Molecular Research on two Orthotospoviruses Infecting Important Legumes in Alabama.

by
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Abstract

Peanut (*Arachis hypogaea*. L) is a major crop in the United States, especially in Georgia, Florida, and Alabama, where they produce two-thirds of the peanut total production in the US. Soybean (*Glycine max* (L.) Merrill) is the fourth most popular crop produced in the state. In Alabama, Tomato spotted wilt virus (TSWV) and Soybean vein necrosis virus (SVNV), can be found in peanuts (TSWV) and soybean (TSWV and SVNV). These viruses are both in the genus *Orthotospovirus* in the family *bunyviridae* and have an ambisense ssRNA genome. To understand the sequences of TSWV in AL, a total of 126 symptomatic peanut samples were collected from three locations in AL over two years, and a protein alignment was conducted. This alignment revealed five compared to the MT2 strain (X61799.1) and seven mutations compared to BR-O1 GenBank strain (NC_002051.1). Eight samples were selected for microscopy and a difference in aggregation speed between samples was described. Although unlikely the cause of rising levels of TSWV, these differences reveal new insights into TSWV-N protein function.

SVNV proteins have never been localized in plant cells. The five ORFs of SVNV (N, NSs, NSm, GN, and GC) were localized, and the images revealed that NSs and N proteins localize to the cell periphery and to the nucleus. NSm causes cell death and the glycoproteins GN and GC localize to the cell membrane and accumulate around the nucleus. To further our work, the genes of SVNV from the field were sequenced and a protein alignment and a phylogeny tree were constructed for each of SVNV's three genes. This revealed mutations in the SVNV of AL compared to the SVNV reference genome (GCF_004789395.1). This is essential to understand the diversity and the distribution of SVNV in AL which will further our understanding of this new emergent orthotospoviurs infecting soybean fields in the southern states of the US.

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List of abbreviations

Abbreviation	Meaning
TSWV	Tomato spotted wilt virus
SVNV	Soybean vein necrosis virus
INSV	Impatiens necrotic spot virus
L1	First instar larvae
RdRp	RNA-dependent RNA polymerase
NSV	Negative-stranded RNA viruses
N	Nucleocapsid protein
NSs	Nonstructural silencing suppressor
NSm	Nonstructural viral movement protein
G_N	Glycoprotein G_N
G_C	Glycoprotein G_C
SNV	Sin Nombre hantavirus
RNPs	Viral ribonucleocapsid proteins
ER	Endoplasmic reticulum
ERES	ER-export sites
IYSV	Iris yellow spot virus
WSMoV	Watermelon silver mottle virus
GYSV	Groundnut yellow spot virus
TCSV	Tomato chlorotic spot virus
ZLCV	Zucchini lethal chlorosis virus
ANSV	Alstroemeria necrotic streak virus
CSNV	Chrysanthemum stem necrosis virus
MSMV	Melon severe mosaic virus
PNSV	Pepper necrotic spot virus
BeNMV	Bean necrotic mosaic virus
ICTV	International Committee on Taxonomy of Viruses
IPAD	International Production Assessment Division
USDA	United States Department of Agriculture
RB	Resistance-breaking
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TMV	Tobacco mosaic virus

FG	Foreguts
MG	Midgut
HG	Hindgut
SG	Salivary glands
PD	Plasmodesmata
ORF	Open reading frame
NCBI	National Center for Biotechnology Information
RNP	Ribonucleoprotein complex
BARU	Brewton Agricultural Research Unit
WGREC	Gulf Coast Research and Extension Center
GCREC	Wiregrass Research and Extension Center
pSITE	Stable Transient Integration Expression
GFP	Green fluorescent protein
RFP	Red fluorescent protein
dpi	Day post infiltration
LSD	least significance difference
CaCV	Capsicum chlorosis virus
GBNV	Groundnut bud necrotic virus
TSV	Tomato spotted virus
SMV	Soybean mosaic virus

Chapter One

Title: Orthotospoviruses in Alabama: Spotlight on Tomato Spotted Wilt Virus and Soybean Vein Necrosis Virus.

1. Importance of soybean and peanut in Alabama agriculture system

1.1. Peanut

Peanuts (*Arachis hypogaea*. L), also referred to as "groundnuts" in certain regions, represent the consumable seeds of a leguminous plant. The United States ranks fifth globally in peanut production, yielding 2.627 million metric tons (5% of the worldwide production) (USDA International Production Assessment Division “IPAD” 2023a). Peanuts boast high levels of protein, oil, and fibers (Suchoszek-Lukaniuk et al. 2011). Beyond their high oil content, peanuts are used in making peanut butter, confections, roasted snacks, and as extenders in meat formulations, soups, and desserts (Arya et al., 2015). In 2022, Alabama produced 560 M lbs of peanut with a value of $145 M (USDA 2022a).

1.2. Soybean

Soybean (*Glycine max* (L.) Merrill) is a significant crop cultivated in the United States, due to its diverse range of applications spanning from textiles to biofuels, as well as being used as a food source. Soybean serves dual purposes, providing both meals and oil. The United States ranks the second globally in soybean production with 113,344 million metric tons annually (28% of the worldwide production) (USDA International Production Assessment Division “IPAD” 2023b). According to data from the United Soybean Board in 2019, approximately 80% of soybeans grown in the United States are utilized for meal production, while the remaining 20% is processed into soybean oil (Stowe, 2022). Soybean production constitutes a substantial portion of the state's agricultural economy. In 2022, Alabama harvested 14.5 M bushels (870 M lbs) of soybean with a value of $206.6 M (USDA 2022b).

2. Overview of the virus family *Bunyviridae*

2.1. History and characterization of *Bunyviridae*

Bunyaviridae is a family of arthropod-borne or rodent-borne viruses. They are spherical enveloped negative sense or ambisense ssRNA viruses. The *Bunyaviridae* family comprises five acknowledged genera: *Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus,* and *Tospovirus* (Plyusnin et al., 2012) (refer to the sidebar titled *Tenuiviruses*). All bunyavirus species possess genomes consisting of three RNA segments, predominantly employing negative-sense coding strategies (Walter and Barr 2011). While an arthropod vector for hantaviruses remains unidentified (Vaheri et al., 2013), generally, viruses within Orthobunyavirus, Nairovirus, and Phlebovirus can replicate in both vertebrates and arthropods. They tend to be cytolytic in vertebrate hosts, exhibiting minimal to no cytopathogenicity in arthropod hosts (Walter and Barr 2011). Based on these observations and recent evolutionary analyses (Marklewitz et al., 2015), an arthropod origin for bunyaviruses has been proposed.

Bunyaviruses are responsible for several febrile diseases in humans and plants (genus: orthotospovirus) and other vertebrates. They have either a rodent or arthropod vector and a vertebrate host (Shope 1996). The majority of arbovirus encephalitis cases in North America are attributed to the La Crosse virus. Additionally, Asia and Europe collectively witness over 100,000 cases of hemorrhagic fever with renal syndrome each year. The Rift Valley fever demonstrated its severe impact in Egypt in 1977, with approximately 200,000 recorded cases and 598 deaths. Although uncommon, the Hantavirus pulmonary syndrome is notable for its high case fatality rate of 50% (Shope 1996).

Notably, orthotospovirus is the sole genus within this family known to infect plants. In 2017, the bunyavirales order was established to encompass viruses sharing similar characteristics including segmented, linear, single-stranded, negative-sense, or ambisense RNA genomes, classified into nine families (Maes et al., 2018). Subsequently, the family

tospoviridae was reestablished for the genus tospovirus, which was then renamed orthotospovirus (Abudurexiti et al., 2019). Currently, the genus Orthotospovirus comprises 26 recognized species **(https://talk.ictvonline.org/taxonomy/).**

2.2. Taxonomy and classification history of orthotospoviruses

Unlike the other four genera within the bunyaviridae family, the unconventional genus orthotospovirus stands out. While orthotospoviruses infect insect vectors like many other bunyaviruses, members of this genus uniquely target plant hosts. The disease known as tomato spotted wilt was initially identified in Australia in 1915 (Brittlebank 1919). Until the early 1990s, TSWV remained the sole recognized species in the genus Tospovirus (a name derived from Tomato spotted wilt virus). However, during that period, the genus was divided into two species after the characterization of Impatiens necrotic spot virus (INSV) (Law and Moyer 1990).

Subsequent molecular analyses of virus isolates have significantly expanded our understanding of the diversity within the genus. By 2015, it was 100 years since the initial identification of orthotospovirus disease, the genus orthotospovirus comprises 11 officially recognized distinct virus species with an additional 17 described species (Oliver and Whitfield 2015). Nearly all of these viruses are transmitted by thrips insects belonging to the order *Thysanoptera* (Oliver and Whitfield 2015). Many of thrips species exhibit broad plant host ranges and have established global distributions.

Interestingly, the primary virus vector species in agriculture appears to have shifted over the years from onion thrips (*Thrips tabaci*), initially identified as the primary vector in early literature, to western flower thrips (*Frankliniella occidentalis*), now recognized as the primary vector of species within the TSWV clade of orthotospovirus (Oliver and Whitfield 2015). In 2015, reports indicate that at least 15 different species of thrips can vector orthotospoviruses (Rotenberg et al., 2015).

2.3. Modes of transmission by the insect vector in orthotopsoviruses

Thrips, tiny insects that function as pests across a wide array of plant species, play a pivotal role as the primary vectors for orthotospoviruses. Their small size and ability to develop resistance to insecticides pose significant hurdles in their effective control, contributing substantially to the success of this pathosystem and resulting in considerable yield losses within agricultural systems (Rotenberg et al., 2015).

Belonging to the insect order *Thysanoptera*, thrips comprise a diverse group encompassing over 7700 species (Mound 2015). Classified within the Hemipteroid assemblage alongside orders such as Hemiptera and Psocodea, Thysanoptera shares certain characteristic features. These include postembryonic remetaboly development, characterized by two non-feeding pupal stages (Heming 1973), and a genetic system termed haplodiploidy, where males are haploid and females are diploid (Crespi 1972). Adult thrips typically measure less than 1 mm in length and possess asymmetrical mouthparts adapted for feeding (Rotenberg et al., 2015).

Many thrips species that serve as vectors for orthotospoviruses exhibit traits conducive to efficient virus transmission and pest behaviour that include high fecundity, short generation time, active mobility, a preference for concealed habitats, and a broad plant diet (polyphagy). Employing piercing-sucking mechanisms, phytophagous thrips extract contents from epidermal and mesophyll cells of a host, resulting in extensive feeding damage (Hunter and Ullman 1989, and Hunter and Ullman 1992)

Orthotospoviruses are uniformly transmitted by thrips vectors in a persistent, propagative manner. The virus undergoes replication within the insect, disseminating throughout its body and remaining viable across various developmental stages. Virus acquisition is contingent upon the developmental stage of the thrips, with only larvae that acquire the virus capable of transmitting the virus as adults. Among larval stages, first instar

larvae (L1) exhibit the highest efficiency in virus acquisition with efficiency decreasing in subsequent developmental stages.

Prepupal and pupal stages, being non-feeding stages, neither acquire nor transmit the virus. Adult thrips equipped with functional wings and increased mobility emerge as the primary agents of virus transmission. The efficiency of virus transmission correlates with the viral titer present within individual insects, with the amount of virus acquired by larval thrips also a determinant of virus titer (Montero-Astua et al., 2014) significantly influencing transmission during adulthood (Rotenberg et al., 2009). This unique stage-dependent process of orthotospovirus acquisition presents a potential avenue for disrupting virus transmission (Rotenberg et al., 2015).

2.4. Genome structure and features of orthotospoviruses

Mature orthotospovirus virions have a size ranging from 80 to 120 nm. These virions consist of a phospholipid membrane embedding glycoproteins (G_N and G_C), along with three viral genomic RNAs bound to an icosahedral nucleocapsid protein (N) and an RNA-dependent RNA polymerase (RdRp) within each virion (Adkins 2000, Whitfield et al., 2005 and Kormelink et al., 2011).

Similar to other members of the *Bunyaviridae* family, orthotospovirus possess a multipartite ambisense genome. The genomes of orthotospovirus are tripartite, single-stranded, and negative-sense or ambisense, with each viral gene transcribed as a distinct mRNA for the translation of its corresponding protein products (Oliver and Whitfield 2015).

These three viral genomic RNAs, referred to as the large (L), medium (M), and small (S) RNAs, are designated based on their respective lengths. Specifically, RNA L segment (measuring 8.9 kb for TSWV and 9.1 kb for SVNV) encodes the RdRp in the negative-sense orientation; RNA M segment (with a length of 4.8 kb for TSWV and 4.9 kb for SVNV) encodes the nonstructural viral movement protein (NSm) in the positive-sense orientation which

functions in plant cells by forming tubules for virion/protein movement through the plasmodesmata. Also, the RNA M segment contains the glycoproteins G_N and G_C in the negative-sense orientation; and RNA S segment (spanning 2.9 kb for TSWV and 2.6 kb for SVNV) encodes the viral N protein in the negative-sense orientation alongside the viral nonstructural silencing suppressor (NSs) in the positive-sense orientation.

The initial nine nucleotides at the 3' end of each genomic RNA are highly conserved and possess inverted complementarity to the 5' ends. This facilitates the base-pairing of the ends and the formation of panhandle structures, resulting in the pseudocircular appearance of each genomic RNA (Dehaan et al., 1989 and Kellmann et al., 2001). For replication within the host cell, each virion must contain an RdRp, which also facilitates the transcription of each viral gene into an orientation conducive to the translation of each viral protein (Oliver and Whitfield 2015). Table 1 presents the small differences in the gene length of the two viruses; TSWV and SVNV.

2.5 Replication cycle of orthotospoviruses

Transcription and replication of the orthotospoviral RNA genome take place in the cytoplasm and requires the RNA-dependent RNA polymerase (RdRp) and N proteins (Kormelink et al., 2021). The viral RdRp exhibits conserved motifs and features typical of RdRp proteins found in segmented negative-stranded RNA viruses (NSV), comprising an N-terminal endonuclease domain essential for cap-snatching and a central domain containing the six motifs of RNA polymerase, including the characteristic "SDD" core motif. However, the presence of a cap-binding domain remains elusive, and no structural sequence homology has been observed with the cap-binding domain found in the PB2 subunit of influenza virus RdRp or in any other RdRp protein from bunya/arenaviral/tenuiviral families (Reguera et al., 2010 and Zhao et al., 2019).

Contrary to nuclear-replicating orthomyxoviruses/influenza viruses, wherein nascent mRNAs generated by RNA polymerase II machinery serve as the source for capped-RNA leader sequences initiating genome transcription (Chan et al., 2006 and Engelhardt et al., 2005), and cytoplasmic replicating orthotospoviruses are likely to utilize cytoplasmic RNA processing bodies (PB) (Kormelink et al., 2021). Arabidopsis thaliana mutants lacking components of the RNA decay machinery, or the nonsense-mediated decay pathway demonstrated heightened susceptibility to TSWV infection (Ma et al., 20019). Moreover, partial co-localization of TSWV N protein with P bodies was observed. These findings align with previous observations involving Sin Nombre (SNV) hantavirus, which implicated PB as a source of capped RNA for cap-snatching (Mir et al., 2008 and Cheng and Mir 2012).

Viral replication is believed to occur at electron-dense sites in the cytoplasm, often referred to as viroplasm. However, it remains unknown whether and how these sites relate to the locations where transcription/cap-snatching occur (i.e., P bodies) (Kormelink et al., 2021). Following replication, progeny viral ribonucleocapsid proteins (RNPs) either move intra/intercellularly or mature into enveloped virus particles. In this process, the viral RNPs receive their envelope membrane at the Golgi complex, similar to most other animal-infecting viruses within the Bunyavirales. This process involves multimeric protein interactions between the three major structural proteins (N, G_N, and G_C) and occurs at the endoplasmic reticulum ER and Golgi (Ribeiro et al., 2009 and Dietzgen et al., 2012).

Proteolytic processing of the GP precursor at the endoplasmic reticulum ER yields viral G_N and G_C, likely facilitated by ER-resident proteases. Both proteins then concentrate at ER-export sites (ERES). G_C, in particular, reaches ERES through condensation with the cytosolic N protein. Escape from ERES occurs via COPII vesicle transport, necessitating heterodimerization of G_C and G_N. The G_N glycoprotein harbors a Golgi localization signal within its transmembrane domain, enabling its escape and movement towards the Golgi

complex (Ribeiro et al., 2009 and Snippe et al., 2007). Intracellular movement of mature envelope glycoproteins and N relies on actin filaments (Ribeiro et al., 2009 and Feng et al., 2013) rather than microtubules, as observed with animal-infecting bunyaviruses (Kikkert et al., 1999 and Kikkert et al., 2001). Particle maturation entails wrapping of RNPs by an entire Golgi stack, differing from the budding process seen in most animal-infecting bunyaviruses. Doubly enveloped virus particles resulting from this process fuse with each other, leading to the accumulation of mature virus particles in large vesicles. These particles await uptake/acquisition by thrips upon feeding on infected plants for further dissemination (Kikkert et al., 1999). Differing from budding in the insect vector, assembled virion of orthotospoviruses move within the infected host plant from cell to another cell through the plasmodesmata (PD). This process requires the interaction of the N-terminal of the NSm protein with the nucleocapsid protein N which assists in the movement of the RNPs toward the plasmodesmata using the actin microfilament/ER transport system (Leastro et al., 2015, Tripathi et al., 2015, and Feng et al., 2016).

Orthotospoviruses are known for genome exchange or reassortment (Webster et al., 2015), which makes genes or segments share an important factor driving these viruses's evolution (Rotenberg et al., 2015). TSWV and SVNV share the same thrips vector (*F. fusca*) and the same host plant soybean in Alabama (Sikora et al., 2011), and in Georgia (Nischwitz et al., 2006). This suggest that the two viruses have the potentials to meet within their shared insect vector or plant host and reassort. If that occurs, SVNV might earn new characteristics from TSWV through the reassortment process which could cause severe infections from SVNV and increased yield losses to legume crops in Alabama.

2.6. Phylogenetic relationship of TSWV and SVNV with other orthotospoviruses

The phylogenetic relationship of *Tomato spotted wilt virus* (TSWV) and *Soybean vein necrosis virus* (SVNV) with other *orthotospoviruses* is typically analyzed through molecular

phylogenetic methods. These methods involve comparing genetic sequences, usually of specific genes or genomic regions, among different viruses to infer their evolutionary relationships.

To determine their phylogenetic relationships with other orthotospoviruses, researchers commonly conduct phylogenetic analyses using sequences from conserved regions of the viral genome, such as the nucleocapsid (N) gene or the RNA-dependent RNA polymerase (RdRp) gene. Species within the orthotospovirus genus are distinguished based on their vector specificity, plant host range, and serological relationships of the N protein (Oliver and Whitfield 2015).

For an isolate to be classified as a distinct species within the genus, its N protein sequence should display less than 90% amino acid identity with that of any other orthotospovirus species (Plyusnin et al., 2012). Recent studies have revealed that known orthotospovirus species group into at least five distinct phylogenetic clades, which can be denoted as the TSWV clade, the Soybean vein necrosis virus (SVNV) clade, the Iris yellow spot virus (IYSV) clade, the Watermelon silver mottle virus (WSMoV) clade, and the Groundnut yellow spot virus (GYSV) clade, based on the type species within each clade (Oliver and Whitfield 2015).

Thrips vectors for species within clades exhibit similarities, and these clades also correspond to the geographical distributions of the species. Additionally, several unassigned species, although not officially approved, are recognized as likely orthotospovirus (Oliver and Whitfield 2015).

In the phylogenetic tree constructed in Oliver and Whitfield's (2015) study, TSWV and SVNV are observed in distinct clades. TSWV clades with eight different orthotospovirus species including Groundnut ringspot virus (GRSV), Impatiens necrotic spot virus (INSV), Tomato chlorotic spot virus (TCSV), Zucchini lethal chlorosis virus (ZLCV), Alstroemeria

necrotic streak virus (ANSV), Chrysanthemum stem necrosis virus (CSNV), Melon severe mosaic virus (MSMV), Pepper necrotic spot virus (PNSV). On the other hand, SVNV groups only with Bean necrotic mosaic virus (BeNMV).

Seeing TSWV and SVNV in different clades introduces the hypothesis that their protein localization in the plant cells might be different and/or their protein interactions with themselves or with either their insect vector or plant host might be different as well.

3. Orthotospoviruses in Alabama

3.1. History and characterization of TSWV

The tomato disease was first identified in Australia in 1915 (Brittlebank 1919), and its causal agent, a virus, was characterized by Samuel et al. (1930). TSWV was previously considered as the type species of the genus orthotospovirus. The first detection of TSWV in peanuts in the U.S. was in Texas in 1974 (Halliwel and Philley 1974) and field epidemics were reported in the same state by 1985 (Black et al., 1986). In Alabama, TSWV was first reported in 1990 (Cudauskas et al., 1993). According to the initial report from the International Committee on Taxonomy of Viruses (ICTV), TSWV was considered as the sole member of the 'Tomato spotted wilt virus group' (Fenner, 1976). However, following the early 1990s, the identification and characterization of numerous viruses similar to TSWV led to the establishment of the genus Tospovirus, subsequently reclassified under the family *Tospoviridae* in the latest proposed taxonomy (Maes et al., 2018). In 2018 a family named *Tospoviridae* was initiated to group orthotospoviruses including TSWV and SVNV (Maes et al., 2018).

Since the discovery of TSWV in Alabama, it has become a major obstacle to peanut production over the past three decades (Srinivasan et al., 2017). The western flower thrips (*Frankliniela occidentalis*) and tobacco thrips (*Frankliniela fusca*) were found to be involved with the transmission of TSWV (Culbreath and Srinivasan 2011). Early infection of peanut

plants yields less than those infected later in the season (Culbreath et al., 1992a). The losses caused by TSWV and other orthotosopoviruses were estimated to be more than tens of millions of dollars worldwide. In the U.S. alone, it was estimated to exceed $1.4 billion in a ten-year period (Culbreath et al., 2003, and Riley et al., 2011).

Significant losses resulting from spotted wilt disease, caused by TSWV, were commonplace from the late 1980s to the mid-1990s (Culbreath et al., 1992a,b, 2000, 2003). In Georgia, an average annual yield reduction of 12% was realized totalling $40 million loss attributed to spotted wilt from the late 1980s through 1997 (Bertrand, 1998). Notably, Georgia, Alabama, and Florida collectively contribute over two-thirds of peanut production in the U.S. (USDA National Agricultural Statistics Service, 2016). However, the losses gradually declined in the 2000s as a result of the adoption of risk reduction practices outlined in Peanut Rx (Sundaraj et al., 2014)

Peanut growers were provided with a Risk Mitigation Index (Peanut Rx) to evaluate risks and offer strategies for risk reduction, such as planting field-resistant cultivars alongside in-furrow insecticides, scheduling planting after the peak thrips incidence, adopting twin-row planting, and increasing seeding rates. These initiatives played a crucial role in reducing losses attributed to spotted wilt. Unfortunately, the TSWV-resistant cultivars that were previously used to manage the disease are showing an increase in susceptibility? in recent years (Sundaraj et al., 2014).

3.2. Symptoms of TSWV

TSWV infection induces spotted wilt disease in peanuts (*Arachis hypogaea* L.), posing a significant challenge to peanut production in the southeastern U.S. (Lai et al., 2021). The impact of TSWV on peanut plants have been extensively documented (Culbreath et al., 2013), encompassing a broad spectrum of symptoms such as concentric ring spots, various chlorotic patterns, and stunted growth (Figure 2, A).

Plants infected early in the season typically yield less than those infected later in the season (Culbreath and Srinivasan, 2011). Additionally, TSW adversely affects the physiology of peanut plants, leading to a decrease in photosynthesis, transpiration, and water-use efficiency (Rowland et al., 2005). Although TSWV is not transmitted through seeds, infection results in shriveled, shrunken, and discolored/reddish seeds (Pappu et al., 1999). Furthermore, TSWV infection leads to malformed pegs and kernels, causing the seed coat or testa to exhibit a reddish or darker hue on infected pods compared to healthy ones. Early infection of peanut plants results in lower yields compared to those infected later in the growing season (Culbreath et al., 1992).

3.3. Host range of TSWV

TSWV is ranked as the most destructive and damaging plant virus, causing estimated global losses reaching approximately one billion dollars annually (Scott 2000). Its economic significance, coupled with its biological and molecular characteristics, has led to extensive research, making TSWV one of the most thoroughly investigated plant viruses (Parrella et al., 2003).

Two important factors contributing to TSWV's status as one of the most damaging and widely spread plant viruses are the broad feeding habits of its thrips vectors and the virus's limited host specificity. As a result, TSWV exhibits an extensive host range, posing significant challenges for its control (Parrella et al., 2003).

Although resistant varieties prove beneficial in mitigating losses, instances of infections by field resistance-breaking (RB) TSWV isolates have been documented in purportedly resistant pepper hybrids (Roggero et al., 1999, 2002) and resistant tomato hybrids (Aramburu and Marti 2003). Nonetheless, the genetic makeup at resistance loci, such as heterozygosity versus homozygosity, may significantly influence the level of resistance.

TSWV is known to infect over 1,000 plant species across more than 85 plant families,

including numerous cultivated important crops such as *peanut* (*Arachis hypogaea*), *pepper* (*Capsicum annuum*), *potato* (*Solanum tuberosum*), *spinach* (*Spinacia oleracea*), *tobacco* (*Nicotiana tabacum*), and *tomato* (*Solanum lycopersicum*) (Brittlebank, 1919; Cho et al., 1987; Costa, 1941; German et al., 1992; Parrella et al., 2003; Sakimura, 1940; Sherwood et al., 2009; Smith, 1931). Additionally, besides economic crops, various weeds serve as natural hosts for TSWV and act as reservoirs for the spread of the virus to other susceptible crops (Chatzivassiliou et al., 2001; Cho et al., 1986; Parrella et al., 2003).

3.4. Thrips species involved in TSWV transmission

Frankliniella occidentalis (Western flower thrips) is the most efficient vector, although *F. fusca* (Tobacco thrips) also contributes to transmission at a percentage exceeding 51% (Arthurs et al., 2018). Additionally, there are at least six other thrips species implicated in the transmission of TSWV. Therefore, TSWV exhibits a broad plant host range infecting over 1000 plant species across 69 families of Dicotyledonous plants and 15 families of Monocotyledonous plants (Parrella, 2003).

3.5. Management strategies of TSWV

During the 1990s and early 2000s, attempts to control the virus mainly involved the chemical management of the thrips vector, applying traditional broad-spectrum insecticides like Organophosphates and organocarbamates (Chamberlin et al., 1992; Todd et al., 1994, 1996, 1997). Additionally, Phorate and aldicarb were utilized to mitigate thrips feeding damage (Culbreath et al., 2008, 2016; Hagan et al., 1991; Herbert et al., 2007; Todd et al., 1997; Tubbs et al., 2013). However, despite efforts to suppress thrips feeding, these insecticide applications often proved ineffective in reducing the incidence of TSWV (Hagan et al., 1991; Herbert et al., 2007; Marasigan et al., 2016; Mulder et al., 2001; Todd et al., 1996, 1997; Weeks and Hagan, 1991; Wells et al., 2002).

In conjunction with insecticides, cultural practices were also employed for TSWV management. Alterations in planting dates were frequently utilized to avoid peak thrips flights, and some growers adopted conservation tillage options, which were associated with reduced thrips populations and feeding damage (Srinivasan et al., 2017).

Host resistance emerged as the most crucial management strategy for peanut growers. However, during the initial emergence of TSWV in the Southeast, prevalent cultivars such as SunOleic 97R, Florunner, GK-7, and Georgia Runner exhibited extreme susceptibility to the virus (Culbreath et al., 1992a, 1996). Subsequently, an extensive screening and breeding program commenced in Georgia and Florida, leading to the identification of tolerance in cultivars like Southern Runner (Black, 1990; Black and Smith, 1987).

To aid growers in selecting TSWV-resistant or tolerant peanut cultivars and implementing appropriate chemical and cultural management tactics, a Risk Mitigation Index known as Peanut Rx was developed (Srinivasan et al., 2017). Adoption of risk reduction practices outlined in Peanut Rx resulted in declining yield losses during the 2000s. However, recent years have seen an increase in the severity and incidence of TSWV in the field (Sundaraj et al., 2014).

3.6. History and characterization of SVNV

Soybean vein necrosis virus (SVNV) was first discovered in Arkansas and Tennessee, U.S., in 2008 (Tzanetakis et al., 2009). SVNV is an orthotospovirus that mainly infects soybean plants. The virus is primarily spread by soybean thrips (*Neohydatothrips variabilis*) in a persistent and propagative manner leading to localized infections on soybean leaves. Being a distinct member of the genus orthotospovirus within the orthotospovirus family, SVNV exhibits minimal similarity to established species within the genus and represents a new evolutionary clade (Zhou et al., 2011; Zhou and Tzanetakis, 2013). Over subsequent years, SVNV was observed not only in the initially reported states but also in various other soybean-

growing regions, including Illinois, Wisconsin, Michigan, Ohio, Pennsylvania, Delaware, Kansas, Oklahoma, Kentucky, Missouri, Mississippi, Louisiana, and Alabama (Zhou et al., 2011; Zhou, 2012; Ali and Abdalla, 2013; Conner et al., 2013; Escalante et al., 2018; Han et al., 2013; Jacobs and Chilvers., 2013; Kleczewski, 2016; Smith et al., 2013). Presently, SVNV has been confirmed in at least 22 states across the U.S., as well as in Canada and Egypt (Abd El-Wahab and El-Shazly, 2017), with vein necrosis emerging as the most prevalent virus disease in North America (Zhou and Tzanetakis, 2013).

