Biology, 24*(6), 430-447.

McDaniel, L. L., Raid, R. N., Elliott, C. L., Tsai, J. H., & Nagata, R. T. (1992). Purification and serological characterization of a tobacco streak virus isolate infecting field-grown escarole and lettuce. *Plant Disease, 76*(9), 966-971.

Mette, M. F., Kanno, T., Aufsatz, W., Jakowitsch, J., van der Winden, J., Matzke, M. A., & Matzke, A. J. (2002). Endogenous viral sequences and their potential contribution to heritable virus resistance in plants. *EMBO Journal, 21*(3), 461-469.

Muller, E., Ullah, I., Dunwell, J. M., Daymond, A. J., Richardson, M., Allainguillaume, J., & Wetten, A. (2021). Identification and distribution of novel badnaviral sequences integrated in the genome of cacao (*Theobroma cacao*). *Scientific Reports, 11*(1), 8270

Naville, M., & Volff, J. N. (2016). Endogenous retroviruses in fish genomes: From relics of past infections to evolutionary innovations? *Frontiers in Microbiology, 7*, 1197.

Ndowora, T., Dahal, G., LaFleur, D., Harper, G., Hull, R., Olszewski, N. E., & Lockhart, B. (1999). Evidence that badnavirus infection in *Musa* can originate from integrated pararetroviral sequences. *Virology, 255*(2), 214-220.

Nizamani, M. M., Zhang, Q., Muhae-Ud-Din, G., & Wang, Y. (2023). High-throughput sequencing in plant disease management: A comprehensive review of benefits, challenges, and future perspectives. *Phytopathology Research, 5*(1), 44.

Pahalawatta, V., Druffel, K., & Pappu, H. R. (2007). Seed transmission of Dahlia mosaic virus in *Dahlia pinnata*. *Plant Disease, 91*(1), 88-91.

Pahalawatta, V., Druffel, K., & Pappu, H. (2008). A new and distinct species in the genus *Caulimovirus* exists as an endogenous plant pararetroviral sequence in its host, *Dahlia variabilis*. *Virology, 376*(2), 253-257.

Pecman, A., Kutnjak, D., Gutiérrez-Aguirre, I., Adams, I., Fox, A., Boonham, N., & Ravnikar, M. (2017). Next generation sequencing for detection and discovery of plant viruses and viroids: Comparison of two approaches. *Frontiers in Microbiology, 8*, 1998.

Pfeiffer, P., & Hohn, T. (1983). Involvement of reverse transcription in the replication of cauliflower mosaic virus: A detailed model and test of some aspects. *Cell, 33*(3),781-789.

Rageshwari, S., Malathi, V. G., Renukadevi, P., & Nakkeeran, S. (2023). Molecular studies on tobacco streak virus (TSV) infecting cotton in Tamil Nadu, India. *3 Biotech, 13*(1), 35.

Randell, T. M., Roberts, P. M., & Culpepper, A. S. (2021). Palmer amaranth (Amaranthaceae) and at-plant insecticide impacts on tarnished plant bug (Hemiptera: Miridae) and injury to seedling cotton terminals. *Journal of Entomological Science, 56*(4), 487-503.

Richert-Pöggeler, K. R., Noreen, F., Schwarzacher, T., Harper, G., & Hohn, T. (2003). Induction of infectious petunia vein clearing (pararetro) virus from endogenous provirus in petunia. *EMBO Journal, 22*(18), 4836-4845.

Richert-Pöggeler, K. R., & Shepherd, R. J. (1997). Petunia vein-clearing virus: A plant pararetrovirus with the core sequences for an integrase function. *Virology*, *236*(1), 137-146.

Roberts, P. M., & Toews, M. (2023). Commercial insect and weed control in cotton. University of Georgia Extension. Retrieved February 10, 2023, from https://extension.uga.edu/publications/detail.html?number=SB28-16

Saito, N., Chen, S., Kitajima, K., Zhou, Z., Koide, Y., Encabo, J. R., Diaz, M. G. Q., Choi, I. R., Koyanagi, K. O., & Kishima, Y. (2023). Phylogenetic analysis of endogenous viral elements in the rice genome reveals local chromosomal evolution in *Oryza* AA-genome species. *Frontiers in Plant Science, 14*, 1261705.

Schepetilnikov, M., & Ryabova, L. (2014). Cauliflower mosaic virus (CaMV) upregulates translation reinitiation of its pregenomic polycistronic 35S RNA via interaction with the cell's translation machinery. In R. K. Gaur, T. Hohn, & P. Sharma (Eds.), *Plant Virus-Host Interaction* (pp. 325-343). Academic Press.

Schmidt, N., Seibt, K. M., Weber, B., Schwarzacher, T., Schmidt, T., & Heitkam, T. (2021). Broken, silent, and in hiding: Tamed endogenous pararetroviruses escape elimination from the genome of sugar beet (*Beta vulgaris*). *Annals of Botany*, *128*(3), 281-299.

Schoelz, J. E. (2008). Caulimoviruses: General features. In B. W. J. Mahy & M. H. V. Van Regenmortel (Eds.), *Encyclopedia of Virology* (Third Edition) (pp. 457-464). Academic Press.

Scholthof, H. B., Gowda, S., Wu, F. C., & Shepherd, R. J. (1992). The full-length transcript of a caulimovirus is a polycistronic mRNA whose genes are trans-activated by the product of gene VI. *Journal of Virology, 66*(5), 3131-3139.

Sedhain, N. P., Bag, S., Morgan, K., Carter, R., Triana, P., Whitaker, J., Kemerait, R. C., & Roberts, P. M. (2021). Natural host range, incidence on overwintering cotton and diversity of cotton leafroll dwarf virus in Georgia, USA. *Crop Protection, 144*, 105604.

Sharman, M., Persley, D. M., & Thomas, J. E. (2009). Distribution in Australia and seed transmission of tobacco streak virus in *Parthenium hysterophorus*. *Plant Disease, 93*(7), 708-712.

Shirley, A. (2023, January 4). Thrips management in cotton. UGA Extension, Tatnall County. Retrieved June 4, 2024, from https://site.extension.uga.edu/tattnall/2023/04/thrips-management-in-cotton

Silva, T. F., Romanel, E. A., Andrade, R. R., Farinelli, L., Østerås, M., Deluen, C., Corrêa, R. L., Schrago, C. E., & Vaslin, M. F. (2011). Profile of small interfering RNAs from cotton plants infected with the polerovirus cotton leafroll dwarf virus. *BMC Molecular Biology, 12*, 40.

Skirmuntt, E. C., Escalera-Zamudio, M., Teeling, E. C., Smith, A., & Katzourakis, A. (2020). The potential role of endogenous viral elements in the evolution of bats as reservoirs for zoonotic viruses. *Annual Review of Virology, 7*, 103-119.

Squires, J., Gillespie, T., Schoelz, J. E., & Palukaitis, P. (2011). Excision and episomal replication of cauliflower mosaic virus integrated into a plant genome. *Plant Physiology, 155*(4), 1908-1919.

Stein, R. A., & DePaola, R. V. (2023). Human endogenous retroviruses: Our genomic fossils and companions. *Physiological Genomics, 55*(6), 249-258.

Suzuki, Y., Baidaliuk, A., Miesen, P., Frangeul, L., Crist, A. B., Merkling, S. H., Fontaine, A., Lequime, S., Moltini-Conclois, I., Blanc, H., van Rij, R. P., Lambrechts, L., & Saleh, M. C. (2020). Non-retroviral endogenous viral element limits cognate virus replication in *Aedes aegypti* ovaries. *Current Biology, 30*(18), 3495-3506.e3496.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution, 38*(7), 3022-3027.

Teycheney, P. Y., Geering, A. D. W., Dasgupta, I., Hull, R., Kreuze, J. F., Lockhart, B., Muller, E., Olszewski, N., Pappu, H., Pooggin, M. M., Richert-Pöggeler, K. R., Schoelz, J. E., Seal, S., Stavolone, L., Umber, M., & Report Consortium, I. (2020). ICTV virus taxonomy profile: Caulimoviridae. *Journal of General Virology, 101*(10), 1025-1026.

Valli, A. A., Gonzalo-Magro, I., & Sanchez, D. H. (2023). Rearranged endogenized plant pararetroviruses as evidence of heritable RNA-based immunity. *Molecular Biology and Evolution, 40*(1).

Veglia, A. J., Bistolas, K. S. I., Voolstra, C. R., Hume, B. C. C., Ruscheweyh, H.-J., Planes, S., Allemand, D., Boissin, E., Wincker, P., Poulain, J., Moulin, C., Bourdin, G., Iwankow, G., Romac, S., Agostini, S., Banaigs, B., Boss, E., Bowler, C., de Vargas, C., Douville, E., Flores, M., Forcioli, D., Furla, P., Galand, P. E., Gilson, E., Lombard, F., Pesant, S., Reynaud, S., Sunagawa, S., Thomas, O. P., Troublé, R., Zoccola, D., Correa, A. M. S., & Vega Thurber, R. L. (2023). Endogenous viral elements reveal associations between a non-retroviral RNA virus and symbiotic dinoflagellate genomes. *Communications Biology, 6*(1), 566.

Wang, Y., & Folimonova, S. Y. (2023). Long noncoding RNAs in plant-pathogen interactions. *Phytopathology, 113*(8), 1380-1386.

Weiss, R. (1967). Spontaneous virus production from "non-virus producing" Rous sarcoma cells. *Virology Journal, 32*(4), 719-723.

Weiss, R. A. (2006). The discovery of endogenous retroviruses. *Retrovirology, 3*(1), 67.

West-Ortiz, M., Stuehler, D., Pollock, E., Wilson, J. R., Preising, S., Larrea-Sarmiento, A., Alabi, O., Fuchs, M., Heck, M., & Olmedo-Velarde, A. (2023). Characterization of cotton virus A, a novel and distinct member of the genus *Caulimovirus* with endogenous viral elements in *Gossypium* spp. *bioRxiv*.

Xin, M., Cao, M., Liu, W., Ren, Y., Lu, C., & Wang, X. (2017). The genomic and biological characterization of *Citrullus lanatus* cryptic virus infecting watermelon in China. *Virus Research, 232*, 106-112.

Yu, H., Wang, X., Lu, Z., Xu, Y., Deng, X., & Xu, Q. (2019). Endogenous pararetrovirus sequences are widely present in Citrinae genomes. *Virus Research, 262*, 48-53.

Yu, J., Jung, S., Cheng, C.-H., Lee, T., Zheng, P., Buble, K., Crabb, J., Humann, J., Hough, H., & Jones, D. (2021). CottonGen: The community database for cotton genomics, genetics, and breeding research. *Plants, 10*(12), 2805.

Zambrana-Echevarría, C., Roth, M. G., Dasgupta, R., German, T. L., Groves, C. L., & Smith, D. L. (2021). Sensitive and specific qPCR and nested RT-PCR assays for the detection of tobacco streak virus in soybean. *PhytoFrontiers, 1*(4), 291-300.

Zhao, H., Zhang, R., Wu, J., Meng, L., Okazaki, Y., Hikida, H., & Ogata, H. (2023). A 1.5-Mb continuous endogenous viral region in the arbuscular mycorrhizal fungus *Rhizophagus irregularis*. *Virus Evolution, 9*(2), vead064.

Tables

Table 3.1 Oligo primer and the targeted virus genes used in this study.

S. No	Oligo	Virus	Gene Targeted	Primer Sequence	References
1	Caulimo MP-F	EVEs	Movement protein	GGACGACTCGAAGGAAACTTAGG	West-Ortiz et al., 2023
2	Caulimo MP-R	EVEs	Movement protein	ACTAGAAGGGTGCTCTACTGGTA	West-Ortiz et al., 2023
3	SB26-F	CLRDV	Partial Capsid protein	CTCAATGGTCTTATTGGAGTTCA	Edula et al., 2023
4	SB26-R	CLRDV	Partial Capsid protein	TTCCTCCCATTCTTGGTGATTCC	Edula et al., 2023
5	SB11-F	CLRDV	Capsid protein	AGGTTTTCTGGTAGCAGTACCAATATCAACGTTA	Sedhain et al., 2021
6	SB11-R	CLRDV	Capsid protein	TATCTTGCATTGTGGATTTCCCTCATAA	Sedhain et al., 2021
7	SB 28-F	CLRDV	P0 protein	CACTTGAGACATAACTCGCTT	Sedhain et al., 2021
8	SB 28-R	CLRDV	P0 protein	GCGGTGAGGAGACCATACTCA	Sedhain et al., 2021
9	SB162F	TSV	Capsid protein	TCAGCCTGACTGTTGGGTTGT	Bag et al., 2019
10	SB162R	TSV	Capsid protein	AGCTATGCATGTTGTTCATAGG	Bag et al., 2019
11	SB164F	TSV	Movement protein	ACGATTTCCAACTTTGAATTCCTACAA	Bag et al., 2019
12	SB164R	TSV	Movement protein	ATCTATCTCTAGAATTCATCAACTTAATACT	Bag et al., 2019

Table 3.2 Long non-coding and small RNAs read coverage, matching, and percent nucleotide identity with different virus sequences suspected in the occurrence of terminal abortion.

(A)												
Sample ID	**Total lncRNA Reads**	**Reads Match to CotV-A**	**Consensus seq**	**Coverage% with CotV-A**	**Nucleotide % Identity**	**Reads Match to eCPRVE**	**Coverage % with eCPRVE**	**Nucleotide % Identity**	**Reads Matching CLRDV**	**Coverage % with CLRDV**	**Reads Matching with TSV**	**Coverage % with TSV**
S2	78,686,152	73,027 (0.09)	7484	100	99.55	47,584 (0.06)	100	99.84	0	-	0	-
S3	57,242,296	29,794 (0.05)	7481	99.99	99.45	19,663 (0.03)	100	99.72	0	-	0	-
S4	83,642,364	33,534 (0.04)	7481	99.99	99.44	20,928 (0.02)	100	99.72	0	-	0	-
S5	83,991,624	43,588 (0.05)	7482	100	99.49	26,587 (0.03)	100	99.77	0	-	0	-

(B)								
Sample ID	**Total sRNA reads**	**% sRNA reads match with CotV-A**	**Coverage % with CotV-A**	**% sRNA reads match with eCPRVE**	**Coverage % with eCPRVE**	**% sRNA reads match with CLRDV**	**Coverage % with CLRDV**	**% sRNA reads match with TSV**	**Coverage % with TSV**
S2	22,664,462	1462 (0.006)	-	1300 (0.005)	-	539 (0.002)	-	330 (0.001)	-
S3	19,209,722	985 (0.005)	-	873 (0.004)	-	395 (0.002)	-	237 (0.001)	-
S4	22,718,020	2138 (0.009)	-	1833 (0.008)	-	944 (0.004)	-	663 (0.002)	-
S5	26,555,305	2169 (0.008)	-	1904 (0.007)	-	893 (0.003)	-	643 (0.002)	-

Notes: The table above represents the coverage of **Table 2**A long non-coding RNAs with eCPRVE (OR269951), CotV-A (OR184923), CLRDV (NC_014545.1), and TSV (KP256522.1) sequences; **Table 2**B small RNA sequences of field samples with eCPRVE (OR269951), CotV-A (OR184923), CLRDV (NC_014545.1) and TSV (KP256522.1). Abbreviation used: eCPRVE: endogenous cotton pararetroviral elements; CotV-A: cotton virus A; CLRDV: cotton leafroll dwarf virus; TSV: tobacco streak virus.

Table 3.3 Endogenous viral elements detection in cotton samples from field and greenhouse conditions.

Cotton Sample-Type	**Number of Samples Tested (*n*)**	**[a] Number of Samples (*n*) Positive in PCR/RT-PCR/qPCR**		
		EVEs	**CLRDV**	**TSV**
Commercial field samples				
Symptomatic (L1&2)	46	32 (70%)	ND	ND
Asymptomatic (L1&2)	8	8 (100%)	ND	ND
Experimental plot				
Symptomatic	40	37 (93%)	1 (2.5%)	NT
Asymptomatic	40	39 (98%)	1 (2.5%)	NT
Induced	20	19 (95%)	ND	
Seed assessment (Greenhouse conditions)				
Seeds	40	38 (95%)	NT	NT
Seedlings	40	30 (75%)	NT	NT

Notes: [a] Sample percentage is rounded off to the nearest decimal; Acronyms used are ND-not detected and NT-not tested. Abbreviation used: EVEs: endogenous viral elements; CLRDV: cotton leafroll dwarf virus; TSV: tobacco streak virus.

Table 3.4 Yield response of two Dyna-Gro varieties: DG3615 and DGH959.

Variety	**Treatment**	**Seedcotton Yield (g plant^{-1})**	**Boll Density plant^{-1}**
DG3615	Asymptomatic	51.97 a	9.85 a
	Symptomatic	43.53 a	8.5 a
	p value	0.4139	0.4933
DGH959	Asymptomatic	60.57 a	12.3 a
	Symptomatic	57.36 a	11.3 a
	Induced terminal abortion	56.55 a	10.95 a
	p value	0.8866	0.7028

Notes: Alphabet (a) represents the compact letter display showing statistical significance.

