Creation of an Infectious Clone of Cotton leafroll dwarf virus (CLRDV), a fluorescent clone of CLRDV, and the transmission determinants of CLRDV by *Aphis gossypii*

by

Wilson Christopher Clark

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science in Plant Pathology

Auburn, Alabama
August 5, 2023

Keywords: Cotton leafroll dwarf virus, infectious clone, green fluorescent protein, transmission, *Aphis gossypii*

Approved by

Dr. Kathleen Martin, Chair, Assistant Professor Department of Entomology and Plant Pathology
Dr. Alana Jacobson, Associate Professor Department of Entomology and Plant Pathology
Dr. Sang Wook Park, Associate Professor Department of Entomology and Plant Pathology

Abstract

Cotton leafroll dwarf virus is an emerging pathogen in the United States. This disease has caused significant damage to the cotton (*Gossypium hirsutum L.*) production in other countries. In order to avoid these significant losses in the United States, a strong molecular understanding of the virus should be developed in order to inform disease and vector management. Two of the most important aspects to understand about viral diseases is their replication and their transmission. The first aim of this thesis is to further our understanding of CLRDV replication and systemic spread in plants by developing an infectious clone of the virus. Two infectious clones were developed, a wild-type virus which infects both cotton and *Nicotiana benthamiana* systemically, and a virus clone which produces Green Fluorescent Protein on a protein produced by the subgenomic RNA. This CLRDV-GFP clone was imaged by fluorescent microscopy and was detected infecting cotton plants systemically 28 days post infiltration. The second aim is to identify the transmission determinants of CLRDV. *Agrobacterium tumefaciens* expression vectors containing the CLRDV coat proteins P3 and P3-5 were produced with GFP, Red Fluorescent Protein, Myc and Flag tags. The major coat protein tagged with GFP (GFP-P3) was infiltrated into cotton leaves and aphids were allowed to feed on the leaf, GFP was then successfully detected in the leaf and aphid by Western blot. The results show that cotton is a suitable host for both agrobacterium expression of proteins and as a method of feeding aphids these expressed proteins. Future work is required to determine which viral protein is responsible for CLRDV transmission by the cotton-melon aphid (*Aphis gossypii* Glover).

Acknowledgments

My completion of this work could not have been accomplished without the help of my fellow graduate students, undergraduate student workers, and supervisor. I would like to thank Dr. Martin for her expert guidance and endless patience with this project. I also thank my lab mates Alec Mayfield, Abdelaal Shehata, and Aasma Sharma, who provided technical guidance and empathy when experiments of mine did not work as intended. Abdelaal and Alec were both incredibly helpful in teaching me how to use the fluorescent microscope. As for undergraduates, I would like to especially thank Rachel Livingston and Nicholas Mueller for their help with this project. Rachel has an incredible eye for detail and the persistence necessary in order to work in this virus system, and without her help on the diagnostic PCRs I would not have gotten anywhere near as far on this project. Nick Mueller was especially helpful, and I commend him for staying consistent on a time-consuming and inconvenient experiment. The data collected from that experiment is not presented in this thesis, howevver the time he put into it on my account should be recognized.

I'd like to make a special note for Kassie Connor and Kellie Bourguignon, who were kind enough to allow me to tour their lab at the Plant Diagnostic Lab in Auburn. They provided incredible advice and instruction on how to manage the diagnostics of CLRDV, especially in regard to eliminating contamination from the diagnostic PCRs.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

CLRDV	Cotton leafroll dwarf virus
CP	Coat Protein
dpi	Days post infiltration
GFP	Green Fluorescent Protein
ORF	Open Reading Frame
mRFP	Red Fluorescent Protein monomer
PCR	Polymerase Chain Reaction
RT	Read-Through
RT*	truncated Read-Through
VpG	Viral genome-linked protein

Chapter 1 -
Literature Review

1.1 Cotton

Upland Cotton (*Gossypium hirsutum* L) is a plant in the Malvaceae family whose fiber is harvested to be used in textiles. *Gossypium* is a genus with species across the world (Viot & Wendel, 2023). *Gossypium hirsutum* (upland cotton) and *G. barbadense* L (Pima cotton) are polyploid species with upland cotton being the primary species of cotton grown around the world (Viot & Wendel, 2023). The top cotton producing countries of the world are India, China, the United States, Pakistan, and Brazil (Khan et al., 2020). In the United States 97% of cotton production uses upland cotton varieties (ERS, 2022). *Gossypium barbadense* produces longer fibers than upland cotton, is used for luxury goods, and is only 3% of cotton grown in the US (ERS, 2022). Cotton production is concentrated in the Southern United States, informally known as the Cotton Belt from Virginia to California (ERS, 2022). In 2022 in Alabama, 430,000 acres of cotton were harvested producing over 39 million pounds of cotton and 236,000 tons of cottonseed (Service, 2017). The cotton produced in the state was worth over $346 million (Service, 2017). Cotton producers in Alabama deal with many pests and pathogens that reduce yields, however this review will focus on a recently introduced polerovirus that began infecting cotton in Alabama in 2016, Cotton leafroll dwarf virus (CLRDV).

CLRDV has severely impacted cotton in other nations, especially Brazil, and its introduction to the US is a cause for concern. One of the best methods of combating a crop disease is to develop a molecular understanding of how it works in the field. A useful tool for understanding how viruses infect their hosts is an infectious clone, which has already been

developed for CLRDV strains in other countries and for related viruses. This is an important and logical first step, as it provides a readily available source of inoculum to researchers studying viral protein function or testing resistant cultivars. One application for an infectious clone is testing viral transmission under controlled circumstances. Transmission of the virus from one plant to another is a key part of the virus life cycle, and understanding the underlying mechanisms can lead to novel control methods. This thesis combines the two goals of developing an infectious clone of CLRDV from Alabama isolates and determining the proteins responsible for its transmission.

1.2 History of Cotton leafroll dwarf virus

CLRDV is the causal agent of Cotton Blue Disease (CBD). This disease was first described in the Central African Republic in 1949, with cotton plants showing viral symptoms in conjunction with infestations of the cotton-melon aphid, *Aphis gossypii* Glover (Cauquil, 1977; Corrêa et al., 2005). During the years between 2003-2010, symptoms of CBD were observed in Brazil, East Timor, India and Thailand and the molecular characterization of these viruses revealed the presence of an aphid-transmitted polerovirus then named Cotton leafroll dwarf virus (CLRDV) (Corrêa et al., 2005; Mukherjee et al., 2012; Ray et al., 2016; Sharman et al., 2015). Symptoms of CLRDV in South America include stunting due to shortened internodes, leaf rolling and an intense green color of foliage (Corrêa et al., 2005). CLRDV initially was devastating to cotton production, causing up to 80% losses in susceptible varieties with inadequate aphid control (Silva et al., 2008).

There are two strains of CLRDV in South America. The original strain (accession number GU167940)(Corrêa et al., 2005; Delfosse et al., 2013; Distéfano et al., 2010) caused

severe damage in Brazilian cotton production in the late 1990s to early 2000s (Galbieri et al., 2017). CBD-resistant cultivars were bred and utilized to control the disease. In 2006 symptoms were recorded in CBD resistant cultivars, and the new strain of CLRDV became known as atypical (Agrofoglio et al., 2017; Silva et al., 2008). The atypical strain of CLRDV was notably less damaging to yield losses (Galbieri et al., 2017), but was economically important because it could break resistance bred into the cotton for the original, now called, typical strain (Agrofoglio et al., 2017). In 2016, farmers in Baldwin County, Alabama first noticed symptoms of disease in their cotton fields, and in 2017 the symptoms returned, and samples were collected (Avelar et al., 2019). This was the first recorded instance of CLRDV in Alabama, and since then the virus has been detected in cotton fields from Texas to North Carolina (See Figure 1.1). Although its initial report (Avelar et al., 2019) cited fairly significant damage, in the years since its discovery its range has increased but its impact has not been as dramatic as South American CLRDV epidemics. The Cotton Board has reported 0 to low numbers of bales lost to this virus in the past six years (Crop Protection Network, 2019; Lawrence, 2022). One reason for this could be the significant difference in symptomology of American CLRDV variants compared to CLRDV in South America. As previously discussed, South American strains of CLRDV cause more severe and noticeable symptoms compared to CLRDV in the US (Agrofoglio et al., 2017; Delfosse et al., 2013; Ramos-Sobrinho et al., 2021; Tabassum et al., 2021).

CLRDV found in Alabama (CLRDV-AL) is notably different from both the typical and atypical isolates found in South America (Tabassum et al., 2021). While CLRDV-AL does not produce the dramatic symptoms of CLRDV-typical and CLRDV-atypical in South America, it can still cause significant yield losses. Symptoms on seedling and young cotton can often be attributed to other issues, such as nutrient deficiency, insect herbivory, or pesticide damage.

CLRDV in the United States is characterized by distorted leaves, reddening petioles and leaf midveins, and stunted growth (Avelar et al., 2020; Edula et al., 2023; Tabassum et al., 2021). Symptoms depend on the growth stage the cotton was infected, and at times the virus can cause cryptic infection with no symptoms. Yield loss is caused by stunted plants infected early in the season or mature plants that produce elongate terminal whips. These either produce no bolls at the top of the plant or will produce squares or blooms and then drop them (Avelar et al., 2020). The host range of CLRDV in America includes 23 identified species in 16 plant families, and the virus was identified in different weeds across multiple seasons (Sedhain et al., 2021). CLRDV overwinters in these weedy species or in remaining cotton stalks post harvest, leading to further infection the next season (Ramos-Sobrinho et al., 2021; Sedhain et al., 2021).

Another major difference described by Tabassum et al 2021, is the genetics of CLRDV strains from South America compared to the US. In particular, the viral silencing suppressor protein P0 has the most genetic differences between virus isolates (Avelar et al., 2020; Cascardo et al., 2015; Tabassum et al., 2021). As P0 acts to block plant defense by knocking down both the plant's viral defense response and important post-translational gene regulation, symptom expression may be tied to this gene (Avelar et al., 2020; Patton et al., 2020). Although the American CLRDV strains are significantly different from both the South American typical and atypical strains and group into their own clade, Tabassum notes that the P0 sequence of US CLRDV contains the same amino acid sequence that South America's atypical strain to break resistance. The prevailing theory is that US CLRDV is an introduced virus due to its sequence similarity to the South American atypical (Avelar et al., 2020; Tabassum et al., 2021). Thus, the CLRDV in the United States presents mild symptoms and is difficult to track.

The result is that CLRDV in the United States has spread rapidly throughout the cotton belt and has the potential to cause significant damage to cotton production. The incidence and severity of CLRDV is affected by a number of factors, including the hosts available, the virus itself and the environment (Figure 1.2). The virus in the United States has already been identified as becoming quite diverse (Ramos-Sobrinho et al., 2021; Tabassum et al., 2021). Numerous weed species have been identified as hosts (Sedhain et al., 2021; Tabassum et al., 2021), so there are many reservoirs for the disease to spread from. The direct effects of the environment on CLRDV infection is not known, but for another polerovirus system hotter temperatures correlate to higher rates of infection (Patel & Parmar, 2023) while also increasing vector populations (Ebert & Cartwright, 1997). . In order to better understand CLRDV infection, I will now cover what is known about replication and transmission for the genus it belongs to, *Polerovirus*.

1.3 Poleroviruses

Polerovirus as a genus is named after the type species *Potato leafroll virus*. These viruses are positive sense, single-stranded RNA viruses encapsidated in icosahedral particles (Sõmera et al., 2021). They encode as many as 9 proteins with many overlapping open reading frames. ORFs 6 and 7 have not been identified or are indicated to be present in CLRDV, so they will not be discussed further. The genomic RNA is capped by a Viral genome-linked protein (VpG), and its 3’ end contains no poly A tail nor tRNA structure seen in other viruses. Poleroviruses are typically low titer in plants and infections are limited to the phloem (Olmedo-Velarde et al., 2023, Peter et al., 2009). The 5’ end of the genome encodes for proteins involved in replication, including the RNA silencing-suppressor (P0), the viral genome-linked protein (P1), and the RNA polymerase (P1-2). There are multiple proteins involved in movement through the plant, including P3a, P4, and the soluble form of P3-5. The virion is composed of two proteins, P3 and

a truncated P3-5 (see Figure 1.3). I will now cover the replication of the virus within the cell and its movement into new plants via insect transmission.

1.3.1 Replication

Once the virus first uncoats inside a host cell, it begins by transcribing its replication machinery. On the 5' end of the genomic RNA there are three genes, P0, P1 and P1-2. P0 encodes the viral silencing suppressor and is produced by leaky scanning of the ribosome (Avelar et al., 2020; Cascardo et al., 2015; Tabassum et al., 2021). Leaky scanning occurs due to a strand of RNA containing multiple start codons, but the ribosome may miss one and move 5'-3' to the next. The other AUG start codon on the 5' end is ORF1, which encodes P1. P1 provides a variety of functions related to replication (Prüfer et al., 1999), including the VpG and a serine protease that releases the VpG from the rest of the protein. The last protein produced at the 5' end is P1-2, which is a -1 frameshift readthrough of P1 and encodes the RNA polymerase (Sõmera et al., 2021). This virus also contains a subgenomic RNA, which encodes the major and minor coat proteins are produced (P3 and P3-5 respectively), along with the movement protein P4 and a small non-AUG start protein P3a which is also necessary for systemic infection (Smirnova et al., 2015).

The replication of poleroviruses requires the production of negative sense copy of the genomic positive sense RNA, which is then used to produce more copies of genomic RNA and subgenomic RNAs. Typically a polerovirus will enter a plant cell by the probing and feeding of a viruliferous aphid, after which it will uncoat and expose the RNA genome to host ribosomes (Figure 1.4). The host ribosomes read off P0, P1 and P1-2. The virus then begins replicating, with the RNA polymerase encoded by P1-2 making negative sense RNA complements to the genomic positive sense RNA. The polymerase either makes full length negative copies, or RNA

transcription is stopped early in order to produce the subgenomic RNA. This is achieved by the 5' end of the positive sense RNA molecule curling around and binding to the intergenic region between ORF 1-2 and 3a, which prevents the RNA polymerase from reading further (Steckelberg et al., 2020). Once negative sense genomic and subgenomic RNAs are produced, the RNA polymerase then begins transcribing them back into numerous copies of positive sense RNA that can either be translated into proteins or packaged into virions. The positive sense subgenomic RNAs produced similarly utilize leaky scanning in order to produce P3a, the major coat protein P3, minor coat protein P3-5 and the movement protein P4. Genomic positive sense RNAs are then encapsidated by P3s and truncated RT*. The virions then either remain in the cell to be ingested by a feeding aphid, or move to surrounding plant cells via the plasmodesmata through the action of P3a, P4 and P3-5.

1.3.2 Transmission

Once a plant's phloem cells have been infected by a polerovirus, the virus can be picked up by aphids feeding on infected tissues. Poleroviruses are circulative, non-propagative viruses (Sõmera et al., 2021), meaning that they pass through the gut, hemolymph and salivary glands in order to be inoculated into a new host. While inside the vector they do not uncoat or replicate (Brault et al., 2007). Depending on the virus, they may be transmitted by a few to single species of aphids (Brault et al., 2007) or whiteflies (Ghosh et al., 2019). Aphids pierce the plant phloem with their stylet and uptake the cell contents, including any virus particles. The virus then moves through the esophagus and enters the midgut. The virus must then pass through the gut cell lining and enter the hemolymph, but different viruses have different points of entry through the gut tissues. Generally, poleroviruses exit the digestive tract at the midgut while luteoviruses enter the hemolymph by the hindgut (Brault et al., 2005), though there are exceptions to this rule such as

Cucurbit aphid-borne yellows virus (Reinbold et al., 2003). From the hemolymph, the virions must travel to the accessory salivary glands (Brault et al., 2007; Brault et al., 2010; Reinbold et al., 2003) where they likely enter through receptor-mediated endocytosis (Brault et al., 2007). This represents two locations in which the virus must be moved through membranes by transcytosis: the gut membrane and the salivary gland membrane (see Figure 1.5). The mechanism that allows for transcytosis of the virions has been theorized to be clathrin-mediated (Brault et al., 2007) and may use proteins specific to each vector and virus (Yang et al., 2008) along with ephrin receptors (Mulot et al., 2018).

