A Molecular and Field Survey Approach to Understanding Legume Viruses in Alabama

by

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A thesis submitted to the Graduate Faculty of

Auburn University

in partial fulfillment of the

requirements for the Degree of

Master of Science

Auburn, Alabama

August 7, 2021

Keywords: thrips, legumes, viruses, vector, survey, microscopy

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Abstract

Legumes grown in Alabama contribute to hundreds of millions of dollars for the state's economy. During the soybean growing season, viruses prevalent in Alabama such as Soybean mosaic virus (SMV), Soybean vein necrosis virus (SVNV), Tobacco streak virus (TSV), and Bean pod mottle virus (BPMV) have the potential to infect crops and cause wide yield losses. A soybean viral survey was performed over the 2020 Alabama soybean growing season to identify viruses present in the field as well as their insect vectors. Tomato spotted wilt virus (TSWV), is another virus in Alabama which was experimented on within a molecular environment. TSWV proteins were fused to GFP/mRFP tags to identify localization when expressed in insect cells. Results from the viral survey identified SVNV being transmitted by thrips in the 2020 soybean growing season. Data from TSWV protein localization reveals where proteins are expressed in insect cells.

Acknowledgments

I would like to thank my parents and my family for their support and motivation throughout my program. I would also like to thank my supervisor, Dr. Kathleen Martin, for allowing me the opportunity to perform research in her lab while advancing my education. I am grateful for the Auburn University department of Entomology and Plant Pathology faculty members, especially Dr. John Beckmann and Dr. Alana Jacobson for being on my graduate committee and helping me work towards obtaining this degree.

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List of Abbreviations

TSWV	Tomato spotted wilt virus
SVNV	Soybean vein necrosis virus
SMV	Soybean mosaic virus
BPMV	Bean pod mottle virus
TSV	Tobacco streak virus
DAS	Double antibody sandwich
ELISA	Enzyme-linked immunosorbent assay
ORF	Open reading frame
L	Large
M	Medium
S	Small
IPM	Integrated pest management

CHAPTER ONE

Five of the Alabama's most important cash crops include peanuts, soybean, cotton, corn, and hay, contributing to over $934,000,000 in combined value of production in 2018 (USDA National Agricultural Statistics Service). Two of these cash crops, soybeans and peanuts, are legumes belonging to the family *Fabaceae.* Plants belonging to this family are susceptible to a variety of pathogens, with viruses having the potential to cause yield loss during the growing season during optimal conditions. Some of these potentially devastating viruses include Tomato spotted wilt virus (TSWV), Bean pod mottle virus (BPMV), Tobacco streak virus (TSV), Soybean mosaic virus (SMV), and Soybean vein necrosis virus (SVNV). These viruses have a history in Alabama, with TSWV being found in 82% of peanut farms surveyed in the state from 1986-1989 (Hagan, 1990). One difficulty when dealing with viruses is their relationship with insect vectors responsible for transmitting the pathogens. By the time most viruses are identified in the field during a growing season, the insect vectors already have established populations and management difficulties arise. Vectors responsible for transmitting the aforementioned viruses commonly found in Alabama include soybean thrips (*Neohydatothrips variabilis*), aphids (Aphididae), sharpshooters (Cicadellidae), and bean-leaf beetles (*Cerotoma trifurcate*).

Tobacco streak virus (TSV) belongs to the family *Bromoviridae*; it can be found worldwide with a host range of at least 200 species. TSV has the potential to infect crops which have economic importance, such as soybean, peanuts, cotton, cranberry, chickpea, and tobacco (Gulati *et al.*, 2016). Symptoms of this virus are distinct on tobacco, but it can be harder to identify visually on alternate crops such as soybean. Typical symptoms of this virus include mosaic patterning, tip deformation, streaking, chlorosis, and stunting (Krishnareddy *et al.*, 2003) (Figure 1 – D ,E ,F); TSV can be difficult to identify due to the range of these symptoms. As with all of the following viruses, TSV has the potential to be a part of a mixed infection with another virus or pathogen. Mixed infections increase the difficulty in a correct diagnoses of a disease due to new symptoms and appearances of previous symptoms. Although TSV can be spread through seed and pollen, the insect vector responsible for spreading the virus is thrips. Multiple species of thrips can spread TSV, with two main species being *Microcephalothrips abdominalis* and *Thrips tabaci* (Rabedeaux *et al.*, 2005). Thrips spread TSV through pollen; thrips feeding on pollen of infected plants carry the virus with them on their mouthparts to new plants where virions enter plant wounds caused by feeding (Spoodee and Teakle, 1987).