Supplementary data

Table 3.5 (S1): *In silico* mining of integrated caulimovirid-like EVE sequences in the various *Gossypium* sps. using cotton databases

Location	Species	Annotation version	E-value	% identity	Align length	Strands	Query from	Query to	Target from	Target to
D04	*G.barbadense*	v1.1	0	81	953	+/+	4898	5849	2028496	2029447
D04	*G.barbadense*	v1.1	0	75	1099	+/+	1336	2431	2026877	2027954
D04	*G.darwinii*	v1.1	0	75	1462	+/+	4632	6083	2168902	2170343
D04	*G.darwinii*	v1.1	0	80	953	+/+	4898	5849	2181416	2182367
A03	*G.tomentosum*	v1.1	0	79	5341	+/+	798	6094	102007327	102012565
D04	*G.tomentosum*	v1.1	0	81	953	+/+	4898	5849	2140473	2141421
D04	*G.tomentosum*	v1.1	0	76	1099	+/+	1336	2431	2138863	2139937
A13	*G.tomentosum*	v1.1	0	75	1112	+/-	4961	6063	103755520	103754420
A04	*G.hirsutum_Coker*	v1.1	0	100	7042	+/-	1	7042	80638708	80631669
A04	*G.hirsutum_Coker*	v1.1	0	99	6229	+/+	1	6225	80623669	80629889
A04	*G.hirsutum_Coker*	v1.1	0	97	394	+/+	7025	7413	80633655	80634048
A04	*G.hirsutum_FM958*	v1.1	0	100	7042	+/-	1	7042	80682226	80675187
A04	*G.hirsutum_FM958*	v1.1	0	99	6229	+/+	1	6225	80667187	80673407
A04	*G.hirsutum_FM958*	v1.1	0	97	394	+/+	7025	7413	80677173	80677566
A04	*G.hirsutum_DeltaPearl*	v1.1	0	100	7042	+/-	1	7042	80667859	80660820
A04	*G.hirsutum_DeltaPearl*	v1.1	0	99	6229	+/+	1	6225	80652820	80659040
A04	*G.hirsutum_DeltaPearl*	v1.1	0	97	394	+/+	7025	7413	80662806	80663199
A04	*G.hirsutum*	v3.1	0	100	7042	+/-	1	7042	80045055	80038016
A04	*G.hirsutum*	v3.1	0	99	6229	+/+	1	6225	80030016	80036236
A04	*G.hirsutum*	v3.1	0	97	394	+/+	7025	7413	80040002	80040395
A04	*G.hirsutum_CSX8308*	v1.1	0	100	7042	+/-	1	7042	80355540	80348501
A04	*G.hirsutum_CSX8308*	v1.1	0	99	6229	+/+	1	6225	80340501	80346721
A04	*G.hirsutum_CSX8308*	v1.1	0	97	394	+/+	7025	7413	80350487	80350880
A04	*G.hirsutum_UA48*	v1.1	0	100	7042	+/-	1	7042	79992710	79985671
A04	*G.hirsutum_UA48*	v1.1	0	99	6229	+/+	1	6225	79977671	79983891

A04	*G.hirsutum_UA48*	v1.1	0	97	394	+/+	7025	7413	79987657	79988050
A04	*G.hirsutum_UGA230*	v1.1	0	100	7042	+/-	1	7042	79933039	79926000
A04	*G.hirsutum_UGA230*	v1.1	0	99	6229	+/+	1	6225	79918000	79924220
A04	*G.hirsutum_UGA230*	v1.1	0	97	394	+/+	7025	7413	79927986	79928379
A04	*G.hirsutum*	v2.1	0	100	7042	+/-	1	7042	80620206	80613167
A04	*G.hirsutum*	v2.1	0	99	6229	+/+	1	6225	80605168	80611387
A04	*G.hirsutum*	v2.1	0	97	394	+/+	7025	7413	80615153	80615546
A04	*G.hirsutum*	v1.1	0	100	7042	+/-	1	7042	74680136	74673097
A04	*G.hirsutum*	v1.1	0	99	6229	+/+	1	6225	74665098	74671317
A04	*G.hirsutum*	v1.1	0	97	394	+/+	7025	7413	74675083	74675476
scaffold_590	*G.hirsutum*	v3.1	0	99	7059	+/+	1	7042	21796	28823
D03	*G.hirsutum_Coker*	v1.1	0	84	4080	+/+	1	4048	4110884	4114884
D03	*G.hirsutum*	v3.1	0	84	4080	+/+	1	4048	4064996	4068996
D03	*G.hirsutum_UA48*	v1.1	0	84	4080	+/+	1	4048	4091889	4095889
D03	*G.hirsutum*	v2.1	0	84	4080	+/+	1	4048	4160650	4164650
D03	*G.hirsutum*	v1.1	0	84	4080	+/+	1	4048	4240736	4244736
D03	*G.hirsutum_FM958*	v1.1	0	84	4080	+/+	1	4048	4073383	4077383
D03	*G.hirsutum_DeltaPearl*	v1.1	0	84	4080	+/+	1	4048	4065268	4069268
D03	*G.hirsutum_CSX8308*	v1.1	0	84	4080	+/+	1	4048	4130947	4134947
D03	*G.hirsutum_UGA230*	v1.1	0	84	4080	+/+	1	4048	4137270	4141270
A05	*G.hirsutum_Coker*	v1.1	0	73	3037	+/+	1422	4363	59981679	59984677
A05	*G.hirsutum_FM958*	v1.1	0	73	3037	+/+	1422	4363	59850004	59853002
A05	*G.hirsutum_DeltaPearl*	v1.1	0	73	3037	+/+	1422	4363	59908995	59911993
A05	*G.hirsutum*	v3.1	0	73	3037	+/+	1422	4363	59778505	59781503
A05	*G.hirsutum_CSX8308*	v1.1	0	73	3037	+/+	1422	4363	59762457	59765455
A05	*G.hirsutum_UA48*	v1.1	0	73	3037	+/+	1422	4363	59337644	59340642
A05	*G.hirsutum_UGA230*	v1.1	0	73	3037	+/+	1422	4363	59691548	59694546
A05	*G.hirsutum*	v2.1	0	73	3037	+/+	1422	4363	60033890	60036888
scaffold_134	*G.hirsutum*	v1.1	0	73	3037	+/+	1422	4363	40325	43323
D07	*G.hirsutum_Coker*	v1.1	0	80	1059	+/-	5044	6097	2848709	2847651

D07	*G.hirsutum_FM958*	v1.1	0	80	1059	+/-	5044	6097	2815839	2814781
D07	*G.hirsutum_DeltaPearl*	v1.1	0	80	1059	+/-	5044	6097	2806525	2805467
D07	*G.hirsutum*	v3.1	0	80	1059	+/-	5044	6097	2822754	2821696
D07	*G.hirsutum_CSX8308*	v1.1	0	80	1059	+/-	5044	6097	2814226	2813168
D07	*G.hirsutum_UA48*	v1.1	0	80	1059	+/-	5044	6097	2658605	2657547
D07	*G.hirsutum_UGA230*	v1.1	0	80	1059	+/-	5044	6097	2843521	2842463
D07	*G.hirsutum*	v2.1	0	80	1059	+/-	5044	6097	2848951	2847893
D07	*G.hirsutum*	v1.1	0	80	1059	+/-	5044	6097	2843658	2842600
A13	*G.hirsutum_Coker*	v1.1	0	76	1254	+/-	4821	6065	105253892	105252652
A13	*G.hirsutum*	v3.1	0	76	1254	+/-	4821	6065	104414249	104413009
A13	*G.hirsutum*	v2.1	0	76	1254	+/-	4821	6065	104661058	104659818
A13	*G.hirsutum*	v1.1	0	76	1254	+/-	4821	6065	97601618	97600378
A13	*G.hirsutum_CSX8308*	v1.1	0	76	1254	+/-	4821	6065	104162913	104161673
D07	*G.mustelinum*	v1.1	0	70	5134	+/+	991	6065	615272	620222
D07	*G.mustelinum*	v1.1	0	71	3254	+/-	2847	6065	628092	624899
D07	*G.mustelinum*	v1.1	0	73	2113	+/+	3970	6065	628483	630565
D07	*G.mustelinum*	v1.1	0	72	1319	+/-	4754	6065	641766	640459
D05	*G.mustelinum*	v1.1	0	78	1333	+/+	4755	6081	7182119	7183438
A07	*G.mustelinum*	v1.1	0	75	1527	+/+	4555	6065	391111	392611
A04	*G. stephensii*	v1.0	0	99	7042	+/+	1	7042	79018976	79011937
A04	*G. stephensii*	v1.0	0	99	6225	+/+	1	6225	79018976	79011937
D03	*G. stephensii*	v1.0	0	84	3686	+/+	35	3702	79003935	79010156
D07	*G. stephensii*	v1.0	0	80	1045	+/+	5044	6084	2841911	2840867

Figures

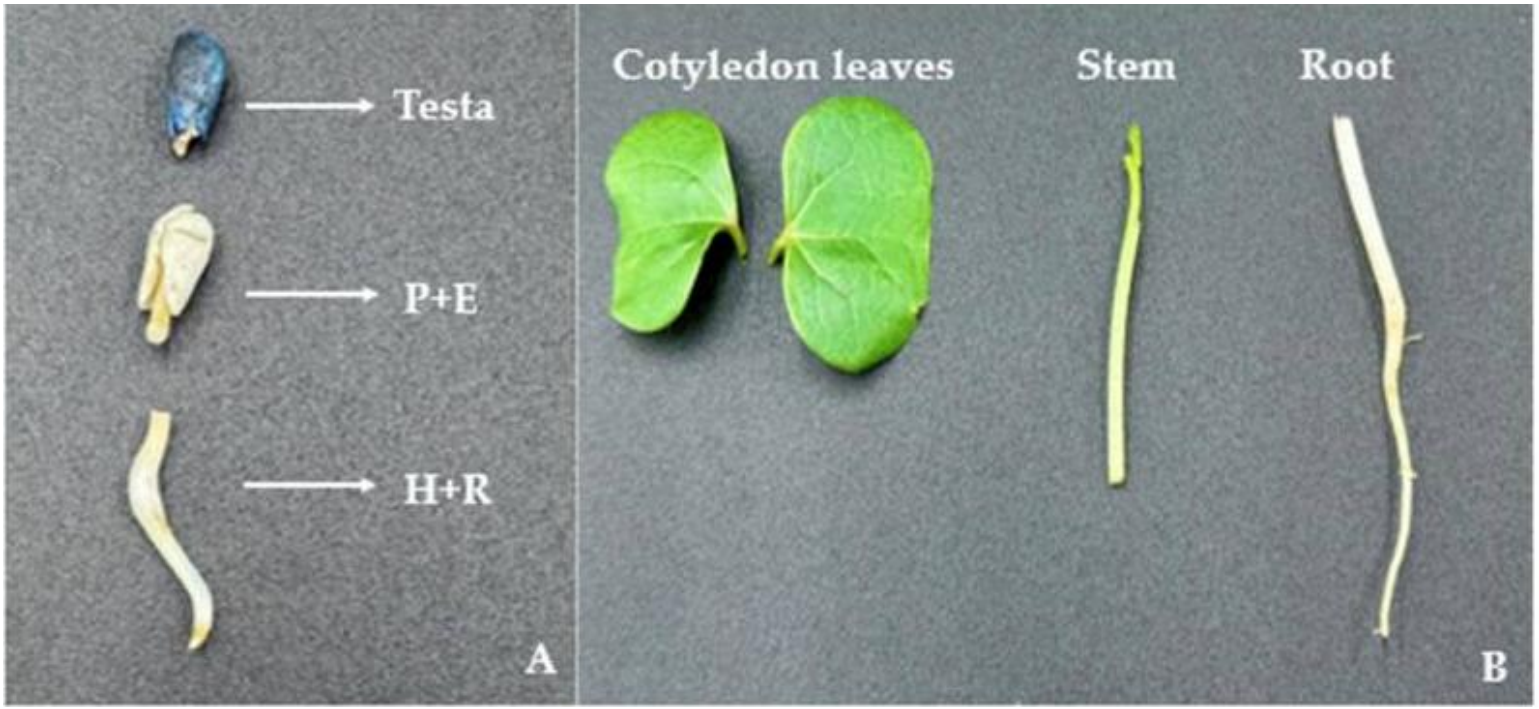


Figure 3.1 Cotton seed and seedlings testing for endogenous viral element (EVE) detection. (**A**) Cottonseed parts (testa, plumule with epicotyl, and hypocotyl with root tissue) and (**B**) cotton seedling parts (cotyledon leaves, stem, and root tissue).

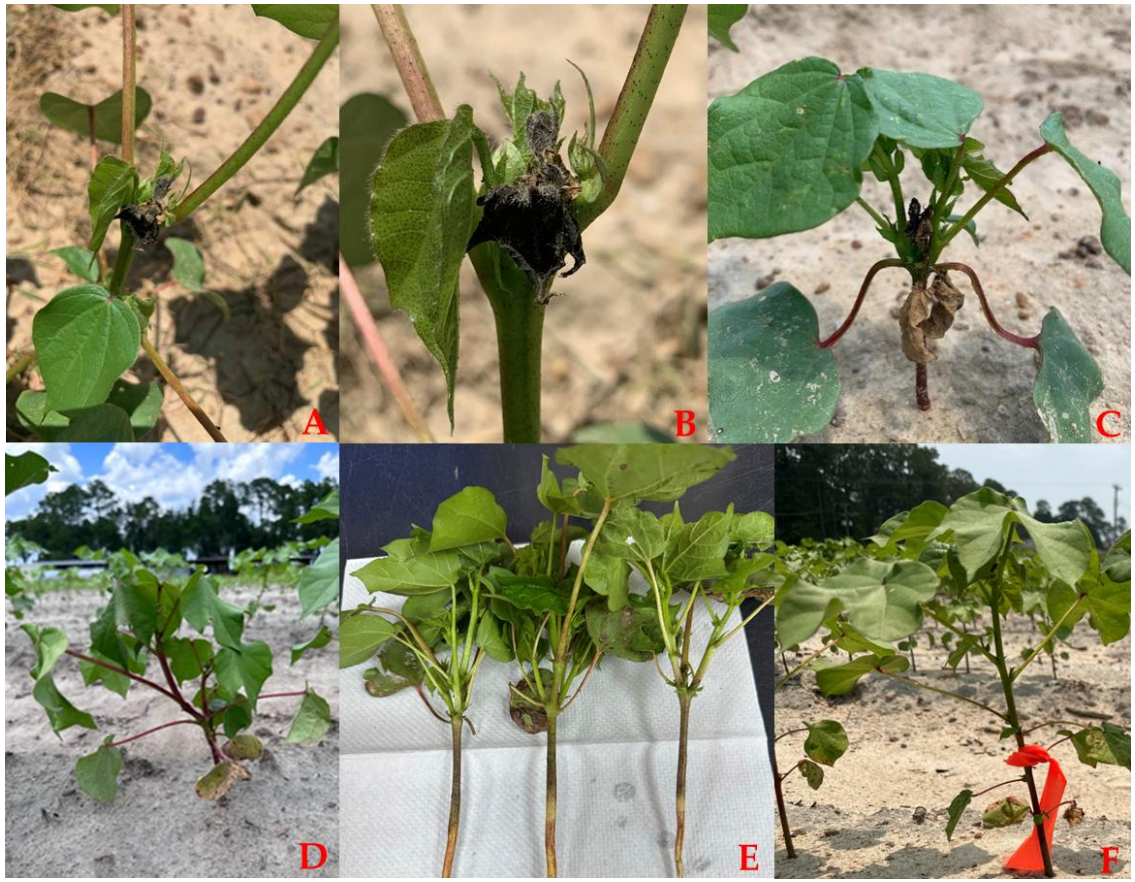


Figure 3.2 Terminal abortion symptoms in cotton plants; (A,B) terminal abortion at primary growth stages (2–4 true leaf stage) of cotton seedlings in the field; (C) initial stage of terminal abortion resulting in profuse vegetative branching; (D) symptomatic plant in the experimental plot in Tift County; (E) vegetative branching from Expo-Colquitt and Hopeful-Mitchell counties, GA; (F) asymptomatic plant without any vegetative branching. Photo Credit: S.E., P.C. and S.B.

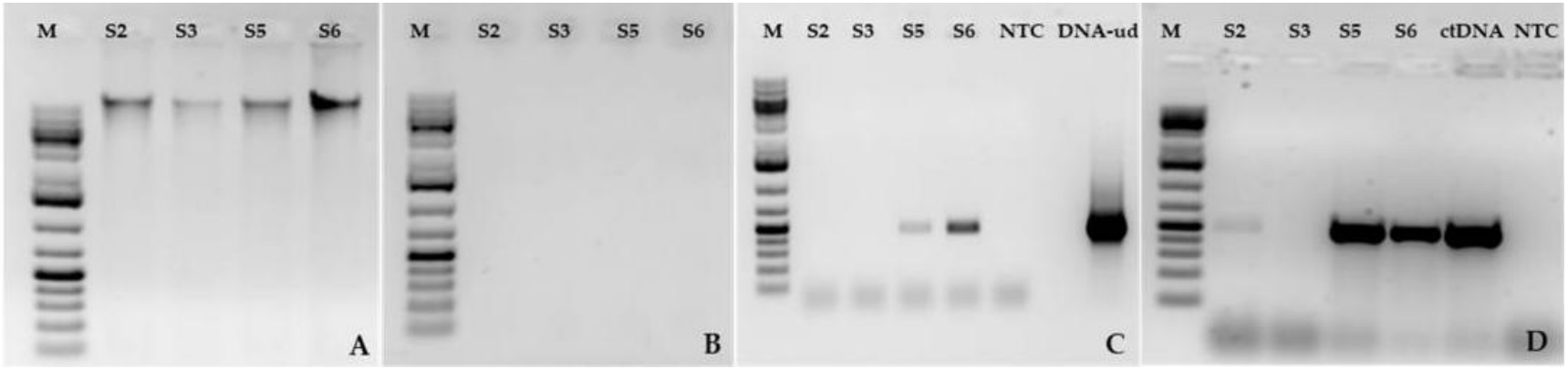
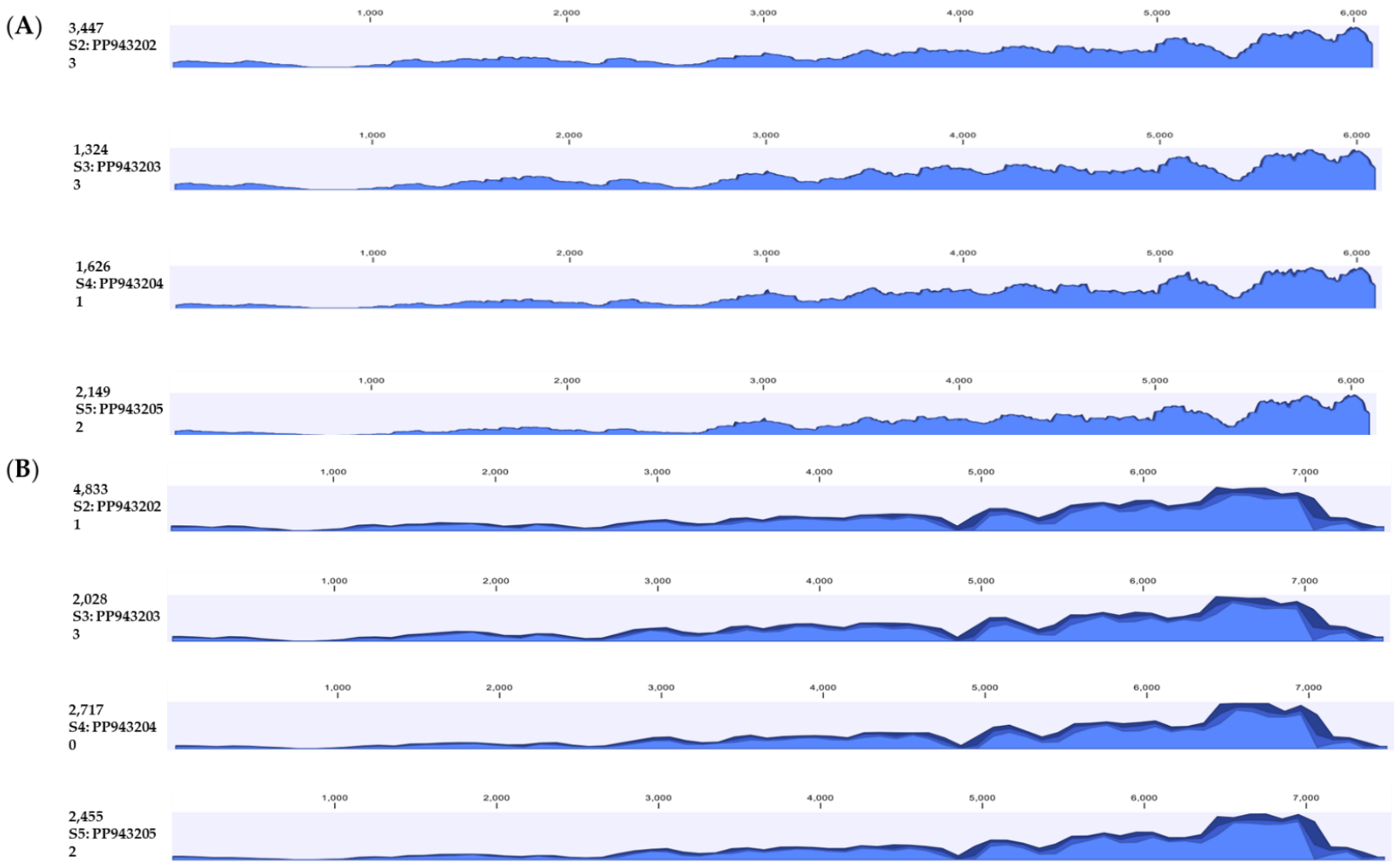


Figure 3.3 Treated nucleic acid PCR with a caulimo movement protein gene primer pair; (A) untreated genomic DNA extracted from pooled field samples; (B) exonuclease V digested DNA; (C) PCR of Exo V digested DNA; (D) DNaseI digested RNA RT-PCR. Lanes are represented as M: Marker; Samples: S2, S3, S5, S6; NTC: No template control; DNA-ud: undigested DNA; ctDNA: cotton DNA.