ORF 3 and ORF 3-5 produce three protein products, each of which provide vital functions during infection. These three products are P3 (the major capsid), a full length P3-5 (RT), and a truncated P3-5 (RT*) (See Figure 1.6). P3 contains a P3 binding domain as well as a RNA binding domain (Brault et al., 2003), which helps the virus encapsidate. P3-5 is produced by an amber stop at the end of P3, as well as two sequences promoting read-through immediately after the amber stop and further into ORF 5 (Bruyere et al., 1997). The C-terminal proteins encoded by ORF 5 are attached to P3 by a sequence containing many prolines that acts as a molecular hinge (Bahner et al., 1990; Brault et al., 2005; Bruyere et al., 1997; Schiltz et al., 2022). P3-5 is either expressed and remains as the full-length protein, or it is further processed after translation into a truncated form. P3-5 as the full RT is likely involved in systemic movement of virions in plant cells, as well as being responsible for the phloem-limitation of poleroviruses (Boissinot et al., 2014; Peter et al., 2009). The RT* version of P3-5 has the C-terminus of the protein cleaved off, and is incorporated into the virion due to the P3 binding domain with the remaining C-terminal end acting as a spike on the virion (Boissinot et al., 2014; Brault et al., 2005; Brault et al., 1995; Schiltz et al., 2022; Wang et al., 1995). It has been found

that poleroviruses can encapsidate without the RT* domain, but the RT* is likely required for efficient uptake of the virus by aphids (Brault et al., 2007; Bruyere et al., 1997; Filichkin et al., 1994). Poleroviruses do not replicate in their vector and the viral RNA is never exposed to insect proteins. The only viral proteins available to interact are P3 (major coat protein) and the RT* (minor coat protein). Either or both of these proteins must be responsible for interacting with host proteins, because the virion must pass through both to be transmissible. The receptors for passage through the gut and the salivary glands appear to be different, as a mutation in the BYWV RT* domain could reduce transcytosis through the gut membrane but not affect salivary gland transcytosis. Once inside the salivary glands, the virus is egested with the saliva into the plants that the aphid feeds on. Poleroviruses are not passed to offspring, so the aphid must feed on an infected plant in order to become viruliferous. The primary method of long-distance spread for these viruses then are alatae, or winged aphids. These winged aphids acquire the virus from an infected host plant, then fly to a new host and inoculate the virus into the new plant.

1.4 Aphis gossypii

CLRDV is spread primarily by *Aphis gossypii* (Heilsnis et al., 2023), though other aphid species have been implicated as possible vectors (Mahas et al., 2023). Aphids are small phytophagous insects in the family Homoptera (Ebert & Cartwright, 1997). *Aphis gossypii* is known as the cotton-melon aphid, and is a cosmopolitan vector found worldwide in tropical and temperate areas. While this aphid can be found on over 900 plant species and causes damage either by direct feeding or as a vector of plant viruses (Ebert & Cartwright, 1997).

1.4.1 Lifecycle

Aphis gossypii can reproduce parthenogenetically (Carletto et al., 2010). Only in colder climates or in preparation for overwintering do aphid populations lay eggs (Ebert & Cartwright,

1997). During the summer, these aphids reproduce with live young parthenogenetically (Kring, 1959). *Aphis gossypii* have four nymphal instars and can produce three different adult morphs (Ebert & Cartwright, 1997). Apterae are wingless morphs and are typically dark green, while alatae develop wings over their nymphal stages and can fly. There appears to be no single factor that causes *A. gossypii* to produce alate morphs, although the number of alatae produced can be influenced by crowding conditions and poor host quality (Ebert & Cartwright, 1997). A third morph is produced at temperatures of 15 and 25 degrees Celsius possibly due to crowding conditions or by unfavorable host plant conditions, and is called a yellow dwarf due to its color and size (Watt & Hales, 1996). The yellow morph is smaller than the dark green morph and does not reproduce as much (Ebert & Cartwright, 1997; Watt & Hales, 1996), and is theorized by Ebert & Cartwright (1997) to be an overcrowding response.

CLRDV in South America was managed based on breeding efforts in cotton, instead of a focus on aphid control. This is partly because of aphid biology. Due to their large host range which includes many weeds (Ebert & Cartwright, 1997; Ma et al., 2019; Pandey et al., 2022) and mobile alate forms, it is hard to prevent them from entering a cotton field. Chemical control is also fairly ineffective, because their generation time and quick population growth means that they adapt and develop insecticide resistance fairly quickly (Carletto et al., 2010). While chemicals may be applied to reduce aphid populations, pesticide application does not cause a reduction in CLRDV incidence (Mahas et al., 2022).

In the US, cotton aphids are also naturally controlled by an entomophagous fungi *Neozygites fresenii*, which reduces aphid infestations below threshold levels in cotton production in the Southeastern US (Abney et al., 2008; Ebert & Cartwright, 1997; Sedhain et al., 2021; Steinkraus et al., 1991). While this fungus prevents aphid populations from growing too large

and causing significant damage to cotton production, CLRDV complicates the matter of aphid control. An alate aphid can inoculate a cotton plant with CLRDV in as few as 15 minutes, though infection is more successful if the aphid feeds for up to 24-48 hours (Heilsnis et al., 2023). There is also evidence that *A. gossypii* is not the sole vector of CLRDV, as virus spread has been detected before and after *A. gossypii* infestations occurred (Mahas et al., 2023). While *N. fresenii* can control aphid populations and reduce the number of viruliferous aphids flying to new plants, it will not prevent aphids from spreading the virus. As previously mentioned, the primary method of control for CLRDV in South America was by the use of infectious clones to help accelerate resistance breeding. It is likely that the US response to CLRDV will also mainly focus on breeding resistance into cotton cultivars.

1.5 Infectious clones of poleroviruses

Infectious clones of poleroviruses have been developed as early as 1995. Poleroviruses have a small size and positive sense RNA genomes which can be made into a clone due to its similarity to already present mRNA in the plant cell. One limitation of polerovirus clones is that their icosahedral particles limit the size of their genome, so any genetic inserts such as a molecular marker must be small enough to not interfere with genome packaging. The most popular method of creating and using polerovirus infectious clones is by a binary agrobacterium system. *Agrobacterium tumefaciens* is an important bacterial plant pathogen that creates tumors in plant tissues using its Ti plasmid system (Escobar & Dandekar, 2003), which cuts out a region of its plasmid and inserts it into a host plant cell (Gelvin, 2003). This system has been repurposed and simplified to allow labs to produce their own agrobacterium expression systems for expression of heterologous sequences in plants (Gelvin, 2003). The Ti plasmid has been split into two plasmids, with one serving as the insert vector containing a gene of interest (or in the

cases of infectious clones, entire sequences of viruses) flanked by the Left and Right Borders and a helper plasmid (Lee & Gelvin, 2008). The left and right borders are conserved sequences of 25 nucleotides that are recognized by the *Agrobacterium* molecular machinery encoded by the helper plasmid (Horsch & Klee, 1986). The helper plasmid contains the genes necessary for the gene of interest to be lifted out from between the left and right borders and placed into a plant's genome. This system greatly simplifies vector construction, as the vector with the left and right borders can be manipulated while the helper plasmid can remain constant, as long as the two plasmids are compatible (Lee & Gelvin, 2008). A full review of the mechanisms behind *Agrobacterium tumefaciens* plasmids is provided in Gelvin (2003). The important features of agrobacterium transformation is that it inserts a chosen DNA construct into the plant genome, which is then under the control of a viral DNA promoter such as Cauliflower mosaic virus (CaMV) 35s (Amack & Antunes, 2020). The DNA promoter will cause the plant to transcribe the DNA insert into mature mRNA. Barring any splicing issues, this transcription will produce a full length and fully processed mRNA of the insert. If the insert is the full sequence of an RNA virus, then this should result in a viral infection. Once the genomic RNA of the virus is present in the cell, host ribosomes will begin translating the virus' own replication machinery and it should begin replicating as in a native infection.

Numerous infectious clones have been made from polerovirus species. The infectious clones of poleroviruses includes Beet western yellows virus (now renamed to Turnip yellows virus TuYV) (Brault et al., 1995; Leiser et al., 1992), Cucurbit aphid-borne yellows virus (CABYV) (Prüfer et al., 1995), Potato leafroll virus (PLRV) (Franco-Lara et al., 1999), Melon aphid-borne yellows virus (MABYV) (Liu et al., 2022), Beet mild yellowing virus (BMYV) (Stevens & Viganó, 2007), and both typical and atypical strains of CLRDV from South America

(Agrofoglio et al., 2017; Delfosse et al., 2013). Of particular interest are the CABYV, PLRV and TuYV infectious clones, which were instrumental in the identification of the function of polerovirus genes, especially the P3-5 fusion protein. Likewise, there have been attempts to create poleroviruses that infect plants systemically while producing a visible molecular marker, namely Green Fluorescent Protein (Shimomura, 2005). These GFP-producing clones include PLRV (Nurkiyanova et al., 2000), BMYV (Stevens & Viganó, 2007), and TuYV (Boissinot et al., 2017). These infectious clones will be discussed further in chapter 2.

Due to the heavily destructive nature of typical and atypical CLRDV infections in Argentina, an infectious clone was created by (Delfosse et al., 2013). The clone of the South American typical strain was assembled in multiple pieces into an agrobacterium expression plasmid under the control of a Cauliflower mosaic virus 35S promoter. The clone was then agroinfiltrated into cotton and *Nicotiana benthamiana* plants, where it infected systemically and recreated symptoms seen in the field in cotton and caused symptoms of vein clearing in *N. benthamiana*. Additionally, an infectious clone of the atypical strain was made in Agrofoglio et al., (2017), and was similarly successful in systemically infecting the plants and recreating the symptoms caused by the wild type atypical strain. The primary response to CLRDV in South America was the development of resistant cultivars of cotton which was accelerated by the development of infectious clones of the two strains of the virus present (Manenti et al., 2023). Due to previously described factors such as the many alternate hosts of CLRDV and the difficulties in controlling aphids, the US response to CLRDV will likely involve the use of resistant cultivars. This breeding effort would likewise be accelerated by the development of an infectious clone of CLRDV from the United States.

1.6 Conclusion

CLRDV is a member of a group of viruses that are difficult to manage. This virus in particular has caused significant damage to yields in Brazil and Argentina, and remains an issue to this day (Edula et al., 2023). The rest of this thesis aims to contribute to the understanding of CLRDV by producing an infectious clone, and by testing which proteins are required for transmission as previously determined for other poleroviruses. The objectives of Chapter 2 are to develop an infectious clone of CLRDV, and to determine its infection efficiency in cotton and *Nicotiana benthamiana* as described for other CLRDV infectious clones (Agrofoglio et al., 2017; Delfosse et al., 2013). If the full-length sequence of CLRDV from Alabama (accession number MN071395.1) is inserted into an agrobacterium expression vector, then it will cause infection with similar symptoms as CLRDV in Alabama. As well as the wild type infectious clone, a version of the clone producing GFP during infection is also produced following the strategies of other GFP polerovirus clones (Boissinot et al., 2017; Nurkiyanova et al., 2000). In Chapter 3, the capability of the wild type infectious clone will be determined, as well as which viral capsid protein (P3 or P3-5) is required for aphid transmission. Because the infectious clone is the same sequence as the wild-type virus, it should be transmitted by aphids at the same rate as the wild-type virus. According to previous research on polerovirus transmission, the RT* domain of ORF 3-5 is necessary for transmission by aphids (Schiltz et al., 2022), so this same result is expected to be true for CLRDV.

1.7 References

Abney, M. R., Ruberson, J. R., Herzog, G. A., Kring, T. J., Steinkraus, D. C., & Roberts, P. M. (2008). Rise and fall of cotton aphid (Hemiptera: Aphididae) populations in southeastern cotton production systems. *Journal of economic entomology*, *101*(1), 23-35.

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

Adams, M. C., Schiltz, C. J., Heck, M. L., & Chappie, J. S. (2022). Crystal structure of the potato leafroll virus coat protein and implications for viral assembly. *J Struct Biol*, *214*(1), 107811. https://doi.org/10.1016/j.jsb.2021.107811

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. https://apsjournals.apsnet.org/doi/pdf/10.1094/PHYTO-09-16-0349-R?download=true

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. (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.

Amack, S. C., & Antunes, M. S. (2020). CaMV35S promoter–A plant biology and biotechnology workhorse in the era of synthetic biology. *Current Plant Biology*, *24*, 100179.

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. https://apsjournals.apsnet.org/doi/pdf/10.1094/PDIS-06-19-1316-RE?download=true

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

Bahner, I., Lamb, J., Mayo, M., & Hay, R. (1990). Expression of the genome of potato leafroll virus: readthrough of the coat protein termination codon in vivo. *Journal of General Virology*, *71*(10), 2251-2256.

Boissinot, S., Erdinger, M., Monsion, B., Ziegler-Graff, V., & Brault, V. (2014). Both structural and non-structural forms of the readthrough protein of cucurbit aphid-borne yellows virus are essential for efficient systemic infection of plants. *PLoS One*, *9*(4), e93448. https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0093448&type=printable

Boissinot, S., Pichon, E., Sorin, C., Piccini, C., Scheidecker, D., Ziegler-Graff, V., & Brault, V. (2017). Systemic Propagation of a Fluorescent Infectious Clone of a Polerovirus Following Inoculation by Agrobacteria and Aphids. *Viruses*, *9*(7). https://doi.org/10.3390/v9070166

Brault, V., Bergdoll, M., Mutterer, J., Prasad, V., Pfeffer, S., Erdinger, M., Richards, K., & Ziegler-Graff, V. (2003). Effects of point mutations in the major capsid protein of beet

western yellows virus on capsid formation, virus accumulation, and aphid transmission. *Journal of Virology*, *77*(5), 3247-3256. https://journals.asm.org/doi/pdf/10.1128/jvi.77.5.3247-3256.2003?download=true

Brault, V., Herrbach, E., & Reinbold, C. (2007). Electron microscopy studies on luteovirid transmission by aphids. *Micron*, *38*(3), 302-312. https://www.sciencedirect.com/science/article/pii/S0968432806000709?via%3Dihub

Brault, V., Périgon, S., Reinbold, C., Erdinger, M., Scheidecker, D., Herrbach, E., Richards, K., & Ziegler-Graff, V. (2005). The polerovirus minor capsid protein determines vector specificity and intestinal tropism in the aphid. *Journal of Virology*, *79*(15), 9685-9693. https://journals.asm.org/doi/pdf/10.1128/jvi.79.15.9685-9693.2005?download=true

Brault, V., Uzest, M., Monsion, B., Jacquot, E., & Blanc, S. (2010). Aphids as transport devices for plant viruses. *Comptes rendus biologies*, *333*(6-7), 524-538.

Brault, V., van den Heuvel, J. F., Verbeek, M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Garaud, J. C., Guilley, H., Richards, K., & Jonard, G. (1995). Aphid transmission of beet western yellows luteovirus requires the minor capsid read-through protein P74. *EMBO J*, *14*(4), 650-659. https://doi.org/10.1002/j.1460-2075.1995.tb07043.x

Bruyere, A., Brault, V., Ziegler-Graff, V., Simonis, M.-T., Van den Heuvel, J., 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.

Carletto, J., Martin, T., Vanlerberghe-Masutti, F., & Brévault, T. (2010). Insecticide resistance traits differ among and within host races in Aphis gossypii. *Pest Management Science: formerly Pesticide Science*, *66*(3), 301-307. https://onlinelibrary.wiley.com/doi/pdfdirect/10.1002/ps.1874?download=true

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*, 1-10.

Cauquil, J. (1977). Etudes sur une maladie d'origine virale du cotonnier, la maladie bleue.

Corrêa, R., Silva, T., Simões-Araújo, J., Barroso, P., Vidal, M., & Vaslin, M. (2005). Molecular characterization of a virus from the family Luteoviridae associated with cotton blue disease. *Archives of Virology*, *150*, 1357-1367. https://link.springer.com/content/pdf/10.1007/s00705-004-0475-8.pdf

Crop Protection Network. (2019). *Cotton Disease Loss Calculator*. Crop Protection Network. Retrieved 7/17/2023 from https://loss.cropprotectionnetwork.org/crops/cotton-diseases?year_start=2016&year_end=2022&diseaseCategory=20&diseases%5B%5D=145&country=1&region=2&cropID=5

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. https://www.sciencedirect.com/science/article/pii/S016817021300124X?via%3Dihub

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*, 1849-1854.