SVNV is the only known orthotospovirus that infects soybeans in the U.S., except for the tomato spotted wilt virus, which was reported to infect soybeans in Alabama in 2008 (Sikora et al. 2011). SVNV is a distinct species of the genus orthotospovirus within the bunyaviridae family (Khatabi et al. 2012; Zhou et al. 2011). SVNV impacts the soybean's growth, seed quality, and oil content.

SVNV was found in 27 of 28 counties in Alabama during a 4-year study (Figure 1) (Sikora et al., 2018). Throughout the four-year study period, every field sampled in northern Alabama tested positive for SVNV, with incidence rates varying from 8% to 100%. The average incidence of SVNV in northern Alabama fields notably escalated from 31.8% in 2013 to 61% in 2014, further increasing to 64% in 2015 and peaking at 82.6% in 2016. Meanwhile, in central Alabama, the average SVNV incidence ranged from 5.1% to 14.8% between 2014 and 2016. Fields in south Alabama exhibited an average SVNV incidence ranging from 0% to 8.8% over the same three-year period (Sikora et al., 2018).

3.7. Symptoms of SVNV

The Symptoms of SVNV appear on infected plants as chlorotic regions on the infected leaves. Over time, these chlorotic regions turn to brown necrotic tissue (tissue death) along the major veins of the upper and lower leaf surface resulting in a scorched appearance (Figure 2, B) (Zhou and Tzanetakis, 2013 and Conner et al., 2013). The impact of SVNV on soybean

yield and quality remains poorly understood with only a limited number of studies reported. These studies have suggested a marginal 0.1% reduction in oil content and alterations in fatty-acid profiles (Irizarry 2016, Anderson et al., 2017, Ullman et al., 2017). SVNV is also known to induce symptoms on soybean seeds (Zhou & Tzanetakis, 2019). Groves et al. (2016) demonstrated that SVNV infection did not impact germination, seed size, protein or fiber content, but it did reduce oil content in soybean seed.

SVNV has been linked to an undesirable increase in the concentration of linolenic acid, while no notable effects on oleic acid have been observed (Anderson et al., 2017). In Indiana, SVNV-infected double crops experienced a significant decrease in oil content but an increase in protein content (Anderson et al., 2017). Enhancing our comprehension of the interconnected relationships among the host plants, viruses, and thrips will facilitate the development of more accurate disease and pest pressure predictions, along with more effective pest management strategies (Ullman et al., 1997 and Belliure et al., 2005).

3.8. Host range of SVNV

SVNV has a narrow host range compared to TSWV. In a study conducted by Zhou and Tzanetakis in 2013, SVNV was inoculated into 25 different plant species belonging to 9 plant families. The presence of the virus was detected using Reverse Transcription Polymerase Chain Reaction (RT-PCR) in nine out of the 25 mechanically inoculated plant species. These positive hosts of SVNV included *Dendrathema grandiflorum*, *Ipomoea hederacea* Jacq, *Cucurbita pepo* L., *Glycine max*, *Vigna unguiculata*, *Vigna radiata*, *Nicotiana benthamiana*, *Nicotiana tabacum*, and *Nicotiana glutinosa*, which comprised plants from the families Asteraceae, Convolvulaceae, Cucurbitaceae, Leguminosae, and Solanaceae. *N. benthamiana* emerged as the most susceptible host and served as an indicator for assessing the efficacy of each inoculation experiment. Notably, virus replication remained confined solely to the inoculated leaves across the three legume species used. In contrast, Hutcheson soybean exhibited

resistance to mechanical inoculation compared to *N. benthamiana* with an infection rate of only 27.3%, markedly lower than the transmission rate via thrips (100%).

Furthermore, the virus was detected in symptomless plants of *Cucurbita pepo* L., *Dendranthema grandiflorum*, and *I. hederacea* Jacq. (commonly known as Ivyleaf morning glory). Ivyleaf morning glory, a native weed species found in soybean fields, prompted field surveys for natural infection. Upon randomly collecting eight plants from SVNaV-infested soybean fields in Arkansas, five were found to be infected, as confirmed by amplicon sequencing following RT-PCR testing.

The localized infection observed in legume species suggests that these species are likely not the original hosts of the virus, as prolonged virus-host interactions typically lead to systemic infections, thus promoting virus survival. However, further testing of additional accessions and cultivars is necessary to substantiate this hypothesis adequately. Additionally, SVNV is reported as the only known orthotospovirus that can be transmitted through soybean seeds (Groves et al., 2016).

3.9. Thrips species involved in SVNV transmission

For SVNV, the soybean thrips, *Neohydatothrips variabilis*, is the most efficient insect vector (Zhou and Tzanetakis, 2013). While other species such as *F. fusca*, *Frankliniella schultzei*, and *Frankliniella tritici* have been identified as potential vectors for SVNV, their transmission rates remain below 5% (Han et al., 2019), contrasting with the considerably higher rates ranging between 68-100% observed in *N. variabilis* (El-Wahab, 2021; Zhou and Tzanetakis, 2013). This indicates a relatively limited vector host range for SVNV compared to TSWV.

2.10. Management strategies of SVNV

Managing diseases caused by *orthotospoviruses* has consistently been a significant challenge (Bag et al., 2015; Pappu et al., 2009; Oliver and Whitfield, 2016). There remains a

lack of comprehensive understanding regarding many aspects of the biology and epidemiology of SVNV which are pivotal for devising effective strategies for virus control and disease management (Zhou, J. 2018).

As primary and secondary vector species have been well documented and additional data has become available regarding potential alternative hosts of the virus (Zhou and Tzanetakis, 2013; Escalante et al., 2018; Sikora et al., 2018; Irizarry et al., 2018; Keough et al., 2016), current management strategies for soybean vein necrosis have concentrated on mitigating the impact of thrips on soybeans and identifying potential virus reservoirs in the field (Zhou, J. 2018).

Numerous studies have been undertaken to assess the composition of thrips species and the population dynamics of SVNV vectors, particularly focusing on *Neohydatothrips variabilis* across different seasons and geographic regions (Zhou, J. 2018). Data indicate that *N. variabilis* is the most prevalent vector species in Northern, Midwestern, and Southern U.S. states (Chitturi et al., 2018; Keough et al., 2018; Bloomingdale et al., 2017).

Interestingly, the absence of distinct seasonal trends in thrips migration based on location in northern states suggests that virus vectors may not migrate from external regions; instead, they may colonize other host plants, particularly perennial species, to overwinter during periods when soybeans are absent or early in the growing season before transitioning to soybeans (Keough et al., 2018; Bloomingdale et al., 2017). This underscores the importance of identifying and eliminating local virus reservoirs (Zhou, J. 2018).

Given that peak activity for the primary vector occurs either simultaneously or prior to the appearance of vein necrosis symptoms (Chitturi et al., 2018; Keough et al., 2018), there is a possibility to reduce disease incidence through managing the planting system or altering planting dates, as suggested by Kleczewski (Kleczewski, 2018).

Currently, no soybean cultivar resistant to SVNV has been identified, although one study indicates differentiation in symptom intensities among cultivars (Anderson, 2017). Besides direct resistance to the pathogen, cultivars resistant to the virus vector could potentially decrease disease incidence (Zhou, J. 2018). Such resistance may arise from physical or biochemical attributes of specific cultivars or a combination thereof (Zhou, J. 2018).

Soybean cultivars were evaluated to explore potential thrips resistance with varying levels of pubescence (Zhou, J. 2018). The study revealed that feeding damage caused by *N. variabilis* varies among selected cultivars and is correlated with their pubescence levels (Zhou, 2018). The efficacy of chemical products on disease incidence, including insecticides and seed treatments, has not been assessed to date (Keough et al., 2018).

4. Summary Thesis

Orthotospoviruses are important group of plant infecting viruses contributing to more than $1.4 billion as a yield loss in a ten-year period (Mainly by TSWV) (Culbreath et al., 2003, and Riley et al., 2011). TSWV was the first described member of orthotospoviruses and can infect more than 1000 plant species including important crops (Rotenberg et al., 2015), and is being transmitted by at least nine species of thrips. These two reasons, TSWV was ranked as the second most destructive plant virus after Tobacco mosaic virus (TMV) (Scholthof et al., 2011). SVNV is another important and new emergent member of the genus orthotospovirus (Tzanetakis et al., 2009). SVNV incidence on soybean increased from 31 % to 82% in a three-year period in AL (Chitturi et al., 2018). In chapter two, we conducted a sequencing study targeting the nucleocapsid protein of TSWV to observe any mutations that might assist in understanding the symptoms increase of TSWV on resistant and non-resistant peanut cultivars in AL. In the third chapter, SVNV proteins were localized in plant cells which has never been shown before and is essential for us to understand how this new virus is functioning in its host

cells. In the fourth chapter, SVNV was isolated from AL and three genes were sequenced and amino acid changes were found which may indicate the increase in the virus virulence.

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Table 1: Gene length of Tomato spotted wilt virus and Soybean vein necrosis virus. * Predicted cleavage sites.

Gene name	TSWV gene length	SVNV gene length
N gene	774 bp	833 bp
NSs gene	1401 bp	1322 bp
NSm gene	906 bp	950 bp
GN gene	1135 bp*	1354 bp*
Gc gene	2272 bp*	2223 bp*
L gene	8897 bp	9010 bp

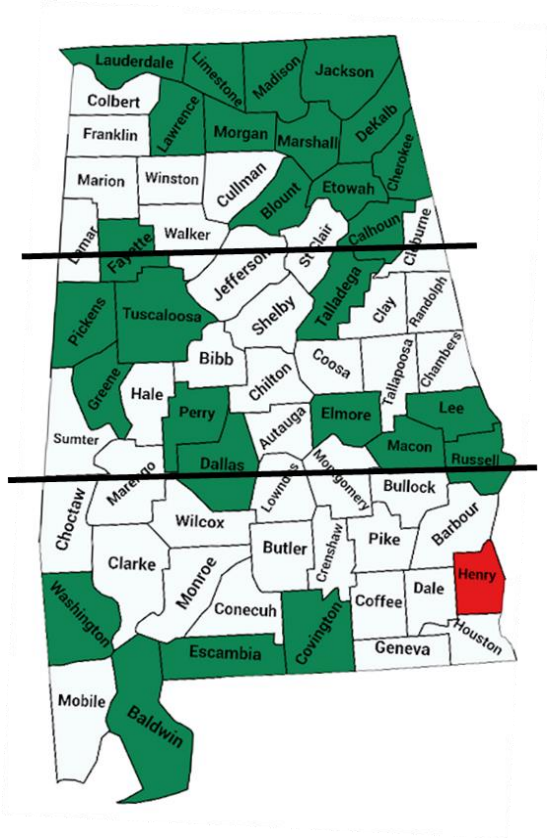

Figure 1: A map of Alabama showing the state's division into northern, central, and southern regions. Counties where Soybean vein necrosis virus (SVNV) was detected in at least one field during the study are highlighted in green. In contrast, a red county indicates that soybeans were sampled and tested but SVNV was not detected (Sikora et al., 2018).

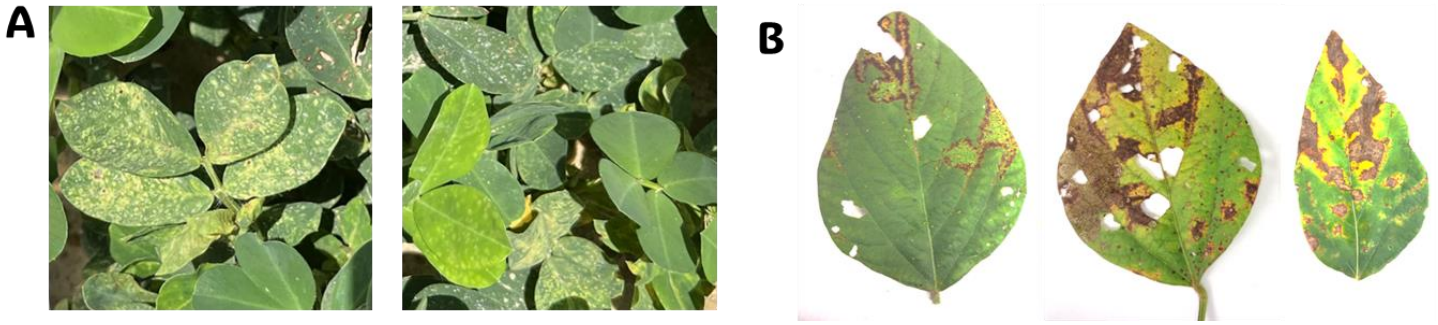

Figure 2: A: Tomato spotted wilt virus symptoms on infected peanut plants in Alabama.

B. Soybean vein necrosis virus symptoms on infected soybean plants in Alabama.

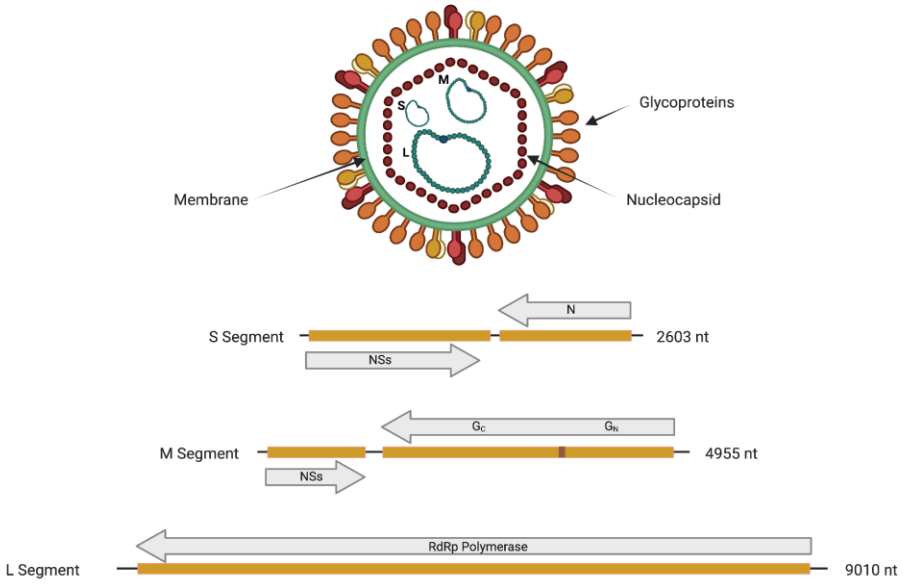

Figure 3: Genome structure of othotospoviruses composed of three segments; small segment S, medium segment M, and large segment L. The S segment is composed of the non-structure silencing protein (NSs) and the nucleocapsid protein (N), the M segment is composed of the non-structure movement protein (NSm), and the two glycoproteins (G_N and G_C), and the L segment is composed of the RNA dependant RNA polymerase protein (RdRp) (Abdelaal Shehata 2024).

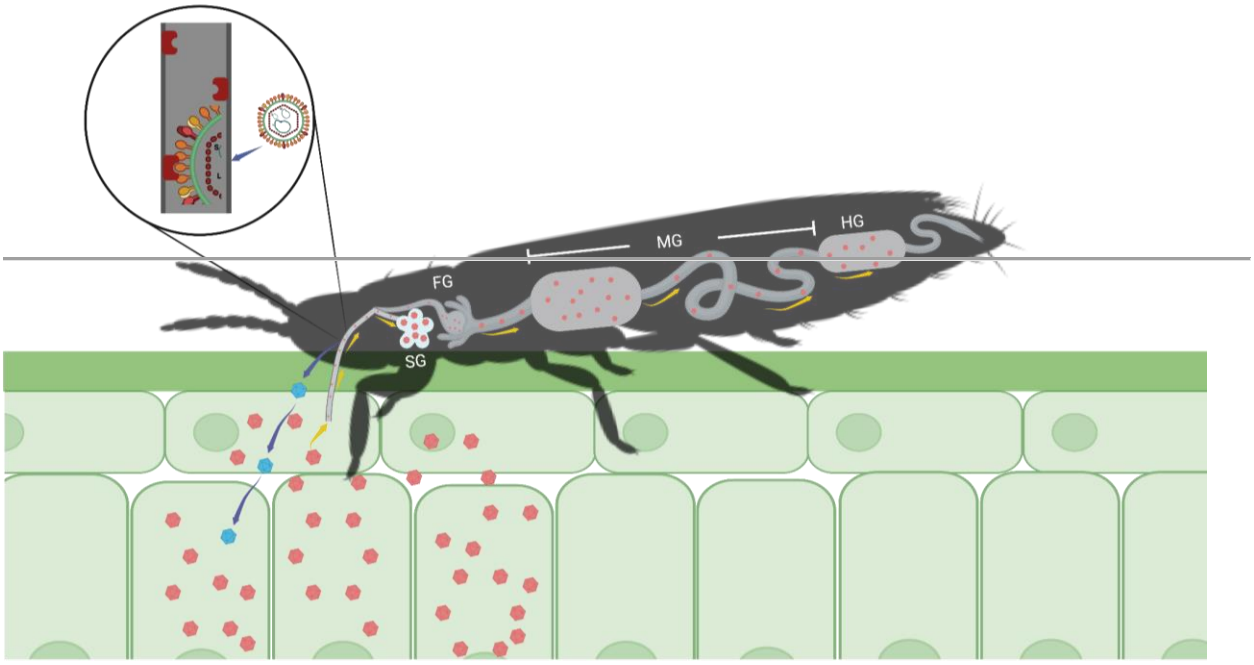

Figure 4: The acquisition of orthotospoviruses in a persistent propagative manner mediated by thrips. Thrips larvae feed on infected plants and the virus is acquired from infected plant tissue. The virus moves throughout their foreguts (FG), midgut (MG), and hindgut (HG). During pupation, the virus replicates within the thrips body and accumulates in their salivary glands (SG). When the adults emerge and feed on healthy plants, the virus moves through their saliva to the healthy plant tissues (Abdelaal Shehata 2024).

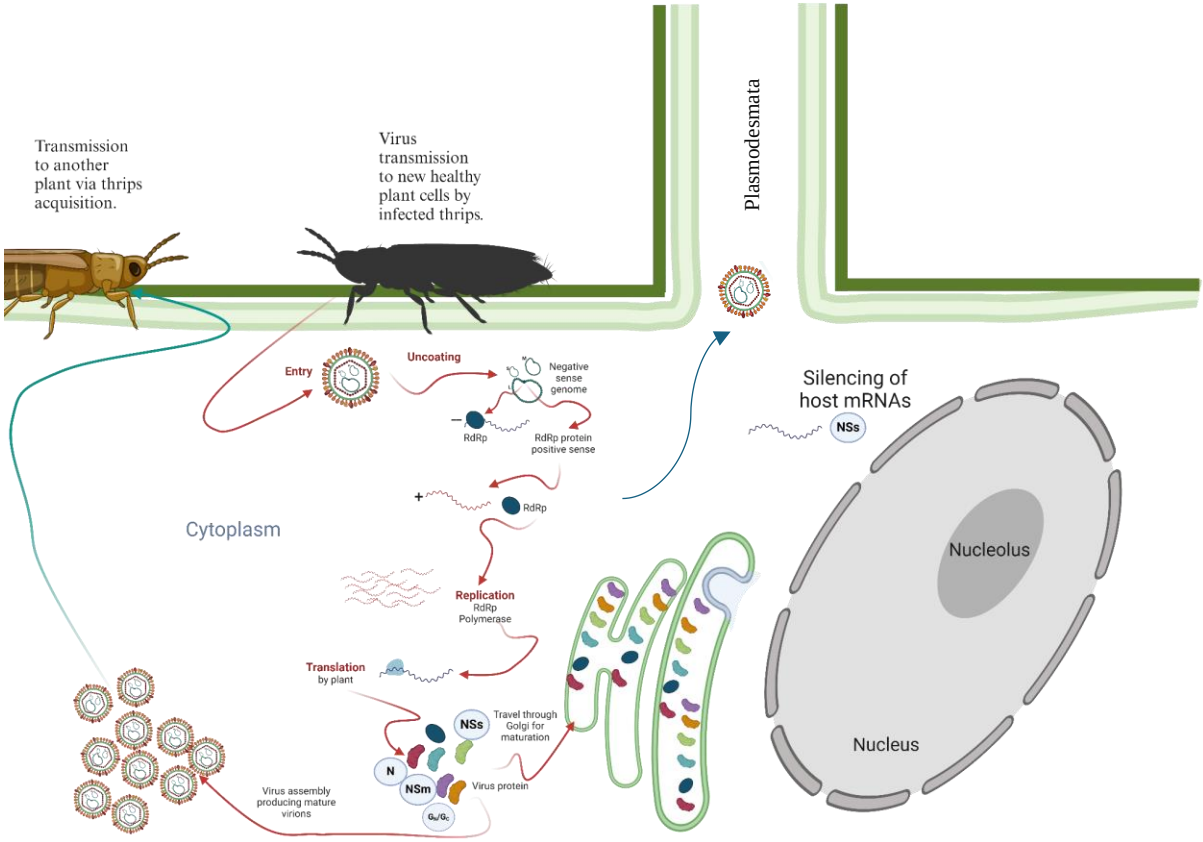

Figure 5: The replication cycle of orthotospoviruses in plant cells and the acquisition by their insect vector thrips. Upon virus entry by thrips, virus un-coat allowing its negative sense RNA material to be converted to positive sense by the virus RNA dependant RNA Polymerase protein (RdRp). Following that process, the positive sense genome of the virus is replicated and made as mRNA by the plant. The viral mRNA is translated into protein which gets into Golgi for maturations and secretion. Finally, the virus assembly is thought to take place in Golgi and the mature virions are being acquired by thrips. Also, mature virions can move between cells using the plasmodesmata using the movement protein of the virus (NSm) which connect plant cells together.

Chapter 2

Title: Sequencing Analysis of Tomato Spotted Wilt Virus Nucleocapsid Protein in Alabama and the Unanswered Questions Surrounding Symptom Increase on Peanut Resistant Cultivars.

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1. Abstract

Tomato spotted wilt virus's nucleocapsid protein (TSWV-N) plays an important role in assembling the genomic RNA of TSWV into ribonucleoprotein complex (RNP). In this study, a total of 126 peanut leaf samples were collected from 2021 to 2022 across three site locations in Alabama. Samples were selected preferentially based on symptoms of wilt and leaf ring spots regardless of the cultivar. The nucleocapsid protein of the TSWV positive samples was sequenced. The protein alignment of 140 nucleocapsid proteins of Alabama strains identifies five conserved amino acid mutations in a comparison with the MT2 strain originally isolated from Hawaii (X61799.1). On the other hand, seven conserved amino acids were detected when the 140 nucleocapsids aligned with the reference genome of TSWV-N from the NCBI strain BR-O1 (NC_002051.1). Conserved and non-conserved mutations were detected in the RNA binding omains of the nucleocapsid. To further understand the impact of the amino acid mutations, eight nucleocapsids were selected based on their mutations to be localized in *Nicotiana benthamiana* as a plant model using the MT2 strain as a reference control. The changes in the localization of the

nucleocapsid protein are an important first step in determining why peanut plants have demonstrated high symptoms of TSWV in the field in recent years.

Keywords

Tomato spotted disease, orthotospoviruses, nucleocapsid protein, sangar sequencing, thrips, protein localization, amino acid mutations, and Illumina MiSeq full genome sequencing.

2. Introduction

Tomato spotted wilt virus (TSWV) is an ambisense virus belonging to the *Orthotospovirus* genus in the Bunyaviridae family that includes more than 350 species of plants and animals (Plyusnin et al., 2010). Viruses that belong to bunyaviruses have a tri-segmented genome including a large segment (L), a middle segment (M), and a small segment (S). In all bunyaviruses, the L segment encodes RNA-dependent RNA polymerase (RdRp). In those members that infect plants, the M segment encodes the glycoproteins (G_N and G_C) and the nonstructural movement protein (NSm); and the S segment encodes the nucleocapsid protein (N) and the nonstructural silencing protein (NSs) (Plyusnin et al., 2010). TSWV infects more than 1000 plant species including many important crops (Rotenberg et al., 2015) in 80 botanical monocot and dicot plant families (Cho et al., 1987, Debreczeni et al., 2011, EPPO/CABI, 1997). In addition to plant hosts, the transmission of TSWV occurs in a persistent, propagative manner meaning that the virus replicates within the insect vector. The larval stages acquire TSWV after hatching and the virus replicates in the gut tissues of the insect. After pupation, the virus moves from gut tissues to the salivary glands where the infected adults transmit the virus for their entire life span (Rotenberg et al., 2015). There are at least nine different species of thrips that transmit TSWV, however, the western flower thrips (*Frankliniella occidentalis*) is the most efficient vector and tobacco thrips (*Frankliniella fusca*) is the most prevalent vector in peanuts in Alabama (Culbreath and Srinivasana et al., 2011). The

extremely wide host range of TSWV and the ubiquitous nature of the thrips were the main reasons to rank TSWV as one of the 10 most devastating plant viruses (Scholthof et al., 2011).

In the United States, virus infection was first reported in peanuts in Texas in 1971 and eventually spread to other southern states (Culbreath et al., 2003; Halliwell and Philley, 1974). TSWV was first recorded in Alabama, Georgia, and Florida in the 1980s and became a major obstacle to peanut production (Srinivasana et al., 2017). TSWV infection causes spotted wilt disease in peanut which has become a significant problem limiting the production of peanut (*Arachis hypogaea* L.) in the southeastern United States (Lai et al., 2021). Symptoms of TSWV infection on peanut include concentric ring spots, various chlorotic patterns, and general stunting of infected plants (Srinivasana et al., 2017). Additionally, TSWV causes misshaped pegs and kernels where the seed coat or testa becomes reddish or darker on infected pods compared to non-infected pods. Early infection of peanut plants yields less than those infected later in the season (Culbreath et al., 1992). The losses caused by TSWV and other *Orthotosopoviruses* were estimated to be more than tens of millions of dollars worldwide and in the U.S. alone, it was estimated to exceed $1.4 billion in a ten-year period (Culbreath et al., 2003, and Riley et al.,2011).

Insecticides were used to manage the virus initially in the 1990's and early 2000s targeting the thrips vector. Typically, broad-spectrum insecticides including Organophosphate insecticides and organocarbamate insecticides (Chamberlin et al., 1992; Todd et al., 1994, 1996, 1997). Moreover, Phorate and aldicarb were also used to reduce thrips feeding damage (Culbreath et al., 2008, 2016; Hagan et al., 1991; Herbert et al., 2007; Todd et al., 1997; Tubbs et al., 2013). Despite suppressing thrips feeding, most insecticide applications were not effective in reducing TSWV incidence (Hagan et al., 1991; Herbert et al., 2007; Marasigan et al., 2016; Mulder et al., 2001; Todd et al., 1996, 1997; Weeks and Hagan, 1991; Wells et al., 2002). Cultural practices have also

been used for TSWV management in conjunction with insecticides. Planting date alterations have long been used to avoid peak thrips flights. Additionally, conservation tillage options have been used by some growers. Conservation tillage practices are mainly associated with fewer thrips to reduce the thrips feeding damage (Srinivasan et al., 2017). Currently, host resistance is one of the most effective management methods available for peanut growers. Initially, at the advent of TSWV in the Southeast, the cultivars grown such as SunOleic 97R, Florunner, GK-7, and Georgia Runner were extremely susceptible to TSWV (Culbreath et al., 1992, 1996). Later, an intensive screening and breeding effort started in Georgia and Florida. The early screening led to identifying tolerance in one of the cultivars − Southern Runner (Black, 1990; Black and Smith, 1987). A Risk Mitigation Index (Peanut Rx) to assess risks and include TSWV resistance cultivars and chemical and cultural management tactics was made available to assist growers in choosing the best peanut cultivars that resist or tolerate TSWV (Srinivasan et al., 2017). Due to the adoption of risk reduction practices described in Peanut Rx, yield losses from TSW disease started to decline in the 2000s. However, an increase in the disease incidence and severity has been observed in recnt years (Sundaraj et al., 2014).

To identify the strains of TSWV present in the field, the viral protein sequence for the nucleocapsid (N) is used for virus detection from field samples. This single open reading frame has the most data available on the NCBI of any other sequence in all TSWV strains, 2100 as at the time of writing (www.ncbi.nlm.nih.gov). The nucleocapsid protein (N) of TSWV is the main structural protein responsible for viral RNAs assembling into ribonucleoprotein complexes (RNPs) (Kormelink 2011) during replication. The genomic RNA of the bunyaviruses is encapsidated by the nucleocapsid protein to form a ribonucleoprotein complex (RNP) which has central roles in virus replication, transcription, and viral movement throughout the cell (Guo et al., 2017). To assist

in this function, the nucleocapsid of TSWV has two RNA-binding domains, the first one is in the N-arm region hanging from 1 – 39 aa, and the second one is in the C-arm from 233 – 248 aa (Uhrig et al., 1999). Although both domains of N were shown to be involved in RNA binding (Richmond et al., 1998), the importance of each of the RNA binding sites on the N protein also remains to be determined. In 2020, an increase in observable symptoms in peanuts in AL was observed and this continued to increase in 2021 and 2022. After this observation, in 2021, symptomatic peanut samples were collected, and the N protein was selected for sequencing. We hypothesize that the amino acid mutations in the N protein of TSWV could indicate the changes in TSWV which may be responsible for the increase in the observance of symptoms in the field. Therefore, our objective is to detect these mutations and investigate them in more detail to determine the localization of the TSWV-N protein in the host plant cells and if this differs from pre-2021 samples from Georgia which were less likely to show symptoms in the field. We will be comparing possible mutations to the management methods to determine if the leading cause of the resistant breaking is due to the mutations, or management methods or both.