(A)
3,447
S2: PP943202
3
1,324
S3: PP943203
3
1,626
S4: PP943204
1
2,149
S5: PP943205
2
(B)
4,833
S2: PP943202
1
2,028
S3: PP943203
3
2,717
S4: PP943204
0
2,455
S5: PP943205
2

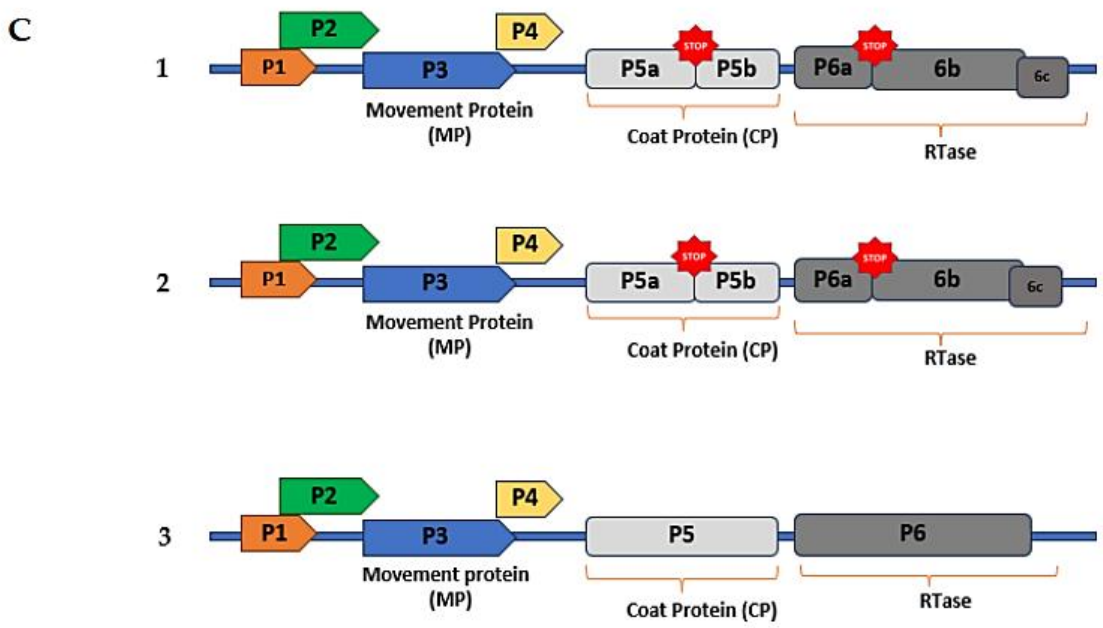


Figure 3.4 Read coverage map at each genome region, showing the maximum, minimum, and average coverage values with the reference sequence. Scaled genome positions of the virus are represented above the histogram and the Y-axis shows the coverage in number of reads. Within the specified peaks, from top to bottom, the colors represent: the maximum coverage (read counts), the average coverage value, and the minimum coverage value. Read coverage map of field samples EVE-GA's (S2: PP943202; S3: PP943203; S4: PP943204; and S5: PP943205) with reference sequences (A) endogenous cotton pararetroviral elements (eCPRVE; OR269951) and (B) cotton virus A (CotV-A; OR184923). Schematic of genome organization of EVE sequences. (C) genome organization of endogenous viral elements, GA (1) compared to a putative eCPRVE (2) and CotV-A (3). Stop codons of the open reading frames (ORFs) coding for putative viral coat protein and reverse transcriptases are shown in red spots.

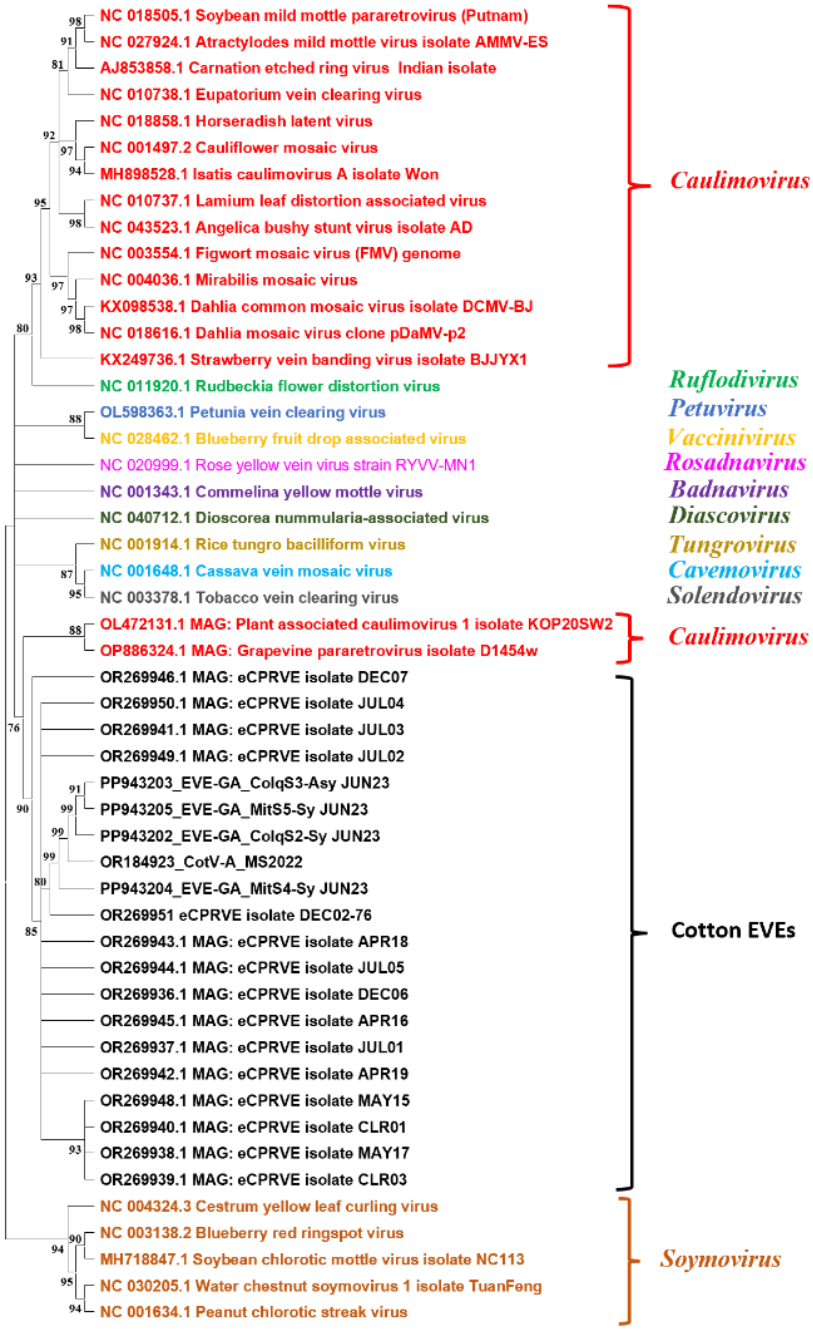


Figure 3.5 Phylogenetic tree comparing near-complete sequences of endogenous viral elements (EVEs) obtained from field samples (S2: PP943202; S3: PP943203; S4: PP943204; and S5: PP943205) of GA in comparison to near-complete sequences of cotton virus A isolate, endogenous cotton pararetroviral elements (eCPRVE) DEC02-76 isolate, and various genera in the family *Caulimoviridae*. Color code represents similar genera.

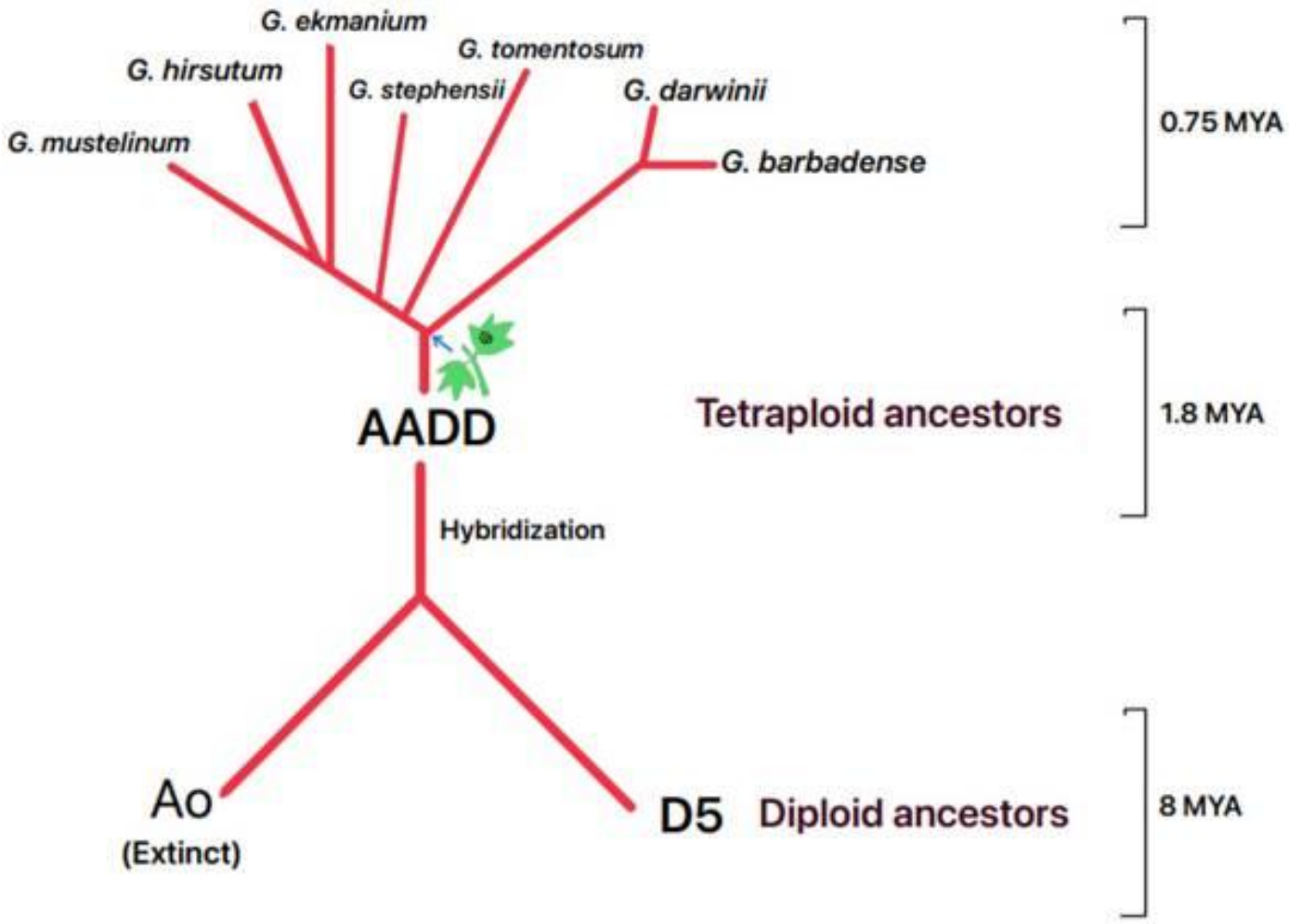


Figure 3.6 Graphic representation of the origin of tetraploid *Gossypium* spp. from their diploid ancestors indicated with timeline and possible virus integration event represented using a green cotton plant and a blue arrow.

Supplementary Figures:

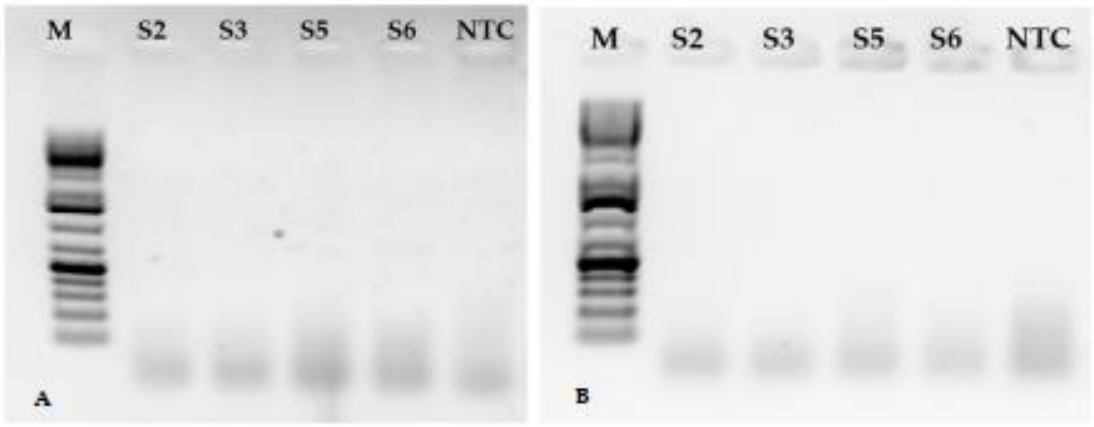


Figure 3.7 (S1) Detection of tobacco steak virus (TSV) using RT-PCR, (A) coat protein gene using primer pair SB162 F/R and (B) movement protein gene using primer pair SB164 F/R; M-Marker, Samples: S2, S3, S5, S6, NTC-No template control

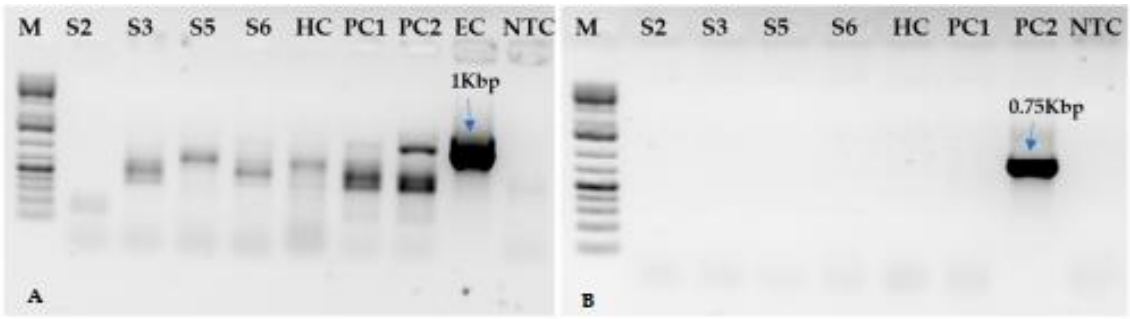


Figure 3.7 (S2) Detection of cotton leafroll dwarf virus (CLRDV) using RT-PCR, (A) P0 protein gene using primer pair SB28 F/R; (B) Coat protein gene detection using primer pair SB11F/R; Lanes were marked as: M-Marker; Samples: S2, S3, S5, S6; HC-Healthy control; PC1 and PC2-plant positive control; EC- P0 amplicons eluted from prior PCR used as positive control for P0 gene, NTC-No template control

CHAPTER 4

COTTON LEAFROLL DWARF VIRUS-INDUCED BRONZE WILT IN UPLAND COTTON: THE ROLE OF CULTIVAR SELECTION IN MITIGATING YIELD LOSS IN GEORGIA, USA.

Manuscript submitted to Plant Pathology.

Edula, S. R., Bag, S., Hand, L. C., Chee, P. W., Snider, J. L., Kemerait, R. C. & Roberts, P. M.

Abstract:

Cotton leafroll dwarf virus (CLRDV), first observed in the USA in 2017, continues to be a concern among the cotton industry. Although its impact has been challenging to quantify, it has the potential to be detrimental. Field diagnosis is difficult due to symptom overlap with abiotic stress and inexplicable disorders, such as bronze wilt, demanding reliable molecular detection for accurate assessment. This study seeks to monitor selected cotton cultivars in Georgia in on-farm variety trials over two years to evaluate host response to CLRDV infection. In 2024, a significant surge in bronze wilt-like symptoms was observed in producer fields, particularly in symptomatic cultivars. Disease severity varied in different locations during the monitoring period. In symptomatic cultivars, plants with wilting symptoms exhibited a significantly higher viral gene copy number and no measurable yield compared to asymptomatic plants. Notably, the yield per unit land area of symptomatic cultivars evaluated in the selected fields at two locations with low disease incidence (Turner and Worth County) exceeded the 2024 trial averages. Conversely, symptomatic cultivars in dryland and irrigated fields in Colquitt County (high disease incidence) experienced yield declines, falling below trial averages. An exception was *DP 2038 B3XF* under irrigated conditions, although tagged plants showed reduced yields per plant, the yield per unit land area remained unaffected. These findings underscore the complex interactions between CLRDV infection, cultivar susceptibility, geographical location, and environmental factors. Consequently, continued epidemiological surveillance and breeding efforts for resistance are crucial for managing this disease.

Keywords: Aphids, bronze wilt, cotton leaf roll dwarf virus, cotton leaf roll dwarf disease, upland cotton.