Ebert, T., & Cartwright, B. (1997). Biology and ecology of Aphis gossypii Glover (Homoptera: aphididae). *Southwest. Entomol*, *22*(1), 116-153.

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. *Mol Plant Pathol*, *24*(6), 513-526. https://doi.org/10.1111/mpp.13335

ERS, U.-. (2022). *Cotton Sector at a Glance*. Retrieved 6/2/23 from https://www.ers.usda.gov/topics/crops/cotton-and-wool/cotton-sector-at-a-glance/

Escobar, M. A., & Dandekar, A. M. (2003). Agrobacterium tumefaciens as an agent of disease. *Trends in plant science*, *8*(8), 380-386. https://www.sciencedirect.com/science/article/pii/S1360138503001626?via%3Dihub

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.

Filichkin, S., Lister, R., McGrath, P., & Young, M. (1994). In vivo expression and mutational analysis of the barley yellow dwarf virus readthrough gene. *Virology*, *205*(1), 290-299. https://www.sciencedirect.com/science/article/pii/S004268228471645X?via%3Dihub

Franco-Lara, L. F., McGeachy, K. D., Commandeur, U., Martin, R. R., Mayo, M. A., & Barker, H. (1999). Transformation of tobacco and potato with cDNA encoding the full-length genome of potato leafroll virus: evidence for a novel virus distribution and host effects on virus multiplication. *Journal of General Virology*, *80*(11), 2813-2822.

Galbieri, R., Boldt, A. S., Scoz, L. B., Rodrigues, S. M., Rabel, D. O., Belot, J. L., Vaslin, M., da Franca Silva, T., Kobayasti, L., & Chitarra, L. G. (2017). Cotton blue disease in central-west Brazil: Occurrence, vector (Aphis gossypii) control levels and cultivar reaction. *Tropical Plant Pathology*, *42*, 468-474.

Gelvin, S. B. (2003). Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiology and molecular biology reviews*, *67*(1), 16-37. https://journals.asm.org/doi/pdf/10.1128/mmbr.67.1.16-37.2003?download=true

Ghosh, S., Kanakala, S., Lebedev, G., Kontsedalov, S., Silverman, D., Alon, T., Mor, N., Sela, N., Luria, N., & Dombrovsky, A. (2019). Transmission of a new polerovirus infecting pepper by the whitefly Bemisia tabaci. *Journal of Virology*, *93*(15), e00488-00419. https://journals.asm.org/doi/pdf/10.1128/jvi.00488-19?download=true

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*. https://doi.org/10.1093/jee/toad080

Horsch, R., & Klee, H. (1986). Rapid assay of foreign gene expression in leaf discs transformed by Agrobacterium tumefaciens: Role of T-DNA borders in the transfer process. *Proceedings of the National Academy of Sciences*, *83*(12), 4428-4432.

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

Khan, M. A., Wahid, A., Ahmad, M., Tahir, M. T., Ahmed, M., Ahmad, S., & Hasanuzzaman, M. (2020). World cotton production and consumption: An overview. *Cotton production and uses: Agronomy, crop protection, and postharvest technologies*, 1-7.

Kring, J. B. (1959). The life cycle of the melon aphid, Aphis gossypii Glover, an example of facultative migration. *Annals of the Entomological Society of America*, *52*(3), 284-286.

Lawrence, K. S., Amanda Strayer-Scherer, Randy Norton, Jiahuai Hu, Travis R. Faske, Robert Hutmacher, John Muller, Ian Small, Zane J. Grabau, Robert Kemerait, Rodrigo Borba Onofre, Paul Price, Tristan Watson, Thomas W. Allen, Kaitlyn Bissonnette, John Idowu, Guy D. Collins, Seth A. Byrd, Heather M Kelly, Terry A. Wheeler, Thomas Isakeit, Cecilia Monclova-Santana, and David B. Langston. (2022). *Cotton Disease Loss Estimate Committee Report, 2021*. National Cotton Council of America. Retrieved 6/2/2023 from https://www.cotton.org/beltwide/proceedings/2005-2022/data/conferences/2022/paper/21402.pdf

Lee, L. Y., & Gelvin, S. B. (2008). T-DNA binary vectors and systems. *Plant Physiol*, *146*(2), 325-332. https://doi.org/10.1104/pp.107.113001

Leiser, R., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Lemaire, O., Guilley, H., Richards, K., & Jonard, G. (1992). Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus. *Proceedings of the National Academy of Sciences*, *89*(19), 9136-9140. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC50080/pdf/pnas01093-0288.pdf

Liu, L., Ren, Q., Peng, B., Kang, B., Wu, H., & Gu, Q. (2022). Construction of an Agrobacterium-mediated infectious cDNA clone of melon aphid-borne yellows virus. *Virus Res*, *315*, 198779. https://doi.org/10.1016/j.virusres.2022.198779

Ma, L., Li, M.-Y., Chang, C.-Y., Chen, F.-F., Hu, Y., & Liu, X.-D. (2019). The host range of Aphis gossypii is dependent on aphid genetic background and feeding experience. *PeerJ*, *7*, e7774. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6768058/pdf/peerj-07-7774.pdf

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.

Manenti, D. C., Araújo, J., Maeda, M. H. K., Tavares-Esashika, M. L., Aguiar, P. H., Meda, A. R., Nagata, T., & de Souto, E. R. (2023). Infectious clones of cotton leafroll dwarf virus strains used for genotype evaluation in cotton. *Annals of Applied Biology*, *182*(2), 216-225.

Mukherjee, A., Chahande, P., Meshram, M., & Kranthi, K. (2012). First report of Polerovirus of the family Luteoviridae infecting cotton in India. *New Dis Rep*, *25*(22), 2044-0588.2012.

Mulot, M., Monsion, B., Boissinot, S., Rastegar, M., Meyer, S., Bochet, N., & Brault, V. (2018). Transmission of turnip yellows virus by Myzus persicae is reduced by feeding aphids on double-stranded RNA targeting the ephrin receptor protein. *Frontiers in microbiology*, *9*, 457. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5859162/pdf/fmicb-09-00457.pdf

Nurkiyanova, K. M., Ryabov, E. V., Commandeur, U., Duncan, G. H., Canto, T., Gray, S. M., Mayo, M. A., & Taliansky, M. E. (2000). Tagging Potato leafroll virus with the jellyfish green fluorescent protein gene. *Journal of General Virology*, *81*(3), 617-626.

Olmedo-Velarde, A., Wilson, J. R., Stallone, M., DeBlasio, S. L., Chappie, J. S., & Heck, M. (2023). Potato leafroll virus molecular interactions with plants and aphids: Gaining a new tactical advantage on an old foe. *Physiological and Molecular Plant Pathology*, *125*. https://doi.org/10.1016/j.pmpp.2023.102015

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. https://mdpi-res.com/d_attachment/viruses/viruses-14-02249/article_deploy/viruses-14-02249-v2.pdf?version=1666685843

Patel, P., & Parmar, R. (2023). Influence of weather parameters on development of leaf roll disease in potato (Solanum tuberosum L.). *Journal of Agrometeorology*, *25*(1), 164-166.

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.

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.

Price, T., Valverde, R., Singh, R., Davis, J., Brown, S., & Jones, H. (2020). First report of cotton leafroll dwarf virus in Louisiana. In: Am Phytopath Society.

Prüfer, D., Kawchuk, L., Monecke, M., Nowok, S., Fischer, R., & Rohde, W. (1999). Immunological analysis of potato leafroll luteovirus (PLRV) P1 expression identifies a 25 kDa RNA-binding protein derived via P1 processing. *Nucleic acids research*, *27*(2), 421-425. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC148195/pdf/270421.pdf

Prüfer, D., Wipf-Scheibel, C., Richards, K., Guilley, H., Lecoq, H., & Jonard, G. (1995). Synthesis of a full-length infectious cDNA clone of cucurbit aphid-borne yellows virus and its use in gene exchange experiments with structural proteins from other luteoviruses. *Virology*, *214*(1), 150-158. https://www.sciencedirect.com/science/article/pii/S004268228579945X?via%3Dihub

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. https://mdpi-res.com/d_attachment/viruses/viruses-13-02230/article_deploy/viruses-13-02230.pdf?version=1636113425

Ray, J., Sharman, M., Quintao, V., Rossel, B., Westaway, J., & Gambley, C. (2016). Cotton leafroll dwarf virus detected in Timor-Leste. *Australasian Plant Disease Notes*, *11*, 1-3.

Reinbold, C., Herrbach, E., & Brault, V. (2003). Posterior midgut and hindgut are both sites of acquisition of Cucurbit aphid-borne yellows virus in Myzus persicae and Aphis gossypii. *Journal of General Virology*, *84*(12), 3473-3484.

Schiltz, C. J., Wilson, J. R., Hosford, C. J., Adams, M. C., Preising, S. E., DeBlasio, S. L., MacLeod, H. J., Van Eck, J., Heck, M. L., & Chappie, J. S. (2022). Polerovirus N-terminal readthrough domain structures reveal molecular strategies for mitigating virus transmission by aphids. *Nature Communications*, *13*(1), 6368.

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*. https://doi.org/10.1016/j.cropro.2021.105604

Service, U. N. A. S. (2017). https://quickstats.nass.usda.gov/data/printable/2E1DB602-DC4C-37DA-BC1B-EA857A099730

Sharman, M., Lapbanjob, S., Sebunruang, P., Belot, J.-L., Galbieri, R., Giband, M., & Suassuna, N. (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*, 1-4.

Sharmeen, L., Kuo, M., Dinter-Gottlieb, G., & Taylor, J. (1988). Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. *Journal of Virology*, *62*(8), 2674-2679.

Shimomura, O. (2005). The discovery of aequorin and green fluorescent protein. *Journal of microscopy*, *217*(1), 3-15.

Silva, T., Corrêa, R., Castilho, Y., Silvie, P., Bélot, J.-L., & Vaslin, M. (2008). Widespread distribution and a new recombinant species of Brazilian virus associated with cotton blue disease. *Virology Journal*, *5*(1), 1-13.

Smirnova, E., Firth, A. E., Miller, W. A., Scheidecker, D., Brault, V., Reinbold, C., Rakotondrafara, A. M., Chung, B. Y.-W., & 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. https://journals.plos.org/plospathogens/article/file?id=10.1371/journal.ppat.1004868&type=printable

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

Steckelberg, A.-L., Vicens, Q., Costantino, D. A., Nix, J. C., & Kieft, J. S. (2020). The crystal structure of a Polerovirus exoribonuclease-resistant RNA shows how diverse sequences are integrated into a conserved fold. *RNA*, *26*(12), 1767-1776.

Steinkraus, D., Kring, T., & Tugwell, N. (1991). Neozygites fresenii in Aphis gossypii on cotton. *Southwestern Entomologist*, *16*(2), 118-122.

Stevens, M., & Viganó, F. (2007). Production of a full-length infectious GFP-tagged cDNA clone of Beet mild yellowing virus for the study of plant–polerovirus interactions. *Virus Genes*, *34*(2), 215-221. https://link.springer.com/content/pdf/10.1007/s11262-006-0046-z.pdf

Tabassum, A., Bag, S., Roberts, P., Suassuna, N., Chee, P., Whitaker, J., Conner, K., Brown, J., Nichols, R., & Kemerait, R. (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. https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0252523&type=printable

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.

Viot, C. R., & Wendel, J. F. (2023). Evolution of the Cotton Genus, Gossypium, and Its Domestication in the Americas. *Critical Reviews in Plant Sciences*, 1-33.

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.

Wang, J., Chay, C., Gildow, F., & Gray, S. (1995). Readthrough protein associated with virions of barley yellow dwarf luteovirus and its potential role in regulating the efficiency of aphid transmission. *Virology*, *206*(2), 954-962. https://www.sciencedirect.com/science/article/pii/S0042682285710185?via%3Dihub

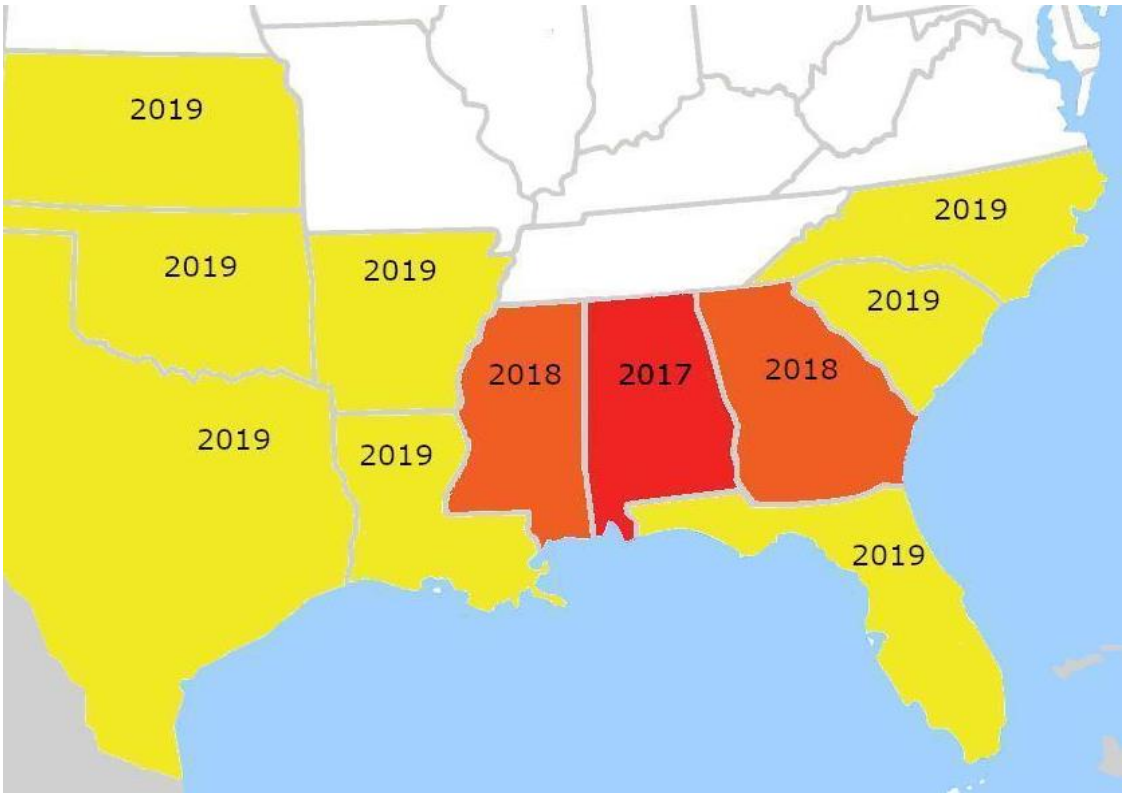
Watt, M., & Hales, D. F. (1996). Dwarf phenotype of the cotton aphid, Aphis gossypii Glover (Hemiptera: Aphididae). *Australian Journal of Entomology*, *35*(2), 153-159.

Xu, Y., Ju, H.-J., DeBlasio, S., Carino, E. J., Johnson, R., MacCoss, M. J., Heck, M., Miller, W. A., & Gray, S. M. (2018). A stem-loop structure in potato leafroll virus open reading frame 5 (ORF5) is essential for readthrough translation of the coat protein ORF stop codon 700 bases upstream. *Journal of Virology*, *92*(11), e01544-01517.

https://journals.asm.org/doi/pdf/10.1128/jvi.01544-17?download=true
Yang, X., Thannhauser, T., Burrows, M., Cox-Foster, D., Gildow, F. E., & Gray, S. M. (2008). Coupling genetics and proteomics to identify aphid proteins associated with vector-specific transmission of polerovirus (Luteoviridae). *Journal of Virology, 82*(1), 291-299. https://journals.asm.org/doi/pdf/10.1128/jvi.01736-07?download=true

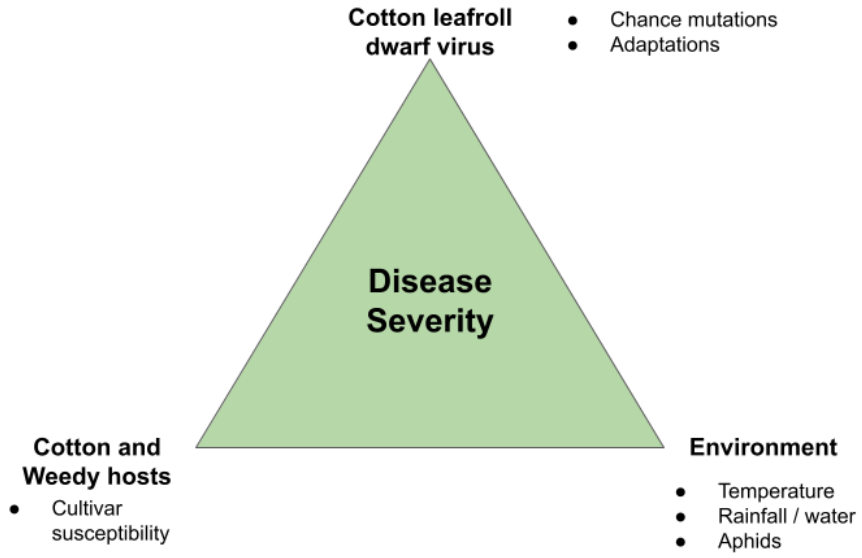
Section 1 Figures

Figure 1.1. Years of first detection of CLRDV from Southeastern cotton fields, based on published first reports.