3. Materials and Methods

3.1. Peanut sample collection and field experiments

Peanut cultivar research trials were established at the Brewton Agricultural Research Unit (BARU) in Brewton, Alabama, the Gulf Coast Research and Extension Center (GCREC) in Fairhope, Alabama, and the Wiregrass Research and Extension Center (WGREC) in Headland, Alabama and surveyed for TSWV. The WGREC trials were planted on 7 May in 2021 and 12 May in 2022 in a non-irrigated field under conventional tillage following a one-year cotton-peanut rotation. Plots consisted of four 7.0 m rows spaced 0.9 m apart arranged in a randomized complete block design with four replications in 2021 and 2022. In 2021, 11 experimental breeding lines (‘ACI-1041’, ‘ACI-1426’, ‘ACI-212’, ‘ACI-3321’, ACI-F104’, ‘ARDG 1’, ‘ARDG 2’, ‘ARDG

3', 'AU14-34', 'AU18-53', and 'UF11x41HO') and 14 commercial peanut cultivars (AU_NPL 17, FloRun™ '331', Georgia-06G, Georgia-07W, Georgia -09B, Georgia-12Y, Georgia-14N, Georgia-16HO, Georgia-18RU, Georgia-20VHO, TifNV-High O/L, and TUFRunner™ 297) were evaluated at WGREC. In 2022, 14 experimental breeding lines ('ACI-222', ACI-1041', 'ACI-212', 'ACI-3321', 'ACI-N104', 'ARDG 1', ARDG 2', 'ARDG 3', 'ARDG 4', 'ARDG 5', 'AU 20-41', 'CB 1', 'CB20', and 'CB7') and 14 commercial peanut cultivars (AU Barkley, SU-NPL 17, FloRun™ '331', FloRun™ 'T61', Georgia-06G, Georgia -09B, Georgia-12Y, Georgia-14N, Georgia-16HO, Georgia-18RU, Georgia-20VHO, TifNV-HG, TifNV-High O/L, and TUFRunner™ 297). The BARU trial was planted on 13 May 2022 and the GCREC trial was planted on 11 May 2022. Both trials were established in irrigated fields under conventional tillage following a one-year cotton-peanut rotation. At BARU and GCREC, plots consisted of 9.1 m rows spaced 0.9 m apart arranged in a randomized complete block design with four replications. At BARU and GCREC, the peanut cultivars included TUFRunner™ '297', FloRun™ 'T61', FloRun™ '331', Georgia-06G, Gerogia-09B, Georgia-12Y, Georgia-14N, Georgia-16HO, Georgia-18RU, Georgia-19HP, Georgia-20VHO, and AU-NPL 17. Recommendations of the Alabama Cooperative Extension System for tillage, fertility, weed, disease, and nematode control were followed. At BARU and GCREC, phorate (Thimet® 20-G EZ Load®; Amvac Chemical Corporation, Newport Beach, CA) at 5.6 kg/ha and 6.7 kg/ha, respectively, in-furrow at planting for thrips and TSW control. No insecticide was applied in-furrow at planting at WGREC in 2021 and 2022. Five to six trifoliate leaves were collected from individual peanut plants displaying symptoms of TSW on 5 Aug 2021 at WGREC, 1 Jul 2022 at BARU, 8 Jul 2022 at GCREC, and 6 Jul 2022 at WGREC. Samples were individually bagged, transported on ice, and stored at -80 C until processing. The center two rows of the four row plots were rated for TSW incidence, which

is expressed as the number of disease loci (≤ 0.30 m of TSW symptomatic plants) per 18.2 m of row at on 5 Aug 2022 BARU and on 26 Jul 2022 at GCREC, and per 14.0 m of row at WGREC on 26 Jul 2021 and 29 Jul 2022. Yield data was collected from the center two rows at <10% moisture and plots were harvested on 30 Sep 2022 at BARU, GCREC, WGREC on 27 Sep and 4 Oct 2021, and WGREC on 17 Oct 2022, 24 Oct 2022, and 1 Nov 2022.

3.2. Total RNA extraction and cDNA synthesis

The total RNA was extracted from the 126 collected peanut leaf samples from 2021 and 2022. Two peanut leaflets that were symptomatic of TSW were used for RNA extraction. The symptomatic tissues were placed in 2 ml screw cap tubes with 5-7 sterilized 2.4 mm metal beads. The tubes were frozen in liquid nitrogen, then the frozen tissues were ground using a Bead Ruptor Elite (Omni International, Kennesaw GA, USA). The samples were ground on two cycles of 30 seconds at 5 m/s and were re-frozen in liquid nitrogen between the two grinding cycles. RNA extraction was then performed using IBI Mini Total RNA Kit (Plants) (IBI Scientific, Dubuque, IA, USA) using the manufacturer's protocol. The concentrations were measured on a Nanodrop 1000. The cDNA synthesis was performed using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham MA, USA) according to the manufacturer's directions. The cDNA synthesis was performed using a segment-specific reverse primer for the S segment of TSWV (TSWV_S_R) (Ruark-Seward et al., 2020) (Table 1). The amount of RNA used in each cDNA synthesis reaction was 1 µg of total RNA.

3.3. Nucleocapsid amplification and cloning

Specific TSWV_N_F and TSWV_N_R PCR primers were used to amplify the nucleocapsid (Table 1) using the Thermocycler PCR on the following settings: 98 °C–30 s; 35X: (98 °C–10 s, 64 °C–45 s, 72 °C–4 m); 72 °C–5 m and infinite hold at 12 °C using Phusion High-

Fidelity PCR Kit (Thermo Fisher Scientific, Waltham MA). Positive samples that showed a band in the size of 774 bp of the nucleocapsid were moved into pJET-T-d-TOPO vector using the CloneJET PCR Cloning kit (Thermo Scientific Baltics UAB) following the manufacturer's recommended instructions. Colonies of N were confirmed using colony PCR using the pJET-F and pJET-R primers (Table 1). Two different colony plasmids from each sample were sent for sequencing at North Carolina State University's Genomic Science Laboratory.

3.4. Sanger sequences, analysis, and constructing the phylogenetic tree of the nucleocapsid

The nucleocapsid sequences were analyzed using Blast Tool (www.ncbi.nlm.nih.gov) by comparing our sequences with all TSWV nucleocapsids available on the NCBI database. Only sequences that have a full read with a start codon and no premature stop codons were considered for the next step. The full read sequences were then translated from nucleotide sequence to protein sequence using Expasy free online tool, Translate (https://web.expasy.org/translate/). Complete protein sequences with no gaps were then used to build the alignment using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to detect the conserved and non-conserved amino acid mutations of the nucleocapsid protein using our X61799.1_MT2 strain as a reference genome (Whitfield et al., 2005) and the NCBI reference genome of strain BR-O1 (NC_002051.1) (de Haan et al., 1990). The protein alignment was converted to a FASTA format using the online converting tool (https://molbiol-tools.ca/Convert.htm) and then the FASTA alignment was saved in Newick format using MEGA11 version 11.0.13. The phylogenetic tree was finally constructed using iTOL (Letunic and Bork 2019) online tool (https://itol.embl.de/tree/13120425486293691674071207) to show the likelihood between the nucleocapsid protein of Alabama and other nucleocapsid sequences available on the GenBank database.

3.5. Design of constructs for visualization of TSWV-N

Eight nucleocapsids were selected for localization in plant cells using *Nicotiana benthamiana* were chosen for the localization using the nucleocapsid of the MT2 strain as a reference control (Whitfield et al., 2005). The eight nucleocapsids were amplified from the pJET sequencing vector using the same method used to amplify the original TSWV-N gene (see above). Then the eight Ns were recombined to the entry clone of pENTR vector using the pENTR D-Topo following manufacturer protocol (Invitrogen^{TM}).

The sequence of the eight nucleocapsids were confirmed with sequencing using the M13 primers present in the vector. The sequences of the eight nucleocapsids were then received and confirmed. The confirmed eight sequences were then recombined in pSITE-2NA destination vectors (Chakrabarty et al., 2007; Martin et al., 2009) as fusions to green fluorescent protein (GFP) using LR clonase following manufacturers recommendations (Invitrogen^{TM}). The recombinant pSITE vectors were transformed into *E. coli* and were incubated at 37 C on LB agar with 50% Spec antibiotic overnght. Then, the colonies were confirmed using the GoTaq PCR (Promega ^{TM}) using the pJET forward and the pJET reverse primers (Table 1). The orientation of the nucleocapsid proteins in pSITE-2NA was confirmed with the GFP reverse primer (GFP_R) and the forward primer of the nucleocapsid (TSWV_N_F) (Table 1).

3.6. Plant Growth and Maintenance

Wildtype and transgenic *N. benthamiana* expressing red fluorescent protein fused to the endoplasmic reticulum (RFP-ER) (Martin et al., 2009) were grown in a growth chamber set at 25^{o}C for 14 hours of daylight at 300 uM light intensity and a 10-hour dark period. Plants were transplanted from seedling pots to individual containers at two weeks of age. After transplanting, plants were allowed to grow for approximately one to two weeks until the leaves reached the size of greater than 24.26 mm. This size corresponded to the best age for the infiltration of plants for microscopy.

3.7. Infiltration of plants with constructs for visualization

After the orientation of the selected eight nucleocapsid proteins was confirmed, they were transformed into *Agrobacterium tumefaciens* competent cells by adding 10 µl of the mini-prepped plasmid to 50 µl of *A. tumefaciens* competent cells in a 1.7 ml tube. The solution was mixed and submerged in liquid nitrogen. A heat block set to 37^{o}C was used to incubate the tubes for five minutes, then 600 µl of LB broth media was mixed with the solution and incubated for 2-3 hours on a shaker-incubator set to 28^{o}C. LB agar plates with both Rifamycins and Spectinomycin antibiotics were used to plate the transformation solution and were allowed to grow for 4 days at 28^{o}C. After 4 days of growth, single colonies were selected and restreaked on new plates. After two days, *A. tumefaciens* was used to both make a glycerol stock and used for the infiltration using the protocol used in (Goodin et al., 2002) with some modifications. The bacteria were dissolved into 0.1 M MES and 0.1M $MgCl_2$ solution and the OD_{600} was measured between 0.6 to 0.7 for all samples. The addition of 2.0 M Acetosyringone was then added to the adjusted bacterial solution at a ratio of 1.5 μL:1 ml. The adjusted bacterial solution was allowed to set at room temperature for two hours before the infiltration. Approximately, 1 ml bacterial solution per leaf was infiltrated in healthy *N. benthamiana* plants (*Benth*).

3.8. Microscopy of samples

Microscopy of plant cells was performed two days post infiltration (dpi) until five dpi as a time course using an Eclipse Ts2R (Nikon) epifluorescence microscope. Images were taken using Nikon Elements software v. 5.21.02 (Build 1487). Figures were assembled using Microsoft Powerpoint v. 2305 (Build 16501.20210 Click-to-Run). A solution of 10% DAPI stain was used to stain the plant cell nucleus by infiltrating it one hour before proceeding with imaging. Four replicates were applied, and three different cells were images for each construct in each replicate.

3.9. Statistical Analysis

For the 2021 and 2022 field trials, TSW incidence and yield data were analyzed using analysis of variance (ANOVA) followed by pair-wise comparison with Fisher's least significance difference (LSD) test ($P \leq 0.05$) using IBM® SPSS® Statistics Version 25 (Armonk, NY) with a p-value of 0.05 and analyzed for outliers (Pallant 2013). Pearson correlation coefficient between TSW incidence and yield were calculated using IBM® SPSS® Statistics Version 25.

4. Results

4.1. Tomato spotted wilt incidence and yield impacts

At BARU in 2022, no significant interaction was observed between peanut cultivars as determined by one-way ANOVA for TSW incidence (F = 1.829, p-value = 0.085) and yield (F = 1.37, p-value = 0.229), and no outliers were identified. TSW incidence was low in this trial (<14.0%) and all cultivars produced yields above 4,400 kg/ha (Table 3). However, there was a weak, but significant (p-value = 0.019), negative correlation between TSW incidence and yield (r = -0.336, N = 48).

At GCREC in 2022, no significant interaction was observed between peanut cultivars as determined by one-way ANOVA for TSW incidence (F = 0.824, p-value = 0.617) and no outliers were identified. Overall, TSW incidence was low at less than seven percent for all peanut cultivars (Table 3). However, there was a significant interaction between peanut cultivars for yield (F = 4.464, p-value = <0.001) and no outliers were identified. Georgia-09B had the highest yields in this trial, which was equaled by FloRun T61, Georgia-18RU, and Georgia-06G (Table 3). In contrast, TufRunner 297 had the lowest yields in this trial, which was equaled by FloRun 331, AU-NPL 17, Georgia-19HP, and Georgia-16HO. However, there was no apparent significant correlation between TSW incidence and yield (r = -0.025, p-value = 0.866, N = 48).

At WGREC in 2021, there was a significant interaction between peanut cultivars as determined by one-way ANOVA for TSW incidence (F = 2.532, p-value = 0.001) and yield (F = 6.968, p-value = <0.001), and no outliers were identified. TSW incidence was low to moderate in this trial and reached up to 19 to 21% for some cultivars (Table 4). The experimental breeding line ARDG 3 had the lowest TSW incidence (4.9%), which was equaled by Georgia-12Y, ACI-3321, ACI-1426, ARDG 1, Georgia-14N, ACI-F104, AU-NPL 17, Georgia-20VHO, and AU 18-53. In contrast, the experimental breeding line ARDG 2 had the highest TSW incidence (20.7%), which was equaled by AU 14-34, Georgia-07W, FloRun 331, TufRunner 297, ACI-212, ACI-1041, Tifguard, Georgia-16HO, Georgia-06G, and TifNV-High O/L. In this trial, peanut yields were highly variable between peanut cultivars, which ranged from 1,318 to 6,652 kg/ha. The experimental breeding line ACI-1426 had significantly lower yield (1,318 kg/ha) than all remaining cultivars due to poor stands. The experimental breeding line ACI-3321 had the second lowest yield (4,052 kg/ha) in this trial, which was equaled by ACI-212 and Georgia-07W. In contrast, Georgia-12Y had significantly higher yields than ten of the cultivars including previously mentioned cultivars as well as Georgia-18RU, Tifguard, Georgia-09B, ACI-F104, Georgia-20VHO, and UF11x41HO. Additionally, there was a weak, positive correlation between TSW incidence and yield, r = 0.196, N = 100; however, the relationship was not significant (p-value = 0.076).

At WGREC in 2022, there was a significant interaction between peanut cultivars as determined by one-way ANOVA for TSW incidence (F = 1.977, p-value = 0.010) and yield (F = 3.506, p-value = <0.001), and no outliers were identified. TSW incidence was moderate in this trial and ranged from 15 to 32% for most of the peanut cultivars (Table 4). Georgia-12Y had the lowest TSW incidence (6.8%), which was equaled by Georgia-20VHO, ACI-N104, CB 1, ACI-

3321, FloRun T61, ARDG 1, ACI-222, Georgia-14N, AU-NPL 17, and CB 20. In contrast, FloRun 331 had the highest TSW incidence (32.1%) in this trial, which was equaled by TifNV-HG, Georgia-09B, ARDG 2, Georgia-16HO, ARDG 5, and AU Barkley. In terms of yield, peanut yields varied between peanut cultivars, which ranged from 2,228 to 4,888 kg/ha. The commercial peanut cultivar Georgia-20VHO had the highest yields in this trial, which was equaled by AU-NPL 17, ARDG 1, Georgia-18RU, Georgia-16HO, Georgia-06G, TufRunner 297, CB 7, ACI-1041, TifNV-High O/L, TifNV-HG, and FloRun T61. In contrast, the experimental breeding line ARDG 4 had the lowest yield, which was equaled by Georgia-14N, ARDG 5, Georgia-09B, FloRun 331, ACI-222, and ARDG 2. Additionally, there was a weak, but significant (*p*-value = 0.003), negative correlation between TSW incidence and yield (r = -0.279, N = 112).

4.2. Peanut sample collection, and nucleocapsid amplification and cloning

Between 2021 and 2022, a total of 126 foliar tissue samples were collected from TSWV symptomatic peanut plants in four cultivar trials at BARU (n = 49 in 2022), GCREC (n = 42 in 2022, and WGREC (n = 11 in 2021 and n = 24 in 2022). Ninety-four of the 126 samples tested positive for the nucleocapsid of TSWV and were identified as TSWV via PCR (n = 31 from BARU, n = 31 from GCREC, n = 11 from WGREC in 2021, and n = 21 from WGREC in 2022). The PCR products of 94 positive samples proceeded to the next step in which each sample was cloned into pJET and transformed into DH5 *E. coli* competent cells. Plasmids were confirmed and two colonies from each sample were sent for Sangar sequencing. This was done to guarantee receiving at least one complete nucleocapsid read from each sample and to check if any of the plant samples had multiple N sequences or if the population was consistent in the same plant.

4.3. Sanger sequences, analysis

Out of 126 peanut samples collected in 2021 and 2022, 94 samples tested positive for TSWV-N protein. Sangar sequences of two plasmids from each sample were received (The total number of plasmids that were sequenced was 188 plasmids). Nucleocapsid sequences were blasted on the NCBI database and only complete nucleocapsid protein sequences with no gaps were obtained. As a result, 140 complete sequences of the nucleocapsid protein of TSWV were identified. The complete 140 N proteins were submitted to GenBank for accession numbers assigning (Table 6).

4.4. Phylogenetic tree of the nucleocapsid

All complete TSWV-N sequences were translated into protein, then the alignment of our nucleocapsids was made in a comparison with the X61799.1_MT2 strain (Whitfield et al., 2005) and the NCBI reference genome of TSWV strain BR-O1 (NC_002051.1) (de Haan et al., 1990) separately. The protein alignment results confirmed five conserved amino acid changes that were found among all of Alabama's nucleocapsids of the three locations (Table 2 and Figure 1). Additionally, seven conserved amino acid mutations were observed when the 140 nucleocapsids aligned with the NCBI strain BR-O1 (NC_002051.1). (Table 2 and Figure 1). Furthermore, other amino acid mutations that were not conserved among the 140 nucleocapsids were detected (Table 6). All complete nucleocapsid proteins of TSWV from Alabama were submitted to GenBank and accession numbers were assigned (Table 6). Samples with incomplete sequences for N were not considered for GenBank submission. The phylogenetic tree was constructed between the 140 complete nucleocapsid proteins that represent Alabama with the complete nucleocapsid protein from the GenBank database of Georgia, North Carolina, Hawaii (The MT2 reference strain), Europe, and Asia (Table 7). The phylogenetic tree indicates one moan clade (A) and two sub-clades (B and C) (Figure 2). In Clade A, the majority of Alabama sequences coming from BARU

and WGREC with only N 7 sequences of GCREC were found closest to the N sequences of Georgia of 2013 and 2021 (Figure 2). In sub-clade B, the majority of N sequences of GCREC with 3 sequences from BARU and 10 sequences from WGREC were found cladding with the N sequences of Georgia of 2013 and 2021 (Figure 2). Lastly, in sub-clade C, two samples from BARU and WGREC and four samples from GCREC share the same roots with the TSWV strains from Georgia of 2013 and 2021, North Carolina, the MT2 strain of Hawaii, and TSWV strains of Europe and Asia (Figure 2). In 2010, the nucleocapsid protein of TSWV was sequenced out of 81 peanut samples from Georgia (Sundaraj et al., 2013). Additionally, in 2021, a total number of 59 TSWV-N complete sequences were released (Lai et al., 2021). The nucleocapsids of Georgia from both studies were used as a reference genome on the phylogenetic tree (Fig.2). The majority of these sequences were shown to be closely related to Alabama sequences, mostly to GCREC. To understand the one main clade (A) and the two sub-clades (B and C), eight nucleocapsids, marked with red stars (Figure 2) were selected for the localization in plants using the MT2 strain as a reference control.

4.5. Mutations present in selected candidates for localization

To observe the amino acid mutations present in the eight samples selected for localization, a protein alignment demonstrating conserved and unique mutation was generated including the MT2 strain (Figure 3). Sample BARU25.1 has an amino acid mutation in the first RNA binding region where amino acid S mutated to N in the tenth aa. Sample WGREC5.1 has an amino acid mutation in the core on TSWV-N where amino acid K mutated to R in 213 aa. Sample GCREC45.1 also has an amino acid mutation in the core on the nucleocapsid where amino acid G changed to R in 127 aa. Sample WGREC.B1 has two amino acid changes, the first one is in the first RNA binding region similar to BARU25.1 and the second mutation is in the core on the nucleocapsid

where amino acid S changed to G in 149 aa. Sample WGREC.E2 has two amino acid changes both were found in the first RNA binding region, in the first mutation K mutated to M at the 3rd aa, and in the second mutation, V changed to D at the 4th aa. Sample WGREC119.3. R2, WGREC106.2.R3, and BARU2.1 have no mutation in comparison with the MT2 strain used as a localization control (Figure 3).

4.6. pSITE plasmid constructs, agroinfiltration, and live cell imaging

To determine the impact of the amino acid mutation on the localization of the nucleocapsid of TSWV, eight nucleocapsids were selected to be localized in *Nicotiana benthamiana* using agroinfiltration using the well-characterized MT2 strain as a reference control (Figure 3). At 2dpi, all nucleocapsids localize to the cell periphery with slight protein aggregation (Figure 5). Sample BARU25.1-GFP, WGREC.B1-GFP, and WGREC.E2-GFP show protein accumulation around the nucleus, especially, in WGREC.B1-GFP where the protein expression was surrounding the nucleus from all sides where it was accumulating from one side in the other two strains (Figure 5). Sample WGREC.B1-GFP, WGREC.E2-GFP, WGREC.119.3.R2-GFP, and WGREC.106.2.R3-GFP were soluble along with the cell periphery more than the rest of the samples (Figure 5). At 5 dpi all localized samples including the MT2 strain show a complete protein aggregation to the cell periphery and to the cytoplasm except for sample WGREC.B1-GFP which still looks soluble exactly as it was at 2 dpi (Figure 5). A non-fused pSITE-GFP vector producing free-GFP was transformed into agrobacterium and infiltrated along with the selected nucleocapsids as a localization control and it localized to the cell periphery and the nucleus from 2 dpi to 5 dpi. The fluorescent percentage for all localized samples was 90 – 95% except for sample WGREC.B1-GFP where it was 5 – 10 % at 2 days post infiltration (dpi) and it became weaker by the time with less than 5% (5 cells shows protein expression out of 100 cells).

When sample BARU25.1 was localized, at 2 dpi it was soluble along to the cell periphery and around the nucleus (figure 5). This sample demonstrated a fast protein aggregation from 3 dpi to 5 dpi compared to the MT2 strain reference control (Figure 4). Sample WGREC5.1 and GCREC45.1 were soluble to the cell periphery and the protein aggregation was classified as wildtype aggregation because it was similar to the MT2 reference strain over the imaging time course from 2dpi until 5dpi (Figure 4). Sample WGREC.B1 has the most unique localization pattern where it was always soluble in the cell periphery, never aggregated over the five imaging days, and localized around the nucleus (Figure 4). Sample WGREC.E2 matches the same localization as BARU25.2 showing faster aggregation from 2 to 5 dpi compared to the MT2 strain (Figure 4 and 5). The last three samples of WGREC.119.3.R2, WGREC.106.2.R3, and BARU2.1 have no unique amino acid mutations compared to the MT2 strain (Figure 3) and their nucleocapsid protein localization does not look different from the wildtype protein aggregation of the MT2 strain at 2dpi to 5dpi (Figure 4 and 5).

5. Discussion

Tomato spotted wilt virus represents the most studied orthotospoviruses, therefore, the genome organization, replication, particle morphology, and transcription strategies are well-described (Kormelink et al., 2011). At onset of this study, it was noticed what appeared to be higher rates of TSWV symptoms than previously seen occurring in the field. This was first observed in Alabama in 2021 when initial sampling was done. It continued in 2022 with a more exhaustive sampling protocol to better capture the differences and similarities of samples also taking into consideration the cultivar of peanuts and the location. Samples from 2021 were only sampled at one location which led us to add two new locations in 2022. The goal was to understand why TSWV symptoms were more prevalent post-2021 than what had been seen previously. The

hypothesis for this study was broken down into three possibilities: 1) that TSWV sequences were changing leading to increased incidence of the disease by breaking the field resistance; 2) that TSWV was not changing, but the interaction between TSWV and thrips may be changing; or lastly, 3) that management practices had changed, and the peanuts were showing more symptoms due to change in agronomic practices. To test the first theory, we sequenced TSWV populations in the field using the N protein sequence as a proxy to measure genetic changes. We then compared the lines of peanuts and the mutations of TSWV found in those lines to determine how this may have impacted the incidence of TSWV.

TSWV was highly problematic in the southern United States prior to 2006 leading to significant yield losses in peanuts. In response, scientists and breeders developed TSWV-resistant and developed a comprehensive guide for management of the disease (Peanut Rx). The current integrated pest management strategy for TSW includes planting date selection, seeding rates, twin-row planting, conservation tillage, applying preventative at-plant insecticides, and cultivar selection (Culbreath et al., 2003; Culbreath and Srinivasana, 2011; Srinivasana et al., 2017). Historically, it has been difficult to breed disease resistance from wild species to cultivated peanuts due in part to diploid vs. tetraploid differences, cross-compatibility barriers, narrow genetic base of wild species, and linkage drag (Bertioli et al., 2011; Chaudhari et al., 2019; Pandey et al., 2016). Despite these challenges, peanut breeders have released several highly tolerant peanut cultivars in the last decade such as Georgia-12Y, Georgia-14N, TifNV-HiOL (Branch, 2013; Branch and Brenneman, 2015; Holbrook et al., 2017). Since there are several peanut cultivars with partial resistance to TSW, cultivar selection is the first line of defense in TSW management for peanut producers. However, no cultivar is completely resistant to TSW and their performance (i.e., yield) can vary depending on pest pressure, cultural practices, and environmental conditions. In 2007,

2018, and 2019, the lines AU-NPL 17 and TUFRunnerTM ‘297’ were reported as resistant to TSW, however, in 2021 the line is considered moderately tolerant (Table 5) based on the Peanut RX program (Lai et al., 2021).

In this study, all the cultivars displayed symptoms of TSW at the time of sampling, but the incidence did vary by cultivar and location. Interestingly, all samples displayed symptoms consistent with TSWV, however, not all tested positive for the N sequence used for diagnostics. This suggests that the symptoms could have either come from a different virus or that the primers were too specific for the N sequences coming from other plants and that a redesign of more universal primers may be warranted. Using the DNA alignment that was constructed between the references on the phylogenetic tree (Table7) and one candidate from Alabama, the reverse primer was found in a non-conserved region (Data not shown). This suggests that the samples that displayed TSW symptoms but tested negative to TSWV may had different strains of TSWV which our specific primers could not bind. TSW incidence was lowest at BARU and GCREC in 2022 and cultivar selection did not significantly impact TSW incidence in contrast to WGREC in 2021 and 2022. This is in part due to the planting date (i.e., early to mid-May planted peanuts are at lower risk for TSW) and application of phorate at-planting at both BARU and GCREC. Phorate is a systemic, acetylcholinesterase-inhibiting organophosphate pesticide that is commonly used as an in-furrow insecticide to reduce transmission of TSW via thrips and TSW incidence in peanuts (Culbreath et al., 2008; Culbreath et al., 2016; Jian et al., 2015; Srinivasana et al., 2017). The reduction in TSW incidence by in-furrow applications of phorate is primarily due to the induction of plant defense responses and the down regulation of viral transcripts (Jain et al., 2015). Thus, cultivar selection had less of an impact on TSW incidence at BARU and GCREC in 2022. Similar

results have also been reported in additional studies as well (Culbreath et al., 2008; Culbreath et al., 2016; Haynes et al., 2019).

Although planted in early May, cultivar selection significantly impacted TSW in both WGREC trials which did not receive an in-furrow application of phorate. In 2021, cultivars with high tolerance to TSW such as Georgia-12Y, Georgia-14N, TifNV-High O/L and moderately tolerant cultivars such as Georgia-16HO and AU-NPL 17 had the lowest TSW incidence and highest yields in this trial. In contrast, despite Georgia-12Y having the lowest TSW incidence in both years, it was not the highest yielding cultivar in 2022. The moderately tolerant cultivars such as AU-NPL 17 and Georgia-20VHO were the highest yielding cultivars. The 2022 production season had lower rainfall (15.5 in) overall when compared to 2021 (24.1 in), which could have negatively impacted cultivar yield performance. Drought is a significant limitation to peanut production worldwide, especially when drought occurs mid-season or terminal droughts (Holbrook and Stalker, 2003; Jongrungklang et al., 2013; Nageswara Roa et al., 1989). Thus, although a limiting factor, TSW incidence is not the only factor that can influence the yield response of peanut cultivars.