Introduction

Plant viruses, a group of rapidly evolving plant pathogens, significantly impact plant health, decreasing both the yield and quality of food and fiber crops. Disease symptoms expressed by the host plants are the preliminary indicators of host-pathogen interactions and are crucial for identifying the causal agent. In the case of plant viruses, these symptom expressions underscore the prime consequences of host-virus interactions modulating key physiological and biochemical processes within the host (Hull 2013; Jiang and Zhou 2023; Wang 2015). The symptoms expressed are often influenced by the host's genetic makeup, geographical locations, and other abiotic factors, including temperatures and precipitation (Jiang and Zhou 2023; Tsai et al. 2022). Monocropping with uniform plant genetics can create optimal conditions for insect pests and emerging viral pathogens to proliferate and persist (Tatineni and Hein 2023). Managing plant viral diseases poses significant challenges due to the absence of direct chemical controls and the viruses' interference with cellular functions, resulting in persistent damage to host tissues during infection.

Cotton leafroll dwarf virus (CLRDV) is an emerging viral pathogen in cotton (*Gossypium hirsutum* L.), especially in the Southeast USA. Since its first report from Alabama (AL) in 2019, the presence of the virus has been confirmed in the majority of the cotton-growing states across the cotton belt in the USA (Aboughanem-Sabanadzovic 2019; Alabi et al. 2020; Ali and Mokhtari 2020; Ali et al. 2020; Avelar et al. 2019; Faske et al. 2020; Ferguson and Ali 2022; Iriarte et al. 2020; Olmedo-Velarde et al. 2025; Price et al. 2020; Tabassum et al. 2019; Thiessen et al. 2020; Wang et al. 2020). This virus is primarily transmitted by the cotton aphid (*Aphis gossypii* Glover) in a circulative and non-propagative manner, causing cotton leafroll

dwarf disease (CLRDD) in cotton (Cauquil and Vaissayre 1971; Michelotto and Busoli 2003; Heilsnis et al. 2023).

CLRDV is a phloem-limited *Polerovirus*, causing CLRDD in the USA also known as cotton blue disease (CBD) in other regions (Edula et al. 2023). The symptom expression varies due to multiple biotic and abiotic factors, including cotton variety, geographic location, planting date, aphid population density, and environmental conditions (Bag et al. 2021). Avelar et al. (2019, 2020) described the foliar symptoms as leaf deformation with bluish-green discoloration, rust-to-red pigmentation, petiole and vein reddening, vein clearing, leaf curling, cupping, rolling, dwarfing, and shortened internodes. In Georgia, late-planted cotton exhibits additional symptoms, such as downward leaf drooping, V-shaped lamina folding, warmth in symptomatic leaves, wilting, and plant collapse, especially in symptomatic cultivars—those more prone to severe wilting (Bag et al. 2021; Parkash et al. 2021; Tabassum et al. 2019). In some plants, these symptoms may diminish later in the season, and affected plants, especially during the early growing season remain stunted with no harvestable bolls while utilizing space and soil nutrients (Bag et al. 2021). Furthermore, CLRDV has been detected in many asymptomatic plants, complicating accurate visual diagnosis (Bag et al. 2021; Edula et al. 2023).

Bronze wilt (BW) or bronze wilt complex (BWC) (Creech 1999) in cotton is also known as sudden wilt, copper top, red top, bronzing, and early fadeout (Bell et al. 1998, 2002). Predominant symptoms associated with BWC were bronzing of leaves, red stems, stunting, rugose leaves, absence of squares, square and boll abortions, reduction in internode length, and wilting (Albers and Guthrie 2001; Phipps 2000). Some plants become necrotic and usually die when infected at the 8-12 leaf nodes stage (Albers and Guthrie, 2001; Phipps 2000; Yan et al. 2002). Symptomatic leaves were also warmer than average leaf temperatures (Gwathmey et al.

2008; Phipps 2000). Bronze wilt often appears severe on plants at the edge of the rows, along the side of the fields or in single isolated plants (Fig 4.1; Bell et al., 2002). Prominently observed in short-season cultivars (Bell et al. 1998; Phipps 2000), BWC was widespread and a major concern for the USA cotton industry from 1995 through early 2000 (Bell et al. 1998; Creech 1999; Brown 2000; Padgett et al. 2004; Phipps 2000). The phenotypic expression of this complex is nearly identical to the symptoms observed in plants infected with CLRDV in Georgia (Edula et al. 2023; Parkash et al. 2021).

Similar symptoms were also described in Argentina, Bolivia, and Brazil, with certain germplasm and cultivars showing more susceptibility and severity (Creech 2000), suggesting a potential cultivar-specific anomaly. Gade et al. (2013) proposed that an aphid-transmitted virus was the likely cause of red leaf disease, also known as BW, observed in India. However, the exact causal agent of BW remained unidentified, while certain cultivars showed heightened sensitivity to this disease (Gade et al. 2013; Mukherjee et al. 2012). The occurrence and economic impact of BW varied across different states in the USA (Bell et al. 1998; Brown 2000; Creech 1999; Creech and Fieber 2000; Padget et al. 2004; Phipps 2000). A strong correlation exists between varietal response and susceptibility to BW, with temperature exacerbating symptom severity (Bell 2000; El-Zik et al. 2001; Nichols 2001). Specifically, early-maturing, high-yielding cultivars demonstrated increased susceptibility (El-Zik et al. 2001). The severe wilt complex that emerged in the late 1990s, particularly affecting susceptible cultivars, was frequently associated with the presence of TAMCOT-SP37 in their genetic background (Bell 1999; Creech 1999). Additionally, Seabrook Sea Island 12B2 (SBSI) was often implicated in cases where hypersensitivity was thought to be a contributing factor (Bell et al. 1999). The

severity and timing of BW symptoms, as well as the susceptibility of different cotton cultivars, exhibited annual variability (Creech and Fieber 2000; Phipps 2000).

While some researchers proposed *Agrobacterium* biovar I as a possible cause of the disease, this hypothesis remained inconclusive and lacked broad acceptance. This was primarily due to the bacterium's common presence as an endophyte in cotton seeds and the failure to satisfy Koch's postulates (Bell 1998, 1999, 2000). Researchers theorized that molecular approaches could elucidate the etiology of BW, but no such methodologies were employed at the time to investigate potential viral involvement, leaving a critical knowledge gap in the understanding of its cause.

Descriptions of both CLRDD and BW show overlapping symptoms, progression, and recovery of symptomatic plants (Fig 4.1; Albers and Guthrie 2001; Tabassum et al. 2019). Since bronzing and wilting can result from both biotic and abiotic stresses, a comprehensive investigation is essential to determine their underlying causes.

The primary goal of this study is to determine whether the wilting symptoms observed in cotton cultivars are associated with CLRDV infection, representing a potential CLRDV-induced bronzing and wilting. By quantifying virus gene copy numbers in symptomatic and asymptomatic plants and assessing potential yield losses over two years in Georgia, this research aims to clarify the relationship between CLRDV infection and associated symptom expression. This study also aimed to determine if the characteristic field symptoms of CLRDV could be reproduced under greenhouse conditions using aphid vectors and the virus cDNA clone. These findings provide critical insights into disease diagnosis and management strategies for cotton growers and other researchers.

Materials and methods

Field and cultivar selection

Trial locations, cultivars, and counties were selected for disease monitoring and data collection was based on observations made by the University of Georgia (UGA) cotton team. Since the emergence of CLRDV in 2018, the disease was predominantly observed in and around Tift County in southwest Georgia (Tabassum et al. 2021). For this study, viral disease incidence and its impact on yield were analyzed using data collected from on-farm variety trials conducted by the UGA cotton team, which evaluated regional commercial cotton cultivars in grower fields. Planting dates across the selected counties in 2022-2024 were from May 1st to June 7th. The cultivars included in these studies vary each year depending on the requests and priorities from cotton seed industries for GA. The disease incidence was monitored during 2022-2024, and the yield impact was evaluated on individual plants. In 2023, the disease incidence was minimal, so the experiment was conducted only in Tift County. For 2022 and 2024, five cultivars were selected, representing different seed sources and potential susceptibilities and tolerance to the virus. The cultivars were planted in a six-row plot in each location with three replications following a randomized complete block design. The data was collected from the middle two rows of each plot and at least 50 meters inside the field from the edge. One hundred plants per cultivar and replication were monitored for symptom development and to evaluate the percentage of disease incidence. Five symptomatic and five asymptomatic plants from each cultivar were tagged 30 days after planting (DAP) in 2022 and at 60 DAP in 2024 for tissue sampling to confirm the presence of the virus as well as to assess boll counts and individual plant yield at harvest. This process was repeated in three replications for each cultivar across at least four fields. In 2022, the study was conducted on five cultivars in Bulloch, Colquitt, Cook, and Worth

counties. In 2023, the study was restricted to only two cultivars in Tift County. In 2024, the study was again expanded to five cultivars in three dryland fields in Colquitt, Worth, and Turner Counties, with an additional irrigated field in Colquitt County. In 2022, the five cultivars selected were *Dyna-Gro (DG) 3615 B3XF* and *DG 3799 B3XF*, *Delta Pine (DP) 1646 B2XF*, *DP 2038 B3XF*, and *Stoneville (ST) 4595 B3XF*. In 2023, the two cultivars were *DG 3615 B3XF* and *DG H959 B3XF*. During 2024, the five cultivars included *DG 3615B3XF, DG 3799 B3XF*, *DG H959 B3XF, NexGen (NG) 5430 B3XF* and *DP 2038 B3XF*.

Disease monitoring and sample collection

Based on the prior studies, a disease progression scale was developed to characterize CLRDD symptoms in *DG 3615B3XF* (Parkash et al. 2021). Briefly, the five stages (S_0 to S_5) of the disease development scale include S_0 (healthy plants of the symptomatic cultivar), S_1 (initial reddening, but no wilting), S_2 (reddening plus initial leaf droop), S_3 (loss of leaf turgor), S_4 (severe wilt and advanced leaf chlorosis), and S_5 (senescent plant) (Parkash et al. 2021). The selected cultivars were monitored periodically at 30-day intervals until 90 DAP in 2022 and at 60 DAP in 2024 to assess CLRDD symptom expression. The percentage of disease incidence was calculated at 60 and 90 days after planting in 2022 and at 60 days in 2023 and 2024.

For CLRDV detection and validation, samples consisting of the third or fourth fully expanded leaf from the apex, along with its petiole were collected. In 2022, samples were collected from the tagged plants at 30, 60, and 90 DAP. At each location, 75 samples were collected at every time point, representing all five varieties. In total, 300 samples were collected per time point and a total of 900 samples across four locations in three time points. In 2023, a total of 100 plants were monitored and evaluated in Tift County among two varieties, *DG 3615*

B3XF (n=40) and *DG H959 B3XF* (n=60). In 2024, the sampling was conducted at 60 DAP. Fifteen symptomatic and fifteen asymptomatic samples were collected from three replications for each of the five cultivars from each location. A total of 480 samples were collected, representing four varieties and 60 samples for the fifth variety. To evaluate yield, the number of harvestable bolls per plant were counted, and seedcotton was harvested at maturity.

Nucleic acid (TNA) extraction

Total nucleic acid (TNA) was extracted from approximately 100 milligrams of leaf and petiole samples using MagMAX 96 viral RNA kit using the KingFisher Flex Purification System (Thermo Fisher Scientific) by following the protocol detailed in Kavalappara et al. (2021).

Total RNA was extracted from individual aphids (n=15) collected in pure ethanol from the greenhouse. Aphids were washed twice with sterile distilled water and homogenized using sterile plastic micro pestles (Corning) in microcentrifuge tubes containing 250µL of TRIzol (Invitrogen), ensuring efficient tissue disruption. 50µL of chloroform was added, incubated at room temperature for 2 minutes, and centrifuged at maximum speed at 4°C for 15 min. The supernatant was collected into a fresh 1.7 mL microcentrifuge tube, and RNA extraction was done following the manufacturer's instructions.

The quality and quantity of the total nucleic acids were determined using a NanoDrop One UV–Vis Spectrophotometer (Thermo Fisher Scientific). RNA, with 260/280 absorbance of ≥1.8, were normalized to the final concentration of 40ng/µl, aliquoted and stored at -80°C until further analysis.

cDNA preparation and qPCR analysis

Complementary DNA (cDNA) was synthesized using 200ng of RNA with 10 µM SB26R (5'TTC CTC CCA TTC TTG GTG ATT CC 3') reverse primer using 200 U of Superscript III

(Invitrogen), following manufacturer's instructions. Five microliters of cDNA were used for virus detection and quantification as described earlier by Edula et al. (2023). Briefly, the partial capsid protein gene was amplified and cloned in a Topo-TA PCR cloning vector (Thermo Fisher Scientific). A standard curve was generated using six 10-fold serial dilutions of the linearized plasmids and viral gene copy number was calculated based on the plasmid DNA concentration and quantification cycle (Cq) values obtained in SYBR Green-based RT-qPCR assay as described in Kavalappara et al. (2022). In qPCR analysis, virus gene copy numbers with $\log_{10}$ values exceeding 4.2 indicate significantly elevated levels, correlating with strong detection at Cq values of 31 or below. In contrast, $\log_{10}$ values of 4.1 or less reflect significantly lower viral loads, ranging from borderline detection (Cq = 32) to undetectable (Cq > 33). Healthy samples and no-template controls typically exhibit $\log_{10}$ values below 3.6, corresponding to Cq values greater than 34.

Validation of molecular assay by sequencing

Samples detected with CLRDV in RT-qPCR analysis for partial capsid protein gene were PCR purified and subjected to bi-directional Sanger sequencing (Azenta Lifesciences) to validate the assay.

Statistical analysis

A one-way analysis of variance (ANOVA) was used to analyze disease incidence in 2022 and 2024. In 2022, a one-way ANOVA was used to analyze the effects of cultivar on viral gene copy number and yield parameters within a location. Mean differences were compared using Tukey's HSD post-hoc test, applied at $\alpha = 0.05$. In 2023, a student's *t*-test was used to compare the effect of cultivar on viral gene copy number, seedcotton weight and boll density. In 2024, due to unavailability of symptomatic plants in cultivar *DP 2038 B3XF*, each combination of cultivar

and plant health was considered a unique treatment. Therefore, a one-way ANOVA was used to analyze the effects of cultivar and plant health (symptomatic and asymptomatic) on virus gene copy number and yield parameters within a location. Mean differences were considered statistically significant at $p < 0.05$ based on Tukey's HSD post hoc test. All statistical analyses were performed using JMP Pro version 16 (SAS Institute).

Virus transmission using aphid vector and symptom monitoring

Attempts were made to establish the virus culture using aphids and to replicate the symptom expression under greenhouse conditions. Two cultivars (*Gossypium hirsutum* cv *DG 3799 B3XF* and *ST 4595 B3XF*) were grown in approximately 4 L pots containing greenhouse potting mix (Pro-MIX). Symptomatic plant samples collected from the field were tested for the presence of the virus. Plants with CLRDV were then transplanted into pots and maintained in a separate insect-proof greenhouse as the source of virus inoculum for transmission assay. To maintain a manageable size, plants were trimmed to an average height of 90cm, with bolls, flowers, and excess mainstem branches removed several times annually. Periodically, they were sampled and tested for CLRDV using RT-qPCR before being used as a virus source in transmission assays. Aphids (*Aphis gossypii* G) were collected from cotton or squash at the UGA research farm at Tifton, Tift County, GA and were reared on *G. hirsutum* cv. *DG 3799 B3XF*, grown from untreated seeds to avoid any chemical effects. The aphid colonies were monitored for parasitoids and transferred to healthy plants at regular intervals. An insecticidal soap solution (1% potassium salts of fatty acids) (Garden Safe) was intermittently used to manage any other occasional insect infestations. A minimum of 100 aphids were transferred to the source plants and allowed for 48-72 h of acquisition access period (AAP). A subset of the aphids was tested for CLRDV using qPCR and 50 aphids were used for transmission to test plants at the 4-5 true leaf stage. Eight

plants of cultivars *DG 3799 B3XF* and *ST 4595 B3XF* were infected with 50 viruliferous aphids for at least a 72h inoculation access period (IAP) on the test plants. Another set of five plants were infected with aviruliferous aphids as a control. After infection, the aphids were killed using acetamiprid (0.92 g/L, UPL), and the plants were monitored for symptom development. The transmission assay was replicated two times. The plants were tested for the presence of the virus after 25-30 days and monitored for symptom development. All samples were tested in qPCR as described earlier in section 2.4. All the plants for aphid transmission were grown and maintained in insect-proof BugDorm insect-rearing cages (W75 x D75 x H115 cm; MegaView) at 26°C with 16 h light/8 h dark period. All transmission experiments were performed during the summer and fall of 2023.

Virus infection using CLRDV cDNA clone and symptom monitoring

cDNA sequence of CLRDV isolate-GA53 (NCBI Accession: MT633122) was used to artificially synthesize the cDNA clone in VB Ultrastable (VB230214-1606uxu-pPBV: PiggyBac Gene Expression Vector) cloning host with kanamycin as antibiotic-resistant gene for clonal selection. CLRDV cDNA cassette was inserted between the plasmid's right and left borders, with an enhanced promoter (CaMV35s) upstream of 5’ UTR of the virus sequence. A hammerhead ribozyme sequence, derived from the satellite RNA sequence (Position 41-137; Accession No: M33001) of Subterranean clover mottle virus, was inserted at the 3′ end of the cDNA clone to facilitate *cis*-preferential cleavage for effective mRNA termination and enhanced infectivity (Jarugula et al. 2018). The recombinant clone was mobilized into *E. coli* stock (VectorBuilder).

The recombinant plasmid was purified from *E.coli* and subsequently introduced into *A. tumefaciens* LBA4404 (Lifeasible) using the freeze-thaw method (Weigel and Glazebrook, 2006). Recombinant transformed colonies were selected by culturing them at 28°C on Luria agar

(LA) plates supplemented with 25 µg/ml Rifampicin (Sigma-Aldrich) and 50 µg/ml Kanamycin (Sigma-Aldrich). Recombinant colonies were confirmed using the colony PCR by diluting isolated colonies in 10µL sterile water. 2µL of diluted colony is processed in end-point PCR process using high fidelity platinum Taq polymerase (Invitrogen) as per manufacturers protocol. The culture of a single colony of *A. tumefaciens* LBA4404 harboring the CLRDV cassette was prepared using the protocol described in Kavalappara et al. (2024). The prepared culture was delivered into young plants of two cultivars *DG 3615 B3XF* (n=5) and *ST 4595 B3XF* (n=5) at the two true leaf stage using two methods, 1) agroinfiltration of the culture using a needleless syringe into young cotyledon leaves on the abaxial surface and 2) microneedle-assisted agroinoculation on two true leaves as described in Kavalappara et al. (2024) along with pin-prick injury on petioles and stem parts. The experiment was performed in five biological replicates, three times using the cotyledon infiltration method and two times for microneedle-assisted agroinoculation method. Healthy controls were maintained for each replication. All plants were maintained in insect-proof BugDorm insect-rearing cages (47.4 cm × 47.5 cm × 93 cm; MegaView) under greenhouse conditions (24°-26°C with 16h daylight) and monitored for symptom development until 45 days post inoculation. Plants were tested at 8 and 16 DAI for virus presence in the inoculated and systemic leaf tissues using SB28F/R1 primer pair using end-point PCR (Sedhain et al. 2021).