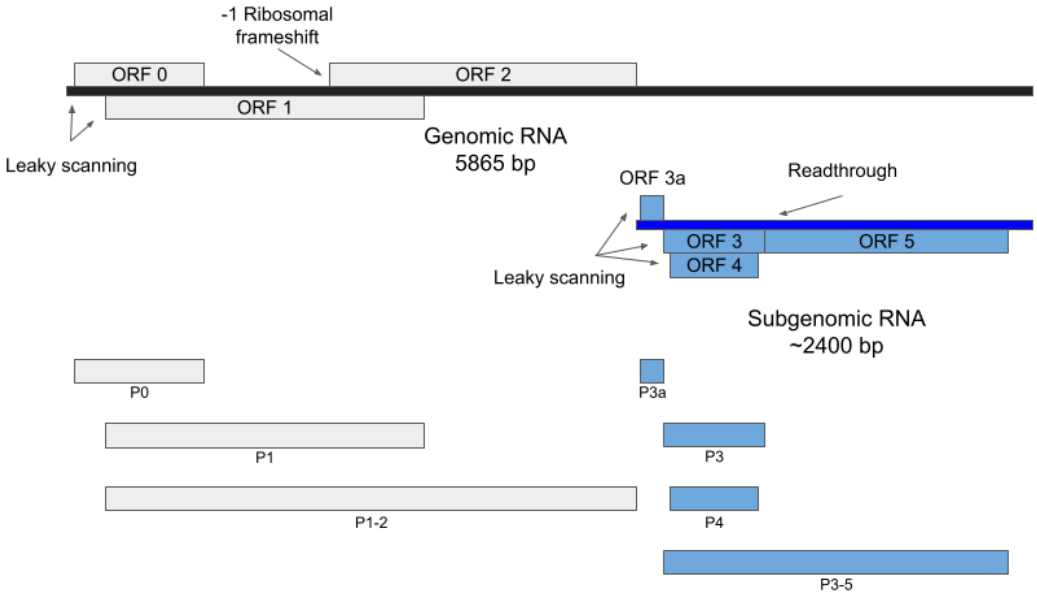
The disease was first recorded in Alabama, then was identified in 2018 in Georgia and Mississipi. In 2019 it had been reported in Kansas, Oklahoma, Texas, Arkansas, Louisiana, Florida, South Carolina, and North Carolina (Aboughanem-Sabanadzovic et al., 2019; Alabi et al., 2020; Ali & Mokhtari, 2020; Ali et al., 2020; Avelar et al., 2019; Faske et al., 2020; Iriarte et al., 2020; Price et al., 2020; Tabassum et al., 2019; Thiessen et al., 2020; Wang et al., 2020)..

Figure 1.2. Disease Triangle for CLRDV.



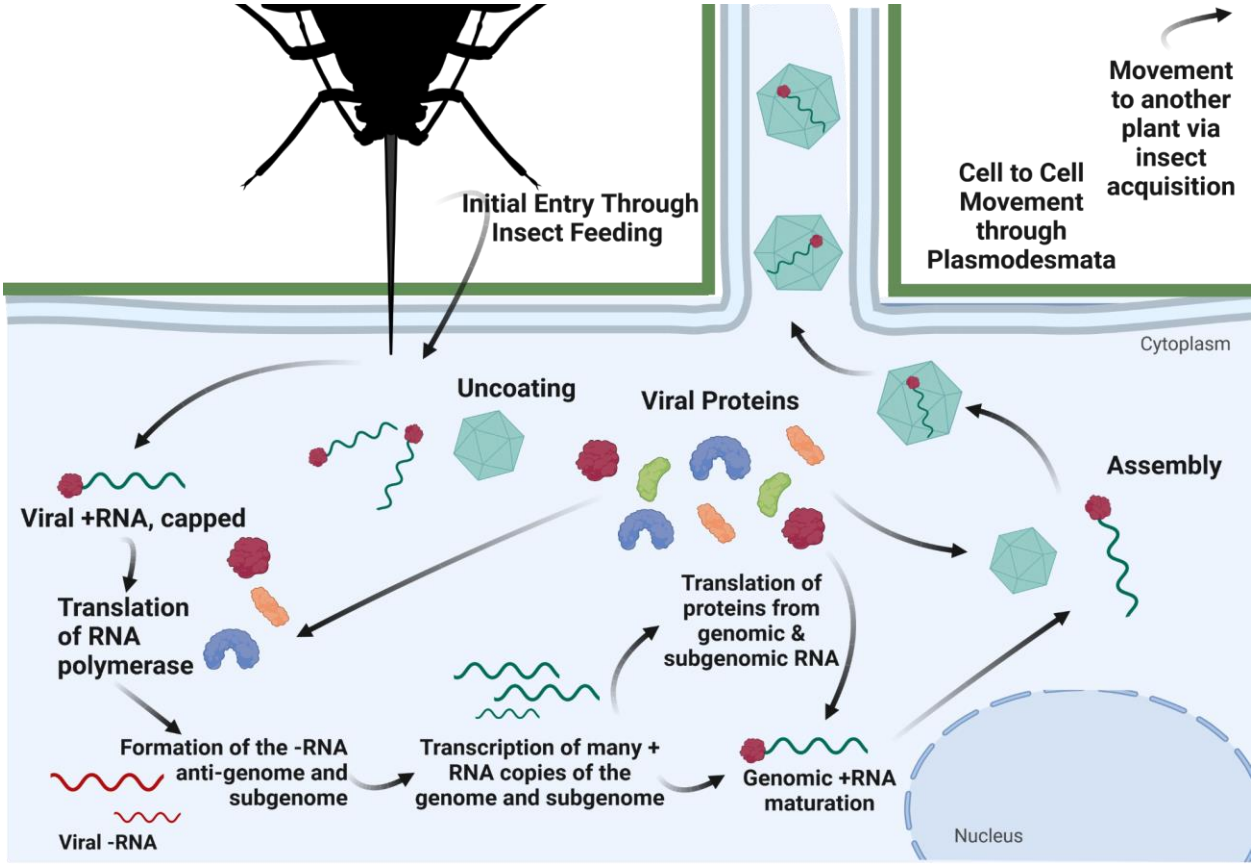
The pathogen itself changes constantly by the adaptations to its host and by chance mutations. The environment affects the disease severity and the aphid population (the vector of CLRDV) by different weather phenomena such as temperature and rainfall. The final aspect of the disease triangle is the host, which can be either cotton or weedy species. Resistant or susceptible cultivars of cotton may decrease or increase disease severity, respectively.

Figure 1.3. Genome structure and proteins of CLRDV.



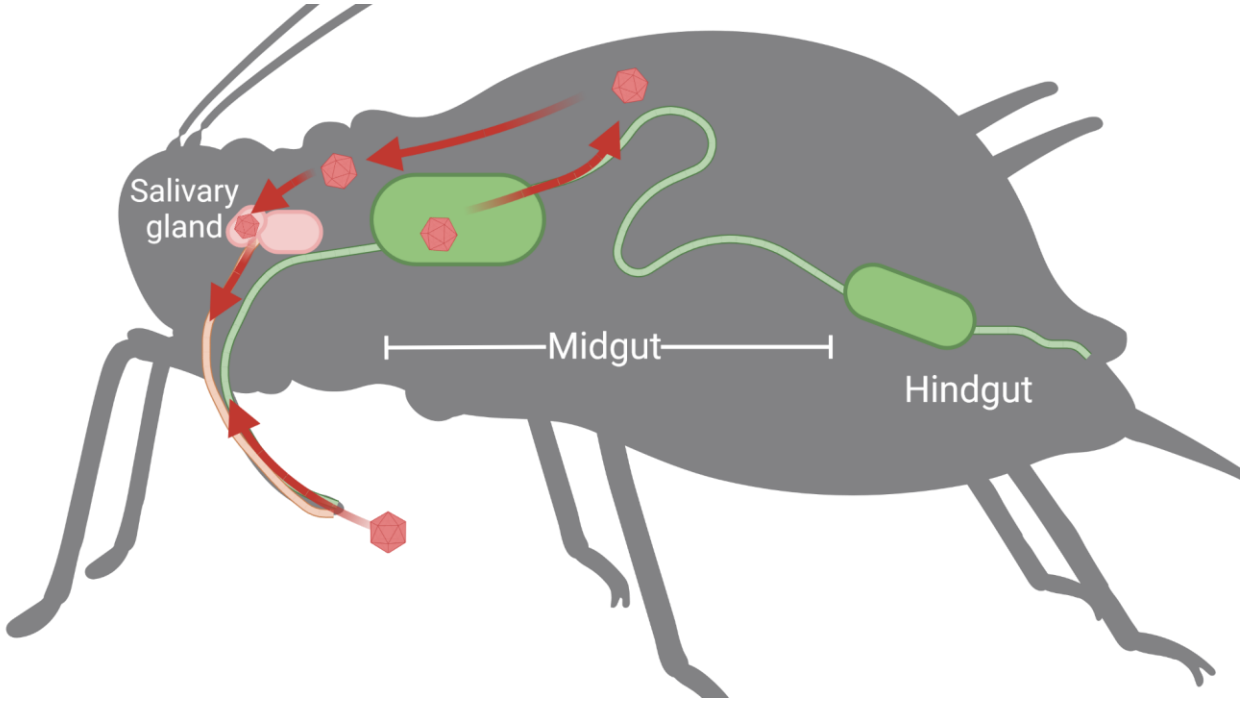
Open reading frames 0 through 2 are translated from genomic RNA during replication, and are represented in gray and black. ORF 0 and ORF 1 are translated by leaky scanning of the ribosome, while ORF 2 is translated by a -1 ribosomal frameshift. ORFs 3a through 5 are shown in light and dark blue, and are only expressed after replication and the formation of subgenomic RNA has occurred. ORF 3a, 3 and 4 are also read by leaky scanning, with ORF 3a being read by a non-AUG start codon (Smirnova et al., 2015). ORF 5 is expressed only as a read through product of ORF 3, which ends with an amber stop.

Figure 1.4. Replication of poleroviruses in plant cells.



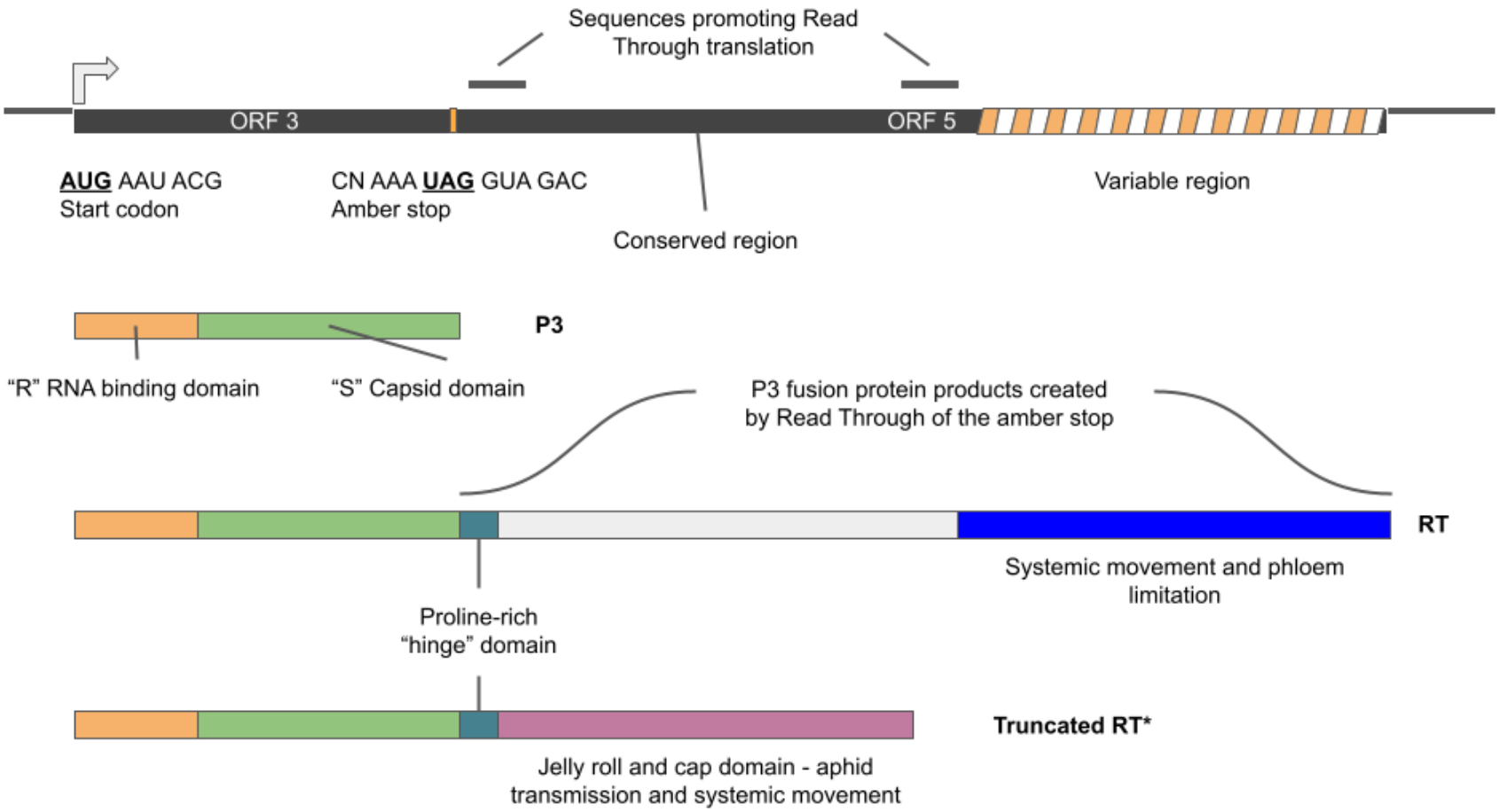
The virus enters either by plasmodesmata or by insect feeding, after which it uncoats. The RNA polymerase is translated from genomic positive sense RNA, and it begins replicating by making negative sense templates of the genome and subgenome. Many positive sense copies are made from these templates, which are then either translated into proteins or packaged into virions. Packaged virions travel to neighboring cells by the plasmodesmata, though poleroviruses are restricted to the phloem. Made with BioRender.com

Figure 1.5. Polerovirus circulation through aphids.



Polerovirus virions are taken up with the phloem sap by aphid feeding. The virions travel up the esophagus, then pass through the gut cell membrane in the midgut to the hemolymph. The virions then travel to the salivary glands, where they undergo a second transcytosis event to get into the aphid saliva. The aphid then egests saliva containing the virus into the phloem tissue of the next plant it feeds on. Made with BioRender.com

Figure 1.6. Polerovirus ORF 3 and ORF 3-5 proteins.