When looking at TSWV-N in the populations of peanuts, we observed distinct subclades of virus sequences (Figure 2). The main clade A and sub-clade B had sequences from AL cladding with just Gorgia, except one sample from Japan (AB038341.1) was found blending with clade A. This suggests that these sequences of N from AL are the closest to Georgia. An interesting sub-clade C was also seen. This sub-clade has samples from AL cladding with sequences from Georgia, North Carolina, Hawaii, Europe, and Asia suggesting the possibility of the evolution of new strains of the virus. *Orthotosopoviruses* members were known for their ability to mutate through the viral RdRp's generation of random mutations (Reguera et al., 2010, Terret-Welter et al., 2020, and

Ruark-Seward et al., 2020) and the ability of TSWV to exchange genome segments through reassortment (Webster et al., 2011, Tentchev et al., 2011, and Webster et al., 2015). The protein alignment of the 140 nucleocapsids of Alabama with the reference genome of TSWV-N from the NCBI (Strain BR-01) (de Hann et al., 1990) and with the MT2 strain indicates that Alabama nucleocapsids are more related to the MT2 strain isolated from Hawaii (Whitfield et al., 2005). Compared to the MT2 strain, Alabama Ns contains five conserved amino acid mutations; however, seven conserved amino acid mutations were observed when compared to the reference strain BR-01 (Table 2 and Figure 1). Two of these conserved mutations were shared between TSWV of AL with the MT2 strain and TSWV of AL with the BR-01 strain. As the MT2 strain originated from Hawaii (Whitfield et al., 2005) and the BR-01 strain is of European origin (de Haan, et al., 1990), these changes seem unlikely to be related to geographic proximity (Savory et al., 2014).

A 3D model of the nucleocapsid protein of orthotospovirus genus-wide was used to compare the unique amino acid mutations of the selected candidate for localization (Olaya et al., 2019). Sample BARU25.1 has amino acid mutation where S changes to N at the 10 aa position, 16 other orthotospovirus show the same change (Olaya et al., 2019). This shows that this mutation is consistent among other orthotospovirus species which might indicate the importance of this mutation as it may play as a motif for RNA binding properties on the N protein of TSWV. Sample WGREC5.1 has a mutation at aa 213 position when aa K changed to R. This change is supported by only two other species of orthotospovirus including ANSV and SVNaV. Amino acid mutations in the samples GCREC45.2, WGREC.B1, and WGREC.E2 were detected in any of the other orthotospovirus in this comparison.

We then selected candidates for localization of TSWV-N to determine if the changes to the sequence had a consequence in the observed phenotypes of the protein after expression.

Previously, it had been observed that the localization pattern of TSWV-N changed over time in MT2 (Montero-Astúa 2012) when comparing the localization at days two through eight post infiltration. To determine if these strains also showed this same phenotype, those sequences from the main clade A and subclades B and C were selected to determine the rate in which they may/may not change their localization pattern. The hypothesis guiding this work is that the change in the phenotype of the N protein may correlate to how the virus is beginning its replication as N functions to assist in this process (Feng et al., 2020). In our observations, we noticed three main phenotypes, those that do not aggregate, those that have wildtype protein aggregation speed matching MT2, and those that are faster than MT2 (Figure 4). Notably, the peanut cultivar AU-NPL 17 has shown both the fast N protein aggregation phenotype and the wildtype aggregation phenotype (Table 5).

In the first phenotype, sample WGREC.B1 had no aggregation and low protein expression. It may be because the two amino acid mutations that happened between the uncharged protein group, the hydrophobic group, and the positively electric-charged group may cause the nucleocapsid protein to be soluble and localize around the nucleus at 2dpi and 5dpi (Figure 4). We also hypothesize that the two amino acid mutations may lead to some kind of interaction in the plant cell which may result in this different localization pattern in this sample. The peanut cultivar that this sample came from is unknown because of the random sampling of some of the 2021 peanut samples, however, the two unique amino acid mutations that were detected in this sample were not observed in the 140 TSWV-Ns from AL. This may suggest that this change is not favorable for the virus. In the second phenotype, samples WGREC5.1, GCREC45.1, WGREC.119.R2, WGREC.106.2.R3, and BARU.2.1 were similar to MT2 strain in terms of the aggregation speed. The amino acid mutation in sample WGREC5.1 was demonstrated in another

sample, and in sample GREC45.1 other three samples had the same mutations (Table 5). This may suggest that there is a selection for these mutations, especially in GCREC45.1. the lowest yield was also demonstrated in the moderately tolerant AU-NPL 17 cultivar with 3737±1230 kg/ha. This could support the hypothesis that the wildtype protein aggregation of TSWV-N is best for this virus resulting in higher disease incidence and yield loss. Other localized samples that demonstrated wildtype protein aggregation with a high yield were found in the WGREC location, which may had different thrips species that do not have high efficiency of transmitting the virus which may resulted in a high yield regardless of the protein aggregation behavior. In the third phenotype, samples BARU25.2 and WGREC.E2, show a faster protein aggregation compared to the MT2 strain. The unique amino acid mutations in these two samples could be responsible for this behavior. The amino acid mutations were unique to these two samples and were not observed in any of the 140 nucleocapsid (Table 5). This may indicate that aggregating faster than the wildtype aggregation (slower) may not be beneficial for the virus replication cycle as N is responsible for encapsulating the viral genome and it may need to move or aggregate at a slower rate to be able to recognize its genome. Also, in the fast protein aggregation phenotype, the cultivar AU-NPL 17 has a high production of 5438±438 kg/ha indicating that the fast aggregation may not be serving a positive purpose for the virus infection cycle.

The five conserved mutations do not change the localization of the selected samples as they appear as the MT2 localization control. Unique mutations in sample 4, 2, and 5 (Figure 3) did change the localization into two phenotypes: no protein aggregations and fast aggregation respectively. At this point, correlating the protein aggregation speed does not tell us about the yield loss, however, in Table 5, slow aggregation showed yield reduction in one location. This suggests that there is an interplay between mutations and the management of TSWV. The impact of these

conserved mutations still need more study. One interesting conserved amino acid mutation occurred in site 21 where aa F mutated to L which happened in the first RNA binding domain of TSWV-N protein (Figure 3). It may have impacted the RNA binding capacity and its ability to recognize its own viral RNA during the viral encapsulation and assembly which may correspond to the virus incidence increase on the resistant peanut lines in AL. Furthermore, the management of the virus and the insecticides used to manage thrips are very important and essential for this disease management. The relationship is complicated between the thrips populations, the peanut cultivars, and the TSWV genome mutations and it requires more studies to reveal this mystery.

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7. Future research

The full genome sequencing of all positive samples is the next step in this project. This will be conducted using the Illumina MiSeq instrument at North Carolina State University. This will allow us to observe the mutations in the entire genome of Tomato spotted wilt virus to be aware of any mutation in the rest of viral genome and their potential impact on the virus symptoms increase in AL.

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Table 1. The segment-specific reverse primer which was used in the cDNA synthesis of the nucleocapsid protein. The pJET forward and reverse primers that were used for PCR colony check of the nucleocapsid protein in pENTR vector and the GFP reverse and TSWV-N-F that were used to check the orientation of the nucleocapsid protein in pSITE-2NA. The specific PCR primers that were used to amplify the nucleocapsid protein.

Primer name	Primer sequence	Amplicon size	Purpose
TSWV_S_R	AGAGCAATTGTGTCAA	2,916 bp	cDNA synthesis
TSWV-N-F	CACCATGTCTAAGGTTAAGCTCACTAAGG	744 bp	Amplifying nucleocapsid protein
TSWV-N-R	AGCAAGTTCTGTGAGTTTTGCCTG		
pJET-F	CGA CTC ACTATAGGGAGAGCGGC	-	Sangar sequencing
pJET-R	AAGAACATCGATTTTCCATGGCAG		
GFP-R	GTGGCATCGCCCTCGC	-	Checking the orientation of TSWV-N gene in pSITE-2NA with TSWV-N-F primer

Table 2: Conserved amino acids mutations of the 140 nucleocapsids of Alabama compared with the NCBI reference genome of TSWV and X61799.1_MT2 strain.

NC_002051.1 (BRO1 reference genome strain)		MT2_X61799.1	
Site	**Mutation**	**Site**	**Mutation**
42	I-L	21	F-L
50	I-V	107	G-E
88	G-A	205*	N-T
187	K-R	222*	S-C
205*	N-T	230	I-V
222*	S-C		
255	A-T		

***The shared amino acid mutations of TSWV-N of AL between the MT2 strain and the BR-O1 strain of the NCBI reference genome.**

Table 3. Impact of cultivar selection on tomato spotted wilt (TSW) incidence and yield in two field trials conducted in 2022 at the Brewton Agricultural Research Unit (BARU) in Brewton, AL and the Gulf Coast Research and Extension Center (GCREC) in Fairhope, AL.

	BARU 2022				GCREC 2022			
Cultivar	**TSW Incidence[z]**		**Yield (kg/ha)**		**TSW Incidence**		**Yield (kg/ha)**	
TufRunner 297	4.4±3.6	a[y]	4958±818	a	4.2±2.2	a	2978±1146	ab
FloRun 331	13.8±4.3	a	5232±519	a	6.7±2.4	a	3355±1342	abc
FloRun T61	2.5±3.54	a	4834±1377	a	3.8±3.2	a	5682±399	fg
Georgia-06G	10.0±7.4	a	4842±800	a	5.4±3.4	a	4911±399	defg
Georgia-09B	6.9±4.3	a	4412±380	a	5.8±2.9	a	6145±525	g
Georgia-12Y	3.1±6.3	a	5694±524	a	2.9±1.6	a	4440±390	b-f
Georgia-14N	3.8±4.8	a	4290±1024	a	5.0±1.4	a	4491±414	cdef
Georgia-16HO	9.4±10.9	a	4716±1008	a	5.4±0.8	a	4011±954	a-e
Georgia-18RU	6.9±7.7	a	5110±902	a	5.0±1.4	a	5211±2112	efg
Georgia-19HP	6.9±3.2	a	5801±570	a	5.8±2.2	a	3831±597	a-e
Georgia-20VHO	1.3±1.4	a	4972±413	a	4.6±2.5	a	2726±875	a
AU-NPL 17	1.9±2.4	a	5438±438	a	4.2±2.2	a	3737±1230	abcd

[z] Percentage of the total plant population with symptoms of tomato spotted wilt (TSW).
[y] Means in each column followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test ($P \leq 0.05$) unless otherwise indicated.

Table 4. Impact of cultivar selection on tomato spotted wilt (TSW) incidence and yield in two field trials conducted in 2021 and 2022 at the Wiregrass Research and Extension Center in Headland, AL.

	2021				2022			
Cultivar	**TSW Incidence[z]**		**Yield (kg/ha)**		**TSW Incidence[z]**		**Yield (kg/ha)**	
ACI-1041	15.5±4.2	d-h[y]	5586±1234	c-h	20.4±8.0	abcd	4199±523	abc
ACI-1426	8.0±4.5	abcd	1318±297	a	--[x]		--	
ACI-212	15.5±5.5	d-h	4878±1081	bc	20.9±12.9	bcde	3374±894	defg
ACI-222	--		--		16.0±4.8	bcde	3136±884	bcde
ACI-3321	7.6±2.0	abc	4052±1007	b	15.2±4.0	abcd	3736±198	b-f
ACI-F104	10.6±5.4	a-e	5350±426	cdef				
ACI-N104	--		--		13.9±7.5	abc	3256±842	bcd
ARDG 1	9.2±4.7	a-e	5744±1190	c-h	15.8±2.9	abcd	4747±614	fg
ARDG 2	20.7±3.2	h	6629±594	fgh	24.7±11.3	cdef	3205±435	abc
ARDG 3	4.9±1.1	a	5842±750	c-h	18.5±5.1	bcde	3616±764	b-f
ARDG 4	--		--		19.3±3.6	bcde	2228±567	a
ARDG 5	--		--		23.4±3.1	cdef	3051±663	abc
AU14-34	19.3±5.8	gh	5783±331	c-h	--		--	
AU18-53	11.4±3.3	a-e	5586±906	c-h	--		--	
AU 20-41	--		--		21.2±4.2	bcde	3559±735	b-f
AU Barkley	--		--		23.1±8.4	cdef	3359±1059	bcde
AU-NPL-17	10.6±4.2	a-e	5783±187	c-h	16.8±6.7	a-e	4816±356	g
CB 1	--		--		14.9±3.9	abcd	3633±712	b-f
CB 20	--		--		17.1±6.8	a-e	3325±575	bcde
CB 7	--		--		19.8±3.9	bcde	4216±319	defg
FloRun 331	18.5±5.7	fgh	5822±294	c-h	32.1±5.7	f	3064±471	abc
FloRun T61	--		--		15.8±2.1	abcd	3931±506	b-g
Georgia - 06G	14.4±5.6	c-h	6039±851	d-h	19.3±7.1	bcde	4356±423	efg
Georgia - 07W	19.3±7.5	gh	4976±793	bcd	--		--	
Georgia - 09B	12.8±7.2	b-g	5252±907	cdef	26.6±22.2	def	3064±1061	abc
Georgia - 12Y	6.0±1.1	ab	6652±133	h	6.8±5.0	a	3308±1248	bcde
Georgia - 14N	9.8±2.3	a-e	5724±621	c-h	16.0±3.9	abcd	2975±725	ab
Georgia - 16HO	15.2±2.1	d-h	6137±970	efgh	24.2±10.5	cdef	4410±571	fg
Georgia - 18RU	13.0±8.3	b-g	4996±1437	cd	17.9±0.6	bcde	4427±538	fg
Georgia - 20VHO	10.6±3.0	a-e	5527±393	cdef	10.9±4.6	ab	4888±375	g
Tifguard	15.2±7.3	d-h	5232±657	cde	--		--	
TifNV-HG	12.8±3.6	b-g	6334±634	fgh	27.2±4.4	ef	4010±309	c-g
TifNV-High O/L	13.9±8.4	c-h	5901±232	c-h	20.1±5.2	bcde	4028±412	c-g
TufRunner 297	16.0±6.6	efgh	5862±681	c-h	21.5±2.9	cde	4286±1241	defg
UF11x41HO	13.3±4.8	c-g	5547±865	c-g	--		--	
p-value	0.001		<0.001		0.010		<0.001	

[z] Percentage of the total plant population with symptoms of tomato spotted wilt (TSW).

[y] Means in each column followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test ($P \leq 0.05$) unless otherwise indicated.

[x] -- = a specific commercial cultivar or experimental breeding line was not present.

Table 5: Comparison of the localization results to both the lines the N samples derived from and the yield losses.

N0	Sample	Cultivar	Year	Yield	Tolerance	Localization type	Is this mutation repeated in any of the 140 TSWV-Ns of AL?
1	BARU25.1	AU-NPL 17	2022	5438±438 kg/ha	Moderately tolerant	Faster protein aggregation	Only in this sample
2	WGREC5.1	AU-NPL 17	2022	4816±356 kg/ha	Moderately tolerant	Slower protein aggregation	One other sample (OR352851)
3	GCREC45.1	AU-NPL 17	2022	3737±1230 kg/ha	Moderately tolerant	Slower protein aggregation	Other three samples (OR364954, OR364955, and OR364956
4	WGREC.B1	Random sampling	2021	Unknown		No protein aggregation	Only in this sample
5	WGREC.E2					Faster protein aggregation	Only in this sample
6	WGREC119.3-R2	AU-NPL 17	2021	5783±187 kg/ha	Moderately tolerant	Slower protein aggregation	No mutations
7	WGREC106.2-R3	Georgia-09B	2021	5252±907 kg/ha	Moderately susceptible	Slower protein aggregation	No mutations
8	BARU2.1	TUFRunnerTM ‘297’	2022	4958±818 kg/ha	Moderately tolerant	Slower protein aggregation	No mutations

Table 6: Assigned accession numbers of the complete protein sequence of 140 nucleocapsids collected from Alabama with the symptoms that were observed while collecting the peanut samples. Amino acid mutations that were unique to each sample were also included.

No	Accession Number	Sample ID	Cultivars and their susceptibility	Symptoms	Amino acids mutations
1	OR335592	BARU2.1	Tuf297 Moderately tolerant	Leaf Distortion, some Ringspots, and Little Speckling	
2	OR335593	BARU3.2		Leaf Distortion, Ringspots, and Chlorosis.	
3	OR335594	BARU4.1		Leaf Distortion, Ringspots, and Little Speckling	
4	OR335595	BARU4.2			
5	OR335596	BARU5.2	Flo331 Moderately susceptible	Leaf Distortion, Ringspots, Chlorosis, and Mild Stunting	
6	OR335597	BARU6.1		Mild Distortion, Chlorosis, and Speckling	
7	OR335598	BARU6.2			
8	OR335599	BARU10.1	FloT61 Moderately tolerant	Light Leaf Distortion, Speckling, and Light Ringspots	
9	OR352836	BARU10.2			E244K
10	OR352896	BARU12.1		Chlorotic Spots, Ringspots, and Leaf Distortion	
11	OR352895	BARU12.2			
12	OR352894	BARU13.1		Ringspots and Chlorosis	V12I
13	OR352893	BARU15.1	GA-O6G Moderately tolerant	Ringspots, Chlorosis, Little Necrosis, Leaf Distortion, and Mottling	
14	OR352892	BARU15.2			
15	OR352891	BARU16.1		Ringspots, Leaf Distortion, and Chlorosis	
16	OR352889	BARU16.2			
17	OR352890	BARU17.1		Little Stunting, Chlorosis, Ringspots, and Leaf Distortion	
18	OR352888	BARU18.1	GA-16HO Moderately tolerant	Sever Leaf Distortion, Ringspots, Chlorosis, and Mild Stunting	
19	OR352887	BARU18.2			
20	OR352886	BARU20.2		Sever Stunting, Chlorosis, Ringspots, and Leaf Distortion	
21	OR352885	BARU22.1	AU-NPL 17 Moderately tolerant	Ringspots, Chlorosis, Little Necrosis, and Speckling	S49N
22	OR352837	BARU22.2			
23	OR352884	BARU25.1		Ringspots, Chlorosis, Necrosis, Leaf Distortion, and Moderate Stunting	S10N

No	Accession Number	Sample ID	Cultivars and their susceptibility	Symptoms	Amino acids mutations
24	OR352883	BARU28.1	GA-20VHO Moderately tolerant	Leaf Distortion, Ringspots, Speckling, Little Necrosis, and Mild Stunting	
25	OR352838	BARU28.2			
26	OR352882	BARU31.1	GA-14N Tolerant	Leaf Distortion, Ringspots, Speckling, Little Necrosis, and Mild Stunting	
27	OR352881	BARU31.2			
28	OR352880	BARU33.1		Ringspots, Speckling, Chlorosis, and Necrosis	
29	OR352879	BARU33.2			
30	OR352878	BARU35.1	GA-12N Tolerant	Leaf Distortion, Ringspots, Speckling, Chlorosis, Necrosis, and Sever Stunting	
31	OR352877	BARU36.1		Leaf Distortion, Ringspots, Speckling, and Mottling	
32	OR352876	BARU37.1		Chlorosis, Ringspots, Necrosis, Leaf Distortion, and Speckling	
33	OR352875	BARU37.2			
34	OR352874	BARU38.2	GA-19HP Moderately tolerant	Ringspots, Chlorosis, Leaf Distortion, and Speckling	
35	OR352839	BARU39A.2		Speckling, Chlorosis, Ringspots, and Small Leaf Distortion	
36	OR352873	BARU39B.1		Speckling, Chlorosis, and Ringspots} Mild Symptoms	
37	OR352872	BARU40.1		Ringspots, Chlorosis, Leaf Distortion, and Speckling	
38	OR352840	BARU40.2			
39	OR352871	BARU41.1	GA-18RU Moderately tolerant	Speckling, Chlorosis, Ringspots, and Leaf Distortion	
40	OR352841	BARU41.2			E24G
41	OR352842	BARU42A.1		Speckling, Necrosis, Ringspots, and Leaf Distortion	
42	OR352870	BARU42A.2			
43	OR352869	BARU42B.2		Mild Stunting, Ringspots, Leaf Distortion, Speckling, and Necrosis	A143V
44	OR352868	BARU45.1	GA-09B Moderately susceptible	Leaf Distortion, Ringspots, Chlorosis, and Speckling	
45	OR352867	BARU45.2			
46	OR349738	WGREC1.1	GA-O6G Moderately tolerant	Mild Stunting, Ring Spots, and Leaf Distortion	D185G
47	OR349739	WGREC2.1		Severe Stunting, Ring Spots, Mottling, and Leaf Distortion	

No	Accession Number	Sample ID	Cultivars and their susceptibility	Symptoms	Amino acids mutations
48	OR349740	WGREC3.2	GA-O6G Moderately tolerant	Severe Stunting, Ring Spots, Mottling, and Leaf Distortion	
49	OR349741	WGREC5.1	AU-NPL 17 Moderately tolerant	Severe Stunting, Ring Spots, Leaf Distortion, and Chlorosis	K213R
50	OR349742	WGREC5.2			
51	OR349743	WGREC7.1		Severe Stunting, Ring Spots, Chlorosis, and Speckling	
52	OR349744	WGREC7.2			
53	OR349745	WGREC8.1		Severe Stunting, Ring Spots, Chlorosis, and Speckling	
54	OR349746	WGREC8.2			
55	OR349747	WGREC9.1	GA-12Y Tolerant	Ring Spots, Chlorosis, and very mild symptoms; ring spotting only one leaflet	
56	OR349748	WGREC10.1		Leaf Distortion, Chlorosis, and Mild leaf distortion	
57	OR349749	WGREC10.2			
58	OR349750	WGREC12.1		Ring Spots, Chlorosis, and Speckling	I51V
59	OR349751	WGREC12.2			
60	OR349752	WGREC13.1	GA-O6G Moderately tolerant	Ring Spots, Mottling, and Chlorosis	
61	OR349753	WGREC13.2			
62	OR352830	WGREC14.1		Mild Stunting, Ring Spots, Mottling, Leaf Distortion, Chlorosis, and Necrosis	
63	OR352831	WGREC14.2			
64	OR352832	WGREC15.1		Severe Stunting, Ring Spots, Mottling, Chlorosis, and Necrosis	L42I
65	OR352833	WGREC15.2			
66	OR352834	WGREC16.1		Ring Spots, Leaf Distortion, Speckling, and Necrosis	M145L
67	OR352835	WGREC16.2			
68	OR352843	WGREC17.1	AU-NPL 17 Moderately tolerant	Mild Stunting, Ring Spots, Leaf Distortion, Mottling, and Chlorosis	
69	OR352844	WGREC17.2			A167D
70	OR352845	WGREC18.1		Severe Stunting, Mottling, Chlorosis, and Speckling	
71	OR352846	WGREC18.2			
72	OR352847	WGREC20.2		Mild Stunting, Ring Spots, Leaf Distortion, Mottling, and Chlorosis	
73	OR352848	WGREC21.1	GA-12Y Tolerant	Moderate Stunting, Ring Spots, Leaf Distortion, Mottling, and Speckling	
74	OR352849	WGREC21.2			
75	OR352850	WGREC22.2		Severe Stunting, Ring Spots, Leaf Distortion, and Mottling	

No	Accession Number	Sample ID	Cultivars and their susceptibility	Symptoms	Amino acids mutations
76	OR352851	WGREC23.1	GA-12Y	Ring Spots, Leaf Distortion, and Mottling	K213R, L254H, E256G
77	OR352852	WGREC23.2			D135V, K213R
78	OR352853	WGREC24.1	Tolerant	Severe Stunting, Ring Spots, Leaf Distortion, Mottling, and Necrosis	
79	OR352854	WGREC24.2			L14S, L21F, and F23Y
80	OR352855	WGREC103.2.1		**2021 strains – Symptoms were recorded.**	
81	OR352856	WGREC103.2.2			
82	OR352857	WGREC104.1.1			
83	OR352858	WGREC104.1.2			
84	OR352859	WGREC105.4.1			
85	OR352860	WGREC106.2.R3	AU-NPL 17 Moderately tolerant		
86	OR352861	WGREC119.3.R2	GA-O9B Moderately susceptible		
87	OR352862	WGREC124.1.1			
88	OR352863	WGREC124.1.2			
89	OR352864	WGREC.A4			K198R
90	OR352865	WGREC.B1			S10I, S149G
91	OR352866	WGREC.E2			K3M, V4D
92	OR364914	GCREC3.2	Tfu297 Moderately tolerant	Speckling and Leaf distortion	
93	OR364915	GCREC4.1		Speckling, Ring spots, Chlorosis, Sever Stunting, and Leaf distortion	
94	OR364916	GCREC4.2			
95	OR364917	GCREC5.2	Flo331 Moderately susceptible	Speckling, Leaf distortion, and Chlorosis	
96	OR364918	GCREC7.1		Speckling, Chlorosis, Necrosis, Leaf distortion, and Sever stunting	
97	OR364919	GCREC7.2			
98	OR364920	GCREC13.1	GA-O6G Moderately tolerant	Speckling, Chlorosis, Necrosis, Leaf distortion, and Sever stunting	
99	OR364921	GCREC13.2			G191R, S236R
100	OR364922	GCREC14.1		Ring spots, Speckling, and Chlorosis	Y174C
101	OR364923	GCREC14.2			
102	OR364924	GCREC15.1		Speckling	
103	OR364925	GCREC15.2			
104	OR364926	GCREC17.2	GA-O9B Moderately susceptible	Ring spots, Speckling, Chlorosis, Leaf distortion, and Mottling	
105	OR364927	GCREC18.1		Speckling, Ring spots, Necrosis, Chlorotic spots, and Leaf distortion	
106	OR364928	GCREC19.1		Speckling, Chlorosis, and Ring spots	
107	OR364929	GCREC19.2			

No	Accession Number	Sample ID	Cultivars and their susceptibility	Symptoms	Amino acids mutations
108	OR364930	GCREC20.1	GA-O9B Moderately susceptible	Ring spots, Chlorosis, Speckling, Necrosis, and Moderate Stunting	
109	OR364931	GCREC20.2			T7S
110	OR364932	GCREC24.1	GA-12Y Tolerant	Ring spots, Speckling, Necrosis, Chlorosis, Moderate Stunting, Leaf distortion, and Mottling	
111	OR364933	GCREC24.2			L54M
112	OR364934	GCREC25.1	GA-14N Tolerant	Ring spots, Leaf distortion, and Mottling	I86T
113	OR364935	GCREC25.2			
114	OR364936	GCREC29.1	GA-16HO Moderately tolerant	Ring spots, Leaf distortion, and Mottling	K138R
115	OR364937	GCREC29.2			
116	OR364938	GCREC30.2		Ring spots, Light Chlorosis, and Single Leaf spot	R103K, E237D
117	OR364939	GCREC31.1		Moderate Stunting, Leaf distortion, Ring spots, Necrosis, and Mottling	
118	OR364940	GCREC31.2			I86T
119	OR364941	GCREC32.1			
120	OR364942	GCREC32.2		Moderate Stunting, Leaf distortion, Ring spots, Chlorosis, and Mottling	R187M, K211N, E237D, K241Q, E244D, Q251L, K253H, T254P, E255D
121	OR364943	GCREC34.1	GA-18RU Moderately tolerant	Ring spots, Necrotic spots, and Mottling	
122	OR364944	GCREC34.2			
123	OR364945	GCREC35.1		Moderate Stunting, Ring spots, Leaf distortion, Chlorosis, Mottling, and Necrosis	
124	OR364946	GCREC35.2			
125	OR364947	GCREC39.1	GA-19HP Moderately tolerant	Leaf distortion, Moderate Stunting, Ring spots, and Chlorosis	
126	OR364948	GCREC39.2			
127	OR364949	GCREC40.1		Leaf distortion, Ring spots, Mottling, and Necrosis	
128	OR364950	GCREC40.2			
129	OR364951	GCREC41.1	GA-20VHO Moderately tolerant	Ring spots, Moderate Stunting, little Necrosis, and Chlorosis	
130	OR364952	GCREC41.2			
131	OR364953	GCREC45.1	AU-NPL 17 Moderately tolerant	Speckling, Chlorosis, Necrosis, Leaf distortion, and Ring spots	I127V for four strains.
132	OR364954	GCREC45.2			
133	OR364955	GCREC46.1		Speckling, Necrosis, Chlorosis, Mottling, Ring spots, and Leaf distortion	K183E
134	OR364956	GCREC46.2			

No	Accession Number	Sample ID	Cultivars and their susceptibility	Symptoms	Amino acids mutations
135	OR364957	GCREC47.1	AU-NPL 17 Moderately tolerant	Chlorosis, Leaf distortion, and Ring spots	
136	OR364958	GCREC47.2			E112Q
137	OR364959	GCREC49.1	FloT61 Moderately tolerant	No symptoms	
138	OR364960	GCREC49.2			
139	OR364961	GCREC52.1	Flo331 Moderately susceptible	No symptoms	
140	OR364962	GCREC52.2			K225E

*** This table is a supplementary table in the original manuscript.**

Table 7: The accession numbers, locations, and citations of the nucleocapsid protein sequences of TSWV obtained from the NCBI database which were used on the phylogenetic tree to compare Alabama sequences with.