Results

Symptomatology and disease monitoring

In 2022, symptoms of CLRDD were not observed at 30 DAP. However, as the plants developed, mild symptoms became evident at 60 and 90 DAP. These symptoms were in S_1 to S_2, which include stem and petiole reddening, bronzing of foliage, and apical leaf drooping. In some cases,

S_4 (severe wilting) was observed. In 2023, disease symptoms were indistinct, making it challenging to assess disease incidence or severity.

However, in 2024, a significantly high incidence of S_3 to S_5 symptoms was observed, mainly from late-planted cotton in southwest Georgia. Affected plants exhibited reddening or bronzing of foliage, maroon-colored stems and petioles, drooping, stunting, wilting, and in severe cases, complete wilting. These symptoms were predominantly observed in young plants of the cultivars *DG 3799 B3XF*, *DG 3615 B3XF*, *DG H959 B3XF*, and *NG 5430 B3XF* (Fig. 4.2A to J). The disease manifestation was in a random pattern and patchy in appearance, affecting clusters of 5–10 plants (Fig. 4.2A to E) or individual plants (Fig. 4.2F to J) within multiple rows. At the time of harvest, canopies appeared normal from the periphery (Fig. 4.3A). However, when observed within the field, tagged symptomatic plants exhibited partial recovery and remained sterile, stunted, and displayed stacked internodes. Some plants showed a delayed flowering stage, producing distorted flowers along with a few unopened or partially opened green bolls that were unharvestable (Fig. 4.3B to C). These plants persisted in the vegetative phase, occupying space and resources without contributing to the production of harvestable bolls (Fig. 4.3D to E). Occasionally, a small number of asymptomatic plants maintained normal height but exhibited boll abortion, with 10–30 unharvestable bolls per plant (Fig. 4.3F to G). In the Colquitt County dryland field, patches of revived symptomatic plants were observed that remained stunted in the vegetative phase were conspicuous among normally grown, mature, asymptomatic plants (Fig. 4.3H to I).

Disease incidence

In 2022, CLRDD incidence remains undetected up to 30 DAP but developed S_1 to S_2 symptoms at 60 and 90 DAP, varying across counties and cultivars (Table 4.1). In Worth County, S_1 to S_2

symptoms were observed in individual plants in selected cultivars at 60 DAP, but not at 90 DAP. Conversely, in Bulloch County, no symptoms were observed at 60 DAP, but S_1 to S_2 symptoms became apparent at 90 DAP (Table 4.1).

In 2024, S_3 and S_4 symptoms were prominent among a few cultivars, and disease incidence varied in each county, ranging from 12-43 percent (Table 4.2). Overall, the disease incidence was highest in Colquitt (irrigated; 43%), followed by Colquitt (dryland 37%), Worth (22%), and Turner (17%) counties. However, statistically, there was no significant difference in the mean percentage of disease incidence between the symptomatic cultivars at all locations (Table 4.2). The disease incidence of the asymptomatic control *DP 2038 B3XF* remained negligible (< 1%) across the monitored counties.

Validation and molecular assay (qPCR analysis) to determine viral gene copy number

In 2022, CLRDV was not detected in any of the tested samples (n=300) on 30 DAP, while it was detected from 64% of samples at 60 DAP and 88% at 90 DAP, irrespective of counties and cultivars analyzed (Table 4.3). Cycle threshold (Cq) values from 90 DAP, representing the final collected samples, were used to estimate viral gene copy numbers. Tukey's HSD post-hoc analysis indicated that the mean copy number was significantly different among cultivars across all counties. Viral gene copy number was generally lower in symptomatic cultivars, *DG 3615 B3XF* and *DG 3799 B3XF* compared to other cultivars at 90 DAP at all monitored locations except Colquitt County (Table 4.3).

In 2023, CLRDV was detected in only two samples from the 100 samples tested from two cultivars, *DG 3615 B3XF* and *DG H959 B3XF*, suggesting a lower prevalence. No statistically significant differences in yield were observed between the cultivars tested, yet boll density was lower in *DG 3615 B3XF* compared to *DG H959 B3XF* (Table 4.4).

In 2024, samples collected from tagged plants at 60 DAP were tested using RT-qPCR to evaluate and understand the difference between virus gene copy number in symptomatic vs asymptomatic plants. Statistical analysis revealed a significant difference in the values of mean copy number, boll density, and other yield parameters across the locations. Tagged symptomatic (S3 to S5) plants from symptomatic cultivars (*DG 3615 B3XF, DG H959 B3XF, DG 3799 B3XF, and NG 5430 B3XF*) exhibited a higher mean virus gene copy number ($\log_{10} > 4.2$) across different counties compared to their asymptomatic plants, which typically had a lower mean virus gene copy number ($\log_{10} < 4.2$). Furthermore, these tagged symptomatic individual plants exhibited negligible to zero boll density plant $^{-1}$, resulting in complete yield loss plant $^{-1}$. Nevertheless, asymptomatic individual plants of symptomatic cultivars had no significant reductions in the yield plant $^{-1}$ at different locations monitored (Table 4.5). Tagged individual plants of the asymptomatic cultivar *DP 2038 B3XF* exhibited a higher viral gene copy number than tagged symptomatic cultivars across the monitored locations. Notably, this cultivar had lower boll density plant $^{-1}$ and reduced yield plant $^{-1}$ compared to other selected cultivars in Colquitt County's dryland and irrigated fields. However, this trend was not observed in Worth and Turner Counties (Table 4.5). The yield per unit land area of all selected cultivars, including symptomatic (*DG 3615 B3XF, DG H959 B3XF, DG 3799 B3XF, and NG 5430 B3XF*), in the monitored fields exceeded the 2024 trial average of 1,398 kg/hectare in Worth and Turner Counties where the disease incidence was lower (Table 4.6). However, in Colquitt County, both dryland and irrigated fields experienced yield losses, with all selected cultivars falling below the 2024 trial averages, except for the asymptomatic cultivar *DP 2038 B3XF* in the irrigated field where the disease incidence was highest (Table 4.6).

To further confirm the presence of CLRDV in these plant samples, the partial capsid protein gene amplicon (141 bp) of thirteen samples was sequenced from the RT-qPCR assay. BLASTn analysis (NCBI) of these sequences revealed 98-100% identity with partial sequences of the CLRDV capsid protein gene regions (Table S1), confirming the virus's presence and the diagnostic assay's accuracy.

Virus transmission

Virus transmission using aphid vector and symptom monitoring

CLRDV-infected plants were uprooted from the field and established in the greenhouse. Initially established plants shed their leaves, and the newly emerged foliage displayed no symptoms as observed in the field. These plants were sustained for a year or two under constant trimming and maintenance in the greenhouse, but the virus titer eventually reduced as the plant grew older, and finally, the inoculum was lost. Individual aphids on these CLRDV-infected plants were tested after acquisition and confirmed for the virus presence using RT-qPCR. Post 25 days after infection (DAI) in the first transmission, CLRDV was detected only in two out of sixteen plants (Table S2). In the second replication, after another 25 days, CLRDV was detected in all the plants (n=14) except two. Notably, Cq values were >25 for *DG 3799 B3XF* and <25 for *ST 4595 B3XF*, indicating a lower viral load in *DG 3799 B3XF* and a higher viral load in *ST 4595 B3XF*. Despite the presence of the virus, all plants remained asymptomatic in the greenhouse.

Virus infection using CLRDV cDNA clone and symptom monitoring

Following agroinfiltration in cotyledon leaves of symptomatic cultivar, *DG 3615 B3XF* and asymptomatic cultivar *ST 4595 B3XF*, at 8 DAI, CLRDV was detected from 73 and 93 percent inoculated leaf tissues, and 60 and 67 percent in systemic tissues, respectively. At 16 DAI, the virus detection was 67 and 80 percent in inoculated tissues but decreased to 20 and 40 percent in

systemic tissues. In agroincoulation of true leaves in the two cultivars, virus detection at 8 DAI was 70 and 80 percent in inoculated tissues and 70 and 50 percent in systemic tissues. At 16 DAI, detection levels rose to 80 percent and 100 percent in inoculated tissues but dropped to 10 and 20 percent in systemic tissues (Table 4.7). No symptoms were observed even after 45 DAI in the inoculated plants under greenhouse conditions. Virus transmission using the agroinfiltration in cotyledon leaves and agroinoculations in the true two leaves using the microneedle method was not efficient for the virus establishment in the cotton host.

Discussion

Cotton is an important fiber crop contributing over $120 billion to the USA economy annually. Beyond its economic impact, it is essential for national fiber security and contributes substantially to the social and economic vitality of rural communities. Georgia is the second leading cotton producer in the USA, with 1.93 million bales of cotton lint and 548,000 tons of cotton seed produced in 2024 (NASS USDA 2024). CLRDV is an emerging viral pathogen causing CLRDD and has been associated with significant but variable yield losses in cotton production in the USA cotton belt. CLRDD symptoms are sporadic and inconsistent, with notable variability across different years and locations (Koebernick et al. 2024). Symptom severity ranged from mild leaf reddening, and severe dwarfing, to complete wilting of young plants, highlighting the complex interaction between CLRDV, the host plant, and possible abiotic factors. This study demonstrated that disease incidence is spatially and temporally variable in the commercial cotton fields monitored in GA. Yield limitations were observed in symptomatic individual plants of some cultivars but not in asymptomatic plants of the same cultivars.

CLRDV is transmitted by the cotton aphid (*A. gossypii*) and exhibits a limited host range among cultivated crops, with cotton as the primary host. CLRDV has been established in alternative hosts like multiple weed species and ratoon cotton, surrounding commercial cotton fields in Georgia and other regions (Pandey et al. 2022; Sedhain et al. 2021), potentially serving as virus reservoirs, facilitating the initial spread (Conner et al. 2021; Pandey et al. 2022; Sedhain et al. 2021). Cotton aphid is an annual pest in southeastern USA cotton production systems, and viruliferous aphids can spread the virus through multiple events of transmission during the crop season (Mahas et al. 2023). A strong correlation has been observed between aphid vector abundance and CLRDV spread, with the virus detected in a high percentage of plant samples tested (Mahas et al. 2022). Investigations into *A. gossypii* seasonal population dynamics and potential role in CLRDV transmission in field plots across GA and AL revealed population peaks in late-June to mid-July, underscoring its role in the primary dispersal of the virus (Heilsnis et al. 2023; Mahas et al. 2023). In Georgia, cotton is planted from late April to Mid-June, when the temperature and rainfall patterns during the initial 30–40 days are critical, as they influence the initial colonization of cotton aphids and the primary virus transmission events in cotton (Mahas et al. 2022). Environmental factors like light and temperature profoundly impact plant physiology, growth, and symptom development during viral infections (Jiang et al. 2023; Jiang and Zhou 2023). A prolonged dry spell with higher temperatures (30-32°C) than normal (29°C) and with no/low amounts of precipitation can influence aphid fecundity and cotton growth (Liu et al. 2021; Ritchie et al. 2004). In contrast, intermittent precipitation, natural enemies, and seasonal activity of the entomopathogenic fungus *Neozygites fresenii* limit vector establishment, thereby influencing CLRDV spread in GA. This highlights the role of

environmental factors in vector dynamics, disease transmission, and symptom expression in cotton.

The disease incidence and CLRDV-induced bronzing and wilting symptoms were minimal in 2022 across GA, including in the symptomatic cultivars, *DG 3615 B3XF* and *DG 3799 B3XF*, which are most likely to show symptoms. Notably, at 30 DAP, in the first week of June, no symptoms or aphid infestations were observed. This might be attributed to early planting dates (4th May to 24th May) enhancing the plant vigor before the primary virus spread and intermittent rains, which could have disrupted the successful establishment of aphid population and associated primary spread of the CLRDV (Knox 2022). In addition, CLRDV was also not detected in samples collected at 30 DAP, emphasizing the influence of virus association in symptom expression. Meanwhile, at 60 and 90 DAP, symptoms were mild, inconsistent, and only evident in some locations and cultivars, but with increased detection of CLRDV by 90 DAP (Table 4.1). This suggests multiple transmission events of the aphids, as described by Mahas et al. (2023). However, plants recovered from these mild symptoms with no yield limitation.

In 2024, high disease incidence was observed in symptomatic cultivars like *DG 3615 B3XF*, *DG 3799 B3XF*, *DG H959 B3XF*, and *NG 5430 B3XF*, with severe symptoms associated with CLRDD. The disease incidence was higher in both dryland and irrigated fields in Colquitt, Worth, Tift and Turner Counties in GA. Symptomatic plants in these cultivars exhibited symptoms ranging from reddening plus initial leaf drooping, loss of leaf turgor, severe wilting and advanced leaf chlorosis, and senescent plants (Parkash et al. 2021). While some senescent plants were in S_5, eventually died and did not recover. Most tagged plants did recover but remained stunted, displaying small, deformed leaves, highly stacked internodes, delayed bloom, and a few unopened/partially opened green bolls (Fig. 4.3B to C). This pattern suggests that the

virus may have significantly disrupted host tissues and their metabolic activities (Jiang and Zhou 2023). Earlier studies demonstrated that CLRDV infections cause characteristic reddening of leaves due to the decreased photosynthetic processes in leaves infected with CLRDV. Specifically, virus infections reduce stomatal conductance (g_s) and photosynthetic activity in individual leaves (Parkash et al. 2021). This, combined with a limited ability to dissipate energy at *PSII*, results in increased oxidative stress. In response, plants produce more anthocyanins as an antioxidant defense, which leads to the reddening or bronzing of cotton leaves infected with CLRDV (Parkash et al. 2023). Consequently, CLRDV infections in symptomatic plants are yield-limiting, as the disease can significantly reduce net photosynthetic rates (A_N) (Parkash et al. 2021). Therefore, all such symptomatic plants noted substantial or complete yield loss (Table 4.5). It is evident that plant growth stage plays a significant role in disease progression, as most symptomatic plants were infected during earlier growth stages and remained stunted until harvest. In contrast, the asymptomatic plants remained unaffected and exhibited a mean boll density ranging from 20-32 bolls plant^{-1}. Our results corroborate this hypothesis, that CLRDV infection is directly associated with severe wilting and bronzing and boll abortion symptoms observed in symptomatic cotton cultivars. The significantly higher viral gene copy numbers in majority of symptomatic plants, particularly in cultivars predisposed to symptom development, demonstrate a clear correlation between viral load and symptom severity. Mature asymptomatic plants of symptomatic cultivars in all the locations studied in 2024 presented with a lower mean virus gene copy number than symptomatic ones; a similar pattern was observed in 2022 in *DG 3615 B3XF* and *DG 3799 B3XF* in all selected locations except in Colquitt County. This response in matured asymptomatic plants may be attributed to plant resistance mechanisms, potentially reducing CLRDV incidence and the likelihood of yield loss. Interestingly, some

cultivars such as *DP 2038 B3XF* remained mostly asymptomatic, suggesting that certain cotton varieties may be resistant to symptom development, even though they could still be infected by and serve as hosts for CLRDV. The high mean viral gene copy number in *DG 3615 B3XF* and *DG 3799 B3XF* asymptomatic plants in Colquitt County can be attributed to elevated virus pressure and transmission events, as CLRDV was detected in most tagged plants as early as 60 DAP.

The yield impact of CLRDD is influenced by cultivars, locations, and plant growth stage. Consistent with studies by Parkash et al. (2021, 2023) on disease progression in *DG 3615 B3XF*, it was observed in 2024 that symptomatic cultivars exhibited CLRDD-associated symptoms (S3 to S4 stages), including leaf bronzing, stem reddening, leaf drooping, and severe wilting, particularly during the early vegetative growth stages. While some plants recovered from severe symptoms, they failed to produce harvestable bolls, resulting in complete per-plant yield loss. In contrast, yield losses were less severe when plants were infected at more mature stages of development (Mahas et al., 2022; Parkash et al., 2021). Despite the high disease incidence in some commercial cotton fields in GA and AL (80%–100%), no significant yield losses were reported (Mahas et al., 2022). However, in 2024, complete yield loss was observed in all tagged symptomatic plants of symptomatic cultivars. Interestingly, a few key questions that merit investigation here to understand this disease better include whether symptom expression is (*i*) a cultivar-specific response to viral infection, or (*ii*) solely a cultivar response to abiotic factors, or (*iii*) a result of both. A plausible hypothesis is that virulent aphids initially establish on a select few young cotton seedlings within the crop, facilitating efficient virus transmission and subsequent proliferation of virus in the young vascular tissues. This is supported by the high viral gene copy number observed in this study and may serve as a key factor in symptom

development, potentially interacting with abiotic conditions that further promote viral replication. This underscores the need for a systematic and comprehensive study into the genetic determinants governing resistance or susceptibility of the host plant during vegetative growth stages underlying these responses.

Notably, in Colquitt dryland field, where yield per unit land area was below 2024 trial average, extensive patches of vegetatively growing plants were observed in symptomatic cultivars at harvest (Fig. 4.4). Beyond the high virus incidence what factors may have exacerbated such crop canopy, and the potential reasons behind such effect in the Colquitt dryland field merit thorough investigation.

In bronze wilt, symptoms were associated with late-planted cotton than with early-planting dates (Phipps et al. 2001). In addition to bronzing and wilting, leaf dropping, fruit shedding, and necrosis of plants were also described in BWC (Gwathmey et al. 2001). Sometimes, it was observed that these symptomatic plants revive, and symptoms diminish (Gwathmey et al. 2001). In Georgia, this abnormality first appeared in 1997 in cotton planted in late May and early June. Symptoms appeared before bloom and continued through mid-August, with random patterns of wilting throughout fields (Brown 2000). Similar symptomatology is observed in the plants of symptomatic cultivars with late planting dates that are subsequently infected with CLRDV in the early growth stages in GA (Bag et al. 2021; Edula et al. 2023). CLRDD symptomatology and disease progression appeared in 2019-2020 and later in 2024 in research farms and commercial cotton fields in GA have striking similarity with the BW symptomatology (Fig 4.1; Bag et al. 2021; Edula et al. 2023). The severity, timing of BW symptoms, and the susceptibility of different cotton cultivars varied yearly (Creech and Fieber 2000; Phipps 2000). Similarly, CLRDD symptomatology among cultivars, severity, and timings

varied across the years and locations as studied since its first identification in 2019 in this region (Bag et al. 2021; Edula et al. 2023).