Soybean mosaic virus (SMV) was first reported in 1915 affecting soybean crops grown in Connecticut (Gardner and Kendrick, 1921). In the modern day it can be found across the globe (Hajimorad *et al.*, 2018), and can lead to yield losses as high as 94% (Hill and Whitham, 2014). Although soybean is the most economically important host, the virus is capable of infecting crops in the families *Fabaceae* and *Leguminosae* among several others. Plants infected with SMV may appear asymptomatic, or they may display mottling/deformation of the leaves, chlorosis, and vein necrosis (University of Minnesota Extension, 2017) (Figure 1 – C). SMV is

transmitted by 32 known species of aphids, with *Aphis gossypii* being one of the most efficient species (Pederson *et al.*, 2007).

Bean pod mottle virus (BPMV) was first identified in Nebraska in 1981 (Lin and Hill, 1983). BPMV belongs to the family *Secoviridae* and most commonly infects soybean and other legumes. The main vector for BPMV are leaf-feeding beetles, with the bean leaf beetle (*Cerotoma trifurcate*) being the most common vector for the virus in Alabama. Symptoms of BPMV include yellow/green splotches throughout the leaves, resulting in a mottling pattern (Figure 1 – B). The severity of the symptoms varies from host to host, with soybeans being the most susceptible for financial losses in both crop yield and seed production.

Soybean vein necrosis virus (SVNV) belongs to the family *Tospoviridae*. SVNV was first characterized in 2008 in Tennessee, it was not described in Alabama until 2013 (Jacobs *et al.*, 2013; Conner *et al.*, 2013). The only known hosts are plants in the families *Leguminosae, Solanaceae*, and *Cucurbitaceae,* with the only known agronomic crop being soybean (Bloomingdale *et al.*, 2014). Symptoms of this virus arise as chlorosis of the leaves and over time chlorotic regions eventually turn necrotic and lead to tissue death (Figure 1 – A). The most known insect vector for SVNV is soybean thrips - *Neohydatothrips variabilis* (Zhou and Tzanetakis, 2013).

Tomato spotted wilt virus, belonging to the family *Tospoviridae*, is considered to be one of the most influential viruses currently affecting the state of Alabama's legume production. Tospoviruses are plant viruses found throughout the globe that have the potential to cause up to 100% yield loss for the infected plants under optimal conditions (Rosello *et al..*, 1996).

Tospoviruses take their name from the discovery of the first virus in the family in 1919: Tomato spotted wilt virus (TSWV) (although the scientific community did not recognize the disease as a virus until 1930). Tospoviruses are transmitted by thrips, specifically those in the family Thripidae and subfamily Thripinae and they have the ability to spread quickly and over large areas (Riley *et al..,* 2011). Tospoviruses infect their insect vector, replicating in the insect in a circulative persistent-propagative manner. Because of the relationship between thrips and tospoviruses, an effective approach to prevent an outbreak is typically through integrated pest management (IPM) strategies such as control of the virus through virus-resistant cultivars of crops and control of thrips populations through pesticide applications.

Tomato spotted wilt virus

The first documentation of a tospovirus took place in 1915 and was published in an Australian journal describing tomato diseases in 1919 (Brittlebank, 1919). The most prevalent symptoms were spotting of the affected plants as well as wilting: giving it the common name of Tomato spotted wilt disease. Fifteen years after its original discovery date, the disease was determined to be from viral origin as compared to fungal or bacterial causes (Samuel *et al.*, 1930). *Tomato spotted wilt virus* (TSWV) did not share any identical characteristics to any other known viral family known at the time, prompting the community to label it as the only member of its own unique family known as *Tospoviridae* in the order of *Bunyavirales.*

Tomato spotted wilt virus has an incredible host range, infecting over 1000 species of plants ranging from monocots to dicots (Sherwood et al, 2009). Some of the most important food crops infected by TSWV include peanuts, tomatoes, zucchinis, capsicums, potatoes, and