No	Accession number	Location	Citation	No	Accession number	Location	Citation
1	DQ777177.1	North Carolina	(Tsompana et al., 2004)	40	DQ777136.1	North Carolina	(Tsompana et al., 2004)
2	DQ777176.1			41	DQ777135.1		
3	DQ777175.1			42	DQ777134.1		
4	DQ777174.1			43	DQ777133.1		
5	DQ777173.1			44	DQ777132.1		
6	DQ777172.1			45	DQ777131.1		
7	DQ777170.1			46	DQ777130.1		
8	DQ777169.1			47	DQ777129.1		
9	DQ777169.1			48	DQ777128.1		
10	DQ777167.1			49	DQ777127.1		
11	DQ777166.1			50	DQ777126.1		
12	DQ777165.1			51	DQ777125.1		
13	DQ777164.1			52	DQ777124.1		
14	DQ777163.1			53	DQ777123.1		
15	DQ777162.1			54	DQ777122.1		
16	DQ777161.1			55	DQ777121.1		
17	DQ777160.1			56	DQ777120.1		
18	DQ777159.1			57	DQ777119.1		
17	DQ777158.1			58	DQ777118.1		
20	DQ777157.1			59	DQ777115.1		
21	DQ777156.1			60	DQ777114.1		
22	DQ777155.1			61	DQ777112.1		
23	DQ777154.1			62	DQ777111.1		
24	DQ777153.1			63	DQ777110.1		
25	DQ777152.1			64	DQ777109.1		
26	DQ777151.1			65	DQ777108.1		
27	DQ777150.1			66	DQ777107.1		
28	DQ777149.1			67	DQ777106.1		
29	DQ777148.1			68	DQ777105.1		
30	DQ777147.1			69	DQ777104.1		
31	DQ777146.1			70	HQ406984.1	Gorgia	(Sundaraj et al., 2013)
32	DQ777145.1			71	HQ406983.1		
33	DQ777144.1			72	HQ406982.1		
34	DQ777143.1			73	HQ406981.1		
35	DQ777142.1			74	HQ406980.1		
36	DQ777141.1			75	HQ406979.1		
37	DQ777140.1			76	HQ406978.1		
38	DQ777139.1			77	HQ406977.1		
39	DQ777138.1			78	HQ406976.1		

No	Accession number	Location	Citation	No	Accession number	Location	Citation
79	HQ406975.1	Gorgia	(Sundaraj et al., 2013)	118	HQ406935.1	Gorgia	(Sundaraj et al., 2013)
80	HQ406974.1			119	HQ406934.1		
81	HQ406973.1			120	HQ406933.1		
82	HQ406972.1			121	HQ406932.1		
83	HQ406971.1			122	HQ406931.1		
84	HQ406970.1			123	HQ406930.1		
85	HQ406969.1			124	HQ406929.1		
86	HQ406968.1			125	HQ406928.1		
87	HQ406967.1			126	HQ406927.1		
88	HQ406966.1			127	HQ406926.1		
89	HQ406965.1			128	HQ406925.1		
90	HQ406964.1			129	HQ406924.1		
91	HQ406963.1			130	HQ406923.1		
92	HQ406962.1			131	HQ406922.1		
93	HQ406961.1			132	HQ406921.1		
94	HQ406960.1			133	HQ406920.1		
95	HQ406959.1			134	HQ406919.1		
96	HQ406958.1			135	HQ406918.1		
97	HQ406956.1			136	HQ406917.1		
98	HQ406955.1			137	HQ406916.1		
99	HQ406954.1			138	HQ406915.1		
100	HQ406953.1			139	HQ406914.1		
101	HQ406952.1			140	HQ406913.1		
102	HQ406951.1			141	HQ406912.1		
103	HQ406950.1			142	HQ406911.1		
104	HQ406949.1			143	HQ406910.1		
105	HQ406948.1			144	HQ406909.1		
106	HQ406947.1			145	HQ406908.1		
107	HQ406946.1			146	HQ406907.1		
108	HQ406945.1			147	HQ406906.1		
109	HQ406944.1			148	HQ406905.1		
110	HQ406943.1			149	HQ406904.1		
111	HQ406942.1			150	HQ406903.1		
112	HQ406941.1			151	DQ777595.1	North Carolina	(Tsompana et al., 2004)
113	HQ406940.1			152	DQ777555.1		
114	HQ406939.1			153	AY856343.1		(Abad et al., 2005)
115	HQ406938.1			154	AY856342.1		
116	HQ406937.1			155	AY856344.1		
117	HQ406936.1			156	MT2 - X61799.1	Hawaii	(Kim, 1991)

No	Accession number	Location	Citation	No	Accession number	Location	Citation
157	MW519186	Gorgia	(Lai et al., 2021)	196	MW519225	Gorgia	(Lai et al., 2021)
158	MW519187			197	MW519226		
159	MW519188			198	MW519227		
160	MW519189			199	MW519228		
161	MW519190			200	MW519229		
162	MW519191			201	MW519230		
163	MW519192			202	MW519231		
164	MW519193			203	MW519232		
165	MW519194			204	MW519233		
166	MW519195			205	MW519234		
167	MW519196			206	MW519235		
168	MW519197			207	MW519236		
169	MW519198			208	MW519237		
170	MW519199			209	MW519238		
171	MW519200			210	MW519239		
172	MW519201			211	MW519240		
173	MW519202			212	MW519241		
174	MW519203			213	MW519242		
175	MW519204			214	MW519243		
176	MW519205			215	MW519244		
177	MW519206			216	Z36882.1	Italy	(Vaira et al., 1995)
178	MW519207			217	MK468469.1	China	(Shuai, 2019)
179	MW519208			218	DQ453158.1	South Korea	(Chung, 2006)
180	MW519209			219	AB010997.1	Japan	(Tsuda et al., 1994)
181	MW519210			220	AB038341.1		(Kato and Hanada 2000)
182	MW519211						
183	MW519212						
184	MW519213						
185	MW519214						
186	MW519215						
187	MW519216						
188	MW519217						
189	MW519218						
190	MW519219						
191	MW519220						
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193	MW519222						
194	MW519223						
195	MW519224						

*** This table is a supplementary table (Table 2) in the original manuscript.**

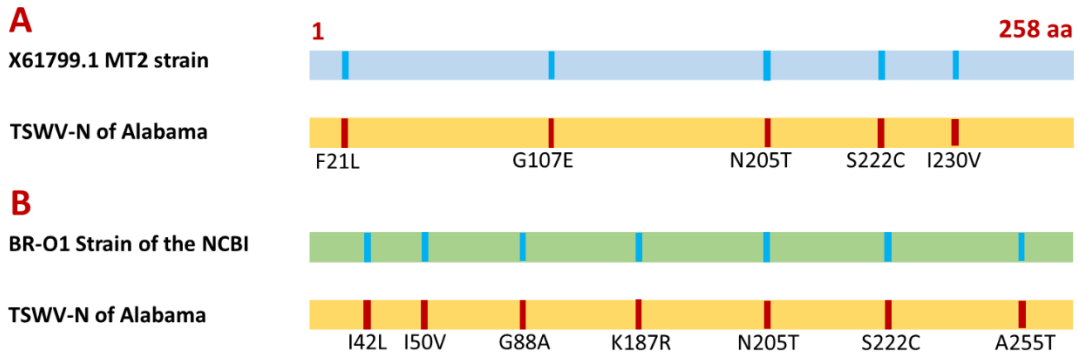

Figure 1: Conserved amino acid mutations in TSWV-N-proteins of Alabama and the sites for each mutation, A, in a comparison with the MT2 strain of Hawaii, and B, in a comparison with the reference genome of TSWV-N from the NCBI (Strain BR-O1).

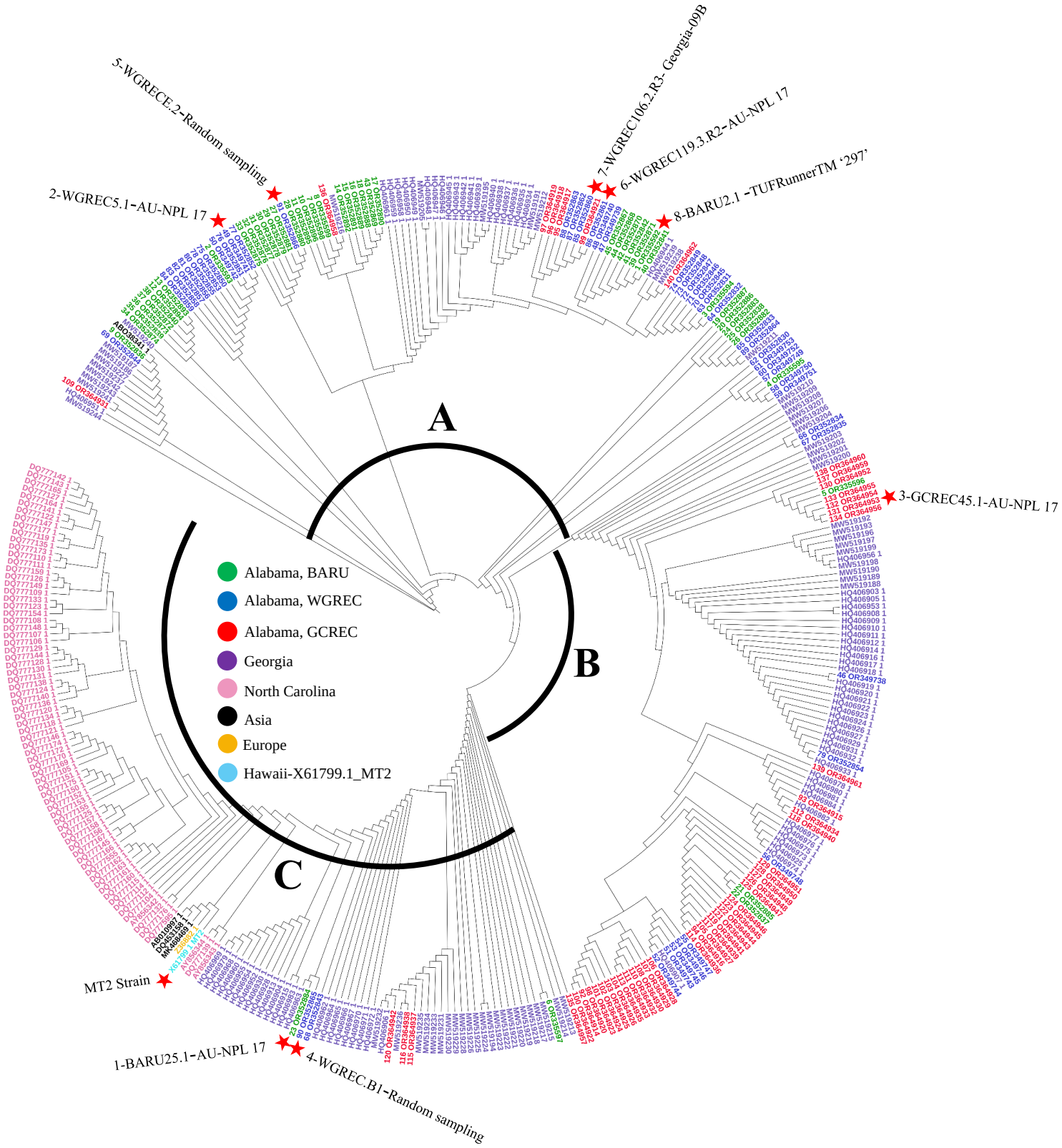

Figure 2: The maximum-likelihood phylogenetic tree constructed from the NCBI database and the nucleocapsid sequences of TSWV from Alabama. The tree is divided into one main clade (A) and two sub-clades (B and C). Samples marked with red stars represent the nine nucleocapsids that were selected for the localization in plant cells including the MT2 strain as a reference control.

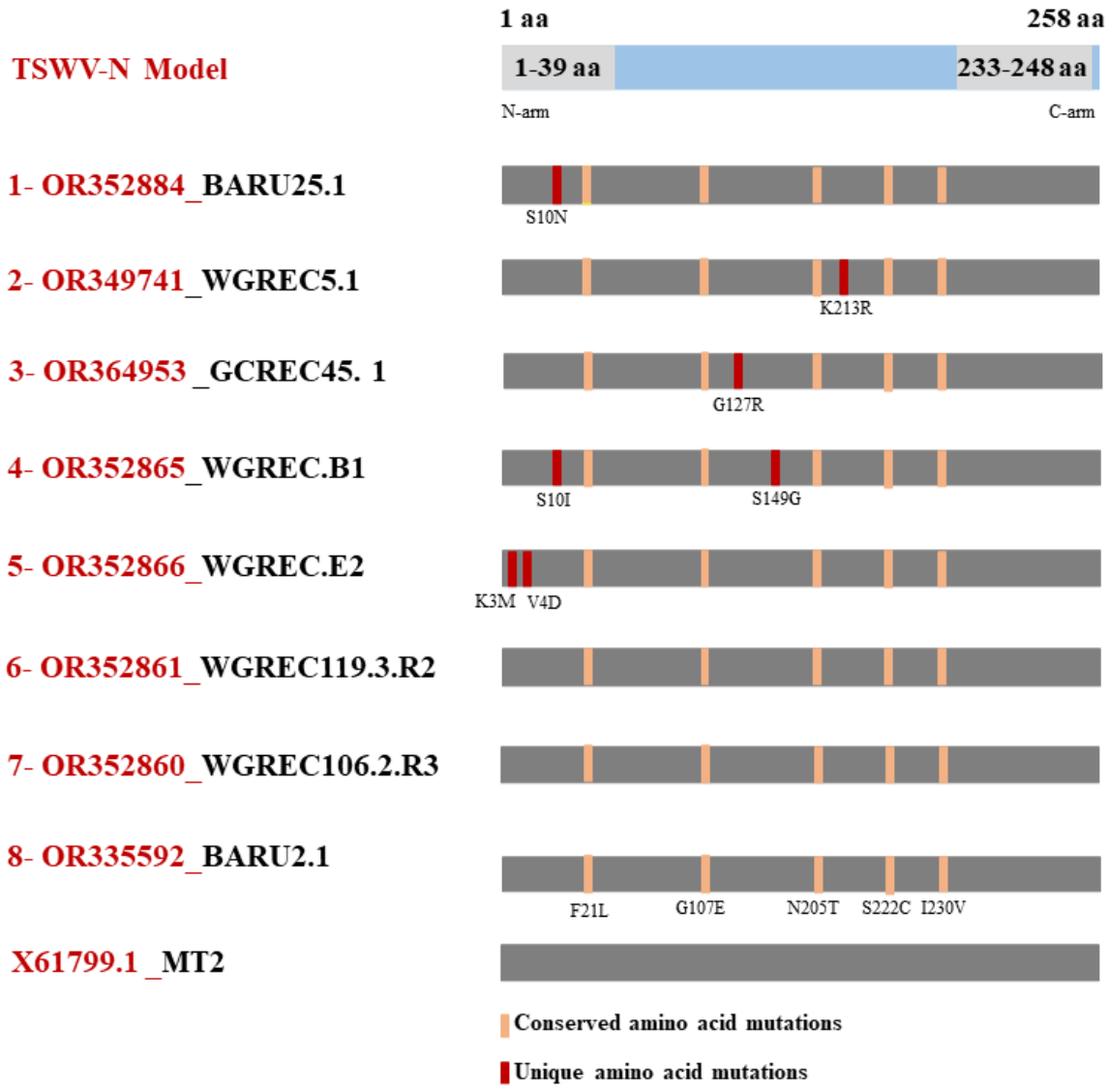

Figure 3: A map of the unique and conserved amino acid mutations that were detected in the selected nucleocapsids for localization that demonstrates the presence of these mutations in the RNA binding domains of TSWV-N protein. The model is adopted from (Uhrig et al., 1999). Rows from top to bottom are, first row is BARU25.1, the second row is WGREC5.1, the third row is GCREC45.1, the fourth row is WGREC.B1, the fifth row is WGREC.E2, the sixth row is WGREC119.3-R2, the seventh row is WGREC106.2.R3, the eighth row is BARU2.1, the ninth row is the localization reference of the MT2 strain.

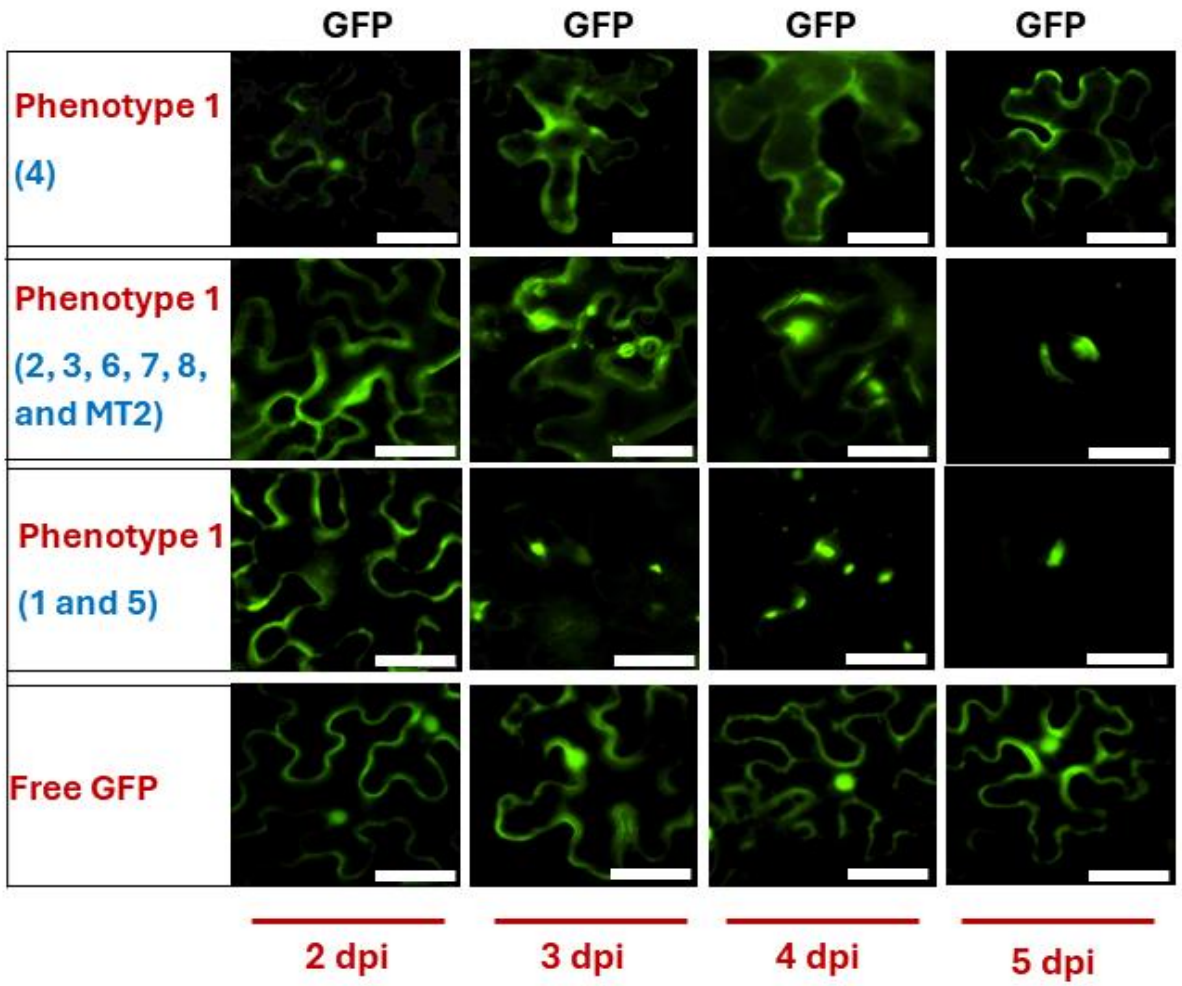

Figure 4: The localization pattern of the selected eight nucleocapsids of Tomato spotted wilt virus (TSWV) in plant cells divided into three main phenotypes. The rows from top to bottom are the first phenotype represented by sample 4: OR352865_WGREC.B1-GFP, the second phenotype represented by sample 1: OR352884_BARU25.1-GFP and 5: OR352866_WGREC.E2-GFP, and the third phenotype represented by samples 2: OR349741_WGREC5.1-GFP, 3: OR364953 _GCREC45.1-GFP, 6: OR335592_BARU2.1-GFP, and 7: and the reference control of X61799.1 _MT2-GFP. The las row is the free GFP as a localization control. Columns from right to left represent the conducted time course imaging, first is 2 bays post infiltration (dpi), second is, 3dpi, third is 4 dpi, and fourth is 5 dpi. The scale bar is 100 μm.

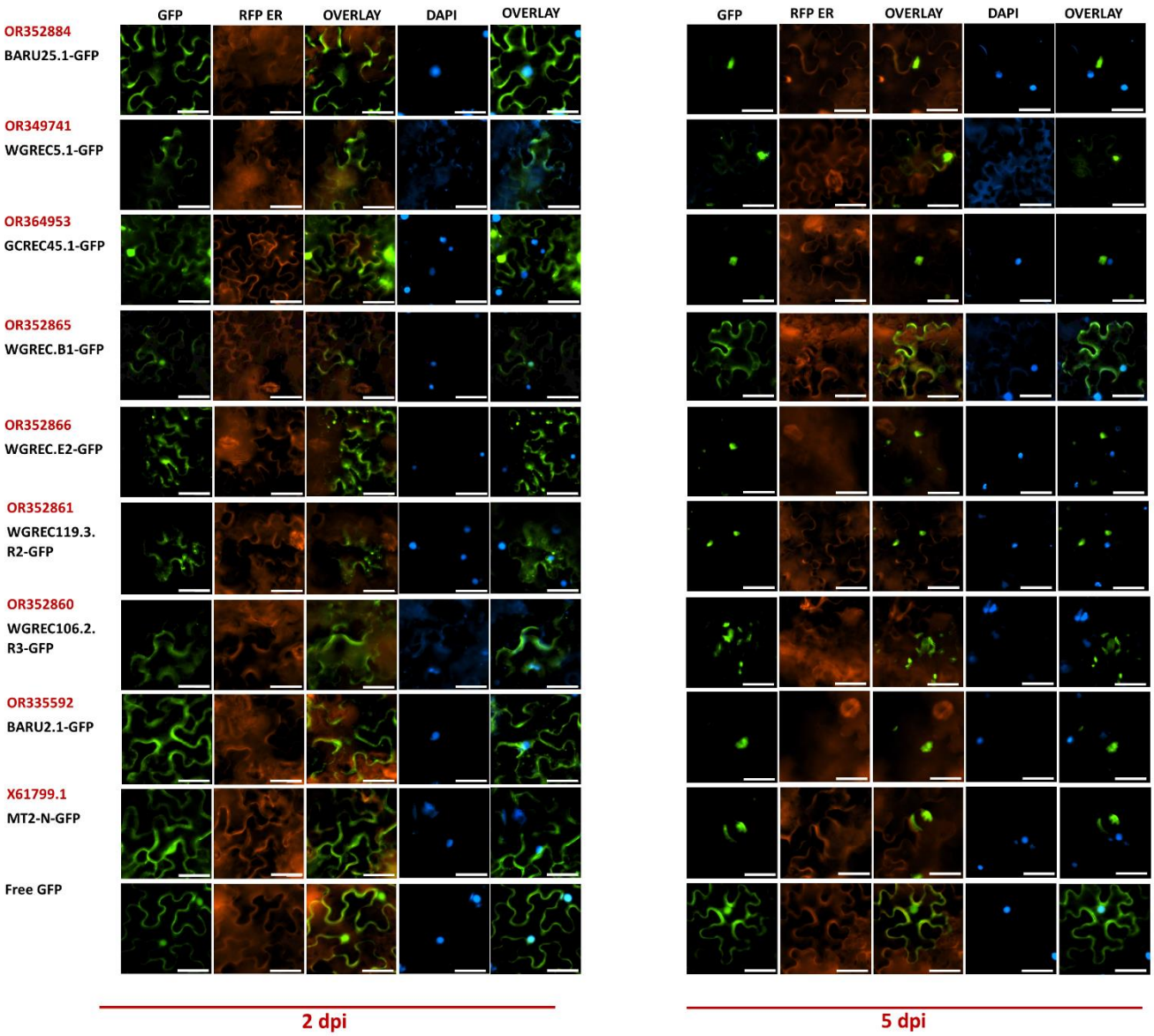

Figure 5: The localization of the nucleocapsids of Tomato spotted wilt virus (TSWV) plant cells. The left block of five images was taken at 2dpi. The right block of five images was taken at 5dpi. In the two blocks from left to right, column 1, is the GFP localization of the nucleocapsid, column 2, is the cellular marker RFP-ER, column 3, is an overlay of columns 1 and 2, column 4, is the DAPI stain to indicate the nucleus, column 5, is an overlay of columns 1 and 4. The rows from top to bottom are, the first row is BARU25.1-GFP, the second row is WGREC5.1-GFP, the third row is GCREC45.1-GFP, the fourth row is WGREC.B1-GFP, the fifth row is WGREC.E2-GFP, the sixth row is WGREC119.3-R2-GFP, the seventh row is WGREC106.2.R3-GFP, the eighth row is

BARU2.1-GFP, the ninth row is the nucleocapsid reference of MT2 strain, and the tenth row is the free GFP. Scale bar is 50 μm.

*** This figure is a supplementary figure (Figure 1) in the original manuscript.**

Chapter 3

Title: Protein Localization and Co-Localization of Soybean Vein Necrosis Virus (SVNV) in Plant Cells compared to other characterized Orthotospovirus, an abundant orthotospovirus.

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1. Abstract

Soybean (*Glycine max* (L.)) is an important crop grown in the United States due to its wide variety of uses. *Soybean vein necrosis virus* (SVNV) is an ambisense ssRNA virus in the genus Orthotospovirus and is transmitted by thrips. SVNV impacts the growth, seed quality, and oil content of the soybean. SVNV proteins have never been localized before, therefore, in this chapter, SVNV five open reading frames (ORFs) (N, NSs, NSm, G_N, and G_C) were amplified from the reference genome of Tennessee (accession: GCF_004789395.1). The five ORFs were cloned in the pENTR D-TOPO vector and after that were fused in frame to GFP or RFP C terminal or N terminal fusions. These were used for transient plant transformation and time course images of 2 days post infiltration (dpi) to 6 dpi was conducted. This revealed that N and NSs localize to the cell periphery and the nucleus. NSm causes cell death to the infiltrated cells. G_N and G_C glycoproteins localize to the cell membrane and accumulate around the nucleus. Free-GFP was used as a localization control. When NSs was present with N, the nuclear localization of NSs was more privilege than when it was localized alone. When G_N and G_C were co-localized with NSm, it caused the aggregation of G_N and G_C. These findings are essential to further our understanding of this new emergent important orthotospovirus in the United States for potential management methods.

Keywords
Soybean vein necrosis virus, orthotospoviruses, nuclear localization, co-localization, cell death, glycoproteins.

2. Introduction

Soybean (*Glycine max*) is a globally significant crop renowned for its versatility and nutritional value. Originating in East Asia, soybeans are now cultivated worldwide, particularly in regions with temperate climates (Hymowitz, 1970). This leguminous plant plays a pivotal role in agriculture serving as a primary source of protein and oil for both human consumption and livestock feed (Wilcox, and Shibles 2001). Soybeans are prized for their adaptability to diverse agricultural practices and their ability to fix nitrogen in the soil through symbiotic relationships with beneficial bacteria (Herridge et al., 2008). With a multitude of applications ranging from food products to industrial uses, soybean remains a cornerstone of modern agriculture and food security initiatives globally (Boehlje and Brorsen, 2009). Soybean is a favourable crop among many farmers in Alabama ranked as the fourth most predominant crop with a production of 14.55 million bushels (870 million lbs) in 2022 with an estimated value of 206.6 million dollars (USDA 2022).

Soybean vein necrosis virus (SVNV) is an ambisense ssRNA virus belonging to the genus Orthotospovirus in the family *Bunyviridae*. The virus was first discovered in Tennessee in 2008 (Tzanetakis et al., 2009) and is transmitted by thrips in a persistent, propagative manner. The competition of thrips species on the acquisition of SVNV was assessed by Zhou, J. 2018, with more focus on soybean thrips, *Neohydatothrips variabilis* compared to other thrips species across different seasons and geographic regions in the United States. It was concluded that *N. variabilis* is the most prevalent vector species vectoring SVNV in Northern, Midwestern, and Southern U.S. states (Chitturi et al., 2018, Keough et al., 2018, and Bloomingdale et al., 2017).

SVNV genome consists of three genomic segments encoding for five ORFs (Figure 3 - chapter 1) (Zhou et al., 2011). The S segment carries two proteins, the non-structural silencing suppressor protein (NSs) and the nucleocapsid protein (N). The NSs ORF length is 1323 bp and functions as a silencing suppressor protein, while the length of the N ORF is 834 bp and is

responsible for encasing the viral genomic RNA into an icosahedral shape (Figure 3 - chapter 1). The M segment contains two ORFs: the non-structural movement protein (NSm) with a length of 951 bp; NSm of SVNV belongs to the '30K superfamily' (Melcher, 2000) which is involved in gating the plasmodesmata (PD) in plant cells allowing the virus to move from one cell to another cell. The second ORF of the M segment includes the combined sequence for the glycoproteins (G_N and G_C) total length is 3588 bp. The two glycoproteins are translated as one polyprotein and then cleaved apart into two proteins G_N and G_C. They are membrane-bound proteins and are located on the outer shape of the virion (Figure 3 - chapter 1). The glycoproteins are necessary for transmission by their insect vector *Neohydatothrips variabilis*. The last genomic segment is the largest genetic segment for SVNV. It is called the L segment, and it only contains a single ORF including an RNA-dependent RNA-polymerase (RdRp) with a length of 8796 bp (Zhou et al., 2011).