Recent evidence indicating the presence of CLRDV in the USA before its official identification in 2019 (Olmedo-Velarde et al. 2024) reinforces the hypothesis of its potential involvement in BWC. A comparable case is seen with grapevine red blotch virus (*Grablovirus Vitis*), which was detected in archival Viti's herbarium specimens collected in California as early as the 1940s, suggesting its presence long before its discovery in 2015 (Al Rwahnih et al. 2015). Similarly, the detection of CLRDV sequences in cotton RNA datasets predating its first official report in 2019 (Olmedo-Velarde et al. 2024) necessitates a reassessment of the possibility that the virus had already been present but remained undetected due to the lack of molecular diagnostic assays at the time. Following concerning reports of BWC from the early 1990s to the mid-2000s, researchers were puzzled by the varying severity of BWC across different cultivars. This observation led both private and public breeding programs to prioritize screening germplasm for BWC-like symptoms and to eliminate susceptible breeding lines, including those with common pedigree. This led to some degree of resistance or tolerance in the varieties developed since then, as evidenced by negligible reports of such anomalies until 2018. However, the reasons behind the re-emergence of these symptoms from 2018 to 2024 remain unclear. The sudden resurgence of symptoms in specific cultivars raises questions about the underlying factors beyond CLRDV, necessitating further investigation into the conditions that may have aggravated symptom expression.

Attempts to replicate field symptoms in cotton plants under greenhouse conditions were challenging and unsuccessful, using both vector (aphids) mediated transmission and agrobacterium mediated CLRDV-cDNA clone. This suggests that environmental factors in the

field conditions may significantly influence host-pathogen-vector interactions and symptom expression. Therefore, future studies that simulate field conditions under controlled environments could provide valuable insights. It is evident in this study that CLRDV is associated with cultivar-specific symptomatology, and individual plants of such cultivars infected at vegetative stages are prone to expressing severe symptoms and complete yield loss in symptomatic plants. Henceforth, we designate the bronzing and such severe wilting symptoms in cotton that follow the symptom progression as "CLRDV-induced bronze wilt." A thorough investigation and evaluation are essential to assess this disease before it becomes a significant threat to the cotton industry in the Southeastern USA.

Conclusion

CLRDD is currently a subliminal threat but could become a significant concern if the epidemic intensifies under favorable environmental conditions. The symptoms described for bronze wilt were indistinguishable from those of CLRDD, supporting the designation "CLRDV-induced bronze wilt." In symptomatic cultivars, early growth stage symptoms inflict yield constraints in individual plants but have a negligible impact on large-scale field production. However, in 2024, yield reductions were observed in symptomatic cultivars, including the asymptomatic control in Colquitt County, GA, where the disease incidence was highest. Additionally, a measurable yield drag was detected in *DP 2038 B3XF* in per plant yield analysis across two counties, suggesting a potential impact of CLRDD. The selection of cotton cultivars with high tolerance to CLRDV-induced bronze wilt, coupled with avoiding late planting, is recommended to mitigate yield impact. Future research should prioritize *i*. understanding cultivar-specific responses to CLRDV infection *ii*. investigating the role of abiotic factors in symptom expression *iii*. identifying genetic resistance mechanisms to enhance breeding programs. A proactive approach integrating

epidemiological surveillance, host resistance, and maintaining plant vigor will likely be critical in mitigating the impact of CLRDV-induced bronze wilt for sustainable cotton production and fiber security.

Author contributions

Conceptualization, S.R.E. and S.B.; methodology, S.R.E., S.B. and L.C.H.; software, S.R.E.; validation, S.R.E., L.C.H., P.M.R., J.L.S., P.W.C. and S.B.; formal analysis, S.R.E.; investigation, S.R.E., and S.B.; resources, L.C.H., P.M.R., R.C.K., P.W.C. and S.B.; data curation, S.R.E., L.C.H., P.M.R., J.L.S., P.W.C. and S.B.; writing—original draft preparation, S.R.E. and S.B.; writing—review and editing, S.R.E., L.C.H., P.M.R., J.L.S., P.W.C. and S.B.; supervision, L.C.H., P.M.R., J.L.S., R.C.K., P.W.C. and S.B.; project administration, S.B.; funding acquisition, S.B., L.C.H. and P.M.R.

References

Aboughanem-Sabanadzovic, N. (2019). First Report of Cotton Leafroll Dwarf Virus in Upland Cotton (*Gossypium hirsutum*) in Mississippi. *Plant disease, v. 103*(no. 7), pp. 1798--2019 v.1103 no.1797.

Al Rwahnih, M., Rowhani, A., & Golino, D. (2015). First Report of Grapevine red blotch-associated virus in Archival Grapevine Material From Sonoma County, California. *Plant disease*, *99*(6), 895-895.

Alabi, O. J., Isakeit, T., Vaughn, R., Stelly, D., Conner, K. N., Gaytán, B. C., Villegas, C., Hitzelberger, C., De Santiago, L., Monclova-Santana, C., & Brown, J. K. (2020). First Report of Cotton leafroll dwarf virus Infecting Upland Cotton (Gossypium hirsutum) in Texas. *Plant disease*, *104*(3), 998.

Albers, D. W., & Guthrie, D. (2001). Field incidence and description of bronze wilt symptoms. *Proceedings of the Beltwide Cotton Conferences*, 1, 104–105. National Cotton Council, Memphis, TN

Ali, A., & Mokhtari, S. (2020). First Report of Cotton Leafroll Dwarf Virus Infecting Cotton (*Gossypium hirsutum*) in Kansas. *Plant disease*, *104*(6), 1880.

Ali, A., Mokhtari, S., & Ferguson, C. (2020). First Report of Cotton Leafroll Dwarf Virus from Cotton (*Gossypium hirsutum*) in Oklahoma. *Plant disease, 104*(9), 2531.

Avelar, S., Schrimsher, D. W., Lawrence, K., & Brown, J. K. (2019). First Report of cotton leafroll dwarf virus associated with cotton blue disease symptoms in Alabama. *Plant disease, 103*(3), 592-592.

Avelar, S., Ramos-Sobrinho, R., Conner, K., Nichols, R. L., Lawrence, K., & Brown, J. K. (2020). Characterization of the complete genome and P0 protein for a previously

unreported genotype of cotton leafroll dwarf virus, an introduced polerovirus in the United States. *Plant Disease, 104*(6), 780–786

Bag, S., Roberts, P. M., & Kemerait, R. C. (2021). Cotton Leafroll Dwarf Disease: An Emerging Virus Disease on Cotton in the U.S. *Crops & Soils*, *54*(2), 18-22.

Bell, A. A., Cui, Y., Magill, C., Orta, H., & Hawkins, M. (1998). Agrobacterium wilt and bronzing: The new challenge. *Proceedings of the Beltwide Cotton Conferences*, 1, 136–137. National Cotton Council, Memphis, TN.

Bell, A. A. (1999). Agrobacterium bronzing and wilt: Cultivar reactions and effects of temperature. *Proceedings of the Beltwide Cotton Conferences*, 1, 117–120. National Cotton Council, Memphis, TN.

Bell, A. A. (2000). Role of Agrobacterium in bronze wilt of cotton. *Proceedings of the Beltwide Cotton Conferences*, 1, 154–160. National Cotton Council, Memphis, TN.

Bell, A. A., Nichols, R. L., Alber, D., Baird, R., Brown, S., Colyer, P., et al. (2002). *Bronze wilt of cotton* (Texas Cooperative Extension Bulletin, L-54122-02, pp. 1–4). Texas A&M AgriLife Extension. https://cotton.tamu.edu/wp-content/uploads/sites/27/legacy-files/Nematodes/bronzewilt.pdf

Brown, S. M. (2000). Bronze wilt field symptoms in Georgia. *Proceedings of the Beltwide Cotton Conferences*, 1, 151. National Cotton Council, Memphis, TN.

Cauquil, J., & Vaissayre, M. (1971). La "maladie bleue" du cotonnier en Afrique: Transmission de cotonnier à cotonnier par *Aphis gossypii* Glover. *Coton et Fibres Tropicales*, *26*, 463–466.

Conner, K., Strayer-Scherer, A., Hagan, A., Koebernick, J., Jacobson, A., Bowen, K., Sikora, E., Graham, S., & Brown, S. (2021). Cotton Leafroll Dwarf Virus. *ANR-2539. Alabama*

Cooperative Extension System. https://www. aces. edu/blog/topics/crop-production/cotton-leafroll-dwarf-virus.

Creech, J. B. (1999). Bronze wilt in the 1998 Mississippi cotton variety trials. In *Proceedings of the Beltwide Cotton Conferences* (*Vol. 1*, pp. 472–473). National Cotton Council, Memphis, TN.

Creech, J. B., & Fieber, J. C. (2000). Bronze wilt complex observations in the 1998 and 1999 Mississippi cotton variety trials. *Proceedings of the Beltwide Cotton Conferences*, 1, 151–152. National Cotton Council, Memphis, TN.

Edula, S. R., Bag, S., Milner, H., Kumar, M., Suassuna, N. D., Chee, P. W., Kemerait, R. C., Hand, L. C., Snider, J. L., Srinivasan, R., & Roberts, P. M. (2023). Cotton leafroll dwarf disease: An enigmatic viral disease in cotton. *Molecular Plant Pathology, 24*(6), 513–526.

El-Zik, K. M., Thaxton, P. M., & Creech, J. B. (2001). A pedigree analysis of cotton cultivars affected by bronze wilt. *Proceedings of the Beltwide Cotton Conferences*, 1, 105–107. National Cotton Council, Memphis, TN

Faske, T. R., Stainton, D., Aboughanem-Sabanadzovic, N., & Allen, T. W. (2020). First Report of cotton leafroll dwarf virus from upland cotton (*Gossypium hirsutum*) in Arkansas. *Plant disease*, *104*(10), 2742.

Gade, R., Tanwar, R., Jeyakumar, P., Kanwar, V., Gade, R., Tanwar, R., Jeyakumar, P., & Kanwar, V. (2013). Leaf reddening and its management in cotton. *Technical bulletin*, *30*, p16.

Gwathmey, C. O., Howard, D. D., Michaud, C. E., & Robison, E. F. (2001). The timing of bronze wilt appearance affects fruit retention. *Proceedings of the Beltwide Cotton Conferences*, 1, 108–111. National Cotton Council, Memphis, TN

Gwathmey, C. O., Newman, M. A., & Canaday, C. H. (2008). Does N or K nutrition affect bronze wilt in field-grown cotton? *Plant Health Progress, 9*(1).

Heilsnis, B., Mahas, J. B., Conner, K., Pandey, S., Clark, W., Koebernick, J., Srinivasan, R., Martin, K., & Jacobson, A. L. (2023). Characterizing the vector competence of *Aphis gossypii*, *Myzus persicae* and *Aphis craccivora* (Hemiptera: Aphididae) to transmit cotton leafroll dwarf virus to cotton in the United States. *J Econ Entomol*, *116*(3), 719-725.

Hull, R. (2013). *Plant virology* (5th ed., pp. 145-198). Academic Press.

Iriarte, F. B., Dey, K. K., Small, I. M., Conner, K. N., O'Brien, G. K., Johnson, L., Savery, C., Carter, E., Sprague, D., Nichols, R. L., Wright, D. L., Mulvaney, M. J., & Paret, M. (2020). First Report of Cotton Leafroll Dwarf Virus in Florida. *Plant disease*, *104*(10), 2744.

Jarugula, S., Gowda, S., Dawson, W. O., & Naidu, R. A. (2018). Development of infectious cDNA clones of Grapevine leafroll-associated virus 3 and analyses of the 5′ non-translated region for replication and virion formation. *Virology, 523,* 89-99.

Jiang, T., & Zhou, T. (2023). Unraveling the Mechanisms of Virus-Induced Symptom Development in Plants. *Plants (Basel)*, *12*(15).

Jiang, T., Du, K., Xie, J., Sun, G., Wang, P., Chen, X., Cao, Z., Wang, B., Chao, Q., Li, X., Fan, Z., & Zhou, T. (2023). Activated malate circulation contributes to the manifestation of light-dependent mosaic symptoms. *Cell Rep*, *42*(4), 112333.

Kavalappara, S. R., Milner, H., Konakalla, N. C., Morgan, K., Sparks, A. N., McGregor, C., Culbreath, A. K., Wintermantel, W. M., & Bag, S. (2021). High throughput sequencing-aided survey reveals widespread mixed infections of whitefly-transmitted viruses in cucurbits in Georgia, USA. *Viruses*, 13, 988.

Kavalappara, S. R., Riley, D. G., Cremonez, P. S. G., Perier, J. D., & Bag, S. (2022). Wild radish (Raphanus raphanistrum L.) is a potential reservoir host of cucurbit chlorotic yellows virus. *Viruses*, 14, 593.

Kavalappara, S. R., Devendran, R., Simmons, A. M., & Bag, S. (2024). Microneedle assisted delivery of the cloned components of cucurbit leaf crumple virus in yellow squash (Cucurbita pepo). *Journal of Virological Methods*, 329, 114992.

Knox, P. (2022, June). June 2022 weather and climate outlook. *UGA Cotton Team Newsletter*, University of Georgia College of Agricultural and Environmental Sciences, June.

Koebernick, J. C., Hagan, A. K., Zaccaron, M., Escalante, C., Jacobson, A. L., Bowen, K. L., Strayer-Scherer, A., Heilsnis, B., Brown, S., Sikora, E. J., Allen, T. W., Faske, T. R., Bourland, F., Greene, J. K., Huseth, A. S., Kelly, H., Kemerait, R. C., Kerns, D., Mulvaney, M., Price, P. P., Small, I., Taylor, S., Wang, H., & Conner, K. (2024). Monitoring the Distribution, Incidence, and Symptom Expression Associated with Cotton Leafroll Dwarf Virus in the Southern United States Using a Sentinel Plot System. *PhytoFrontiers™*, *4*(4), 671-681.

Liu, J., Wang, C., Desneux, N., & Lu, Y. (2021). Impact of Temperature on Survival Rate, Fecundity, and Feeding Behavior of Two Aphids, Aphis gossypii and Acyrthosiphon gossypii, When Reared on Cotton. *Insects*, *12*(6).

Mahas, J. B., Ray, C., Kesheimer, A., Conner, K., & Jacobson, A. L. (2023). Seasonal Dynamics of Aphid Flights and Cotton Leafroll Dwarf Virus Spread in Alabama. *Insects*, *14*(7), 604.

Mahas, J. W., Hamilton, F. B., Roberts, P. M., Ray, C. H., Miller, G. L., Sharman, M., Conner, K., Bag, S., Blythe, E. K., Toews, M. D., & Jacobson, A. L. (2022). Investigating the effects of planting date and Aphis gossypii management on reducing the final incidence of cotton leafroll dwarf virus. *Crop Protection*, *158*, 106005.

Mukherjee, A. K., Chahande, P. R., Meshram, M. K., & Kranthi, K. R. (2012). First report of Polerovirus of the family Luteoviridae infecting cotton in India. *New Disease Reports*, *25*(1), 22-22.

Nichols, R. L. (2001). Introduction to two newly recognized disorders of cotton – bronze wilt and seed rot. *Proceedings of the Beltwide Cotton Conferences*, 1, 104. National Cotton Council, Memphis, TN.

Olmedo-Velarde, A., Shakhzadyan, H., Norton, R., & Heck, M. (2025). First Report of Cotton Leafroll Dwarf Virus Infecting Upland Cotton Plants in Arizona. *Plant disease*, *109*(1), 237.

Olmedo-Velarde, A., Shakhzadyan, H., Rethwisch, M., West-Ortiz, M. J., Waisen, P., & Heck, M. L. (2024). Data mining redefines the timeline and geographic spread of cotton leafroll dwarf virus. *Plant disease*.

Padgett, B., Garber, B. W., Rea, W., & Price, J. (2004). Epidemiology of bronze wilt in Northeast Louisiana. *Proceedings of the Beltwide Cotton Conferences*, 105–110. National Cotton Council, Memphis, TN.

Pandey, S., Bag, S., Roberts, P., Conner, K., Balkcom, K. S., Price, A. J., Jacobson, A. L., & Srinivasan, R. (2022). Prospective Alternate Hosts of an Emerging Polerovirus in Cotton Landscapes in the Southeastern United States. *Viruses*, *14*(10), 2249.

Parkash, V., Sharma, D. B., Snider, J., Bag, S., Roberts, P., Tabassum, A., West, D., Khanal, S., Suassuna, N., & Chee, P. (2021). Effect of Cotton Leafroll Dwarf Virus on Physiological Processes and Yield of Individual Cotton Plants. *Front Plant Sci*, *12*, 734386.

Parkash, V., Snider, J. L., Sintim, H. Y., Hand, L. C., Virk, G., & Pokhrel, A. (2023). Differential sensitivities of photosynthetic processes and carbon loss mechanisms govern N-induced variation in net carbon assimilation rate for field-grown cotton. *J Exp Bot*, *74*(8), 2638-2652.

Phipps, B. J. (2000). Bronze wilt symptoms. *Proceedings of the Beltwide Cotton Conferences*, 1, 688. National Cotton Council, Memphis, TN.

Phipps, B. J., Phillips, A. S., & Tanner, B. J. (2001). Development of Bronze Wilt during the growing season. In P. Dugger & D. Richter (Eds.), *Proceedings of the 2001 Beltwide Cotton Conferences* (pp. 108-108). National Cotton Council.

Price, T., Valverde, R., Singh, R., Davis, J., Brown, S., & Jones, H. (2020). First Report of Cotton Leafroll Dwarf Virus in Louisiana. *Plant Health Progress*, *21*(2), 142-143.

Ritchie, G. L., Bednarz, C. W., Jost, P. H., & Brown, S. M. (2004, September). Cotton growth and development (Bulletin 1252). The University of Georgia College of Agricultural and Environmental Sciences, Cooperative Extension Service.

Sedhain, N. P., Bag, S., Morgan, K., Carter, R., Triana, P., Whitaker, J., Kemerait, R. C., & Roberts, P. M. (2021). Natural host range, incidence on overwintering cotton and diversity of cotton leafroll dwarf virus in Georgia USA. *Crop Protection*, *144*, 105604.

Tabassum, A., Bag, S., Roberts, P., Suassuna, N., Chee, P., Whitaker, J. R., Conner, K. N., Brown, J., Nichols, R. L., & Kemerait, R. C. (2019). First Report of Cotton Leafroll Dwarf Virus Infecting Cotton in Georgia, U.S.A. *Plant disease, 103*(7), 1803.

Tabassum, A., Bag, S., Suassuna, N. D., Conner, K. N., Chee, P., Kemerait, R. C., et al. (2021). Genome analysis of cotton leafroll dwarf virus reveals variability in the silencing suppressor protein, genotypes, and genomic recombinants in the USA. *PLOS ONE, 16*(7), e0252523.

Tatineni, S., & Hein, G. L. (2023). Plant Viruses of Agricultural Importance: Current and Future Perspectives of Virus Disease Management Strategies. *Phytopathology®, 113*(2), 117-141.

Thiessen, L. D., Schappe, T., Zaccaron, M., Conner, K., Koebernick, J., Jacobson, A., & Huseth, A. (2020). First Report of Cotton Leafroll Dwarf Virus in Cotton Plants Affected by Cotton Leafroll Dwarf Disease in North Carolina. *Plant disease, 104*(12), 3275.