P3 is the major capsid of polerovirus virions, and has a RNA binding domain and the S domain that makes up most of the structure (Adams et al., 2022). ORF 5 contains two regions of sequences that enhance readthrough of the amber stop, one immediately after and

one 600-700 bases downstream (Bruyere et al., 1997; Xu et al., 2018). The complete ORF 3-5 is represented by the RT, and is a soluble protein that is involved in systemic movement (Boissinot et al., 2014) and limits infection to the phloem (Peter et al., 2009). A truncated form of P3-5 is formed post translation, and this RT* protein is incorporated into the virion by the action of P3. The C terminal of RT* forms spikes on the virions and is responsible for aphid transmission (Schiltz et al., 2022). Mutated viruses without the ability to form RT* are unable to move systemically, so it is also involved in systemic movement (Boissinot et al., 2014). P3 is attached to either the full RT domain or RT* by a proline-rich region, which acts as a molecular hinge (Bahner et al., 1990; Brault et al., 2005; Bruyere et al., 1997; Schiltz et al., 2022).

Chapter 2 -
Agro-infiltration of two infectious clones of Cotton leafroll dwarf virus, one wild-type and one producing GFP, produces systemic infection.

2.1 Abstract

Cotton leafroll dwarf virus (CLRDV) is a virus that causes yield loss in cotton (*Gossypium hirsutum* L) crops. This virus was recently introduced to the United States, and in this study two infectious clones based on the original Alabama isolate were developed in order to further research on this emerging pathogen. Both are *Agrobacterium tumefaciens* expression constructs which are agroinfiltrated into plants to cause infection. CLRDV-Macon1 was created by inserting the full sequence of CLRDV into pJL89, an agrobacterium expression vector. This virus clone systemically infects both cotton and *Nicotiana benthamiana*. CLRDV-GFP was the second infectious clone created by replacing a C-terminal section of ORF3-5 from CLRDV-Macon1 with EGFP. This clone produces EGFP and is visible by fluorescence microscopy in infiltrated cotton cotyledons. This study presents the first infectious clone of Cotton leafroll dwarf virus in the United States and adds to the growing knowledge of *Poleroviruses*.

2.2 Introduction

In 2017, symptoms of Cotton leafroll dwarf virus was first reported in coastal Alabama counties and has since been detected in cotton-producing states throughout the Southeastern United States (Avelar et al., 2019; Avelar et al., 2020; Tabassum et al., 2021). Symptoms of CLRDV include vein clearing, downwards leafroll, shortened internodes and stunting, which

together cause a reduction in yield (Avelar et al., 2020). Cotton production is a major part of Alabama's economy, and was valued at $288,655,000 in 2021 (USDA Quickstats). In the first report of CLRDV in Alabama, the disease incidence rate was estimated to be from 3-30% and caused an average loss of 560 kg/ha (Avelar et al., 2019).

Cotton leafroll dwarf virus is a positive sense, monopartite, single stranded RNA virus in the *Polerovirus* genus of the *Solemoviridae* (Distéfano et al., 2010; Sõmera et al., 2021). This virus produces seven proteins from seven open reading frames (ORFs). ORF 0 encodes an RNA silencing suppressor (Avelar et al., 2020; Delfosse et al., 2021). ORF 1 encodes a Viral Genome-linked Protein (VPg), and ORF 1-2 produces the RNA-dependent RNA polymerase by a -1 ribosomal frameshift. ORFs 3a, 3, 4, and 3-5 are produced by a subgenomic RNA, with overlapping ORFs for 3a, 3 and 4 that are transcribed by leaky scanning (Delfosse et al., 2021). ORF 3 and 3-5 encode P3 and P3-5 which are the major and minor coat proteins respectively. P3-5 is a readthrough protein that contains the major capsid protein, an amber or “leaky” stop, and a proline-rich “hinge” that links the coat protein with the read-through domain (Boissinot et al., 2014; Guilley et al., 1994). This Read-through domain (RT) is further processed during infection, with the full RT (estimated at 77kD for CLRDV) and a C-terminal truncated RT* being necessary for successful infection. The full RT protein functions non-structurally, while the RT* protein is incorporated into virions (Brault et al., 1995; Bruyere et al., 1997). ORF 5 has been implicated as being the determinant for aphid transmission of poleroviruses as well as acting as a supporting movement protein and the cause of the phloem-limitation of polerovirus infection (Boissinot et al., 2014; Boissinot et al., 2017; Brault et al., 1995; Bruyere et al., 1997; Peter et al., 2009).

Research into this virus has proven difficult because poleroviruses cannot be mechanically inoculated and are strictly transmitted by aphids (Brault et al., 2010; Brault et al., 1995; Olmedo-Velarde et al., 2023). In order to expedite research into this study, we have developed multiple *Agrobacterium tumefaciens* infectious clones. The first clone contains the original CLRDV-AL virus sequenced from Macon County in 2017 (Avelar et al., 2020), and the second was created by replacing the C-terminal end of CLRDV-Macon1 with EGFP. If CLRDV-Macon1 successfully integrates into the plant genome and is expressed, then it should produce a virus that infects cotton and *N. benthamiana* using its own viral machinery. If CLRDV-Macon1 infects plants successfully, then the CLRDV-GFP clone should also infect plants systemically while producing a molecular marker which can be both visualized by fluorescent microscopy and has many commercially available antibodies. Both infectious clones are important steps forward in the research of CLRDV-AL.

2.3 Materials and Methods

2.3.1 Development of the CLRDV infectious clone

The sequence for CLRDV-AL (Genbank MN071395) was synthesized by GenScript (Piscataway, USA) with the addition of a single 35S promoter, an HDV sequence and a 35S terminator sequence, and the addition of two restriction enzymes, SbfI on the 5' end and SphI on the 3'end for insertion into pJL89 (Lindbo et al., 2007). The synthesized construct was placed in pUC57 by Genscript. From pUC57, the SbfI and SphI was used to digest both the pUC57-CLRDV and the pJL89 vector and the two were then ligated together and transformed into *E. coli* strain Top10 (Thermo Fisher Scientific, Waltham MA). Once completed, the vector was confirmed by PCR and sequencing. The plasmid was then moved into LBA 4404 for *Agrobacterium tumefaciens* transformation. The full construct is presented in Figure 2.1.

2.3.2 Development of the CLRDV-GFP infectious clone

CLRDV-GFP was created in order to provide a visual confirmation of the infectious clone being expressed and replicating in plant cells. In order to insert a fluorescent tag without adding unnecessary nucleotides, EGFP was inserted into the CLRDV-Macon1 clone using Gibson Assembly (Gibson et al., 2009). EGFP was amplified out of pSITE-2CA (Chakrabarty et al., 2007) and pJL CLRDV was amplified using the primers from Table 2.1, and then assembled with the GeneArt™ Gibson Assembly HiFi Master Mix kit (Invitrogen, Waltham USA). The assembly product was then transformed into Top10 *E. coli* and selected for using the Kanamycin resistance cassette in the pJL89 backbone. Colonies were then checked using primers specific to GFP, and colonies positive for CLRDV-GFP were grown overnight in liquid culture. Positive colonies were then miniprepped, and the GFP insert location was confirmed by sequencing. LBA4404 agrobacterium was then transformed with the construct and glycerol stocks were made from the *A. tumefaciens* LBA 4404 colonies. The location of the EGFP insert in the ORF 5 region of CLRDV-GFP is presented in Figure 2.2.

2.3.3 Plant Growth and Maintenance

All cotton used in this study was DeltaPine 1646 B2XF (Bayer, Leverkusen, Germany). Cotton plants were grown indoors with a 14 hour photoperiod, and were infiltrated at one to two weeks of age. One week post infiltration they were transplanted into 10 cm pots and kept in a greenhouse with the same photoperiod. The plants were watered regularly and fertilized weekly. *N. benthamiana* plants were grown in similar conditions. The plants were seeded in a small pot, then transplanted to individual pots at two weeks of age. Once the plants had leaves about two centimeters in diameter they were used for infiltration. After infiltration they were transplanted into similar 10 cm pots as the cotton.

2.3.4 Infiltration with clone + VSRs

Infiltrations were performed by adjusting the protocol used in (Goodin et al., 2002). Infiltrations were performed by streaking *A. tumefaciens* onto Rifampin/Kanamycin-amended LB agar plates and allowing the bacteria to grow for 4 days. The bacteria was then dissolved into 0.1 M MES and $MgCl_2$ media at an OD_{600} measurement of 2.0. Acetosyringone was then added to the media at a ratio of 1.5uL:1mL to activate the *A. tumefaciens* and prepare it for infiltration. The bacterial mixture was then allowed to sit at room temperature for two hours before being infiltrated, at a rate of 2mL per plant for cotton and 1mL for *N. benthamiana*.

2.3.5 CLRDV-Macon1 Infiltration Assay

To determine the efficacy of the infectious clone, many replications of agro-infiltration were performed on both cotton and *N. benthamiana*. In each replication ten plants were infiltrated with CLRDV-Macon1, and two plants were infiltrated with *Agrobacterium* carrying an empty pJL89 vector as a control. Each repetition was completed three times in both cotton and *N. benthamiana*. All infiltration experiments were performed using various Viral Silencing Suppressors (VSRs) such as Potyvirus Hc-Pro and Closterovirus P19. VSRs were used in order to increase the infection efficiency of the infectious clone. Infiltrations with both the original CLRDV-Macon1 clone and CLRDV-GFP clone were done in a 1:1 ratio of clone:VSR. The negative controls receiving the empty vectors were similarly infiltrated with a 1:1 ratio of empty vector:VSR.

Plants were allowed to recover for a week before being repotted into larger pots and moved to a greenhouse to allow the virus time to systemically infect the plants. CLRDV-Macon1 plants and the negative control plants were grown in separate locations in order to reduce the chance of greenhouse transmission. Leaf and petiole tissue samples were collected from infiltrated *N. benthamiana* plants at 30 days post infiltration (dpi), while cotton tissue samples

were collected from the roots 30 days post infiltration. Around 100 mg of the uppermost tissue was collected from *N. benthamiana* plants. Due to concerns for RNA quantity, 100 mg of root tissue was collected from the cotton plants at 30 dpi.

In order to run diagnostic PCRs on the RNA virus, reverse-transcription of the viral sequence was performed. RNA extraction was performed on approximately 100 mg of tissue, which was frozen in liquid nitrogen and then pulverized with RNAse-free metal beads in a Bead Ruptor Elite (Omni International, Kennesaw GA, USA). The samples were ground on two cycles of 30 seconds at 5 m/s, with a re-freeze in the liquid nitrogen between cycles. RNA extraction was then performed using IBI Mini Total RNA Kit (Plants) (IBI Scientific, Dubuque, IA, USA) using the manufacturer's protocol.

cDNA synthesis was performed using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham MA, USA) according to the manufacturer's directions. The cDNA synthesis was performed using the Luteo4 Reverse primer, which binds near the C terminus of Luteovirus and Polerovirus P3 ORF (Chomič et al., 2010). The amount of RNA used in each cDNA synthesis reaction was 1 µg of total RNA, however for samples with a low RNA quantity the total amount of RNA used was 200-500 ng.

Nested PCR was performed on cDNA samples in order to amplify viral sequences to a level that can be visualized by agarose gel electrophoresis (Mahas et al., 2022). The PCR method used to detect virus in the samples was a nested set of PCRs using primers created by Sharman et al 2015 to detect CLRDV from plant samples (Sharman et al., 2015).

2.3.6 Fluorescent Microscopy of CLRDV-GFP

Infiltrated cotton cotyledons and *N. benthamiana* leaves were analyzed by fluorescent microscopy in order to confirm the transcription and translation of CLRDV-GFP. Cotton

cotyledons were infiltrated with CLRDV-GFP as described and imaged 4 days post infiltration at 10x magnification.

2.3.7 Detection of CLRDV-GFP mutants

RT-PCR of the 3-5 region containing GFP were performed on CLRDV in order to determine if the GFP sequence was mutated out of the virus (as seen in the PLRV-GFP clone) (Nurkiyanova et al., 2000) or if the GFP sequence was maintained after replication (as in the case of TuYV-GFP) (Boissinot et al., 2017). Twenty-five cotton plants were planted and split into groups of five. In each group four plants were infiltrated with the CLRDV-GFP clone along with HcPro as described in previous infiltration experiments. For each group the fifth cotton plant was infiltrated with the empty pJL89 vector and HcPro to serve as a negative control. At 3, 7, 14, 21, and 28 days post infiltration tissue samples were collected and RT-PCR performed using the CLRDV-GFP 3-5F and CLRDV-GFP 3-5R primers (see Table 2.1). Phusion High-Fidelity PCR kit (Thermo Fisher Scientific, Waltham MA) was used to ensure the amplified sequences were true to the virus sequence. The location from which tissue samples were collected is shown in Table 2.2. Amplified sequences from positive plants were sequenced from the PCR products using the same primers used to amplify them.

2.4 Results

2.4.1 CLRDV-Macon1 Infiltration Assay

Systemic infection of the CLRDV-Macon1 clone was detected in both *N. benthamiana* and cotton. The clone was overall more successful in infecting cotton at 64% than in *N. benthamiana* (25%)(Table 2.3). In both infiltration assays of cotton, at least one of the six negative controls were positive.

2.4.2 CLRDV-GFP Microscopy

Negative controls did not fluoresce. CLRDV-GFP is faintly visible in the margins of infiltrated cotton cotyledon cells (Figure 2.3). The GFP fluorescence was visible in small clusters of cells.

2.4.3 CLRDV-GFP Mutants

The region of 3-5 containing the GFP insert was successfully detected in each round of plants, but was not detected in all tissues equally (Table 2.4). From leaf tissues, the amplified product was always the full length GFP region compared to the positive control. The virus was only detected in the infiltrated cotyledons, and the first and second true leaves. Root tissues were tested for the 14dpi, 21dpi, and 28dpi sets, and the virus was only detected in the roots for the 21dpi set. The amplified products from these root samples varied from 800 bp to the full 1490 bp of the positive control. Two plants in the 14dpi group accidentally became infested with aphids and were removed from the study. Only one plant out of four tested positive at 28dpi, and only the first and second true leaves were positive.

2.5 Discussion

2.5.1 CLRDV-Macon1

CLRDV-Macon1 has a similar infection efficiency rate as compared to the typical strain clone of South American CLRDV, which was reported to vary from 53-92% at 4-5 weeks post infiltration (Delfosse et al., 2013). The negative controls for the CLRDV-Macon1 infiltrated cotton produced a positive result 17% of the time, though this is considered to be a false positive. One factor that may raise our rate of false positives is that our tests were performed using two PCRs and a nested set of primers from (Sharman et al., 2015). In our tests, a single PCR of CLRDV-Macon1 infiltrated cotton would not produce any positive samples. Nested PCR, which

involves using the product of one PCR as the template for another, is used for detection of CLRDV in Alabama as it is more sensitive than a single PCR (Mahas et al., 2022). None of the plants infiltrated with CLRDV-Macon1 or CLRDV-GFP appeared to show obvious symptoms, which aligns with observations that CLRDV-AL does not produce symptoms in greenhouse settings.

2.5.2 CLRDV-GFP

The construction of CLRDV-GFP was inspired by the Potato leafroll virus clone developed by Nurkiyanova et al. 2000. Both viruses are in the same genus of Polerovirus, therefore the insertion of GFP into the C-terminus of ORF 3-5 of CLRDV would likely produce similar results to the PLRV clone. There have been numerous infectious clones of poleroviruses engineered to produce GFP by inserting GFP into the readthrough domain of the virus, with varying strategies and results. In 2000 the fluorescent PLRV clone was created (Nurkiyanova et al., 2000), followed by the Beet mild yellowing virus in 2007 (Stevens & Viganó, 2007) and the Turnip yellows virus fluorescent clone in 2017 (Boissinot et al., 2017). The GFP insert location is typically chosen to be in-frame for translation and avoid interference with the proline rich region, though the PLRV and BMYV do not mention the GFP sequence being placed in a way that avoids interfering with the truncation mechanism.

The PLRV clone was created by a restriction enzyme cutting near the C terminus of 3-5 in their PLRV infectious clone. They then cut and ligated in the GFP sequence using the same enzyme, and the result was a clone that contained all of the original sequence with GFP inserted near the C-terminal end of P3-5. This clone in particular only produced GFP in the initially infiltrated cells and the GFP sequence was excised out in PLRV mutants found infecting the plant systemically. It is speculated that the increased size of the genome did not allow the virus

to encapsidate properly, therefore only mutants with a smaller genome size could assemble into virions and move to other cells. The BMYV clone was created similarly by inserting GFP into the read-through domain, though was only tested in single celled protoplasts.