melons. Ornamental species are also hosts, causing losses to flower farms that specialize in impatiens, chrysanthemums, irises, and calla lilies among others. Typical symptoms include stunting: causing the plants to grow at a reduced rate with reduced vigor. Stunting is often more severe when the plant is infected as a seedling causing a delay in the developing at a natural rate and lowering its overall health. Wilting is also a common symptom, resulting in the loss of water through the plant and reduced vitality. The symptom by which TSWV takes its name is the appearance of yellow or brown ringspots that form on many of its hosts. These necrotic ringspots most often appear on the leaves of infected plants, although they may also form on fruits or foliage. While this symptom might seem like an easy way to diagnose the virus, spots and rings are a symptom which can be attributed to other diseases such as bacterial spots caused by bacteria in the *Xanthomonas* genus (Kyeon *et al.*, 2016). Spots/rings that appear might not always result in necrosis or harm of the plant and may only act as a visual symptom, rendering these plants and fruit unmarketable if being purchased for aesthetic qualities. Although it cannot infect seeds, TSWV has the potential for seed deformation, causing the seeds to stray from their natural color in some infected plant species. TSWV has cultivar dependent effects as well as host range limitations; in one cultivar of tomato, the symptoms may be severe, however, in a more resistant cultivar the virus infection may be less severe. This is due to the genetic composition of the plants including the presence of resistance genes within cultivars of tomato including *Sw-5*. TSWV does not react the same in each host; some plants are more susceptible to the virus and display more prevalent symptoms, consequently suffering higher losses.

As would be expected with a disease that can cause large yield losses in a variety of plants, management strategies are of importance in the control of this system (Roselló *et al.*, 1996). Host-plant resistance is the most efficient method of reducing TSWV in field conditions, and the discovery of the *Sw-5* gene in tomato led to the development of lines of tomato with resistance to TSWV (Oliviera *et al.*, 2018). The *Sw-5* gene encodes protein receptors that can recognize microbial products and activate necessary signal pathways to prevent the microbes from spreading (Oliviera *et al.*, 2018). This gene performs these functions through a hypersensitive response, causing programmed cell death in the healthy plant cells bordering the infected cells limiting the spread of virus throughout the plant. Cultivars of plants grown commercially offer the *Sw-5* gene as protection against TSWV, however, there have been cases of resistance breaking strains of TSWV found in Australia, Spain, and the US (Rienzo *et al.*, 2018).

Due to the warm weather climate and large variety of crops grown such as lettuce, tobacco, eggplant, cucurbits, tomato, pepper, peanuts, and ornamental flowers, TSWV as well as thrips can be found throughout the Southeast. Georgia, Florida, and Alabama produce two-thirds of peanuts grown in the US, and TSWV has a history of infecting peanuts in Alabama. Surveys performed from 1986-1989 resulted in data showing that out of 288 peanut fields, 82% were found to contain plants testing positive for TSWV (Hagan, 1990). In the early 1990's Alabama was suffering from what was termed a "multivirus epidemic of tomatoes". In 1992 major tomato growing regions of Alabama had their production reduced by as much as 25%, with some yield losses estimating 100% from mixed infections (Sikora *et al.*, 1998). Mixed infections occur when two or more viruses are actively infecting the same plant; there is

potential for a virus to make the infection process easier for future viruses by causing damage to the plant and increasing its susceptibility. Plants infected with multiple viruses may also exhibit more severe symptoms, often resulting in greater yield loss than if one individual virus was present. Some viruses are even capable of "disabling" resistance genes, allowing potential mixed infections for viruses that were previously unable to cause infection. It was found in a study that when tomatoes with the *Sw-5* gene were first infected with Tomato chlorosis virus (ToCV), these previously protected plants were then susceptible to TSWV despite containing the resistance gene (García-Cano *et al.*, 2006).

TSWV is able to survive and maintain its presence through overwintering in weeds or other plants. Due to its large host range TSWV can infect weeds near crop fields or in other agricultural hosts which do not suffer the same yield losses, thus are not managed as exhaustively. When suitable conditions apply (such as colonization of infected weeds by thrips vectors), the virus moves with the insect vector to the agricultural hosts. The main plant TSWV overwintered in with peanuts during outbreaks in the 1980s and 1990s was the Florida beggar weed (Hagan 1990). Removing weeds in nearby areas where crops are grown, as well monitoring for new possible weedy hosts can be an effective strategy to prevent TSWV from reaching a field.