Tsai, W. A., Brosnan, C. A., Mitter, N., & Dietzgen, R. G. (2022). Perspectives on plant virus diseases in a climate change scenario of elevated temperatures. *Stress Biol, 2*(1), 37.

U.S. Department of Agriculture, National Agricultural Statistics Service. (2024). *2024 State agriculture overview: Georgia*. https://data.nass.usda.gov/

Wang, A. (2015). Dissecting the molecular network of virus-plant interactions: the complex roles of host factors. *Annu Rev Phytopathol, 53*, 45-66.

Wang, H., Greene, J., Mueller, J., Conner, K., & Jacobson, A. (2020). First report of cotton leafroll dwarf virus in cotton fields of South Carolina. *Plant Disease, 104*(6), 2532.

Weigel, D., & Glazebrook, J. (2006). Transformation of Agrobacterium using the freeze-thaw method. *Cold Spring Harbor Protocols*, 2006, pdb.prot4666

Yan, Z., Kluepfel, D., Mueller, J., Jones, M., & Rothrock, C. (2002). Studies towards the identification of a causal agent of cotton bronze wilt. In *Proceedings of the Beltwide Cotton Conferences* (*Vol. 1*, pp. 105–106). National Cotton Council.

Tables

Table 4.1 Mean percentage of disease incidence in 2022 across the Counties monitored in Georgia in 60 and 90 DAP

2022	**Mean percent disease incidence**							
	Bulloch		**Colquitt**		**Cook**		**Worth**	
Cultivar	60 DAP	90 DAP	60 DAP	90 DAP	60 DAP	90 DAP	60 DAP	90 DAP
DG3615	0 A	1 B	1.33 A	0 A	0 A	3.33 A	2.67 A	1 A
DG3799	0 A	1 B	0 A	0.33 A	0 A	0.67 A	3 A	0.33 A
DP2038	0 A	5.33 AB	0 A	0 A	0.33 A	0 A	1.67 A	0 A
DP1646	0 A	9 A	0.67 A	0 A	0 A	0 A	5.33 A	0 A
ST 4595	0 A	8.33A	0 A	0 A	0 A	0.33 A	2.33 A	0 A
DF	4	4	4	4	4	4	4	4
Prob > F	1	0.0118	0.4609	0.4609	0.4609	0.0923	0.5204	0.4609

Note: Different letters indicate significant differences ($P < 0.05$) based on Tukey's honestly significant difference test.

Table 4.2 Mean percentage of disease incidence in 2024 across the Counties monitored in Georgia at 60 DAP

2024	**Mean percent disease incidence**			
	Colquitt dry	**Colquitt irrigated**	**Turner**	**Worth**
Cultivar	**60 DAP**	**60 DAP**	**60 DAP**	**60DAP**
DG3615	24 AB	32.67 A	17 A	21.67 A
DG3799	38 A	42.33 A	16 A	16.67 AB
DGH959	20 AB	41 A	16 A	21.33 A
NG5430	36.67 A	42.7 A	17.3 A	12 AB
DP2038	0 B	0 B	0.3 A	0 B
DF	4	4	4	4
Prob > F	0.0338	0.0055	0.0434	0.0264

Note: Different letters indicate significant differences ($P < 0.05$) based on Tukey's honestly significant difference test.

Table 4.3 2022 CLRDV monitoring: Mean Cq, viral gene copy number, and yield parameters (boll number, seed cotton weight, lint and seed weight) assessment

County	Cultivar	Mean viral gene copy Number	Mean boll density	Mean Seed cotton weight (gm)
Bulloch	DG3615 B3XF	3.91 BC	12.13 AB	60.13 AB
	DG 3799 B3XF	1.74 C	12.2 AB	61.24 AB
	DP 2038 B3XF	4.08 ABC	12.79 AB	50.07 AB
	DP 1646 B2XF	6.41 A	10.53 B	38.25 B
	ST 4595 B3XF	5.08 AB	17.07 A	68.38 A
	DF	4	4	4
	P>F	<.0001	0.0527	0.0290
Colquitt	DG3615 B3XF	6.51 AB	9.67 A	50.43 A
	DG 3799 B3XF	5.12 B	6.67 A	36.18 A
	DP 2038 B3XF	6.87 A	7.13 A	36.24 A
	DP 1646 B2XF	6.8 A	7.6 A	33.43 A
	ST 4595 B3XF	5.04 B	6.4 A	33.94 A
	DF	4	4	4
	P>F	0.0021	0.2694	0.237
Cook	DG3615 B3XF	4.41 B	8.27 B	40.08 A

	DG 3799 B3XF	2.17 C	11.73 AB	60.48 A
	DP 2038 B3XF	6.68 A	12.8 AB	54.88 A
	DP 1646 B2XF	6.07 AB	12 AB	48.47 A
	ST 4595 B3XF	6.91 A	15.4 A	63.73 A
	DF	4	4	4
	P>F	<.0001	0.0965	0.2332
Worth	DG3615 B3XF	3.7 C	12.6 A	61.79 A
	DG 3799 B3XF	4.88 B	15.07 A	65.39 A
	DP 2038 B3XF	7.24 A	16.73 A	76.64 A
	DP 1646 B2XF	6.69 A	19.53 A	77.14 A
	ST 4595 B3XF	6.81 A	17 A	76.98 A
	DF	4	4	4
	P>F	<.0001	0.1487	0.577

Note: Different letters indicate significant differences ($P < 0.05$) based on Tukey's honestly significant difference test.

Table 4.4 2023 CLRDV assessment: Mean quantification cycle, viral gene copy number, and yield parameters (boll number, seed cotton weight, lint and seed weight)

Cultivar	Mean quantification cycle	Mean viral gene copy number	Mean boll density	Mean seed cotton weight in gm
DGH959B3XF	33.62 A	2.64 A	11.52 A	58.17 A
DG3615B3XF	33.28 A	2.94 A	9.18 B	46.00 A
DF	1	1	1	1
Prob > F	0.338	0.4506	0.0470	0.0530

Note: Different letters indicate significant differences ($P < 0.05$) based on student t-test

Table 4.5 2024 CLRDV monitoring: Mean viral gene copy number, and yield parameters (boll number, seed cotton weight, lint, and seed weight) assessment in Symptomatic and asymptomatic plants**.**

2024		**Mean viral gene copy number**		**Mean boll density**		**Mean seed cotton weight (gm)**	
County	Cultivar	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
Colquitt Dryland	DG3615 B3XF	5.18 B	1.08 E	0.13 D	31.87 AB	0.6 D	162.1 A
	DG3799 B3XF	4.62 BC	2.64 CDE	0 D	25.8 ABC	0 D	131.9 AB
	DGH959 B3XF	5.06 BC	3.18 BCDE	0 D	20.6 BC	0 D	88.6 BC
	NG5430 B3XF	4.07 BCD	2.11 DE	0.13 D	35.46 A	0.4 D	175.8 A
	DP 2038 B3XF	-	8.06 A	-	14.53 C	-	63.3 C
	DF	8		8		8	
	P-value	<.0001		<.0001		<.0001	
Colquitt Irrigated	DG3615 B3XF	5.52 AB	1.63 C	0.6 D	29.3 BC	2.36 D	144.93 B
	DG3799 B3XF	5.21 B	3.71 B	0 D	34.1 AB	0 D	158.53 B
	DGH959 B3XF	5.32 B	4.73 B	0.5 D	35.3 AB	1.79 D	164.89 AB
	NG5430 B3XF	5.37 B	3.94 B	0.1 D	39.9 A	0.05 D	213.12 A
	DP 2038 B3XF	-	7.27 A	-	20.2 C	-	84.70 C
	DF	8		8		8	

	P-value	<.0001		<.0001		<.0001	
Worth	DG3615 B3XF	4.49 BC	3.36 BCD	0.80 B	19.40 A	3.32 B	97.84 A
	DG3799 B3XF	4.6 BC	2.77 D	0.53 B	20.20 A	2.14 B	97.87 A
	DGH959 B3XF	4.83 B	3.85 BCD	1.07 B	18.67 A	1.80 B	89.52 A
	NG5430 B3XF	4.67 BC	3.16 CD	0.33 B	21.47 A	0.78 B	108.28 A
	DP 2038 B3XF	-	7.5 A	-	22.40 A	-	98.35 A
	DF	8		8		8	
	P-value	<.0001		<.0001		<.0001	
Turner	DG3615 B3XF	5.58 AB	3.78 CD	0.60 C	29.13 A	2.78 C	164.85 A
	DG3799 B3XF	5.21 BC	2.97 DE	0 C	20 B	0 C	107.12 B
	DGH959 B3XF	5.9 AB	3.43 D	0.47 C	31.93 A	0.96 C	165.03 A
	NG5430 B3XF	4.58 BCD	1.61 E	0.07 C	24.67 AB	0.13 C	136.34 AB
	DP 2038 B3XF	-	7.19 A	-	27 AB	-	117.63 B
	DF	8		8		8	
	P-value	<.0001		<.0001		<.0001	

Note: Different letters indicate significant differences ($P < 0.05$) based on Tukey's honestly significant difference test.

Table 4.6 2024 On-farm cotton variety trial yield results of selected varieties in Georgia

Variety	Overall lint yield (kilogram/hectare)			
	Colquitt dryland	**Colquitt irrigated**	**Worth**	**Turner**
DG 3615 B3XF	766	1162	1556	1593
DG 3799 B3XF	759	1039	1551	1754
DG H959 B3XF	694	1058	1401	1525
NG 5430 B3XF	824	974	1569	1814
DP 2038 B3XF	1028	1770	1732	1613

Note: Lint yield values have been rounded to the nearest whole number

Table 4.7 Virus infection using CLRDV cDNA clone and symptom monitoring

	Variety	Days after inoculation	No of plants detected with CLRDV/ samples tested
Agroinfiltration (needle less syringe)	DG 3615 B3XF-Inoc	8	11/15
	DG 3615 B3XF -Syst	8	9/15
	ST 4595 B3XF-Inoc	8	14/15
	ST 4595 B3XF-Syst	8	10/15
	DG 3615 B3XF -Inoc	16	10/15
	DG 3615 B3XF -Syst	16	3/15
	ST 4595 B3XF-Inoc	16	12/15
	ST 4595 B3XF-Syst	16	6/15
Agroinoculation (Microneedle)	DG 3615 B3XF -Inoc	8	7/10
	DG 3615 B3XF -Syst	8	7/10
	ST 4595 B3XF-Inoc	8	8/10
	ST 4595 B3XF-Syst	8	5/10
	DG 3615 B3XF -Inoc	16	8/10
	DG 3615 B3XF -Syst	16	1/10
	ST 4595 B3XF-Inoc	16	10/10
	ST 4595 B3XF-Syst	16	2/10

Footnote: Agroinfiltration is performed on cotyledon leaves with a needleless syringe; Agroinoculation is performed with microneedle in true leaves and pin pricking on the petiole and stem.

Table 4.8 (Supplementary Table S1) Validation of qPCR molecular assay by sequencing

Sample	County	Variety	Sample type	Sequence match @NCBI	% match
1	Colquitt -Dryland	DG3799B3XF	Symptomatic	CLRDV-MT559404.1	100
2	Colquitt -Dryland	DG3799B3XF	Symptomatic	CLRDV-MT559404.1	100
3	Colquitt -Dryland	DG3799B3XF	Symptomatic	CLRDV-MT559404.1	100
4	Colquitt -Dryland	DG3799B3XF	Asymptomatic	CLRDV-MT559404.1	100
5	Colquitt -Dryland	DG3799B3XF	Asymptomatic	CLRDV-MT559404.1	100
6	Colquitt -Dryland	DP2038B3XF	Asymptomatic	CLRDV-MT559404.1	100
7	Colquitt -Dryland	DP2038B3XF	Asymptomatic	CLRDV-MT559404.1	100
8	Colquitt -Dryland	Positive control	Under greenhouse conditions	CLRDV-MT559404.1	100
9	Colquitt -Dryland	NG5430B3XF	Symptomatic	CLRDV-MT559404.1	100
10	Colquitt -Dryland	NG5430B3XF	Symptomatic	CLRDV-MT559404.1	99.29
11	Colquitt -Dryland	NG5430B3XF	Asymptomatic	CLRDV-MT559404.1	100
12	Colquitt -Dryland	NG5430B3XF	Asymptomatic	CLRDV-MT559404.1	98.58
13	Colquitt -Dryland	DG3615B3XF	Symptomatic	CLRDV-MT559404.1	99.29
14	Colquitt -Dryland	DG3615B3XF	Symptomatic	CLRDV-MT559404.1	100

Table 4.9 (Supplementary Table S2) CLRDV detection using endpoint PCR during 25 DAI, and 50 DAI plants

Plant no	Variety	PCR reaction 25 DAI/1st round using qPCR	PCR reaction 50 DAI/2nd round infection)
1	DG 3799 B3XF	-	+
2	DG 3799 B3XF	-	+
3	DG 3799 B3XF	-	+
4	DG 3799 B3XF	-	+
5	DG 3799 B3XF	-	+
6	DG 3799 B3XF	-	-
7	DG 3799 B3XF	-	-
8	DG 3799 B3XF	-	+
9	ST 4595 B3XF	-	+
10	ST 4595 B3XF	-	+
11	ST 4595 B3XF	+	+
12	ST 4595 B3XF	+	+
13	ST 4595 B3XF	-	+
14	ST 4595 B3XF	-	+
15	ST 4595 B3XF	-	+
16	ST 4595 B3XF	-	+

Figures

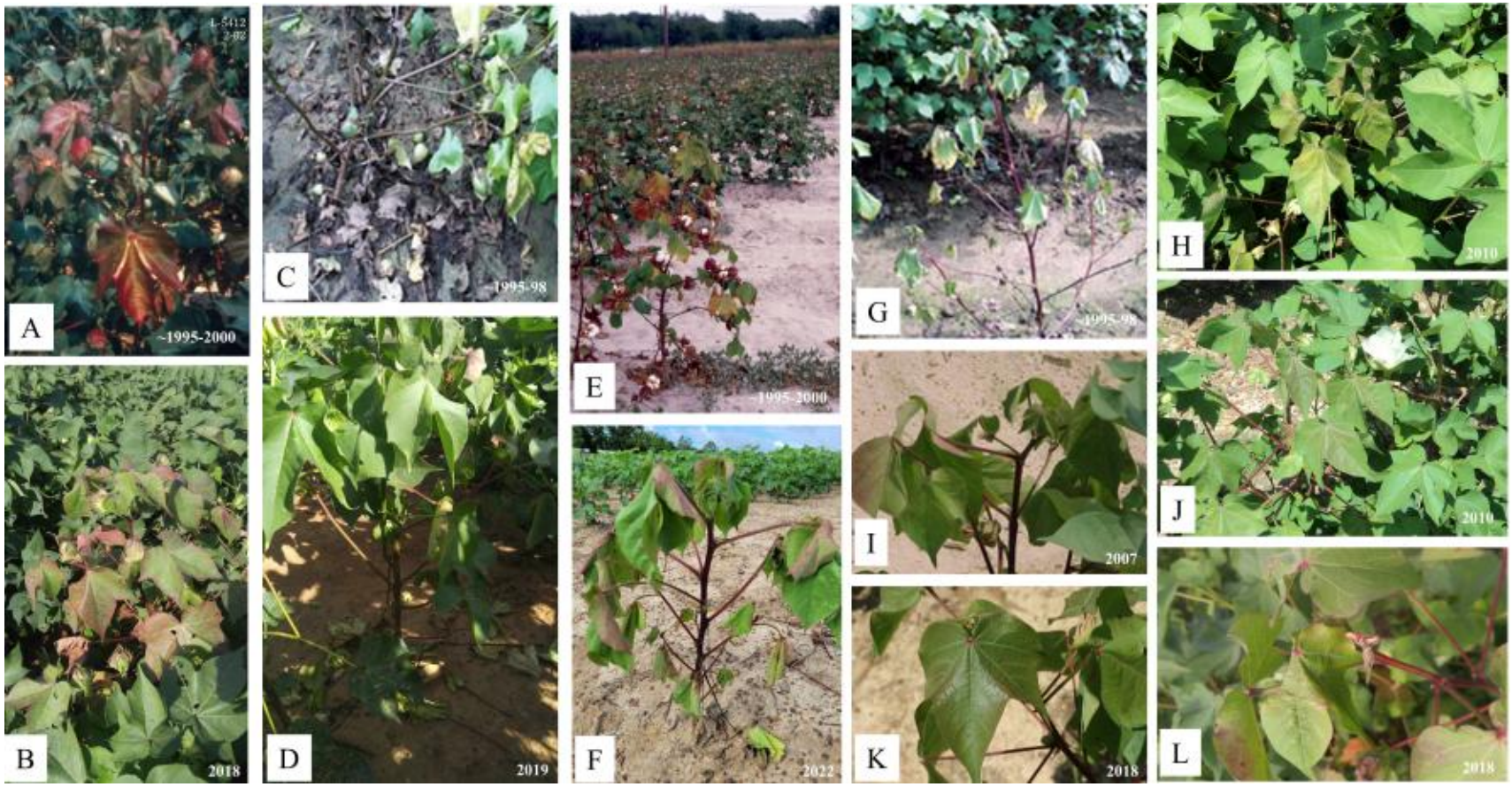


Fig 4.1: Symptoms of bronze wilt (BW) as described in the late 1990s and early 2000s closely mirror the symptoms that have been observed and associated with cotton leaf roll dwarf disease (CLRDD) in the US starting in 2019. Similarity of the most common symptoms between BW and CLRDD is illustrated here. (A-B) reddening, bronzing and drooping of leaves; (C-D) leaves defoliation

and boll abortion; (E-F-G) plant at the edge of the rows exhibiting leaf bronzing, wilting, marooning of stem and petiole symptoms; (H-L) deep red or maroon color of stem and petiole, along with leaf reddening or bronzing, inverted V shaped leaves. Image (A&E) adopted from Bell et al. 2002; (C&G) adopted from Mississippi State University Extension Bulletin (https://extension.msstate.edu/content/bronze-wilt); (B) September 2018, Sumter County GA; (D) July 2019, Dooly County, GA; (F) August 2022, Tift County GA; (I) August 2007, Tift County GA; (H &J) August 2010, Tift County, GA; (K-L) August 2018, Tift County GA. These symptoms were monitored and observed during 2018-2024. Photo Credits: B, D, F, K & L by Sudeep Bag and H-J shared by Steve Brown, Auburn University.