In poleroviruses and luteoviruses, ORF 3-5 encodes the minor capsid protein which is composed of a fusion of the coat protein and the read-through domain (Wang et al., 1995). The read-through domain (RT) is further processed, with the full RT being nonstructural and the truncated RT* is incorporated into the virion (Boissinot et al., 2014). While the RT* is incorporated into virions, it has been shown for other Luteoviruses and Poleroviruses that mutants unable to produce RT* will still encapsidate and do not affect the particle shape when viewed with electron microscopy (Boissinot et al., 2014; Brault et al., 1995; Filichkin et al., 1994). This truncation most likely occurs near a conserved DE motif (Guilley et al., 1994; Revollon et al., 2010) and trims off the C-terminal of the protein. Both the PLRV-GFP and TuYV-GFP infectious clones created in (Nurkiyanova et al., 2000) and (Boissinot et al., 2017) were designed to produce GFP only by the non-truncated full protein. Similarly the EGFP sequence for CLRDV-GFP was inserted after all DE domains present in ORF 5, and thus expresses GFP only on non-truncated P3-5 proteins. After truncation of P3-5 the C terminal end that is cut off is likely degraded (Boissinot et al., 2014; Brault et al., 1995) and so it is expected that GFP cut off from processed RT* in CLRDV-GFP is degraded and does not accumulate in cells.

2.5.3 CLRDV-GFP Microscopy

CLRDV-GFP produces extremely faint GFP fluorescence only detectable by microscopy in cotton. CLRDV is a low titer virus which produces ORF3-5 using an amber stop mechanism, so it was expected to have low GFP production. The C-terminus of ORF 3-5 that contains the

GFP sequence has also been theorized to be degraded after being clipped off of P3-5s incorporated into virions (Boissinot et al., 2014; Brault et al., 1995). Due to these factors the only GFP visibly present in CLRDV-GFP infected cells are either un-truncated P3-5s or possibly the waste product of P3-5 truncation.

2.5.4 CLRDV-GFP Mutants

CLRDV-GFP was able to move systemically to upper leaves without modifying the size of the tested region. This result is similar to the TuYV-GFP clone, which the authors note as not infecting systemically at the same rate as their wild-type clone (Boissinot et al., 2017). Further tests may be done to determine if our clone loses some ability to infect systemically. CLRDV-GFP was also detected in the roots to have mutated out some sequence from the region tested. This finding suggests that the virus required some adaptation in order to move to the roots that was not necessary for systemically infecting the upper leaves. Sequencing of the CLRDV-GFP mutants detected in this study would determine what parts of the genome was excised.

In this study two infectious clones are produced. The self-replication and systemic movement of both clones has been confirmed by PCR. CLRDV-Macon1 produces infection similar to wild type infections, and thus can be used as a molecular tool for future projects involving CLRDV. CLRDV-GFP provides a virus that produces a molecular marker such that infection by this clone can be determined by fluorescent microscopy. Further understanding of CLRDV infection and transmission may be developed from these infectious clones.

2.6 References

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. https://apsjournals.apsnet.org/doi/pdf/10.1094/PHYTO-09-16-0349-R?download=true

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. https://apsjournals.apsnet.org/doi/pdf/10.1094/PDIS-06-19-1316-RE?download=true

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

Boissinot, S., Erdinger, M., Monsion, B., Ziegler-Graff, V., & Brault, V. (2014). Both structural and non-structural forms of the readthrough protein of cucurbit aphid-borne yellows virus are essential for efficient systemic infection of plants. *PLoS One*, *9*(4), e93448. https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0093448&type=printable

Boissinot, S., Pichon, E., Sorin, C., Piccini, C., Scheidecker, D., Ziegler-Graff, V., & Brault, V. (2017). Systemic Propagation of a Fluorescent Infectious Clone of a Polerovirus Following Inoculation by Agrobacteria and Aphids. *Viruses*, *9*(7). https://doi.org/10.3390/v9070166

Brault, V., Uzest, M., Monsion, B., Jacquot, E., & Blanc, S. (2010). Aphids as transport devices for plant viruses. *Comptes rendus biologies*, *333*(6-7), 524-538.

Brault, V., van den Heuvel, J. F., Verbeek, M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Garaud, J. C., Guilley, H., Richards, K., & Jonard, G. (1995). Aphid transmission of beet western yellows luteovirus requires the minor capsid read-through protein P74. *EMBO J*, *14*(4), 650-659. https://doi.org/10.1002/j.1460-2075.1995.tb07043.x

Bruyere, A., Brault, V., Ziegler-Graff, V., Simonis, M.-T., Van den Heuvel, J., 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.

Chakrabarty, R., Banerjee, R., Chung, S.-M., Farman, M., Citovsky, V., Hogenhout, S. A., Tzfira, T., & Goodin, M. (2007). PSITE vectors for stable integration or transient expression of autofluorescent protein fusions in plants: probing Nicotiana benthamiana-virus interactions. *Molecular Plant-Microbe Interactions*, *20*(7), 740-750.

Chomič, A., Pearson, M., Clover, G., Farreyrol, K., Saul, D., Hampton, J., & Armstrong, K. (2010). A generic RT-PCR assay for the detection of Luteoviridae. *Plant Pathology*, *59*(3), 429-442.

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. https://doi.org/10.1111/ppa.13368

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. https://www.sciencedirect.com/science/article/pii/S016817021300124X?via%3Dihub

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*, 1849-1854.

Filichkin, S., Lister, R., McGrath, P., & Young, M. (1994). In vivo expression and mutational analysis of the barley yellow dwarf virus readthrough gene. *Virology*, *205*(1), 290-299. https://www.sciencedirect.com/science/article/pii/S004268228471645X?via%3Dihub

Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., Hutchison III, C. A., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature methods*, *6*(5), 343-345. https://www.nature.com/articles/nmeth.1318

Goodin, M. M., Dietzgen, R. G., Schichnes, D., Ruzin, S., & Jackson, A. O. (2002). pGD vectors: versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. *Plant J*, *31*(3), 375-383. https://doi.org/10.1046/j.1365-313x.2002.01360.x

Guilley, H., Wipf-Scheibel, C., Richards, K., Lecoq, H., & Jonard, G. (1994). Nucleotide sequence of cucurbit aphid-borne yellows luteovirus. *Virology*, *202*(2), 1012-1017. https://www.sciencedirect.com/science/article/pii/S0042682284714292?via%3Dihub

Lindbo, J. A. (2007). TRBO: a high-efficiency tobacco mosaic virus RNA-based overexpression vector. *Plant Physiol*, *145*(4), 1232-1240. https://doi.org/10.1104/pp.107.106377

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. (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.

Nurkiyanova, K. M., Ryabov, E. V., Commandeur, U., Duncan, G. H., Canto, T., Gray, S. M., Mayo, M. A., & Taliansky, M. E. (2000). Tagging Potato leafroll virus with the jellyfish green fluorescent protein gene. *Journal of General Virology*, *81*(3), 617-626.

Olmedo-Velarde, A., Wilson, J. R., Stallone, M., DeBlasio, S. L., Chappie, J. S., & Heck, M. (2023). Potato leafroll virus molecular interactions with plants and aphids: Gaining a new tactical advantage on an old foe. *Physiological and Molecular Plant Pathology*, *125*. https://doi.org/10.1016/j.pmpp.2023.102015

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.

Revollon, S., Strub, J. M., Fitchette, A. C., Wiss, L., Gomord, V., Van Dorsselaer, A., & Brault, V. (2010). A reinvestigation provides no evidence for sugar residues on structural proteins of poleroviruses and argues against a role for glycosylation of virus structural proteins in aphid transmission. *Virology*, *402*(2), 303-314. https://doi.org/10.1016/j.virol.2010.03.037

Sharman, M., Lapbanjob, S., Sebunruang, P., Belot, J.-L., Galbieri, R., Giband, M., & Suassuna, N. (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*, 1-4.

Sharmeen, L., Kuo, M., Dinter-Gottlieb, G., & Taylor, J. (1988). Antigenomic RNA of human hepatitis delta virus can undergo self-cleavage. *Journal of Virology*, *62*(8), 2674-2679.

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

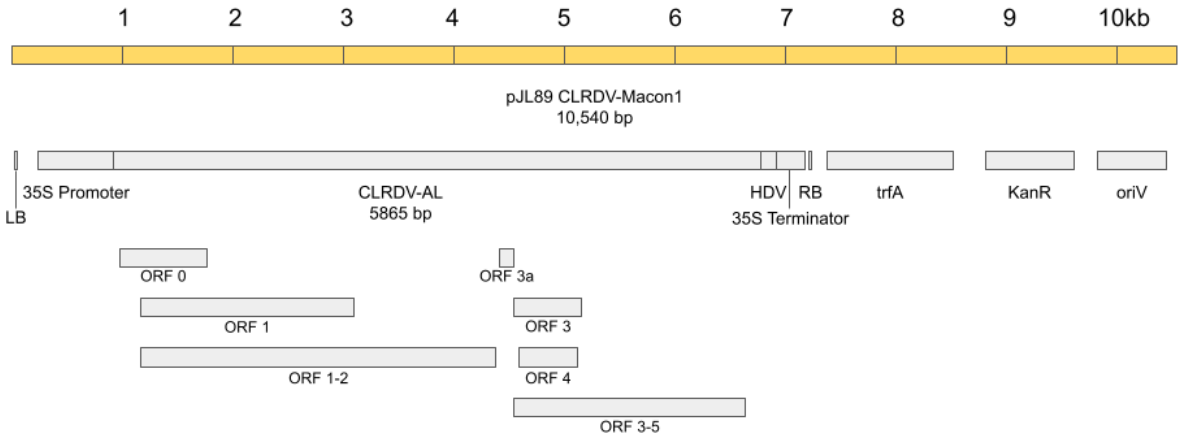
Stevens, M., & Viganó, F. (2007). Production of a full-length infectious GFP-tagged cDNA clone of Beet mild yellowing virus for the study of plant–polerovirus interactions. *Virus Genes, 34*(2), 215-221. https://link.springer.com/content/pdf/10.1007/s11262-006-0046-z.pdf

Sunilkumar, G., Mohr, L., Lopata-Finch, E., Emani, C., & Rathore, K. S. (2002). Developmental and tissue-specific expression of CaMV 35S promoter in cotton as revealed by GFP. *Plant molecular biology*, *50*(3), 463-474.

Wang, J., Chay, C., Gildow, F., & Gray, S. (1995). Readthrough protein associated with virions of barley yellow dwarf luteovirus and its potential role in regulating the efficiency of aphid transmission. *Virology*, *206*(2), 954-962. https://www.sciencedirect.com/science/article/pii/S0042682285710185?via%3Dihub

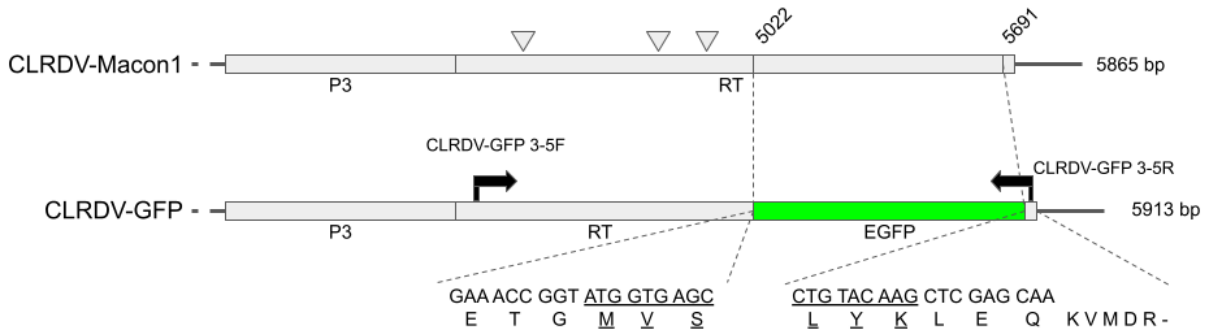
Section 2 Figures

Figure 2.1. Vector map of CLRDV-Macon1 infectious clone.



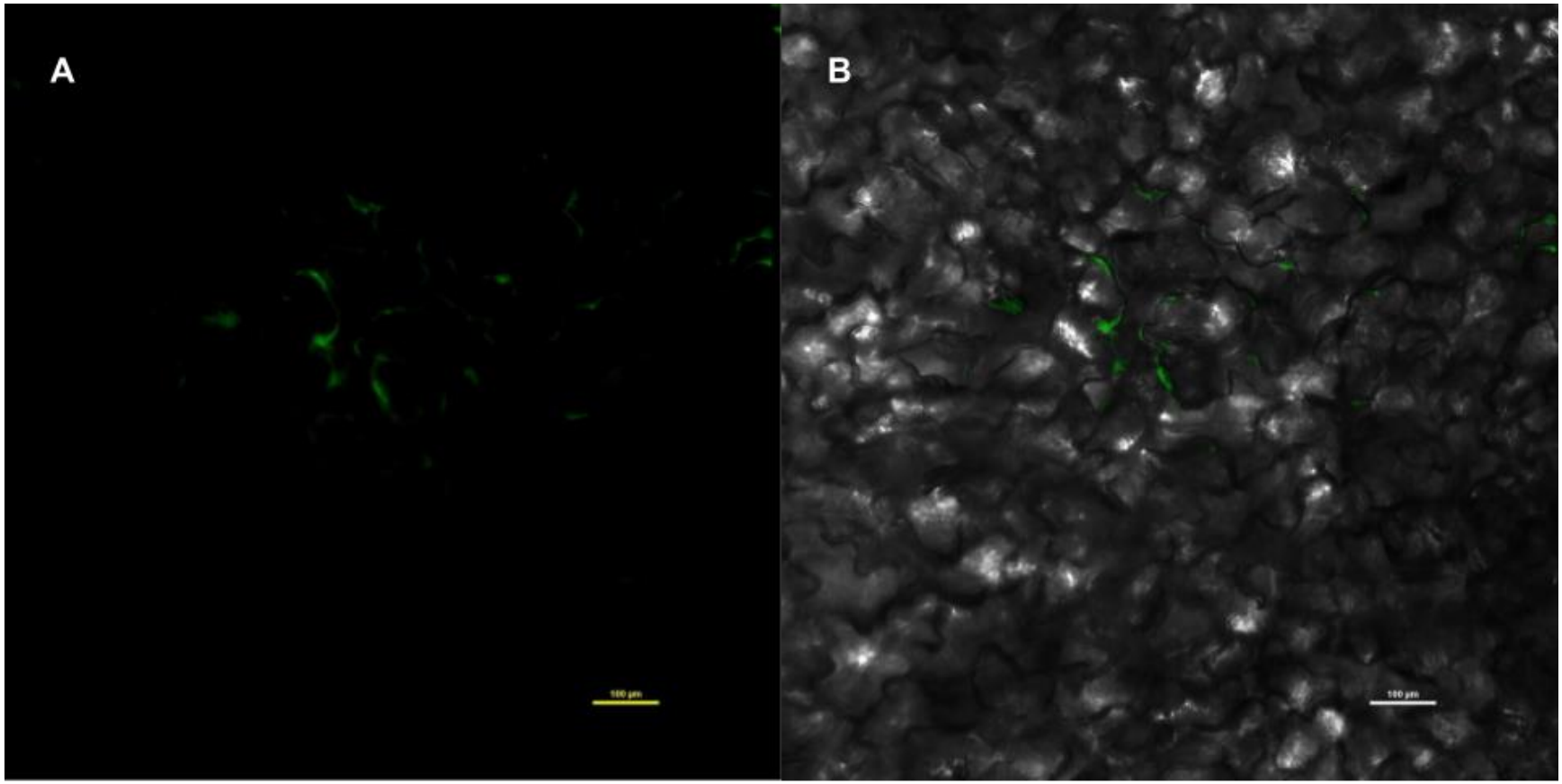
Open reading frames of CLRDV are shown below the viral insert. The sequence of CLRDV-AL (accession number MN071395.1) is under the control of a 35s promotor and terminator. The HDV is an insert that self-cleaves as RNA, leaving the genomic CLRDV RNA with no poly-A tail (Sharmeen et al., 1988). The trfA, Kanamycin resistance (KanR) and origin (oriV) are required for replication and selection of the plasmid in *E. coli* and *A. tumefaciens*.

Figure 2.2. Construction of CLRDV-GFP from CLRDV-Macon1.