In Alabama, SVNV was first reported in 2012 (Conner et al., 2013). Symptoms of the virus include brown necrotic tissues along the major veins of the upper and lower leaf surface which results in a scorched appearance of damaged leaves (Conner et al., 2013). SVNV impacts the growth, seed quality, and oil content of soybean (Anderson et al., 2017). A survey conducted in Alabama from 2013 to 2016 reported an increase in the average incidence of SVNV on soybean from 31.8% to 82.6% (Chitturi et al., 2018).

SVNV has never been localized in plant cells, therefore, the protein localization behavior of this important emergent orthotospovirus remains unknown. In a study by Oliver and Whitfield in 2016 they generated a phylogenetic tree for all known orthtospoviruss sequenced based on the N protein. This tree showed Tomato spotted wilt virus (TSWV) was phylogenetically closely related to (Impatiens necrotic spot virus) INSV, however both (Capsicum chlorosis virus) CaCV and (Iris yellow spot virus) IYSV were in a separate clade with other orthotospoviruses. Most interestingly, SVNV was in a separate clade with only Bean

necrotic mosaic virus (BeNMV) (Oliver and Whitfield 2016). This agrees with another larger study of *Orthotospoviridae*, *Fimoviridae*, *Phenuiviridae*, *Nairoviridae*, *Arenaviridae*, *Phasmaviridae*, and *Hantaviridae* based on the RdRp polymerase (Kormelink et al., 2021). Moreover, the same phylogenetic association between SVNV and BeNMV based on all coding regions of SVNV have indicated that SVNV was most closely related to BeNMV (Zhou and Tzanetakis 2018). This may indicate differences in SVNV protein homology and expected protein localization of SVNV in plant cells compared to other characterized orthotospoviruses. The placement of SVNV with BeNMV suggest that SVNV is part of a novel evolutionary lineage of Fabaceae-infecting orthotospoviruses (de Oliveira et al., 2012 and Zhou and Tzanetakis 2019). This phylogeny association may indicate that the two othrotospoviruses, SVNV and BeNMV, may have evolved together and are distinctly different from other members.

In this study, we were interested in understanding the relationship of SVNV with other orthotospoviruses and to determine why SVNV is found distinct in the phylogeny studies that were conducted previously. To answer this question, we localized and co-localized SVNV proteins in plant cells and documented differences in SVNV protein localization compared to other characterized species. These findings will further our understanding of this important virus and represent the first steps for potential management methods to control a threat to soybean production in Alabama and in the southern states planting soybeans.

3. Materials and Methods

3.1. The source of SVNV strain and five ORFs amplification

To test the protein localization and co-localization of SVNV, the reference genome of the SVNV strain of Tennessee (accession: GCF_004789395.1) was used to clone the five ORFs of SVNV's (N, NSs, NSm, G_N, and G_C). 1 μl of the gene template (50 ng) was combined with specific primers contain CACC before the start codon to amplify each gene (Table 1). The

amplification was carried out using the Thermocycler PCR and Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific, Waltham MA). Positive amplifications showing the expected band size for each gene (Table 1) were purified and recombined into the pENTR vector using the pENTR D-TOPO kit following manufacturer's protocols (Invitrogen^{TM}). For the glycoproteins (G_N and G_C), reverse primers (G_N-S-R and G_C-S-R) (Table 1) were designed to trim the transmembrane domain (TM). The constructs for each gene were sent for Sangar sequencing to confirm the clones.

3.2. Design of the pSITE constructs for visualization of SVNV ORFs and *Agrobacterium* transformation

To visualize the ORFs of SVNV and after the confirmation of all SVNV ORFs sequences, each gene was recombined in pSITE-2CA (C terminal of GFP), pSITE-2NA (N terminal of GFP), pSITE-4CA (C terminal of RFP), and pSITE-4NA (N terminal of RFP) as destination vectors (Chakrabarty et al., 2007; Martin et al., 2009) as fusions to green fluorescent protein (GFP) using LR Clonase following the manufacturers recommendations (Invitrogen^{TM}). The recombinant pSITE vectors were transformed into *E. coli* competent cells and colonies were confirmed after the fusions in the four orientations. For constructs recombined in the C terminal of GFP and RFP, forward primers GFP_F and RFP-F were used with the reverse primer of each gene. For constructs recombined in the N terminal of GFP and RFP, reverse primers GFP_R and RFP-R were used with the forward primer of each gene (Table 1). After the orientation of SVNV ORFs was confirmed, they were transformed into *Agrobacterium tumefaciens* competent cells by adding 10 µl of the mini-prepped plasmid of each construct to 50 µl of *A. tumefaciens* competent cells. The solution was thoroughly mixed by gently and submerged in liquid nitrogen. The tubes were incubated in a head block set to 37°C for five minutes to thaw. LB broth media (600 µl) was added to the solution and incubated for 2-3 hours at 28°C with shaking. LB agar plates containing both Rifamycins and

Spectinomycin antibiotics were used to plate the transformation solution, and the plates were allowed to incubate for 4 days at 28°C. Following the 4-day incubation period, a single colony was selected and streaked onto new plates. After an additional two days, Agrobacterium growth was used to make a glycerol stock and that was stored in -80.

3.3. Plant Growth and Maintenance

Wildtype and transgenic *N. benthamiana* plants expressing red fluorescent protein fused to the endoplasmic reticulum (RFP-ER) and green fluorescent protein fused to the endoplasmic reticulum (16_C-ER) (Martin et al., 2009) were cultivated in a growth chamber maintained at 25°C with a light intensity of 300 uM for 14 hours of daylight, followed by a 10-hour dark period. At two weeks of age, the seedling plants were transferred to individual containers. Subsequently, the plants were allowed to grow for approximately one to two more weeks until their leaves reached the size of greater than 24.26 mm, which is the best optimal age for plant infiltration for microscopy.

3.4. Infiltration of plants with constructs for protein visualization.

After growth, plants were infiltrated using the protocol used in (Goodin et al., 2002) with some modifications. The bacteria were dissolved into 0.1 M MES and 0.1M $MgCl_2$ solution and the OD_{600} was measured between 0.6 to 0.7 for all samples. The addition of 2.0 M Acetosyringone was then added to the adjusted bacterial solution at a ratio of 1.5µl per 1 ml bacterial solution. The solution was incubated at room temperature for two-three hours before the infiltration. Approximately, 1 ml bacterial solution per leaf was infiltrated in healthy *N. benthamiana* plants (Benth).

3.5. Microscopy of samples

Microscopy of plant cells was conducted from two days post infiltration (dpi) up to five dpi as a time course, using the Eclipse Ts2R (Nikon) epifluorescence microscope. A 10% solution of DAPI stain was utilized to stain the plant cell nucleus, with infiltration occurring

one hour prior to imaging. Images were captured using Nikon Elements software v. 5.21.02 (Build 1487). Each construct was subjected to three replicates, with three different cells imaged in each replicate. Figures were compiled using Microsoft PowerPoint v. 2305 (Build 16501.20210 Click-to-Run).

4. Results

4.1. Localization of SVNV ORFs in *N. benthamiana*

To conduct this experiment, RFP-ER *N. benthamiana* plants were used to image the individual SVNV proteins. GFP-N protein (C terminal of GFP) localized to the cell periphery and in the cell nucleus (Figure 2). RFP-N protein (C terminal of RFP) localized to the cell periphery but not to the cell nucleus (Data not shown). N-GFP protein (N terminal of GFP) localized to the cell periphery but not to the cell nucleus (Data not shown). N-RFP protein (N terminal of RFP) localized to the cell periphery and to the cell nucleus (Data not shown). GFP-NSs and RFP-NSs protein (C terminal of GFP and RFP) localized to the cell periphery and aggregates to the cytoplasm (Data not shown). NSs-GFP and NSs-RFP (N terminal of GFP and RFP) localized to the cell periphery and in/around the cell nucleus (Data shown for NSs-GFP in Figure 2). GFP-NSm and RFP-NSm protein (C terminal of GFP and RFP) and NSm-GFP and NSm-RFP (N terminal of GFP and RFP) did not show protein localization. After 5dpi, cell death symptoms started to develop on the infiltrated cells under the microscope (Figure 3). The cell death was increasing after one week of the infiltration as the infiltrated tissues started showing yellowing symptoms (Figure 3). Due to the presence of the single peptide in both G_N and G_C (Figure 1), they were only fused with GFP and RFP N terminal. For G_N-GFP and G_N-S-GFP it localized to the cell membrane and in the cell nucleus (Figure 2). For G_C-GFP and G_C-S-GFP it localized to the cell membrane and around the cell nucleus (Figure 2). The Free-GFP was used as a localization control where it always localized to the cell periphery and to the nucleus.

4.2. Co-Localization of SVNV ORFs in *N. benthamiana*

To conduct this experiment, wild-type of *N. benthamiana* plants were used to image the co-localized SVNV proteins. The purpose of this step is to test the impact of the presence of one protein with other SVNV proteins. All co-localizations were done for SVNV proteins using the N terminal fusion of GFP and RFP. When NSs-GFP was co-localized with N-RFP, both proteins showed similar localization behaviour where both co-localized to the cell periphery and to the cell nucleus (Figure 4). The nuclear localization of both proteins when they were together was more privilege than when each of them was localized individually (Figure 2). When N-RFP was co-localized with G_N-GFP and G_C-GFP, the same protein expression was observed for both proteins, however, there was not a clear difference between the co-localization of both proteins and when they were localized individually. (Figure 2 and 4).

When NSs-RFP was co-localized with G_N-GFP and G_C-GFP, there was no change between the co-localization of both proteins in the presence of NSs-RFP and the individual localization for each of them (Figure 2 and 4). Finally, the two glycoproteins were co-expressed together in their soluble form where G_C-S-GFP was co-localized with G_N-S-RFP. This is because the protein expression of the full-length form of both G_N and G_C was very weak. Both soluble version glycoproteins co-localized to the cell membrane/periphery. Additionally, G_C-S-GFP localized to the cell nucleus (Figure 4).

NSm protein was co-infiltrated with all SVNV proteins (N, NSs, G_N, G_N-S, G_C, and G_C-S), However, NSm protein did not show fluorescence and therefore it was not observed (Data not shown). When NSm protein was localized alone, the cell death symptoms appeared after 5dpi (Figure 3), interestingly, when NSm-RFP was co-infiltrated with G_N-GFP and G_C-GFP, there was an over-protein expression and aggregations at 2 dpi for both G_N-GFP and G_C-GFP proteins (Figure 5). This glycoprotein over-expression and aggregation was not recorded

before either when they were localized individually (Figure 2) or when they were co-localized with N- RFP or with NSs-RFP (Figure 4).

5. Discussion

The protein localization of plant viruses accumulates in building an understanding of how the virus proteins function in their plant host cells. Therefore, it helps us in understanding the infection cycle of plant viruses which is essential to manage any virus disease. Since SVNV proteins have never been localized before, we synthesized the SVNV Tennessee strain, and the ORFs were fused in pSITE-2NA, pSITE-2CA, pSITE-4NA, and pSITE-4CA. The GFP-N and N-RFP showed nuclear protein localization which has never been shown before in any localized orthotospovirus (Dietzgen et al., 2012, Tripathi et al., 2015, Widana Gamage and Dietzgen 2017, and Martin et al., 2024 - submitted). The N protein of the four characterized orthotospoviruses was localized to the cell periphery and aggregates in the cytoplasm. Additionally, in IYSV and CaCV the N protein was localized around the nucleus (Tripathi et al., 2015 and Widana Gamage and Dietzgen 2017). To support the nuclear localization of SVNV nucleocapsid protein, the N protein was subjected to the Nuclear Localization Singal Mapper NLS (https://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). SVNV nucleocapsid protein showed three bipartite NLS signals one of them with a score of 5 (Kosugi et al., 2009a, Kosugi et al., 2009b, Kosugi et al., 2008). These nuclear domains may be implicated in the nuclear localization of SVNV-N protein. Another support for the nuclear localization of the nucleocapsid protein of SVNV is when NSs-GFP was co-localized with N-RFP, the nuclear localization of N was very evident and more privileged to localize in the nucleus (Figure 4) than when it was alone (Figure 2) but in both cases, the nuclear locaization was recoreded. The nuclear loclaization of SVNV-N protein may play an important role for the virus replication or infection cycle. Moreover, this nuclear localization might be necessary for the SVNV virion assembly, and since SVNV is the only orthotospovirus that have shown

that so far, there are suspicions that SVNV could have a different infection route and a different virion assmebly mechanism in plants. Furthermore, SVNV has been recently discovered and it may still adjusting to the plant systems versus the insect system that the virus seems to vafore more than the plant system.

The RNA silencing suppressor activity of the bunyavirus NSs has been shown in orthotospoviruses, while its role in animal-infecting bunyaviruses remains a subject of debate (Hedil and Kormelink, 2016). The NSs proteins of the four localized orthotospoviruses (INSV, IYSV, CaCV, and TSWV) were localized to the cell periphery, additionally, for TSWV it localizes around the nucleus as well (Martin et al., 2024 - submitted). The first documentation of the nuclear loclization of the NSs protein among the characterized orthotospoviruses was in CaCV (Widana Gamage and Dietzgen 2017). Our SVNV-NSs protein in agreement with CaCV-NSs protein, loclaized to the cell periphery and to the nucleus of the cell in GFP-N terminus. The NLS Mapper detected one nuclear domain in SVNV-NSs protein which may suggest that this NLS domain play a role in the nuclear localization. The TSWV-NSs protein has NLS domain, it did not localize in the nucleus but to the cytoplasm (Kormelink et al., 1991; Kikkert et al., 1997), However, the INSV GFP-NSs protein that does not have predicted NLS domains did not loclaize to the cell nucleus (Dietzgen et al., 2012).

The NSm protein of the four orthotospoviruses (INSV, IYSV, CaCV, and TSWV) showed localization in the plant plasmodesmata (PD) which helps the virus to move between plant cells. The NSm protein of IYSV was also localized around the cell nucleus (Tripathi et al., 2015). Not following all four viruses, SVNV-NSm proteins (in both GFP and RFP terminals) did not localize normally and caused cell death on infiltrated leaves. SVNV-NSm protein could indicate the reason this virus causes symptoms of necrosis in in soybean as it was shown to cause cell death in *N. benthamiana* infiltrated leaves (Figure 3). The two phylogenetic trees constructed in Oliver and Whitfield 2016 and Kormelink et al., 2021 have confirmed that

SVNV is phylogenetically closest to BeNMV and distinctly different from other orthotospovirus members. Interestingly, in the BeNMV protein localization study (Leastro et al., 2017), BeNMV-NSm protein was shown to be inefficient in systemically transporting Alfa-Alfa mosaic virus (AMV) in infiltrated plants as the other compared orthotospoviruses in their study (TSWV and Tomato chlorotic spot virus (TCSV)). Since SVNV was inefficient in localizing to the PD, this might indicate the phylogenitic relationship between SVNV and BeNMV and that the two viruses' NSm protein is different from other orthtospoviruses.

The SVNV-NSm protein belongs to the 30K movement protein superfamily, distinguished by the presence of the highly conserved LxDx40G motif (Zhou and Tzanetakis 2019) implicated in cell-to-cell movement (Mushegian and Koonin 1993 and Silva et al., 2001). However, a notable substitution was observed at the beginning of this motif, where the Leu residue was replaced by an Ile (Zhou and Tzanetakis 2019). Unlike some orthotospovirus orthologs such as tomato spotted virus (TSWV) and groundnut bud necrosis virus (GBNV), the SVNV-NSm protein lacks the "P/D-L-X motif" and phospholipase A2 catalytic sites (Silva et al., 2001 and Kormelink 1992). This discrepancy may explain why localized SVNV-NSm protein induces cell death and does not localize to the plasmodesmata like other localized orthotospoviruses.

SVNV does not systemically move on its own in soybeans, and it was shown to be associated in soybean with Bean pod mottle virus (BPMV) (Zhou and Tzanetakis 2020). BPMV is implicated in the SVNV systemic movement in soybeans, however, SVNV did not move systematically in a co-infection of SMV (Zhou and Tzanetakis 2020). The mechanism in which BPMV acts as a helper virus for SVNV remains unknown. The dysfunctionality of SVNV-NSm protein may also be responsible for the inability of SVNV to move systematically on its own in soybeans. Additionally, SVNV is the only orthotospovirus that showed seed transmission and BPMV might be responsible for this phenomenon a well. In order to

understand the reason of the unexpected localization of SVNV-NSm protein and its impact on the virus replication in real infection, further studies focusing on the tubule domain formation of SVNV-NSm are warranted.

The glycoprotein G_N localized in the cell membrane and to the membrane of the nucleus in INSV, IYSV, and TSWV but for CaCV it localized in the cell nucleus. For SVNV, G_N was localized to the cell membrane and around the cell nucleus. This association with the nuclear membrane might be due to the presence of the TM domain which embedding G_N the nuclear membrane. The glycoprotein G_C is localized to the cell membrane in TSWV only and to the cell membrane and nucleus in INSV, IYSV, and CaCV. For SVNV, G_C was localized to the cell membrane and around the cell nucleus which might be embedded in the nuclear membrane due to the presence of the TM domain. Based on these protein localization results, SVNV has shown distinct localization characteristics comparing to other discussed orthotospoviruses, especially, for its nucleocapsid that localized to the cell nucleus contradicting all characterized orthotospoviruses. This may indicate the unique phylogeny cladding of SVNV from other orthotospoviruses.

The protein interaction of many important orthotospoviruses including TSWV, INSV, TCSV, BeNMV, IYSV, CaCV, Chrysanthesmum stem necrotic virus (CSNV), and Groundnut bud necrotic virus (GBNV) was conducted. A schematic map of protein-protein homologou and heterologous interactions was discussed in Widana Gamage and Dietzgen 2017. The N and NSm protein of all viruses, except for GBNV, have shown homologous (self) interaction. NSs protein was only shown to self-interact in CaCV. In TSWV, N protein interacted with G_N, G_C, and NSm. Out of these mapped eight orthotospoviruses, six viruses including INSV, TCSV, BeNMV, IYSV, and CSNV; their N protein interacted with NSm protein which highlight that the interaction between these two proteins is very important and necessary for viral replication and virion assembly cycle. Through conducting the co-loclaization experiments of SVNV

proteins, it has been shown that all SVNV proteins co-localized in the plant cells, suggesting that protein-protein interactions may be observed in the next studies.

Bunyavirus members have the ability to use different receptors to enter the cell, including DC-SIGN, which is also used by other viruses such HIV or HCV, L-SIGN or glycosaminoglycans such heparan sulphate (Albornoz et al., 2016). The interaction of the cellular receptor with the surface glycoprotein of the bunyavirus initiates endocytosis and membrane fusion in a clathrin-dependent manner. This process results in the liberation of the bunyavirus ribonucleoproteins (RNPs) into the cellular cytoplasm, where transcription and replication occur (Ferron et al., 2017). The membrane snatching in bunyaviruses is well studied and well known in animal/insect cells, however, it still unknown in the plant system. Based on the protein over expression and aggregation of the G_N-GFP and G_C-GFP glycoproteins when they were co-infiltrated with NSm-RFP (Figure 5), a predicted virion assembly of the glycoproteins bounded to the cell membrane was developed. We predict that in real viral infection cycle, the glycoproteins after maturation and secretion from Golgi, they bind to the cell membrane. In the presence of NSm protein, it facilitates the movement of the icosahedral shape of SVNV through the cell membrane bounded to the glycoproteins to form the spherical shape around the icosahedral shape. This was seen as aggregations of G_N-GFP and G_C-GFP glycoproteins (Figure 5). This may result in forming a mature virion and due to the presence of the cell wall which represent a barrier against the virions to exit the cells, the formed mature virions may accumulate between the cell membrane and the cell wall. Thrips may be attracted to the accumulated virion pockets and chew in the cell wall during their feeding resulting in the virus acquisition. Some literature that might support this hypothesis is what was reported that the NSm proteins of the bunyavirus family are membrane-bound proteins (Ferron et al., 2017), and that NSm has been linked to viral assembly in bunyviruses (Shi et al., 2006).

6. Conclusion

Soybean vein necrosis virus has never been localized and plant cells and the information regarding this was unknown at the onset of this study. We showed that SVNV-N and NSs protein localized to the cell periphery and to the cell nucleus. The NSm protein caused cell death and might be responsible for the virus necrotic symptoms. The glycoproteins G_N and G_C localized to the cell and nucleus membrane. These findings will further our understanding of SVNV and present important steps towards managing this emergent orthotospovirus.

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Table 1. The specific primers that were used in amplifying SVNV genes, The specific reverse primers of SVNV-G_N and G_C to produce a soluble version of the glycoproteins (G_N and G_C), The M13 primers that were used for PCR colony check of SVNV genes in pENTR vector, and the GFP/RFP forward and reverse primers that were used with the appropriate SVNV gene-specific primer (Forward or reverse) to check the orientation of the constructs in pSITE-2NA, pSITE-2CA, pSITE-4NA, and pSITE-4CA.

Primer name	Primer sequence	Amplicon size	Purpose
SVNV-N-F	CACCATGCCACAAACAGCAGGACCAAG CAATGC	831 bp	Amplifying SVNV-N
SVNV-N-R	AACAGAAAACTCCTTTGATTTCTTGCC		
SVNV-NSs-F	CACCATGCAGAGCTTGATTACTCTTGGC AG	1320 bp	Amplifying SVNV-NSs
SVNV-NSs-R	TTCAAAATAGGTTGCAAGATCTCC		
SVNV-NSm-F	CACCATGTCAAACTTTGTAGCAAAGGCT ATTGAAATGGGACAGG	948 bp	Amplifying SVNV-NSm
SVNV-NSm-R	AATCGGTTTCATTTCCACCAAAGAAGGG		
SVNV-G_N-F	CACCATGAGGATTAAGGATTTCTTTGTT ATAATGC	1354 bp	Amplifying SVNV-G_N Full-length
SVNV-G_N-R	CTGAACTTAGAACTTGCTTTCTTTGG		
SVNV-G_N-S-R	GCCCTTGTATTTTACCAAACAA	935 bp	Amplifying a soluble version of SVNV-G_N
SVNV-G_C-F	CACCATGCTGGTTGTGAATACCAAAATA AGCACAG	2223 bp	Amplifying SVNV-G_C Full-length
SVNV-G_C-R	AACCCTAATTATTAAATCATCAAAAGG		
SVNV-G_C-S-R	AAAGAATGACGCAACCCAGTTA	1934 bp	Amplifying a soluble version of SVNV-G_C

M13-F	GTA AAA CGA CGG CCA GTG	Depending on the sequenced SVNV gene used to sequence in pENTR vector.	Colony check and Sangar sequencing
M13-R	CAGGAAACAGCTATGAC		
GFP-F	ATCACTCTCGGCATGGACG	Depending on the SVNV gene used to check its orientation in pSITE vector.	Check the orientations of SVNV constructs in the destination vector of pSITE
GFP-R	GTGGCATCGCCCTCGC		
RFP-F	CATCGTGGAACAGTACGAG		
RFP-R	CCTCGCCCTCGATCTC		

Table 2: the predicted Nuclear Localization Signals (NLS) of Soybean vein necrosis virus proteins.

SVNV protein	Presence NLS?	Location	NLS type	Domain score
N	Yes	aa 51 aa 60 aa 236	Bipartite	4 4.3 5
NSs	Yes	aa 333	Bipartite	4.8
NSm	Yes	aa 97	Bipartite	4.6
G_N	Yes	aa 104 aa 416	Bipartite	4.5 4.7
G_N-S		aa 104	Bipartite	4.5
G_C	Yes	aa 35 aa 58 aa 58 aa 483 aa 687	Bipartite	3.3 3.8 3.2 3 3.6
G_C-S	Yes	aa 35 aa 58 aa 58 aa 483	Bipartite	3.3 3.8 3.2 3

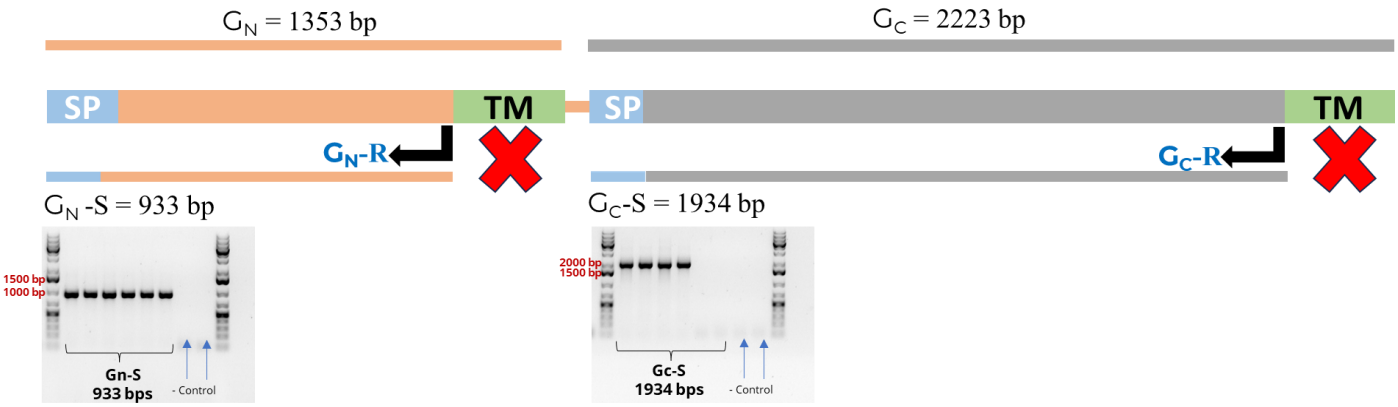

Figure 1: A map of the SVNV glycoproteins G_N and G_C using PredictPrtoein online tool (https://predictprotein.org/).

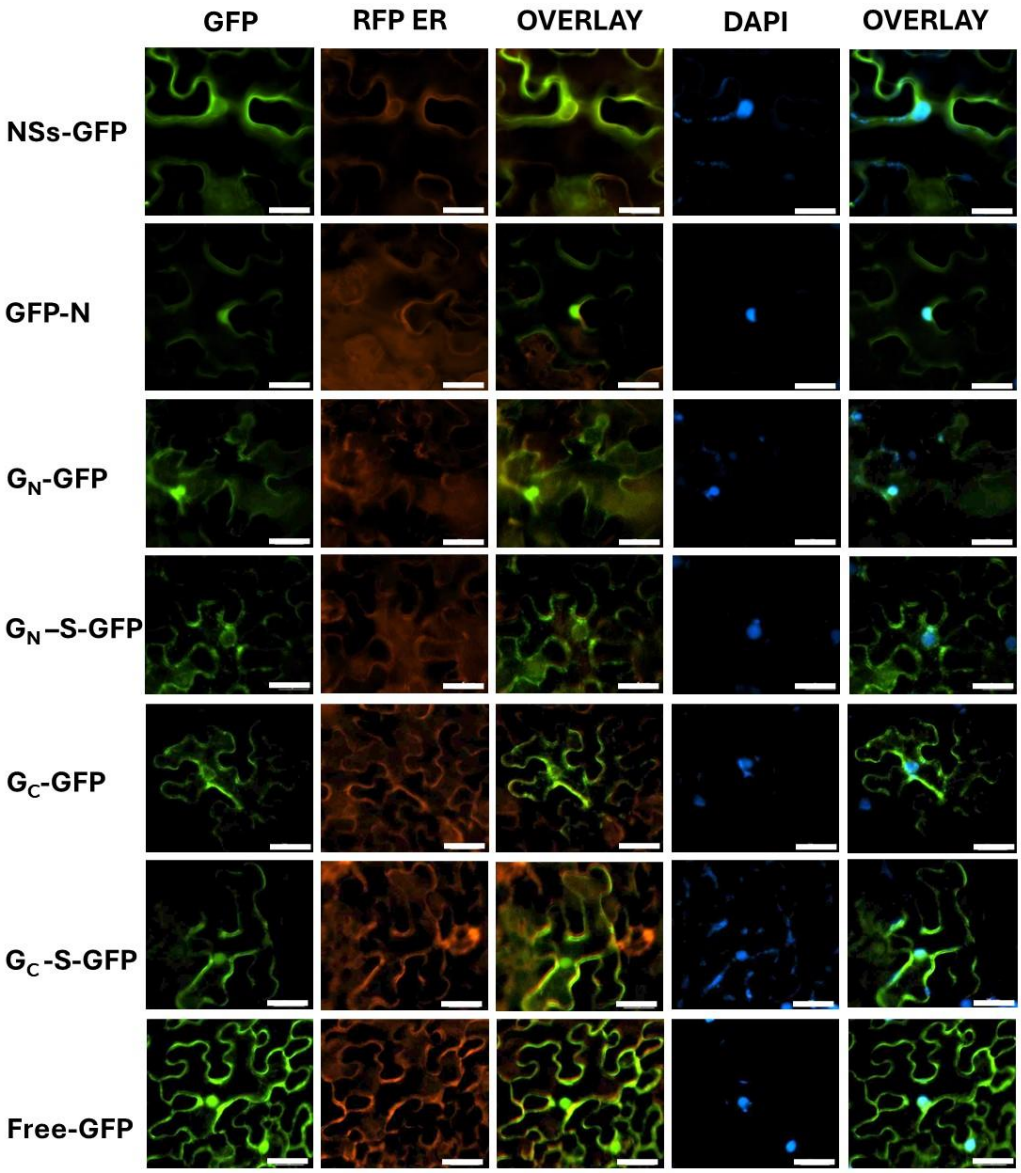

Figure 2: The localization of the soybean vein necrosis virus proteins (SVNV) in plant cells. from left to right, column 1, is the GFP localization for each of SVNV proteins, column 2, is the cellular marker RFP-ER, column 3, is an overlay of columns 1 and 2, column 4, is the DAPI stain to indicate the nucleus, column 5, is an overlay of columns 1 and 4. The rows from top to bottom are, the first row is NSs-GFP, the second row is GFP-N, the third row is G_N-GFP, the fourth row is G_N-S-GFP, the fifth row is G_C-GFP, the sixth row is G_C-S-GFP, and the last row is the localization control of free-GFP. Scale bar is 50 μm.