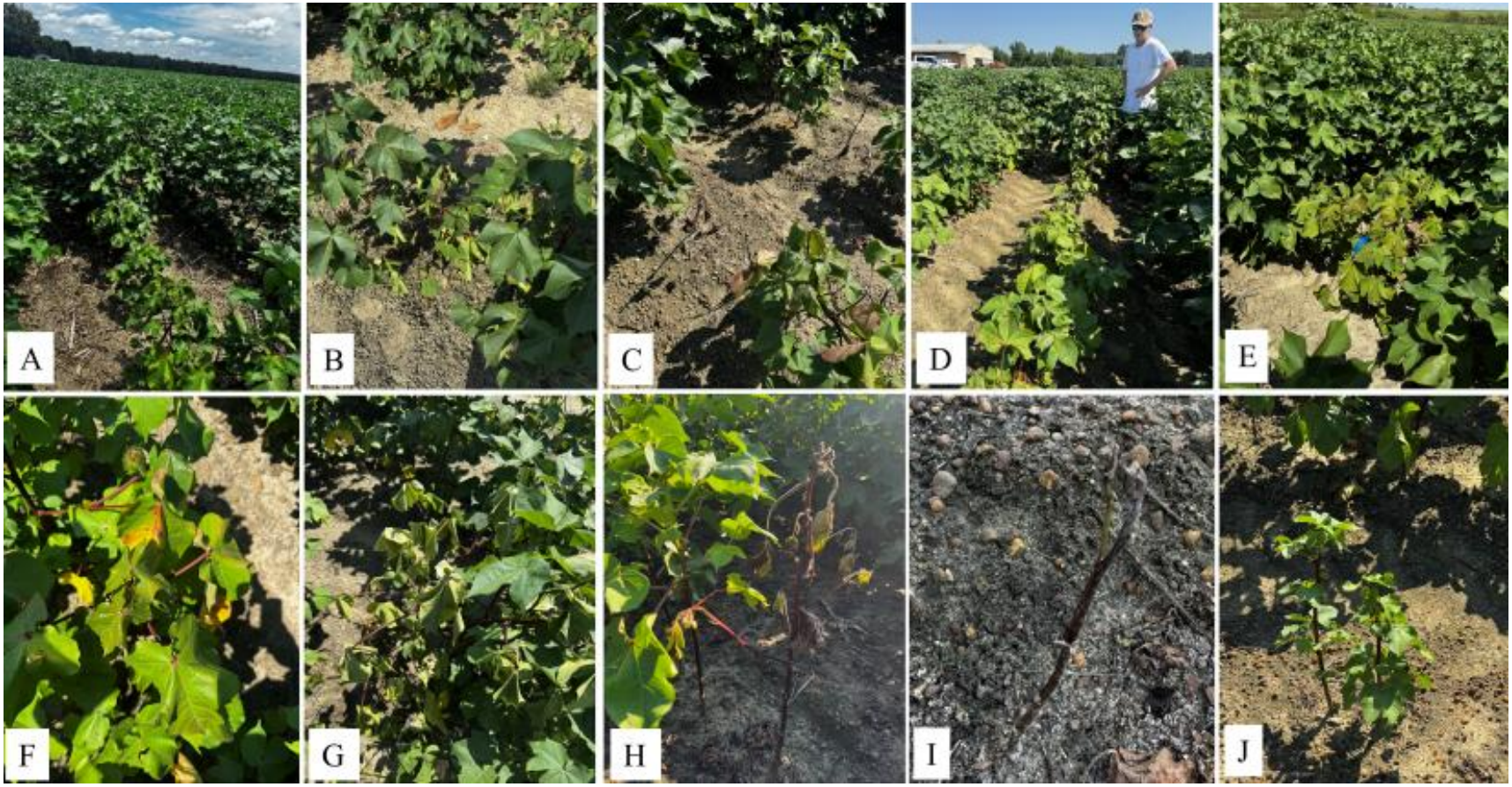


Figure 4.2 Cultivar responses and symptom observed at 60 days after showing: Top row (A-E), representative images of the random pattern and distribution of CLRDV-induced bronze wilt symptoms in the Fall of 2024. (A) Colquitt County dryland NG5430, (B) Stages S_3 to S_4, DG3615 plants in between the rows in Worth County, (C) Stages S_3 to S_5, DGH959 plants in between the rows, (D) Stages S_3 to S_5, DG3799 Plants in Colquitt County irrigated field, person standing for height reference (5 ft 9 inches), (E) Stage S4,

DGH959, in Turner County. The bottom row (F-J) represents individual plants at different stages: (F) Stage S_3, NG5430 plants in between the rows Colquitt irrigated County, (G) Stage S_5, DG3615 plant in Colquitt Irrigated County, (H) Stage S5, DG3799 plant in Worth County, (I) DG3799 dead plant in Worth County, (J) Recovery in DGH959, Colquitt County Irrigated.

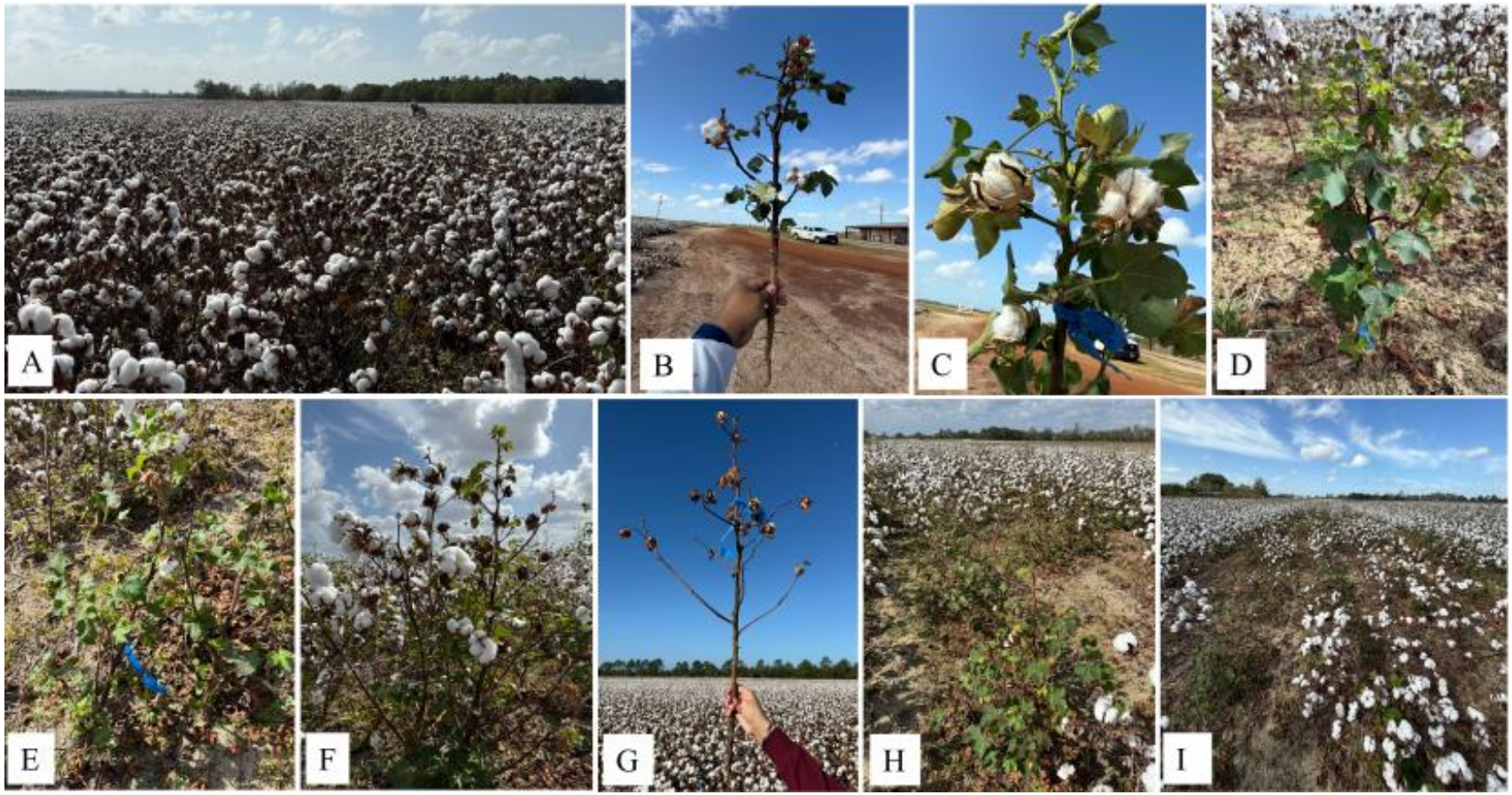


Figure 4.3 Cultivar responses at harvest during late Fall of 2024: Top row (A-D): CLRDV induced bronze wilt symptoms during harvest. (A) Normal appearance of the fields at on-farm variety trial's (OVT's) from the periphery, (B) Stunted plant in vegetative state with late bloom, (C) Plant with stacked internodes, unopened green bolls and partially opened unharvestable bolls in Colquitt County, irrigated field; (D) Plant from Turner County showing stunting and vegetative growth at harvest. The bottom row (E-H) Colquitt County

dryland field, (E) Stunted plants in vegetative stage with no harvestable bolls, (F) Boll abortion in asymptomatic plant remains green, (G) Aborted and unharvestable bolls from a tagged plant in Worth County, (H) vegetative patches of the symptomatic recovered plants at single spot, (I) vegetative patches at multiple spots. Symptomatic plants are tagged using a blue ribbon.

Figure 4.4 Drone image showing vegetative patches at time of harvest in Colquitt County dryland field in the Fall of 2024, exhibiting potential yield impact.

CHAPTER 5

CONCLUSIONS

This study was aimed at enhancing our understanding of emerging viral diseases in cotton, with a primary focus on the cotton leafroll dwarf virus (CLRDV), the causative agent of cotton leafroll dwarf disease (CLRDD), which poses a significant threat to cotton production in Georgia (GA). Additionally, CLRDV's role in inducing bronzing and severe wilting in specific cultivars was investigated and explored the potential contribution of endogenous viral elements (EVEs) to terminal abortion in cotton. Through a combination of molecular and epidemiological analyses, this research has provided valuable insights into the virus's biology, transmission mechanisms, and involvement in disease progression, contributing critical information for the development of targeted control strategies to mitigate CLRDV's impact on cotton production.

Among the significant contributions of this study, one was the characterization of the silencing suppressor ORF P0 gene in CLRDV strains found in the southeastern USA, particularly in Georgia. The P0 protein plays a crucial role in viral pathogenicity by suppressing host RNA silencing mechanisms. Earlier analysis revealed genetic variations in Georgia isolates that closely resemble South American resistance-breaking strains, especially the amino acid substitution at position 72 (I72V) in the F-box domain of the P0 protein. These mutations are believed to influence symptom severity and viral virulence, though their exact role remains to be fully elucidated. A more in-depth analysis of ORF0 of various CLRDV isolates was performed with the sequence information available as of 10 January 2023 (n = 76) using MEGA-XI and iTOL, which revealed four major clades primarily based on geographical location. The Asian

clade included three isolates: two infecting Malvaceae species in South Korea and one found in soybean aphids in China. South American cotton isolates formed three clades: (i) “typical” CLRDV genotypes (mainly from Brazil), (ii) “atypical” genotypes (mostly from Argentina), and (iii) two “atypical” isolates from cacao trees in Brazil. The most diverse clade contained 53 North American sequences, with U.S. isolates from cotton (n=43) and weeds (n=10) forming distinct clusters. This segregation suggests genetic recombination, potentially expanding host range and aiding virus establishment in ecosystems. The same set of sequences was also used to study the amino acid substitution rates and evolutionary pattern of CLRDV using BEAST v. 1.10.4 and Tracer v. 1.7.1. Further analysis showed that the amino acid substitution rate of P0 protein from different hosts combined was lower (2.194×10^{-3} substitutions per site per year) compared to P0 from weed species (6.313×10^{-10} substitutions per site per year). Genome-wide mutations and recombination in viruses allow them to jump from one host to another. The higher amino acid substitution rate in cultivated and non-cultivated host species emphasizes the potential threat to cotton cultivation and other crops in the future.

During the 2023 growing season in Georgia, USA, the intermittent appearance of terminal abortion in young cotton plants with no apparent cause raised concerns among industry and academic scientists alike. Therefore, the cotton samples were assessed for multiple aspects to address these concerns. In further examination of EVEs in the cotton genome, seeds, and seedlings, no correlation was found between their presence and terminal abortion. Furthermore, cotton EVEs’ episomal forms and messenger RNA (mRNA) transcripts were detected in both symptomatic and asymptomatic plants collected from cotton fields and in cotton seeds and seedlings. The high-throughput sequence analysis of symptomatic and asymptomatic plant tissue samples revealed near-complete EVE-Georgia (EVE-GA) sequences closely related to

caulimoviruses. The analysis of EVE-GA's putative open reading frames (ORFs) compared to cotton virus A and endogenous cotton pararetroviral elements (eCPRVE) revealed their similarity in putative ORFs 1–4. However, in the ORF 5 and ORF 6 encoding putative coat protein and reverse transcriptase, respectively, the sequences from EVE-GA have stop codons similar to eCPRVE sequences from Mississippi. In silico mining of the cotton genome database using EVE-GA as a query uncovered near-complete viral sequence insertions in the genomes of *G. hirsutum* species (~7 kb) but partial sequences in *G. tomentosum* (~5.3 kb) and *G. mustelinum* (~5.1 kb) species. No significant yield difference was observed between symptomatic and asymptomatic plants of the two varieties evaluated in the experimental plot. This study emphasizes the need for future research on EVE sequences, their coding capacity, and any potential role in host immunity or pathogenicity.

In CLRDV's tissue tropism study, the highest viral titer was found in the top branches of infected plants. This localization enhances accurate sample selection for diagnostics and highlights the virus's preference for actively growing tissues, impacting symptom development and disease progression. CLRDV was first observed in the USA in 2017 and continues to be a concern for the cotton industry. Field diagnosis is difficult due to symptom overlap with abiotic stress and inexplicable disorders, such as bronze wilt, demanding reliable molecular detection for accurate assessment. In this study, cotton cultivars in Georgia on-farm variety trials were monitored over two years to evaluate host response to CLRDV infection. In 2024, a significant surge in bronze wilt-like symptoms was observed in producer fields, particularly in symptomatic cultivars (cultivars prone to severe wilting) (*DG 3615B3XF, DG 3799 B3XF, DG H959 B3XF, NexGen (NG) 5430 B3XF).* In these symptomatic cultivars, it was observed that symptomatic plants had a significantly higher viral gene copy number compared to the asymptomatic plants of

the same cultivar, and the symptoms described for bronze wilt were indistinguishable from those of CLRDD. Therefore, the bronzing and severe wilting symptoms in CLRDV-infected cotton plants that follow the symptom progression are designated as “CLRDV-induced bronze wilt.” In symptomatic cultivars, early growth stage symptoms inflict yield constraints in individual plants but have a negligible impact on large-scale field production. However, in 2024, yield reductions were observed per unit land area in symptomatic cultivars, including the asymptomatic control in Colquitt County, GA, where the disease incidence was highest. Additionally, a measurable yield drag was detected in *DP 2038 B3XF* in per plant yield analysis across two counties, suggesting a potential impact of CLRDD. Currently, CLRDD is a subliminal threat but could become a significant concern if the epidemic intensifies under favorable environmental conditions. The selection of cotton cultivars with high tolerance to CLRDV-induced bronze wilt, coupled with avoiding late planting, is recommended to mitigate yield impact. Future research should prioritize *i*. understanding cultivar-specific responses to CLRDV infection *ii*. investigating the role of abiotic factors in symptom expression *iii*. identifying genetic resistance mechanisms to enhance breeding programs. A proactive approach integrating epidemiological surveillance, host resistance, and maintaining plant vigor will likely be critical in mitigating the impact of CLRDV-induced bronze wilt for sustainable cotton production and fiber security.

The knowledge generated has been shared with growers, stakeholders, and the broader scientific community, aiding in informed decision-making and the development of sustainable management practices for CLRDV. These findings will contribute to the long-term sustainability of cotton production by informing breeding programs, disease surveillance, and extension initiatives aimed at mitigating the impact of this emerging viral disease in the USA.

PROFESSIONAL AND PUBLIC OUTREACH

1. Edula, S. R., Hand, L. C., Snider, J. L., Chee, P. W., Kemerait, R. C., Roberts, P. M., & Bag, S. (2023, November 5–8). *Aphid-transmitted cotton leafroll dwarf virus and host response in Georgia* [Abstract]. Entomological Society of America 2023: Recent Progress in Ecology, Diversity, Virus Transmission, and Management Session, Gaylord National Resort & Conference Center, National Harbor, MD, USA.
2. Edula, S. R., Hand, L. C., Snider, J. L., Chee, P. W., Kemerait, R. C., Roberts, P. M., & Bag, S. (2024, March 5–6). *Characterization of Caulimovirid-like sequences in upland cotton (Gossypium hirsutum L.) in Georgia, USA* [Abstract]. Oral presentation at the 3-Peat Georgia Association of Plant Pathologists 2024, Savannah-North Historic District, GA, USA.
3. Edula, S. R., Hand, L. C., Snider, J. L., Chee, P. W., Kemerait, R. C., Roberts, P. M., & Bag, S. (2025, March 3–5). *CLRDV-induced bronzewilt: Host response and yield loss in Georgia cotton cultivars* [Abstract]. Oral presentation at the Georgia Association of Plant Pathologists 2025, Brasstown Valley Resort, Young Harris, GA, USA.
4. Edula, S. R., Hand, L. C., Snider, J. L., Chee, P. W., Kemerait, R. C., Roberts, P. M., & Bag, S. (2025, March 9–13). *Is enigmatic cotton leafroll dwarf virus a threat to the cotton crop in Georgia, USA?* [Abstract]. Oral presentation at the Southern Division APS-2025, Gainesville, FL, USA.
5. Edula, S. R., Hand, L. C., Snider, J. L., Chee, P. W., Kemerait, R. C., Roberts, P. M., & Bag, S. (2024). *Characterization of Caulimovirid-like sequences in upland cotton*

(Gossypium hirsutum L.) in Georgia, USA [Abstract]. *Phytopathology*, *114*(11S), P-429. https://doi.org/10.1094/APS-PH24-250

6. Edula, S. R., Hand, L. C., Snider, J. L., Chee, P. W., Kemerait, R. C., Roberts, P. M., & Bag, S. (2024, October 25). *Characterization of Caulimovirid-like sequences in upland cotton (Gossypium hirsutum L.) in Georgia, USA* [Poster symposium]. Conference Center Tifton Campus, Tifton, GA, USA.
7. Edula, S. R., Hand, L. C., Snider, J. L., Chee, P. W., Kemerait, R. C., Roberts, P. M., & Bag, S. (2025, January 29). *CLRDV-induced bronze wilt: Host response and yield loss in Georgia cotton cultivars* [Oral presentation]. Georgia Cotton Commission 2025 Annual Meeting, UGA Tifton Campus Conference Center, Tifton, GA, USA.
8. Participated in the Cotton and Peanut Research Field Day to disseminate scientific findings to growers on September 4, 2024, in Tifton, Georgia.