The ORF 3-5 is shown with the location of the EGFP replacement. Upside down triangles represent conserved DE domains in ORF 5 near which P3-5 is truncated to be incorporated into virions. Underlined nucleotide and protein sequences represent EGFP; the non-underlined sequences are viral. Black arrows indicate the primers used to detect deletion mutants of CLRDV-GFP.

Figure 2.3. Microscopy of CLRDV-GFP 4 days post infiltration in cotton cotyledons.



GFP produced by CLRDV-GFP by epifluorescence (A). Combined epifluorescence and brightfield of CLRDV-GFP in the boundaries of infected cells (B).

Table 2.1. Primers used in Ch. 2

Primer name	Sequence (5'-3')	Citation	Use
CLRDV-FULL (R)	GGGAGGGGGGACTCTCCCTGGGGTTTCGGT		
CLRDV-GFP 3-5F	CTCCTTCCCCTCCTCCTCCAG		Amplification of the RT region of CLRDV-GFP from infected plants
CLRDV-GFP 3-5R	CTTACCTATCCATCACCTTTTGCTCGAG		
Pol3628F	TAATGAATACGGYCGYGGSTAG	Sharman et al. 2015	
Pol4021R	GGRTCMAVYTCRTAAGMGATSGA	Sharman et al. 2015	
CLRDV3675F	CCACCTAGRCGCAACAGGCGT	Sharman et al. 2015	Detection of CLRDV-Macon1
CLRDV Pol3982R	CGAGGCCTCGGAGATGAACT	Sharman et al. 2015	
Lu4	GTCTACCTATTTGG	Chomič et al. 2010	
pJLCLRDV-FW	TGTACAAGCTCGAGCAAAAGGTGA		
pJLCLRDV-RV	CTCACCATACCGGTTTCAGATTCG		Gibson assembly primers used to amplify and assemble CLRDV-GFP
GFP-FW	AAACCGGTATGGTGAGCAAGGGC		
GFP-RV	TGCTCGAGCTTGTACAGCTCGTCC		

Table 2.2. Tissue samples collected from CLRDV-GFP based on time post infiltration.

	Tissues collected				
Days Post Infiltration	**Roots**	**Cotyledons**	**1st leaf**	**2nd leaf**	**3rd leaf**
3		+			
7		+	+		
14	+		+	+	
21	+		+	+	
28	+		+	+	+

Table 2.3. Infection rate of CLRDV-Macon1 in cotton and *N. benthamiana.*

Host plant	**Experiment**	**# Systemic Infections / # Infiltrated**	**% Infection**
Gossypium hirsutum	Negative control A	1/6	17%
	Negative control B	1/6	17%
	A 1	7/10	70%
	A 2	4/10	40%
	B 1	4/10	40%
	B 2	8/10	80%
	B 3	9/10	90%
Nicotiana benthamiana	Negative control A	0/6	0%
	A 1	4/10	40%
	A 2	1/10	10%

Table 2.4. Infection rate of CLRDV-GFP

Days Post Infiltration	Positive Plants / Total
3	4/4
7	3/4
14	2/2
21	4/4
28	1/4

Chapter 3 -
Uncovering the transmission determinants of Cotton leafroll dwarf virus.

3.1 Abstract

Cotton leafroll dwarf virus (CLRDV) is a virus spread by *Aphis gossypii* to many weeds and cotton (*Gossypium hirsutum* L). It is in the genus *Polerovirus*, which requires transcytosis of the virions through the gut and accessory salivary glands in order to be transmitted. This mechanism requires a protein-protein interaction of the virion and membrane proteins, and this interaction can be prevented by feeding the aphid a soluble form of the interacting protein on the outside of the virion. In this paper, a system of feeding aphids viral proteins by feeding them on *Agrobacterium tumefaciens* transformed cotton cotyledons is established. This is the first step in determining the transmission determining protein of CLRDV.

3.2 Introduction

Cotton leafroll dwarf virus is a *Polerovirus* recently introduced to the United States which infects and reduces yield of cotton (*Gossypium hirsutum* L.). This virus historically has been found in many major cotton producing countries, including Brazil, Argentina, India, Thailand, East Timor (Corrêa et al., 2005; Mukherjee et al., 2012; Ray et al., 2016) and has been found in soybean aphids in China (Feng et al., 2017). While this virus has been found worldwide, strains and symptoms vary wildly. South American typical CLRDV, identified in (Corrêa et al., 2005) was estimated to cause almost 80% yield loss for susceptible cultivars, while damage in the United States from this virus so far has been limited (Lawrence, 2022). CLRDV in the United States can often cause minor to cryptic symptoms, causing disease tracking to be difficult and may represent an invisible loss across cotton production in America.

CLRDV is in the genus *Polerovirus*, in the family *Solemoviridae* (Sõmera et al., 2021). These viruses are positive sense, single stranded RNA and are encapsidated in icosahedral virions. They are low titer, phloem limited, and are solely transmitted by aphids (Sõmera et al., 2021). In aphids, poleroviruses are not thought to uncoat nor replicate, and must pass through the aphid gut, the hemolymph, and into the salivary glands in order to be transmitted to new host plants, making them circulative, yet non-propagative viruses (Brault et al., 2007; Brault et al., 2010; Reinbold et al., 2003). Poleroviruses are known to interact with host proteins in order to pass through two cellular barriers, the gut and salivary glands (Brault et al., 2007; Reinbold et al., 2003). Once the aphids acquire a circulative virus they are considered to be viruliferous for the rest of their lives (Brault et al., 2010), which for *A. gossypii* is about 27 days as an adult (Heilsnis et al., 2023). Virus acquisition is by feeding on infected plants, and no poleroviruses have been identified as being passed to offspring through vertical transovarial transmission.

As poleroviruses do not replicate inside their vector, the only proteins which interact with vector proteins are the major and minor capsid proteins. The major coat protein (P3) is encoded on a subgenomic RNA, and protein translation is terminated by an amber stop codon (Distéfano et al., 2010; Sõmera et al., 2021). P3-5 is a fusion protein containing P3 and a Read-Through domain (RT) (Boissinot et al., 2014; Brault et al., 1995; Bruyere et al., 1997), which is attached to the P3 coat protein by a linker peptide sequence (Bahner et al., 1990; Brault et al., 2005) and dimerizes with nearby RTs in order to form spikes on the outside of the virion (Schiltz et al., 2022). Translation of P3-5 is caused by the leaky amber stop, along with two regions that translate the read-through domain (Bruyere et al., 1997; Xu et al., 2018). P3-5 is post-translationally modified as well. The C-terminal end is truncated post-translationally to form a shorter RT (notated as RT*) which is incorporated into virions (Boissinot et al., 2014; Brault et

al., 2000; Brault et al., 1995; Bruyere et al., 1997; Schiltz et al., 2022). The N-terminal region of the RT is responsible for interacting with aphid receptors to move the virion from the gut lumen to the salivary glands (Schiltz et al., 2022). The full P3-5 is also expressed as a soluble protein and acts as a secondary movement protein, limits infection to the phloem tissue, and may be implicated in causing symptoms (Boissinot et al., 2014; Bruyere et al., 1997).

For many plant viruses, they must interact with their vector by a protein-protein interaction in order to be spread by that vector (Brault et al., 2010). This has led to the theory that feeding an insect a transiently expressed form of that viral protein could act as a competitor to virion uptake (See figure 3.2). This interaction has been shown before with Tomato spotted wilt virus glycoprotein, Gn, which when fed to thrips provided competition for uptake and reduced the infection of thrips by TSWV (Whitfield et al., 2008). This concept has also been tested and shown in Potato leafroll virus, in which the N-terminal region of the RT* was identified to be the protein responsible for virus circulation through the aphid gut and circulatory systems (Schiltz et al., 2022). Schiltz et al. performed this experiment by feeding aphids (*Myzus persicae*) on both artificial media containing bacteria-produced RT*, and by feeding aphids on agro-infiltrated leaves expressing the PLRV RT* in *Nicotiana benthamiana*. While this paper demonstrates a long-theorized aspect of polerovirus transmission, this may be a specific interaction and not a conserved mechanism. While transcytosis of virions is thought to be clathrin-mediated endocytosis (Brault et al., 2007), the receptor proteins responsible for binding are likely unique to each virus-vector system due to their evolutionary divergence (Brault et al., 2005). The aims of this paper are to determine if *Aphis gossypii* fed on infiltrated cotton cotyledons will uptake agrobacterium-expressed proteins, and if feeding aphids CLRDV coat proteins P3 or P3-5 will block or reduce transmission of CLRDV.

3.3 Materials and Methods

3.3.1 Growth and maintenance of plants

Cotton plants were grown under UV light with a 14 hour photoperiod. All plants were germinated under UV lights in artificial soil at room temperature. Cotton plants (DeltaPine 1646 B2XF (Bayer, Leverkusen, Germany)) were used for infiltration at 1-2 weeks old (seedling stage cotton with no true leaves), as only the cotyledons can be infiltrated. *N. benthamiana* plants were grown under similar conditions, however they were allowed to germinate in a small pot before being transplanted to a larger tray. The plants were watered regularly and kept free of insects.

3.3.2 Construction of expression vectors for coat proteins of CLRDV.

In order to test blocking transmission by CLRDV P3 and P3-5 coat proteins, the sequence for these proteins were cloned out of the CLRDV-Macon1 infectious clone produced by (Chapter 2) and inserted into pSITE 2CA, 2NA, 4CA and 4NA expression vectors (Chakrabarty et al., 2007). Both P3 and P3-5 were amplified from the CLRDV-Macon1 infectious clone (based on Genbank Accession MN071395.1) by Phusion PCR using the primers presented in (Table 3.1). The sequences were then cloned into pENTR D-Topo following manufacturer protocols, and transformed into Top10 *Escherichia coli* and grown overnight on selective media. Positive colonies were identified and grown in liquid media overnight with shaking at 37°C. These were then miniprepped using ZymoPURE Plasmid miniprep kit (Zymo research Irvine, USA) following manufacturer's protocols. The constructs were sequenced to confirm that there were no mutations before continuing. They were then transformed into pSITE vectors with either N-terminal or C-terminal GFP or mRFP by LR reaction. C-terminal Myc and Flag-tagged versions of both proteins were also created (Min et al., 2010). The pSITE plasmids containing P3 or P3-5 were then transformed into Top10 *E. coli* and grown on selective media overnight. Positive colonies were identified, grown overnight and miniprepped as previously described. Completed

pSITE vectors were sequenced and confirmed to be correct before being transformed into *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium* transformation was performed by mixing 10 µL of pSITE plasmid with 50 µL of competent agrobacterium, which were then mixed and heat-shocked by dropping the tube into liquid nitrogen. The tubes were then thawed at 37°C for five minutes, then 1mL of LB broth was added to the tube. The cells were allowed to incubate at 28°C for 2 hours, then plated on selective media and set in a 28°C incubator. Individual colonies were identified 3 days after transformation, then streaked to a new selective LB agar plate. The plates were set back into the incubator to allow the single colonies to cover the plates, then glycerol stocks were prepared of the transformed agrobacterium. An empty pSITE 2CA vector producing free-GFP was also transformed into agrobacterium to be used as a positive control.

3.3.3 Microscopy of P3 and P3-5 vectors

The resulting pSITE constructs were then checked by fluorescent microscopy to ensure that tagged proteins were being expressed by the plasmid. Both cotton cotyledons and *N. benthamiana* leaves were infiltrated with each construct and analyzed by epifluorescent microscopy. Plants were grown in at room temperature and leaves were infiltrated with each pSITE construct and photographed 3 days post infiltration.

Infiltrations were performed based on the protocol described in (Goodin et al., 2002). *A. tumefaciens* was grown on selective plates for three days, then suspended in .01 M MES/$MgCl_2$ media at a OD_{600} absorbance of OD_{600} of between 0.6-1.0. 0.1M acetosyringone was then added to the infiltration media, and the media was incubated for two hours at room temperature before infitrating with a needless syringe on the underside of cotton and *N. benthamiana* leaves.

3.3.4 Feeding aphids on plants infiltrated with constructs.

Aphids were fed on GFP-P3 infiltrated cotton cotyledons in order to determine if they can acquire by feeding a protein expressed in cotton cotyledons. Cotton was infiltrated as previously described at an OD600 of 0.6-1.0, and three days after leaf disks were cut from the infiltrated area. The treatments included a leaf infiltrated with an empty pJL89 vector, as well as a pSITE vector producing free GFP as a positive control for Western blots. Ten aphids were placed on each leaf disk and allowed to feed for 24 hours, after which they were immediately collected and ground into 4x Laemmli buffer. Aphids and the leaf disks they fed on were collected as separate samples, and this experiment was performed three times. Each replicate included three leaf disks infiltrated with GFP-P3 and one infiltrated with the negative control.

3.3.5 Detection of coat proteins of CLRDV in Aphids

Immunoblots of P3 and P3-5 GFP fusions were performed using the Bio-Rad Mini-PROTEAN® Tetra Cell and Tetra Blotting Module (Hercules, CA USA) following manufacturer's protocols. Western blot samples were collected in 4x Laemmli Sample buffer (Bio-Rad Hercules, CA USA) mixed with 2-mercaptoethanol as instructed by the manufacturer. For leaf samples, leaf disks approximately 1 cm in diameter were ground in 200 µl of sample buffer. Ten aphids were collected from the leaf disk and ground in 100 µl of sample buffer. Wells were loaded with 10 µL of sample and run at 150 volts for 50 minutes. Transfer of the proteins to the Amersham Protran 0.1 NC nitrocellulose Western blotting membrane (Marlborough, USA) was performed following manufacturer's protocols. After transfer, the membrane was blocked in 1% w/v dried milk dissolved in 20 mM Tris, 150 mM NaCl blocking buffer for one hour following the protocol in (Russell & Sambrook, 2001). The membrane was then exposed to NB100-56401 GFP Antibody (Centennial, CO USA) in the blocking buffer overnight, then was rinsed three times in 1X TBS (20 mM Tris, 150 mM NaCl) buffer before

being exposed to Anti-Rabbit IgG (Fc), AP Conjugate (Promega, Madison, WI USA) for 1 hour in blocking buffer. The membrane was again rinsed three times in TBS buffer then exposed in AP substrate as described for AP development (Russell & Sambrook, 2001).

3.4 Results

All pSITE constructs were confirmed at least once by sequencing with specific GFP or mRFP primers, or by M13 primers (primer table). GFP-P3 showed promising initial results with strong expression in plants, and therefore was chosen for following experiments. GFP-P3 was infiltrated into cotton cotyledons three separate times, and is shown in Figure 3.1. The protein localizes on the cell periphery, and appears to form punctate spots in a few cells.

The empty pSITE 2CA vector producing free GFP did not appear to fluoresce in the infiltrated cotton cotyledons.

GFP-P3 was detected from all aphid samples that fed on GFP-P3 infiltrated leaf disks, however the protein was not detected in the GFP-P3 leaf disks themselves. No bands were detected in aphids or leaves infiltrated with pJL empty.

3.5 Discussion

Western blots of infiltrated GFP-P3 show that the protein is detectable from aphids that fed on infiltrated leaf disks. While GFP-P3 was not detected in infiltrated leaf disks, it is thought that the overall expression level of the protein in the transformed leaf disks is below the detection threshold of the Western blot method used. The protein was detectible in the aphids likely because the protein accumulated in their digestive tract, becoming more concentrated than the leaf disks they fed on. It is shown in this paper that cotton is a suitable plant for protein expression and aphid feeding experiments. This system is advantageous because it allows future work involving *A. gossypii* on cotton to use the native host plant instead of using *Nicotiana*

benthamiana. This is especially useful for the CLRDV-*A. gossypii*-*G. hirsutum* because it is unknown how well these insects feed on *N. benthamiana*. Initially it was unknown if aphids would acquire a protein agroinfiltrated into cotton leaf tissue. Aphids are phloem-feeders, and while agroinfiltration often can spread through the whole leaf, it is unknown how well phloem cells are successfully transformed by the agro-infiltration.

The result that GFP-P3 can be detected from aphids fed on infiltrated cotyledons is likely not due to protein-protein binding. The N-terminal of the RT* domain has been shown to be responsible for binding in Potato leafroll virus, and the authors of that paper state that this is likely a conserved function. It is anticipated that GFP-P3 is simply present in the digestive system and would be eliminated if the aphids were allowed to feed on a non-infiltrated plant for a few hours. Further experiments need to be performed to confirm this hypothesis.