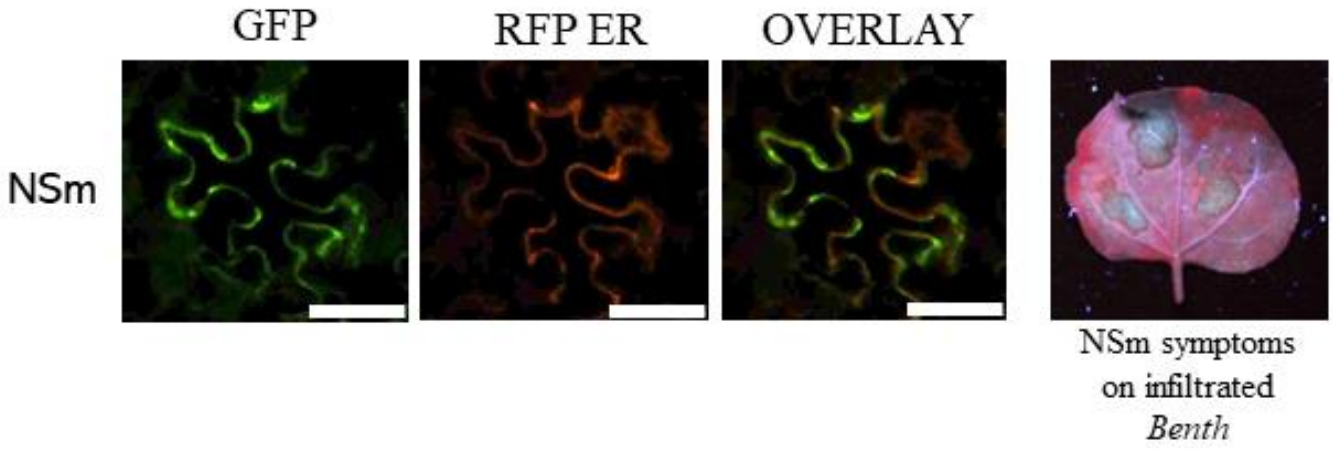

Figure 3: The localization of the soybean vein necrosis virus proteins movement protein (SVNV-NSm) in plant cells showing the cell death observed during imaging after 5 days post infiltration. From left to right, column 1, is the GFP localization for each of SVNV-NSm protein, column 2, is the cellular marker RFP-ER, and column 3, is an overlay of columns 1 and 2. The scale bar is 50 μm.

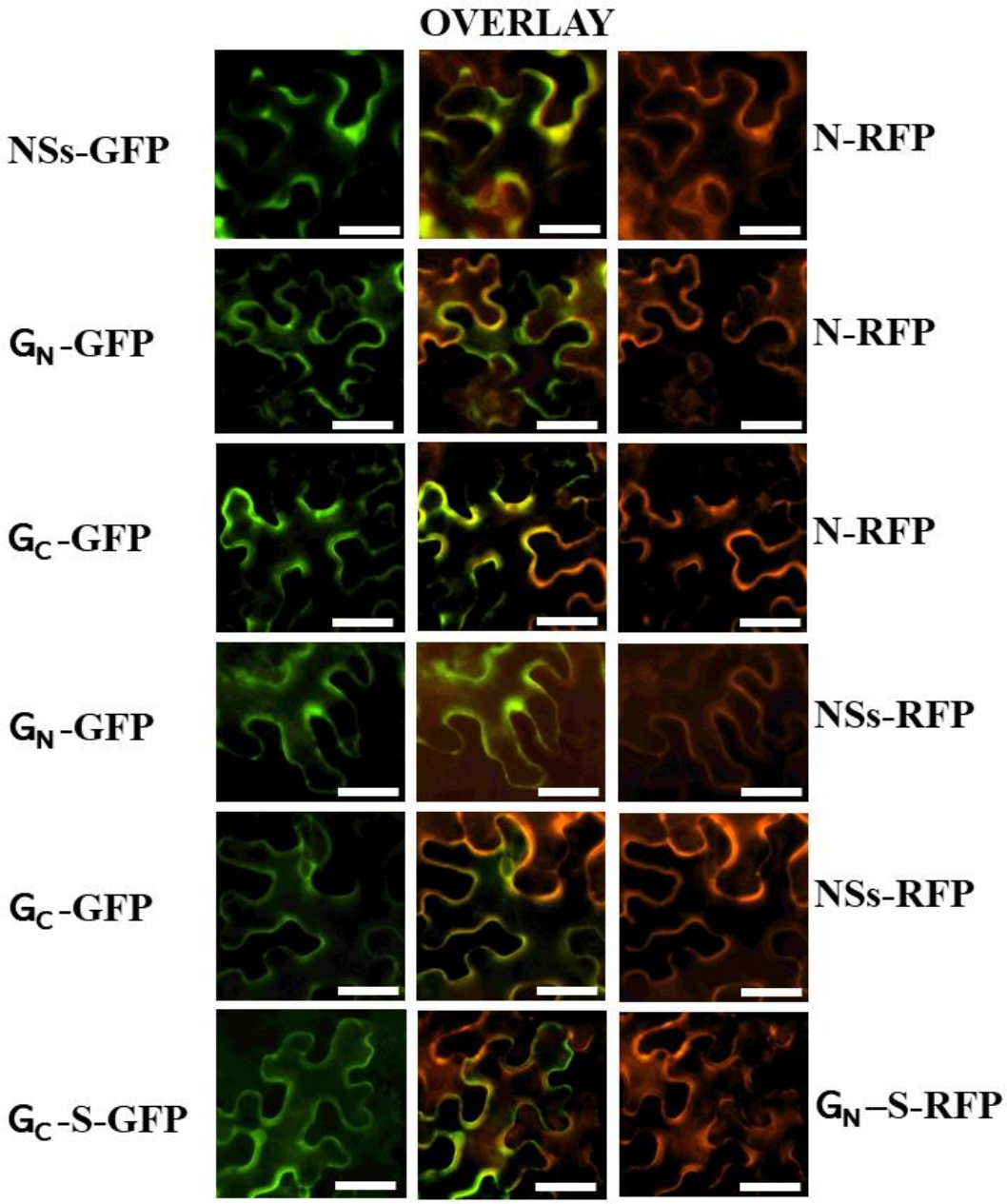

Figure 4: The co-localization of the soybean vein necrosis virus proteins (SVNV) in plant cells. from left to right, column 1, is the first SVNV protein tagged with GFP, column 2, is an overlay with columns 1 and 3, and column 3, is the second SVNV protein tagged with RFP. In the first block of images to the left, the rows from top to bottom are, the first row is NSs-GFP with N-RFP, the second row is N-RFP with G_N-GFP, the third row is N-RFP with G_C-GFP, the fourth row is NSs-RFP with G_N-GFP, the fifth row is NSs-RFP with G_C-GFP, and the sixth row is G_C-S-GFP with G_N-S-RFP. Scale bar is 50 μm.

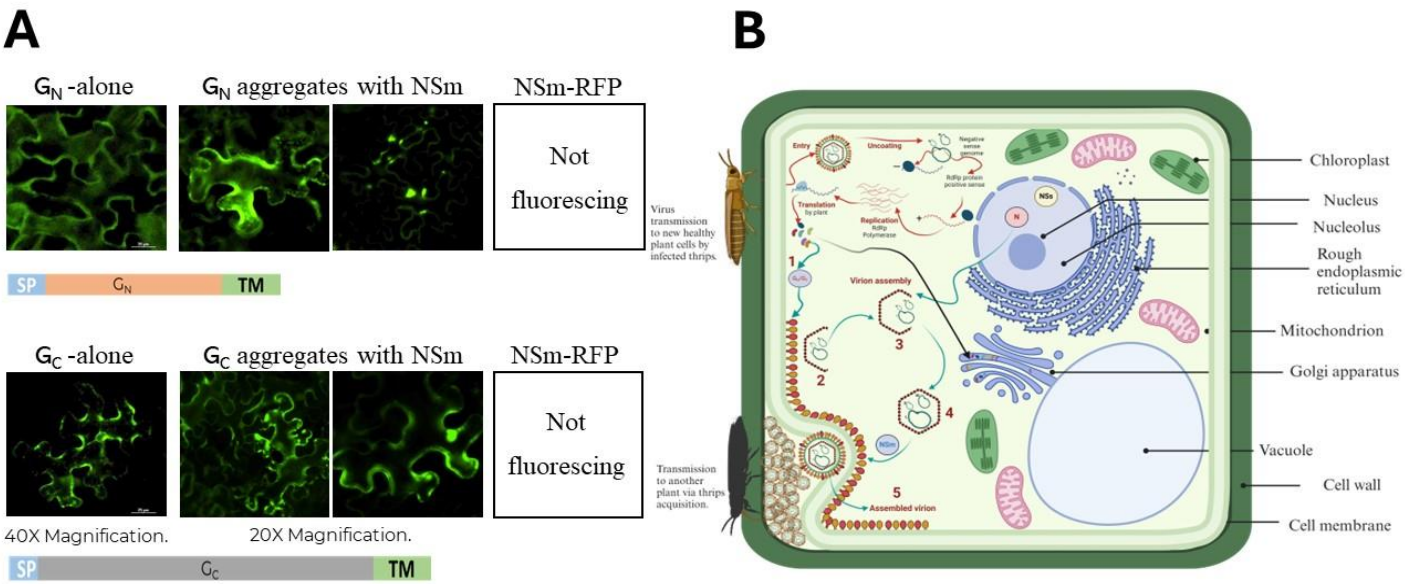

Figure 5: A predicted virion assembly model of soybean vein necrosis virus is based on the co-localization results seen when the NSm protein was infiltrated along with the glycoproteins G_N & G_C proteins. A) is the changes of the protein expression and protein aggregation in G_N-GFP and G_C-GFP with NSm-RFP. B) is model, 1: after the glycoproteins are being made in the cell they travel to Golgi for maturation and secretion then they bind to the cell membrane due o the presence of the TM domain, 2: The nucleocapsid protein (N) localizes to the nucleus and then moves to the cytoplasm preparing for virion assembly, 3: N protein recognizes its viral RNA and encapsidate it into an icosahedral shape, 4: complete icosahedral virions embedded into the cell membrane and in the presence of NSm protein, it facilitates the embedding process, and 5: is the accumulation of assembled virions between the cell membrane and the cell walls allowing thrips to chew them and acquire the virus.

Chapter 4

Title: Characterization of Soybean Vein Necrosis Virus (SVNV) sequences in Alabama.

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1. Abstract

Soybeans (*Glycine max*) have a significant importance globally as a vital crop due to their versatile applications in the food, feed and industrial sectors. Soybeans are known for their high protein and oil content and contribute extensively to human and animal nutrition while also serving as a sustainable resource for biofuel production. Additionally, soybeans play a crucial role in crop rotation practices, enhancing soil fertility and reducing nitrogen depletion. 355,000 acres was planted in Alabama in 2022 (USDA 2022). Soybean vein necrosis virus (SVNV) is a major threat to soybean production in the state. SVNV infects soybean and able to cause symptoms of necrosis which appears between the veins of the infected leaves as patches of dead cells with brown to dark color. To determine the sequence variability in the field, we collected 33 soybean samples expressing symptoms for SVNV. We first determined the sequences for N, NSs, and NSm genes. The amino acid sequences for each gene were aligned to the reference genome of Tennessee (accession: GCF_004789395.1). Conserved amino acid mutations were found in each gene and a phylogenetic tree was constructed for each gene comparing to the reference genome of TN. The findings of this study are essential to determine the evolution and distribution of this virus which will assist in SVNV management in AL.

Keywords

Soybean vein necrosis virus, orthotospoviruses, nucleocapsid protein, movement protein, sangar sequencing, soybean thrips.

2. Introduction

Soybean (*Glycine max*) is one of the most economically significant crops globally, valued for its high protein and oil content, making it a crucial component in human and animal diets (Hymowitz, 1970). Beyond its nutritional value, soybean plays a pivotal role in sustainable agriculture due to its ability to fix atmospheric nitrogen through symbiotic relationships with nitrogen-fixing bacteria, enhancing soil fertility and reducing the need for synthetic fertilizers (Herridge et al., 2008). Additionally, soybean cultivation contributes significantly to the economy, serving as a vital source of income for farmers and supporting numerous industries, including food processing, animal feed, biofuels and pharmaceuticals (Boehlje and Brorsen, 2009). Over the years, extensive research efforts have focused on improving soybean varieties through breeding programs aimed at enhancing yield, disease resistance, stress tolerance, and nutritional quality, contributing to its continued importance in global agriculture (Wilcox, 2001). Soybean is ranked as the fourth most cultivated crop in Alabama. In 2022, soybean production in AL. reached 14.55 million bushels (870 million pounds), valued at approximately 206.6 million dollars (USDA 2022).

Classified within the Orthotospovirus genus of the Bunyviridae family; *Soybean vein necrosis virus* (SVNV) is a single-stranded RNA virus with ambisense genome organization (Tzanetakis et al., 2009). The SVNV genome comprises three genomic segments encoding five open reading frames (ORFs) (Figure 3 - chapter 1) (Zhou et al., 2011). The S segment encodes the non-structural silencing suppressor protein (NSs) and the nucleocapsid protein (N). The NSs protein, spanning 1323 bp, functions as a silencing suppressor, while the N protein, 834 bp in length, is responsible for encapsulating the viral genomic RNA into an icosahedral shape (Figure 3 - chapter 1). In the M segment, two ORFs are found; the first encodes the non-structural movement protein (NSm), a 951 bp protein belonging to the '30K superfamily' (Melcher, 2000), facilitating plasmodesmata (PD) gating in plant cells, enabling intercellular virus movement. The second ORF in the M segment encodes the glycoproteins (G_N and G_C)

within a combined sequence of 3588 bp. These glycoproteins, translated as a polyprotein and then cleaved into G_N and G_C, are membrane-bound and located on the virion's outer surface (Figure 3 - chapter 1), crucial for transmission by *Neohydatothrips variabilis*, the insect vector. The largest genomic segment of SVNV is the L segment, containing a single ORF encoding an RNA-dependent RNA polymerase (RdRp) spanning 8796 bp (Zhou et al., 2011).

SVNV was initially identified in Tennessee in 2008 (Tzanetakis et al., 2009). It was first reported in Alabama in 2012 (Conner et al., 2013). SVNV is predominantly transmitted by thrips in a persistent and propagative manner. A study conducted by Zhou, J. in 2018 examined the competitive abilities of various thrips species in acquiring SVNV, with a specific focus on *N. variabilis*. The study revealed that *N. variabilis* is the primary vector species for SVNV transmission in Northern, Midwestern, and Southern U.S. (Chitturi et al., 2018; Keough et al., 2018; Bloomingdale et al., 2017).

Symptoms caused by SVNV include brown necrotic tissues along the primary veins of both the upper and lower leaf surfaces leading to a scorched appearance of affected leaves (Conner et al., 2013). SVNV detrimentally affects the growth, seed quality, and oil content of soybean plants (Anderson et al., 2017). Notably, in Alabama, the average incidence of SVNV on soybean has increased from 31.8% to 82.6% between 2013 and 2016, indicating a significant increase in the virus's prevalence (Chitturi et al., 2018). It can be found both in and in combination with other viruses such as Soybean mosaic virus (SMV), Been pod mottle virus (BPMV), and Tomato spotted virus (TSV) (Mayfield et al., unpublished)

SVNV has been previously reported to not systemically move in soybean, and it was first reported as the only seed transmitted orthotospovirus (Groves et al., 2016). However, Groves et al. studied the vertical seed transmission of SVNV and the seed transmission in their study occurred at relatively low levels (6%). The SVNV isolate they utilized were asymptomatic on the soybean plant but still reduced total oil and protein content of soybean seeds. Mixed

infection of other viruses with SVNV was not tested, however, three fungal organisms were found parasitizing the same seed. They concluded that it was not clear if SVNV or mixed virus infections have increased the chance of these seeds to increased infection by fungal pathogens (Groves et al., 2016). In 2020, a study by Zhou and Tzanetakis investigated systemic transmission of SVNV in the presence of Bean pod mottle virus (BPMV). They were able to confirm the presence of BPMV in soybean samples infected with SVNV (Zhou and Tzanetakis 2020). However, the SVNV systemic movement in the presence of BPMV remains unanswered.

In a 2016 study by Oliver and Whitfield, a phylogenetic analysis was conducted on all orthotospovirus members using the N protein, revealing that while Tomato spotted wilt virus (TSWV) clustered with Impatiens necrotic spot virus (INSV), both Capsicum chlorotic virus (CaCV) and Iris yellow spot virus (IYSV) formed a distinct clade with other orthotospoviruses. Interestingly, SVNV appeared as the most distinct virus on the tree, forming a separate clade alongside only Bean necrotic mosaic virus (BeNMV) (Oliver and Whitfield, 2016). Another phylogenetic study compromised various virus families, including *Orthotospoviridae*, *Fimoviridae*, *Phenuiviridae*, *Nairoviridae*, *Arenaviridae*, *Phasmaviridae*, and *Hantaviridae*, based on the RdRp polymerase sequence. This study corroborated Oliver and Whitfield's findings by showing SVNV distinctly cladding with BeNMV (Kormelink et al., 2021). These phylogeny results highlight differences in SVNV protein homology and suggest the evolutionary association between SVNV and BeNMV and that they might share protein similarities.

We are interested in understanding the sequence variation and diversity of SVNV from field isolates collected from Alabama compared to other othrotospoviruses. Moreover, we are interested in studying the evolutionary dynamics of SVNV in Alabama and the impact of the virus mutations on the incidence increase on soybean in AL. The discoveries of this study will contribute significantly to our comprehension of this significant virus, marking initial strides

towards devising potential management strategies aimed at mitigating a notable threat to soybean cultivation, particularly in Alabama and other southern states where soybeans are cultivated.

3. Materials and Methods

3.1. Soybean sample collection

Soybean samples were collected from 33 fields in 14 counties in AL representing the northern and central part of the state (Figure 1). Counties that represent north AL are Lawrence, Limestone, Madison, Jackson, Marshall, Cullman, Blount, Etowah, and Dekalb. Counties that represent central AL are Talladega, Chilton, Elmore, Chambers, and Lee county. Samples were collected preferentially based on vein necrosis symptoms suspected to be caused by SVNV regardless of the soybean cultivar. After the samples collection, all samples were stored at -80 degrees until the total RNA was extracted.

3.2. Total RNA extraction and cDNA synthesis

The total RNA was extracted out of the 33 collected symptomatic soybean leaves. Taking into consideration that SVNV is not systemically moving in soybean by its own (Zhou and Tzanetakis 2020), the samples used for RNA extraction were preferentially collected from the necrotic tissues to guarantee SVNV is included. The symptomatic tissues were placed into 2 ml screw cap tubes containing 5-7 sterilized 2.4 mm metal beads. Tubes were then frozen in liquid nitrogen, and the frozen tissues were subsequently grounded using a Bead Ruptor Elite (Omni International, Kennesaw GA, USA). Grinding was carried out in two cycles, each lasting 30 seconds at 5 m/s, with the samples being re-frozen in liquid nitrogen between the cycles. RNA extraction was then conducted using the IBI Mini Total RNA Kit (Plants) (IBI Scientific, Dubuque, IA, USA) following the manufacturer's protocol. The cDNA synthesis was performed using 1000 ng of total RNA and the Verso cDNA Synthesis Kit (Thermo Fisher

Scientific, Waltham MA, USA) and the segment-degenerate reverse primer (Table 1)following the manufacturer's instructions.

3.3. Gene amplification and cloning

Specific SVNV-N, NSs, and NSm PCR primers were used to amplify the three targeted genes (Table 1) using the Thermocycler PCR on the following settings: 98 °C–30 s; 35X: (98 °C–10 s, (for N: 64 °C–40 s, for NSs: 60 °C–1 m, and for NSm: 67 °C–45 s), 72 °C–4 m); 72 °C–5 m and infinite hold at 12 °C using Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific, Waltham MA). Positive samples for each of SVNV genes were purified using the DNA purification kit (ZYMO RESEARCH DNA Clean & Concentrator-5 (Uncapped), Cat #: D4003). The purified PCR products were cloned into pJET-T-d-TOPO vector using the CloneJET PCR Cloning kit (Thermo Scientific Baltics UAB) following the manufacturer's recommended instructions. Colonies of each gene were confirmed using colony PCR using the pJET-F and pJET-R primers (Table 1). One positive colony plasmid from each sample was sent for Sangar sequencing at North Carolina State University's Genomic Science Laboratory. The pJET cloning was not successful for some samples, therefore, these samples were attempted for cloning in the pENTR vector using the pENTR D-TOPO kit following manufacturer's protocols (Invitrogen^{TM}). The M13-F and M13-R primers, were used to confirm the colonies (Table 1).

3.4. Soybean thrips collection and SVNV detection

In 2023, soybean thrips (*Neohydatothrips variabilis*) were collected during the growing soybean in August 2023 from the Cullers location managed by Auburn University. Eighty insects were moved from their original container into a sterile 1.7mL tube containing 250uL of Trizol (ambion life technologies, REF: 15596026) and ground thoroughly. An additional 750uL of Trizol was added while grinding and then incubated for 3 minutes using a rotating mixer. Samples were spun down at 12,000 x g at 4C for 15 minutes. Trizol was collected while

carefully avoiding the insect debris and placed in a new sterile 1.7mL tube. 200uL of chloroform was added to the sample and shaken vigorously for 15 seconds before incubating at room temperature for 5 minutes. Samples were then spun down at 12,000 x g at 4C for 10 minutes. The aqueous phase (top layer) was carefully collected and placed in a new sterile 1.7mL tube with 500uL of isopropanol. Samples were then shaken gently by hand and incubated at room temperature for 10 minutes. After incubation, the samples were spun down at 12,000 x g at 4C for 10 minutes. The supernatant was removed, taking caution not to disturb the small white RNA pellet at the bottom. The pellet was washed with 500uL of 80% ethanol and spun down at 7,500 x g at 4C for 5 minutes. All ethanol was removed and after two minutes at room temperature the pellet was redissolved in 10 µL of molecular grade water. Sample concentrations were checked on the Nanodrop 1000 and stored in a -20 freezer. The total RNA from thrips was used to synthesize the cDNA using the Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham MA, USA) with 1000 ng of total RNA following the manufacturer's instructions and random hexamers reverse primer. To amplify the three SVNV genes from thrips (N, NSs, and NSm), 1 µl from the thrips cDNA was used using the same Phusion settings mentioned above.

3.5. Sanger sequences, analysis, and constructing the phylogenetic trees

The sequences of SVNV three genes (N, NSS, and NSm) were analyzed using Blast Tool (www.ncbi.nlm.nih.gov), by comparing SVNV-AL three gene sequences with all SVNV gene sequences available on the NCBI database. Sequences that have a full read with a start codon and no premature stop codons were only considered for the next steps. The sequences with full read were translated from nucleotide sequence (DNA) to protein sequence (amino acids) using Expasy free online tool, Translate (https://web.expasy.org/translate/). Samples of complete protein sequences with no gaps were then used to build the alignment using the Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) to detect the conserved and

non-conserved amino acid mutations of SVNV of AL three gene with the Tennessee reference genome strain (GCF_004789395.1). The protein alignment was converted to a FASTA format using the online CONVERT tool (https://molbiol-tools.ca/Convert.htm). Finally, to indicate the likelihood between SVNV of Alabama and other SVNV sequences available on the GenBank database, the phylogenetic tree was constructed using MEGA11 version 11.0.13. References available on the NCBI including the TN genome for each gene were used to be compared to our sequences. Additionally, three important out grouping orthotospoviruses including Tomato spotted wilt virus (TSWV), Capsicum chlorosis virus (CaCV), and Bean necrotic mosaic virus (BeNMV) were also used to be compared to our SVNV of AL on the phylogeny trees. This will allow us to understand the similarity of SVNV of AL with other orthotospovirus members.

4. Results

4.1. Soybean sample collection, gene amplification and cloning, and Sanger sequence analysis

In 2023, a total of 33 soybean samples were collected from SVNV symptomatic soybean fields in 14 different counties in AL (Figure 1). All 33 samples tested positive for the three targeted genes of SVNV. The soybean thrips tested positive and the three genes were amplified. Sangar sequences were received for each of SVNV genes and were analyzed. For SVNV-N gene, 31 complete sequences were obtained, and the other two samples had incomplete sequence of N. For SVNV-NSs gene, 24 complete sequences were obtained, and the other nine samples had incomplete sequence. For SVNV-NSm gene, 30 complete sequences were obtained, and the other three samples had incomplete sequence. Moreover, the complete gene sequence of SVNV-N and NSm was obtained from the soybean thrips.

4.2. Protein alignment of SVNV genes

The protein alignment of SVNV-N protein confirmed the presence of eight conserved amino acid mutations in SVNV-N protein of AL compared to the reference genome of TN (Figure 2). The same eight amino acid mutations were detected in the N protein of SVNV sequenced out of soybean thrips. Four conserved amino acid mutations were detected in SVNV-NSs protein of AL compared to the reference genome of TN (Figure 4). Three partially conserved amino acid mutations were detected in SVNV-NSm protein of AL compared to the reference genome of TN (Figure 6). The first amino acid mutation in SVNV-NSm protein of AL was when amino acid S mutated to G at position 19 and this change was detected in 10 samples out of 29 sequenced NSms. For the NSm protein sequenced out of soybean thrips, it did not show this mutation. The second amino acid mutation was when amino acid S mutated to N at position 63 and this change was detected in 15 samples out of 29 sequenced NSms. The third amino acid mutation was when amino acid V mutated to I at position 143 and this change was detected in 17 samples out of 29 sequenced NSms. The second and third amino acid mutations were also detected in the NSm protein coming from *N. variabilis*.

4.3. Phylogenetic analysis of the N protein

Sequences that represent the northern of AL were phylogenetically closest to each other. The same trend was seen in sequences that came from the central of AL, except for sample SVNV.AL.N5 from the north was found closely related to sample SVNV.AL.N17 from the central of AL. Moreover, sample SVNV.AL.N23 from the north was closely related to sample SVNV.AL.N20 from the central of AL. The TN reference genome was phylogenetically closest to the samples came from the northern of AL. Furthermore, the NCBI reference including Fayetteville, Delaware, and Maryland were also closest to SVNV from the northern of AL. The three out grouping orthotospoviruses were found in a clade that has both SVNV sequences from the northern and central of AL. Additionally, the SVNV-N protein

sequence came from soybean thrips which represent the central of AL was closely related o sample SVNV.AL.N30 which came from the north of AL. (Figure 3).

4.4. Phylogenetic analysis of the NSs protein

The same trend seen previously in SVNV-N tree was observed in the NSs tree where sequences from northern AL were grouping together, and sequences that came from the central AL were also grouping together, except for sample SVNV.AL.N3 was more related to sample SVNV.AL.N33 from the central of AL. Additionally, sample SVNV.AL.N27 from the north was closely related to sample SVNV.AL.N32 from the central of AL. The TN reference genome and the NCBI references including Iowa and Illinois were phylogenetically closest to the SVNV of northern AL. The three out grouping orthotospoviruses found closest to SVNV sequences came from the northern of AL. (Figure 5).

4.5. Phylogenetic analysis of the NSm protein

In the phylogenetic tree of NSm protein, sequences that represent the northern of AL were grouping together, however, less sequences from the central of AL were seen together. Similarly with the phylogenetic of N protein, NSm sequence that came from soybean thrips was closely related to sequences from the north of AL. In contradiction with the other two genes N and NSs, the TN reference genome was found in a mixed clad with both samples from the northern and central of AL. Moreover, the NCBI reference including sequence from Iowa and Illinois were also in this mixed clade. Following the same trend with NSs phylogeny tree, the three out grouping orthotospoviruses were found closest to SVNV sequences came from the northern of AL**.** (Figure 7).