3.6 Future work

It is likely that both the CLRDV-Macon1 clone and CLRDV-GFP clone are aphid transmissible, though this remains to be tested. CLRDV-Macon1 appears to act very similarly to wild infection in greenhouse settings, and is true to the viral sequence posted in (accession MN071395.1). The CLRDV-GFP clone was produced in such a way that EGFP replaced the C-terminal region of ORF3-5, and it is theorized that this clone produces an un-adulterated RT* on the virion. Aphid transmission will not be affected, however compromised the clone is in systemic infection due to the loss of the C-terminal region (Boissinot et al., 2014).

One barrier to testing if P3 or P3-5 is responsible for aphid uptake is in the production of a pure RT* region. Infiltrating an expression plasmid containing P3-5 as the sequence is in the infectious clone would produce many P3s, and only a few read-throughs. In order to prove that blocking comes solely from P3 or from the RT* region, these must be separated. Schiltz et al

isolated the N-terminal region of ORF 5, with the truncated section and P3 region removed. In normal infection, this protein dimerizes to form a “cap” structure on the virion, but the dimerization is caused by P3 proteins interacting with each other to force the RT* domains together. Schiltz et al. got around this issue by producing a YFP N-terminal tagged RT*, which eliminated the P3 region while causing a force for dimerization in planta. It is also unknown where the CLRDV P3-5 protein is truncated, and this truncation has been shown in other poleroviruses to not be sequence dependent but based on secondary structure (Boissinot et al., 2014). A similar approach should be followed for our experiment, in order to confirm that the N-terminal region alone is responsible for aphid uptake and P3 is not involved.

Further experiments should be performed to deduce how long a fed protein such as GFP-P3 is present in the aphids. The blocking experiment, where the aphids are fed on a P3 or P3-5 (RT* only) construct may not work if the proteins are passed through the system in a few hours. The time that the aphids are allowed to acquire the virus should be adjusted to how long the previously fed proteins are in the gut in order to achieve the greatest difference between fed-protein controls and no fed-protein controls. The transmission assays using just the clones would also need to use the same acquisition access period, so that transmission rates between the two groups can be compared.

3.7 References

Bahner, I., Lamb, J., Mayo, M., & Hay, R. (1990). Expression of the genome of potato leafroll virus: readthrough of the coat protein termination codon in vivo. *Journal of General Virology*, *71*(10), 2251-2256.

Boissinot, S., Erdinger, M., Monsion, B., Ziegler-Graff, V., & Brault, V. (2014). Both structural and non-structural forms of the readthrough protein of cucurbit aphid-borne yellows virus are essential for efficient systemic infection of plants. *PLoS One*, *9*(4), e93448. https://journals.plos.org/plosone/article/file?id=10.1371/journal.pone.0093448&type=printable

Brault, V., Herrbach, E., & Reinbold, C. (2007). Electron microscopy studies on luteovirid transmission by aphids. *Micron*, *38*(3), 302-312. https://www.sciencedirect.com/science/article/pii/S0968432806000709?via%3Dihub

Brault, V., Mutterer, J., Scheidecker, D., Simonis, M., Herrbach, E., Richards, K., & Ziegler-Graff, V. (2000). Effects of point mutations in the readthrough domain of the beet western yellows virus minor capsid protein on virus accumulation in planta and on transmission by aphids. *Journal of Virology*, *74*(3), 1140-1148. https://journals.asm.org/doi/pdf/10.1128/jvi.74.3.1140-1148.2000?download=true

Brault, V., Périgon, S., Reinbold, C., Erdinger, M., Scheidecker, D., Herrbach, E., Richards, K., & Ziegler-Graff, V. (2005). The polerovirus minor capsid protein determines vector specificity and intestinal tropism in the aphid. *Journal of Virology*, *79*(15), 9685-9693. https://journals.asm.org/doi/pdf/10.1128/jvi.79.15.9685-9693.2005?download=true

Brault, V., Uzest, M., Monsion, B., Jacquot, E., & Blanc, S. (2010). Aphids as transport devices for plant viruses. *Comptes rendus biologies*, *333*(6-7), 524-538.

Brault, V., van den Heuvel, J. F., Verbeek, M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Garaud, J. C., Guilley, H., Richards, K., & Jonard, G. (1995). Aphid transmission of beet western yellows luteovirus requires the minor capsid read-through protein P74. *EMBO J*, *14*(4), 650-659. https://doi.org/10.1002/j.1460-2075.1995.tb07043.x

Bruyere, A., Brault, V., Ziegler-Graff, V., Simonis, M.-T., Van den Heuvel, J., 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.

Chakrabarty, R., Banerjee, R., Chung, S.-M., Farman, M., Citovsky, V., Hogenhout, S. A., Tzfira, T., & Goodin, M. (2007). PSITE vectors for stable integration or transient expression of autofluorescent protein fusions in plants: probing Nicotiana benthamiana-virus interactions. *Molecular Plant-Microbe Interactions*, *20*(7), 740-750.

Corrêa, R., Silva, T., Simões-Araújo, J., Barroso, P., Vidal, M., & Vaslin, M. (2005). Molecular characterization of a virus from the family Luteoviridae associated with cotton blue disease. *Archives of Virology*, *150*, 1357-1367. https://link.springer.com/content/pdf/10.1007/s00705-004-0475-8.pdf

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*, 1849-1854.

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.

Goodin, M. M., Dietzgen, R. G., Schichnes, D., Ruzin, S., & Jackson, A. O. (2002). pGD

vectors: versatile tools for the expression of green and red fluorescent protein fusions in agroinfiltrated plant leaves. *Plant J*, *31*(3), 375-383. https://doi.org/10.1046/j.1365-313x.2002.01360.x

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*. https://doi.org/10.1093/jee/toad080

Lawrence, K. S., Amanda Strayer-Scherer, Randy Norton, Jiahuai Hu, Travis R. Faske, Robert Hutmacher, John Muller, Ian Small, Zane J. Grabau, Robert Kemerait, Rodrigo Borba Onofre, Paul Price, Tristan Watson, Thomas W. Allen, Kaitlyn Bissonnette, John Idowu, Guy D. Collins, Seth A. Byrd, Heather M Kelly, Terry A. Wheeler, Thomas Isakeit, Cecilia Monclova-Santana, and David B. Langston. (2022). *Cotton Disease Loss Estimate Committee Report, 2021*. National Cotton Council of America. Retrieved 6/2/2023 from https://www.cotton.org/beltwide/proceedings/2005-2022/data/conferences/2022/paper/21402.pdf

Min, B.-E., Martin, K., Wang, R., Tafelmeyer, P., Bridges, M., & Goodin, M. (2010). A host-factor interaction and localization map for a plant-adapted rhabdovirus implicates cytoplasm-tethered transcription activators in cell-to-cell movement. *Molecular Plant-Microbe Interactions*, *23*(11), 1420-1432.

Mukherjee, A., Chahande, P., Meshram, M., & Kranthi, K. (2012). First report of Polerovirus of the family Luteoviridae infecting cotton in India. *New Dis Rep*, *25*(22), 2044-0588.2012.

Ray, J., Sharman, M., Quintao, V., Rossel, B., Westaway, J., & Gambley, C. (2016). Cotton leafroll dwarf virus detected in Timor-Leste. *Australasian Plant Disease Notes*, *11*, 1-3.

Reinbold, C., Herrbach, E., & Brault, V. (2003). Posterior midgut and hindgut are both sites of acquisition of Cucurbit aphid-borne yellows virus in Myzus persicae and Aphis gossypii. *Journal of General Virology*, *84*(12), 3473-3484.

Russell, D. W., & Sambrook, J. (2001). Molecular cloning: a laboratory manual (Vol. 1). Cold Spring Harbor Laboratory Cold Spring Harbor, NY.

Schiltz, C. J., Wilson, J. R., Hosford, C. J., Adams, M. C., Preising, S. E., DeBlasio, S. L., MacLeod, H. J., Van Eck, J., Heck, M. L., & Chappie, J. S. (2022). Polerovirus N-terminal readthrough domain structures reveal molecular strategies for mitigating virus transmission by aphids. *Nature Communications*, *13*(1), 6368.

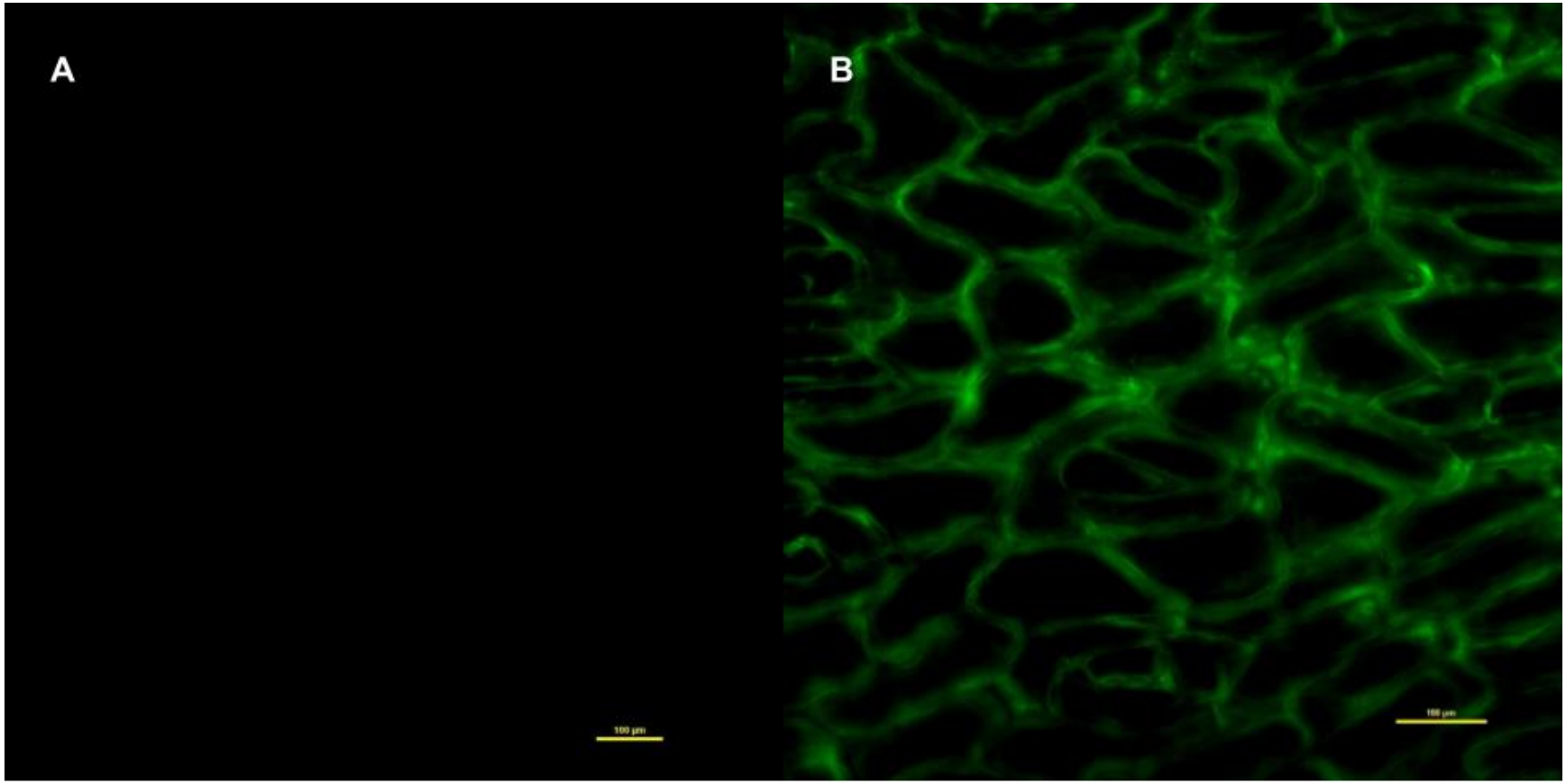
Sõmera, M., Fargette, D., Hébrard, E., Sarmiento, C., & Consortium, I. R. (2021). ICTV virus taxonomy profile: Solemoviridae 2021. *The Journal of General Virology*, *102*(12).

Whitfield, A., Kumar, N. K., Rotenberg, D., Ullman, D., Wyman, E., Zietlow, C., Willis, D., & German, T. (2008). A soluble form of the Tomato spotted wilt virus (TSWV) glycoprotein GN (GN-S) inhibits transmission of TSWV by Frankliniella occidentalis. *Phytopathology*, *98*(1), 45-50.

Xu, Y., Ju, H.-J., DeBlasio, S., Carino, E. J., Johnson, R., MacCoss, M. J., Heck, M., Miller, W. A., & Gray, S. M. (2018). A stem-loop structure in potato leafroll virus open reading frame 5 (ORF5) is essential for readthrough translation of the coat protein ORF stop codon 700 bases upstream. *Journal of Virology*, *92*(11), e01544-01517. https://journals.asm.org/doi/pdf/10.1128/jvi.01544-17?download=true

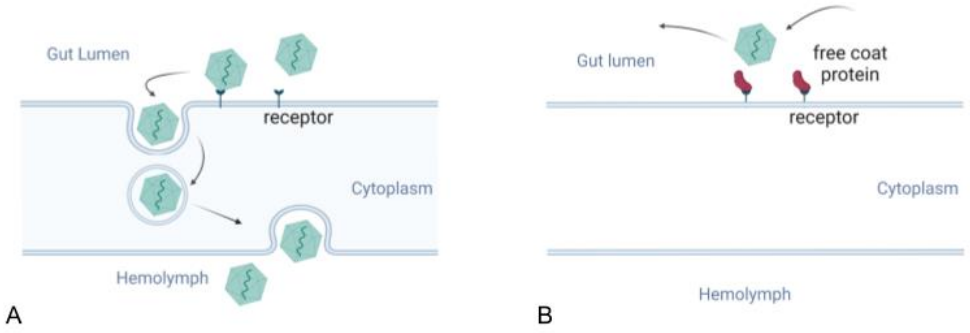
Section 3 Figures

Figure 3.1. Fluorescent microscopy of pJL empty and GFP-P3.



Fluorescent microscopy of pJL empty (A) and GFP-P3 (B). The free GFP vector is not shown due to it not producing fluorescence when infiltrated in cotton. GFP-P3 clusters around the cell periphery, and often forms aggregations.

Figure 3.2. Transcytosis diagram.



The virus under typical conditions would bind to a receptor and be engulfed in a vesicle in order to be moved through the cell (A). If the protein responsible for this interaction was fed to the insect beforehand, then it would theoretically compete for the target site and prevent transcytosis (B).

Table 3.1. Primers used in Ch. 3

Primer name	**Sequence (5'-3')**	**Use**
CLRDV ORF3 F	ATGAATACGGTCGTGGGTAGAAGAACGATC	
CLRDV ORF3 R	TTTGGGGTTATGGAATTGGCACCG	Cloning into pENTR
CLRDV-3-5R	CCTATCCATCACCTTTTGCTCGAG	
M13 F (-20)	GTAAAACGACGGCCAGTG	Sequencing pENTR clones
M13 R	CAGGAAACAGCTATGAC	

Chapter 4 -

Summary

Cotton leafroll dwarf virus is an introduced polerovirus which causes yield loss in cotton. This virus has been previously described in other cotton producing nations, where it caused significant damage to yields. While losses in the US are not as significant as other nations, research into this virus is necessary to ensure that the virus remains under control in the United States. The aims of this thesis were to develop tools to understand the two basic functions of this virus: its replication and transmission. One advantage that the US has is that CLRDV and the genus which it belongs to (*Polerovirus*) have already extensively been researched. The past infectious clones made for these viruses informed the approach taken in our work. In this thesis two infectious clones are presented, a clone of the wild type virus and a clone which has been modified to produce EGFP. Both virus clones infect host plants systemically and can provide a source of inoculum for future researchers or serve as a model for viral replication of a polerovirus. In order to better understand the transmission determinants of CLRDV, a system of feeding aphids viral proteins by way of agrotransformation was devised. This system was shown to be effective in delivering proteins to aphids while keeping the aphids on their preferred host. This system will be used in the future to determine which CLRDV protein is required for the transmission of the virus. Both the infectious clones and the system of feeding aphids agro-expressed proteins are useful tools in aiding the research into Cotton leafroll dwarf virus.