5. Discussion

SVNV genome sequencing and analysis are important in revealing changes that occur in the virus genome. This can help us to understand important events such the incidence increase of SVNV in AL. Additionally, the phylogenetic analysis provides insights regarding the virus evolution. In previous studies done by Kormelink et al., 2021, Oliver and Whitfield

2016, and Zhou and Tzanetakis 2019, it was shown that SVNV is phylogenetically divergent. In this study, we tested 33 soybean samples collected from Alabama in 2023 and all samples tested positive for SVNV. To understand the sequence diversity of SVNV of AL, three genes were sequenced including N, NSs, and NSm. Conserved amino acid mutations were detected in all three genes when they were compared to the reference genome of Tennessee strain of SVNV. When SVNV was first reported in AL, it was confirmed by sequencing 250 nucleotides in the RNA dependent RNA polymerase (RdRp) (Accession number: KC431808). The sequences obtained in this study provide a total number of 86 complete gene sequences of three important genes of SVNV from Alabama (Table 2).

The N protein is the second ORF on the S segment spanning 2533–1700 and is a 31 kDa (Zhou and Tzanetakis 2019). The reference genome of SVNV-N of TN has an RNA-binding Lysine-rich motif KKDGKGKKSK264–273, in addition to several RNA-interacting amino acids (PSN7–9, RK51–52, RY54–55, and KK73–74) (Zhou and Tzanetakis 2019). These domains may the allow nucleoprotein to take part in the RNA synthesis process with the RdRp as shown for members of the *Bunyavirales* (Dunn et al., 1995 and Kainz et al., 2004). By looking into our eight conserved amino acid mutations that were observed in SVNV-N protein of AL, they do not fall at any of the reported motifs and the reported RNA binding regions. This might suggest that these motifs are crucial for the SVNV-N protein function. Since 80% of our sequences of N show these eight amino acid mutations, These mutations may play an important role in the SVNV virion assembly cycle in plant and insect cells. Moreover, these mutations might positively impact the self-interaction of the SVNV-N protein as the self-interaction of orthotospovirus members has previously been reported for TSWV, CaCV-AIT (Thailand), and INSV (Lacorte et al., 2007, Zilian and Maiss 2011, Dietzgen et al., 2012) additionally, for BeNMV, Chrysanthemum stem necrosis virus (CSNV), Iris yellow spot virus (IYSV), and Tomato chlorotic spot virus (TCSV) (Leastro et al., 2015; Tripathi et al., 2015).

The NSs protein is the first ORF on the S segment spanning 59–1381and is a 50-kDa (Zhou and Tzanetakis 2019). It is predicted to be an RNAi suppressor (Takeda et al., 2020). The reference genome of SVNV-NSs protein of TN contains conserved $GK_{178–179}$ and $DE_{148–149}$ comprise the Walker A and B motifs (Zhou and Tzanetakis 2019). These important motifs interact with ATP/ADP phosphates and coordinate/bind $Mg2^{+ions}$ during the ATP hydrolysis process (Caruthers and McKay 2002 and Lokesh et al., 2010). However, the four amino acid mutations that were detected in our SVNV-NSs protein of AL did not appear in these motifs and that the Walker A and B motifs remained conserved in our sequences. This might indicate the importance of these motifs for the functionality of NSs protein of SVNV. However, the documented four amino acid mutations in our sequences were conserved among 95% of the sequenced samples (. This indicates that these mutations are being selected by the virus which may indicate that these mutations could play an important role in suppressing the plant defense mechanism. The detected mutations may also play a role in the protein-protein interactions with SVNV-NSs self-interaction (Dietzgen et al., 2017) or with other SVNV proteins during the virus infection cycle.

The NSm protein is the first ORF on the M segment spanning 58–1008 and it codes for a 35-kDa non-structural movement protein protein (NSm) (Zhou and Tzanetakis 2019). SVNV-NSm protein is considered one of the 30K movement protein superfamily due to the presence of the highly conserved LxDx40G motif (Zhou and Tzanetakis 2019) implicated in cell-to-cell movement (Mushegian and Koonin 1993 and Silva et al., 2001); however, a substitution at the beginning of the motif was observed where the Leu residue substituted by an Ile (Zhou and Tzanetakis 2019). The ‘‘P/D-L-X motif’’ and phospholipase A2 catalytic sites, present in some orthotospovirus orthologs such as tomato spotted virus (TSWV) and groundnut bud necrosis virus (GBNV) are absent from the SVNV counterpart (Silva et al., 2001 and Kormelink 1992). The mutations that we detected in our SVNV-NSm protein were not found to be at any of the

reported motifs suggesting that these motifs are highly conserved among orthotospoviruses. The three detected mutations in NSm were less conservative among the three genes with 46% compared to 95% for N and 80% for NSs. This indicates that SVNV-NSm is undergoing mutations which allows the gene to evolve and function better because as mentioned by Zhou and Tzanetakis 2019, the motifs in SVNV-NSm either have amino acid substitutions or are absent. This might be the reason why localized SVNV-NSm protein cause cell death and that it did not localize to the plasmodesmata as other localized orthotospoviruses (Shehata and Martin 2024-in progress).

The SVNV-N and NSm proteins that were identified from thrips collected from the central AL in 2023 show the same conserved mutations that were mentioned above except for the first mutation. This indicates that these mutations were successful in both the plant and the insect system. Because only the mature assembled SVNV virions will be acquired by thrips. In the phylogeny of N and NSs proteins, the SVNV Tennessee reference genome was found closely related to the northern SVNV samples possibly due to the proximity of the northern region of Alabama which borders Tennessee. However, in NSm protein, the TN reference genome was seen in a mixed group of samples from the north and central AL. Relating this phylogenetic of SVNV-NSm with the SVNV-NSm protein localization study conducted in chapter 3, it helps us in understanding why SVNV-NSm did not localize to plasmodesmata as it was expected. And it may support our hypothesis that SVNV-NSm is undergoing mutations which might allow the virus genes to evolve and function better in terms of the virus movement in the cells during the virus infection cycle. Furthermore, in the phylogenetic trees of the three genes, a clear trend was observed, where samples that came from the northern part of AL were cladding close to each other and samples that came from the central part of AL were also cladding together. This phenomenon was the clearest in the N protein followed by the NSs protein, however, in the NSm protein, most clades were mixed of samples from the north and the central of AL. A topographic map of Alabama showed that there are mountains and hills between the north and the central of AL (Figure 8). These mountains and hills may work as natural barriers between the north and central AL allowing SVNV to evolve distinctly in each of the two parts of the state. These discovery

findings are very important revealing conserved and no conserved mutations in SVNV genome of AL and will assist in understanding of SVNV evolution. In future research, SVNV naturally mutated proteins of Alabama will be localized in plant cells and compared with the localization results of SVNV of Tennessee discussed in chapter 3. We also will be looking for BPMV detection with SVNV.

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7. References

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Table 1. The segment-degenerate reverse primer which was used in the cDNA synthesis of the SVNV S and M segments. The SVNV, N, NSs, and NSm gene specific primers were used to amplify the three genes. The pJET forward and reverse primers that were used for PCR colony check of the pJET vector cloning and for Sangar sequencing.

Primer name	Primer sequence	Amplicon size	Purpose
SVNV-S-R Degenerate	VRVMWGAGCAATCGGCACGATTAAAG	2603 bp	cDNA synthesis
SVNV-M-R Degenerate	TCGGCGCAACAAGAAAGATWCAYAAG	4955 bp	cDNA synthesis
SVNV-N-F	CACCATGCCACAAACAGCAGGACCAAG CAATGC	831 bp	Amplifying SVNV-N
SVNV-N-R	AACAGAAAACTCCTTTGATTTCTTGCC		
SVNV-NSs-F	CACCATGCAGAGCTTGATTACTCTTGGC AG	1320 bp	Amplifying SVNV-NSs
SVNV-NSs-R	TTCAAAATAGGTTGCAAGATCTCC		
SVNV-NSm-F	CACCATGTCAAACTTTGTAGCAAAGGCT ATTGAAATGGGACAGG	948 bp	Amplifying SVNV-NSm
SVNV-NSm-R	AATCGGTTTCATTTCCACCAAAGAAGG G		
pJET-F	CGA CTC ACTATAGGGAGAGCGGC	Depending on the length of each sequenced gene.	Sangar sequencing
pJET-R	AAGAACATCGATTTTCCATGGCAG		
M13-F	GTA AAA CGA CGG CCA GTG	Depending on the sequenced SVNV gene	PCR colony check and Sangar sequencing
M13-F	GTA AAA CGA CGG CCA GTG		

Table 2: The cloned three genes of Soybean vein necrosis virus of Alabama NSs, N, and NSm. From right to left, column one refers to the samples name, column two refers to SVNV-N gene, column three refers to SVNV-NSs gene, and column four refers to SVNV-NSm gene.

Sample #	SVNV-N	SVNV-NSs	SVNV-NSm
Soybean 1	✓	✓	✓
Soybean 2	✓	✓	✓
Soybean 3	✓	✓	✓
Soybean 4	✓	✓	✓
Soybean 5	✓	✓	✓
Soybean 6	✓	✓	✓
Soybean 7	✓	✓	✓
Soybean 8	✓	✓	✓
Soybean 9	✓	✓	✓
Soybean 10	✓	✓	✓
Soybean 11	✓	✓	✓
Soybean 12	✓	✓	✓
Soybean 13	✓	✓	✓
Soybean 14	✓		✓
Soybean 15	✓	✓	✓
Soybean 16	✓		✓
Soybean 17	✓		✓
Soybean 18	✓		✓
Soybean 19	✓	✓	
Soybean 20	✓	✓	✓
Soybean 21	✓		✓
Soybean 22			✓
Soybean 23	✓	✓	✓
Soybean 24			✓
Soybean 25	✓	✓	
Soybean 26	✓	✓	✓
Soybean 27	✓	✓	
Soybean 28	✓		✓
Soybean 29	✓	✓	
Soybean 30	✓	✓	✓
Soybean 31	✓		✓
Soybean 32	✓	✓	✓
Soybean 33	✓	✓	✓
Thrips tRNA	✓		✓
Total sequenced	32	24	30

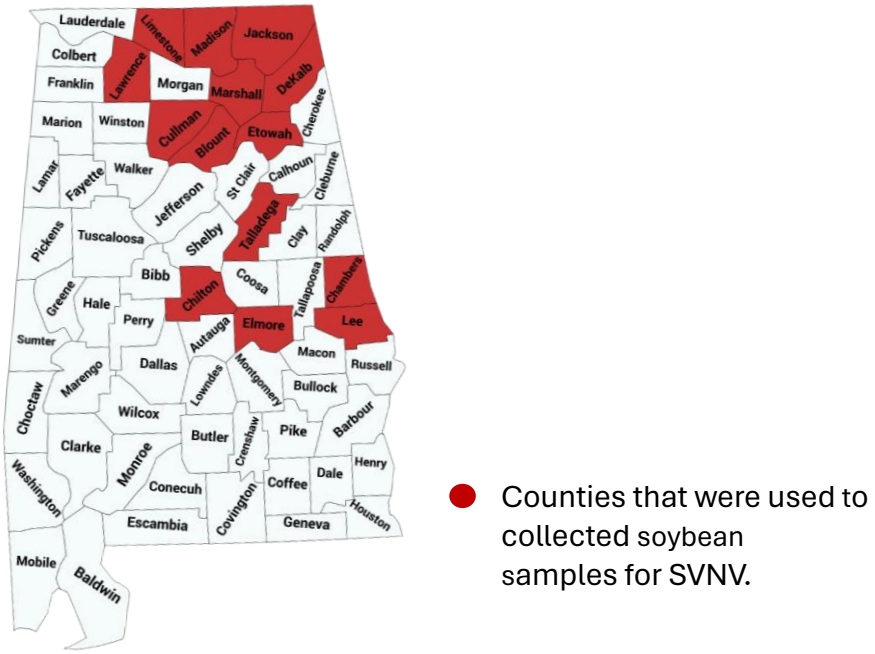

Figure 1: A map of the fields that were used to collect soybean samples that were symptomatic to the Soybean vein necrosis virus (SVNV) in 2023 from Alabama.

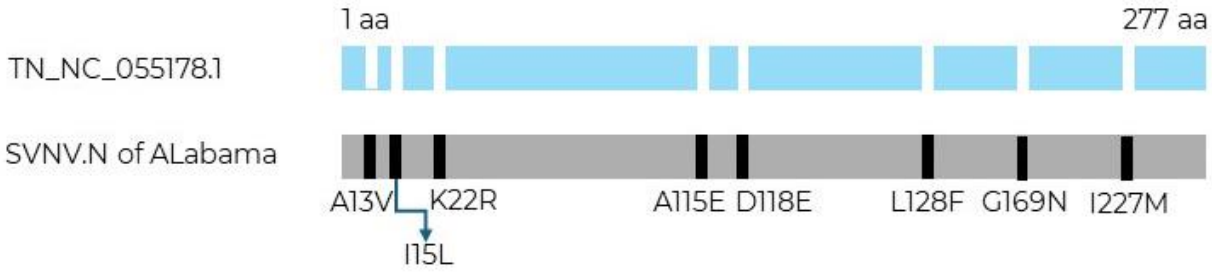

Figure 2: Conserved amino acid mutations in SVNV-N-proteins of Alabama and the sites for each mutation, in a comparison with the reference genome of Tennessee-N from the NCBI.

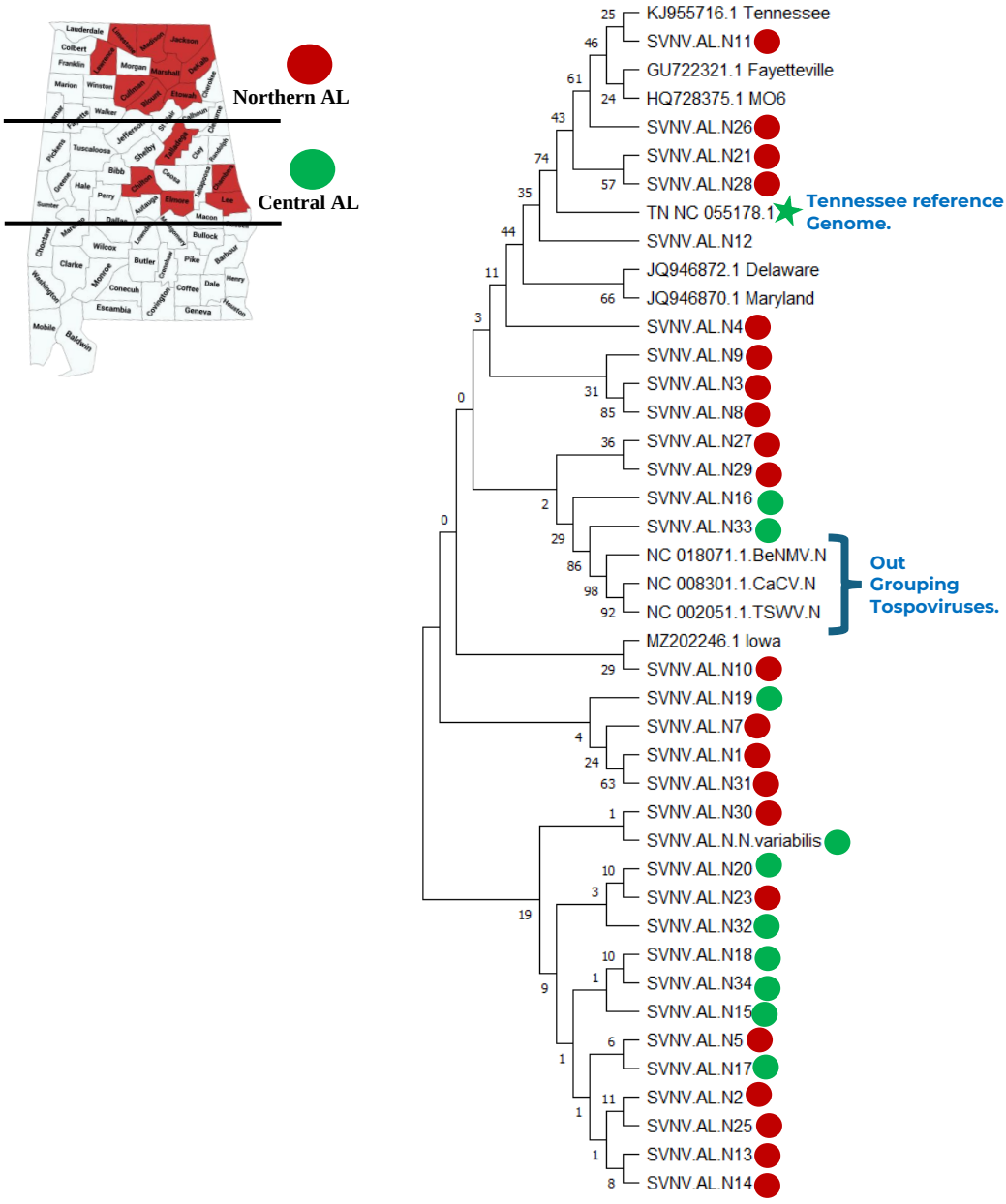

Figure 3: Phylogenetic tree of SVNV-N proteins of Alabama with the NCBI references including the reference genome of Tennessee marked with a green star. N protein of Bean necrotic mosaic virus (BeNMV), Tomato spotted wilt virus (TSWV), and Capsicum chlorosis virus (CaCV) were used as out-groups.

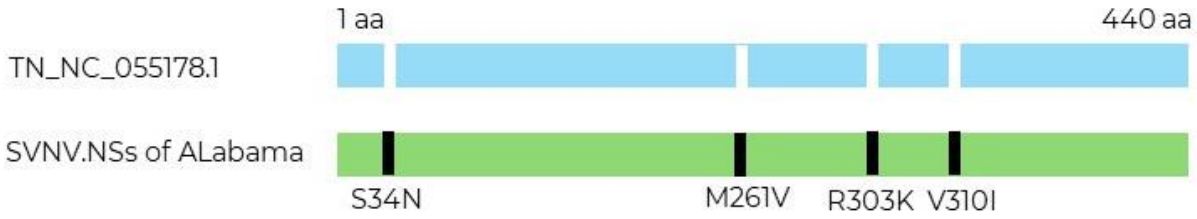

Figure 4: Conserved amino acid mutations in SVNV-NSs-proteins of Alabama and the sites for each mutation, in a comparison with the reference genome of Tennessee-NSs from the NCBI.

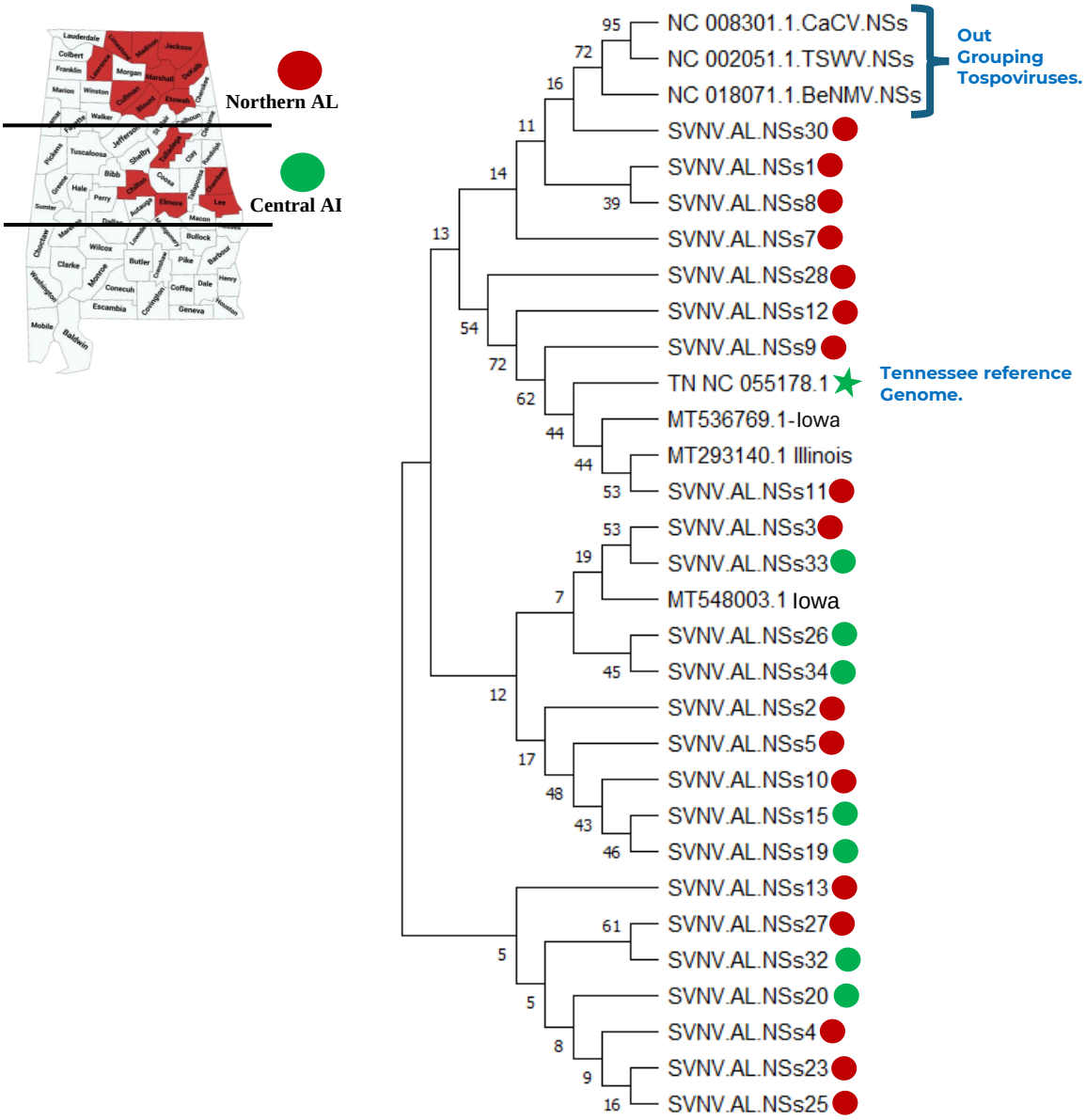

Figure 5: Phylogenetic tree of SVNV-NSs proteins of Alabama with the NCBI references including the reference genome of Tennessee marked with a green star. NSs protein of Bean necrotic mosaic virus (BeNMV), Tomato spotted wilt virus (TSWV), and Capsicum chlorosis virus (CaCV) were used as out-groups.

Figure 6: Conserved amino acid mutations in SVNV-NSm-proteins of Alabama and the sites for each mutation, in a comparison with the reference genome of Tennessee-NSm from the NCBI.

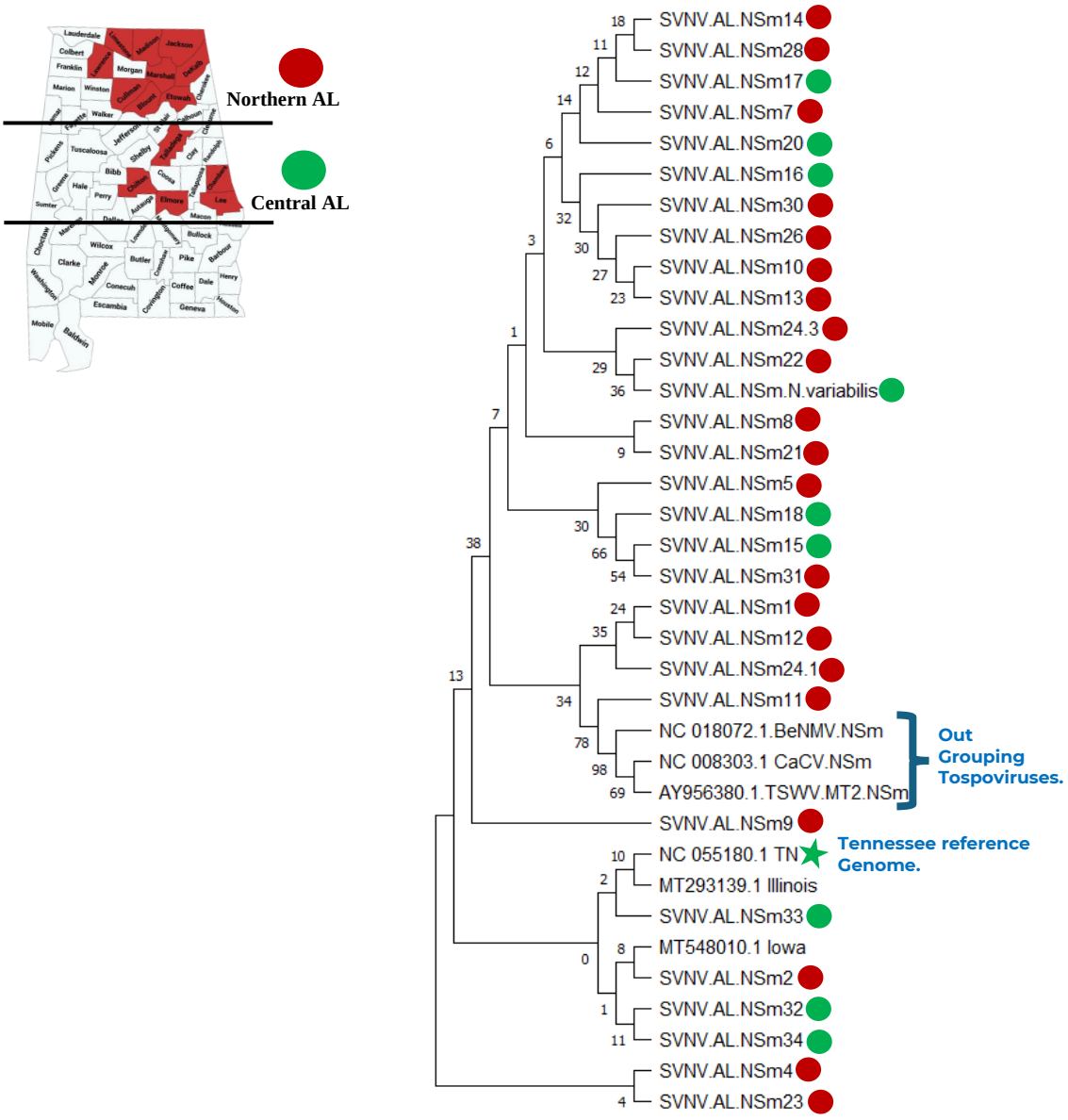

Figure 7: Phylogenetic tree of SVNV-NSm proteins of Alabama with the NCBI references including the reference genome of Tennessee marked with a green star. NSm protein of Bean necrotic mosaic virus (BeNMV), Tomato spotted wilt virus (TSWV), and Capsicum chlorosis virus (CaCV) were used as out-groups.

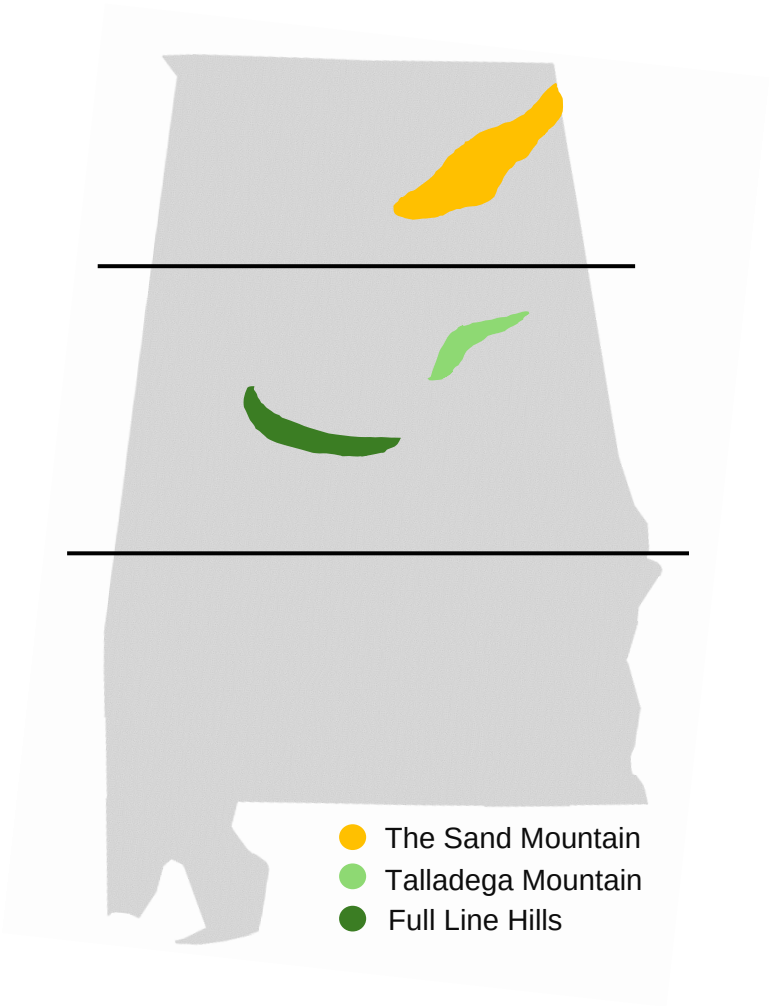

Figure 8: A map of Alabama indicating the geographic natural barriers including the Sand Mountain in the northern of AL, Talladega Mountain, and the Full Line Hills both in the central part of AL.

(https://www.nationsonline.org/oneworld/map/USA/alabama_map.htm#google_vignette)