Thrips vectors of Tospoviruses

Tospoviruses are spread by thrips: minute insects in the order Thysanoptera with narrow bodies and fringed wings. These insects take their name from a Greek word for "fringed wings" which translates to the English common name "thrips", and when talking about these insects

the word “thrips” is correct for both singular and plural use. Their fringed wings can be used for both conventional and non-conventional flight, helping the insects travel through the air in any vortices or currents they encounter. Males throughout the order are often around 1mm in size, with the females averaging slightly larger at 1.4mm in comparison. This small body size can result in a field of crops harboring thousands of thrips, as the stippling damage from thrips feeding can be easy to overlook. Adults typically prefer to feed on soft tissue such as the reproductive organs or flowers, causing the already small insects to be obscured. There are ten known species of thrips that vector TSWV, with some of the most common in Alabama being *Frankliniella occidentalis* (western flower thrips), *Frankliniella fusca* (tobacco thrips), and *Sericothrips variabilis* (soybean thrips). The most important species that transmits TSWV throughout the world is *Frankliniella occidentalis*. These three pests are native to the Southwestern US but have been identified as far as Europe, Australia, or South America. Thrips feed with unique asymmetrical piercing/sucking mouthparts, penetrating the tissue of their host plant and secreting the virus into the wounded tissue. Depending on the species of thrips, the female will lay her eggs directly into soft, susceptible tissue such as leaves, flowers, or fruit. The process of growing from hatched larvae to adult can take approximately 18-20 days at a temperature of 20°C (Murai, 2000), with the adult stage living for potentially 30-45 days. Thrips in the suborder Terebrantia consist of a lifecycle of four instar stages before reaching adulthood, while the suborder Tubulifera contains a fifth instar stage. In Terebrantia, the first two instar stages spend their time feeding on the leaves or soft tissue near where they hatched. The third and fourth instar stage leave their original host plant, and do not feed for these two stages. The third and fourth instar stages are typically referred to as the pupal

stages, and complete their development on/in the ground litter. Adults then emerge and use their fringed wings to travel through air currents to find a new plant where they will begin feeding.

Thrips have a unique way of transmitting tospoviruses, with many specific conditions which must be met in order to not only obtain the virus, but transmit it as well. Although the acquisition time of the virus is rather short (as little as 5 minutes for *Frankliniella occidentalis)* (Wijkamp *et al.*, 1996), the virus can only be acquired during the thrips larval stage, specifically during its first or second instar for viral transmission to occur. Eggs hatching on a host plant that contains the virus will result in the larval thrips acquiring the virus through feeding. TSWV will stay with these thrips for their lifetime, replicating inside their bodies as they progress through their instar stages into adulthood. It is important to note that although these larval thrips are carrying the virus, they are unable to spread TSWV until they have matured into adults (Badillo-Vargas *et al.*, 2018). First and second instars will continue to feed on infected plants and accumulate virus titer before reaching their pupal stages. No feeding occurs in the pupal stage, although a metamorphosis takes place where the body becomes soft and organs reorganize themselves as the adult body is formed. It is during this crucial stage in the thrips life cycle where the virus obtains the ability to now be passaged to plants. Virions which had originally entered through the esophagus of the thrips made their way to the insect midgut before replicating and increasing viral titer. During the pupal stage, the virus is transferred from the nearby midgut and visceral muscle cells into the tubular salivary gland through contact (Badillo-Vargas *et al.*, 2018). These virions then enter the primary salivary gland where they exit the thrips body in saliva secreted into the host plant during feeding. Since the virions

can only spread if they had been acquired during the larval stage, adult thrips which feed on infected plants will not be able to transmit the virus to other plants (although they can still obtain the virus and harbor the virions in their midgut). It may seem that adult thrips on an infected plant pose no threat with TSWV since they cannot transmit the virus unless they already obtained it while in their larval stage, but these adult thrips might choose to lay their eggs in the tissue of the infected plant which would result in the offspring eating the host and obtaining the virus.

Management of thrips and viruses

Management of thrips can be a difficult task, due to their small body size and ability to be transported freely by taking advantage of small air currents. Cultural control methods can be useful to manage these insects, although the most beneficial approach would be through integrated pest management: combining cultural, chemical, and biological control methods for maximum efficiency. Trying to manage thrips in an area through these methods can often reduce numbers greatly and prevent/reduce viral outbreak in a field. One sampling method to collect thrips in an area is by placing a light colored sheet on the ground and beating nearby crops gently to knock off any insects which were feeding or living on the plants. This method of sampling indicates which thrips if any are currently present on the plant/crop of interest and is preferential for collection instead of monitoring. Another method for sampling is to walk a pre-determined amount of steps while swinging an insect collection net; use of an insect collection net can be used to determine which thrips are in the field during that specific collection date. Since thrips have an enormous range of host plants, many weeds are alternate hosts; this can be managed by eliminating weeds near crop fields as well as any other plants which could

potentially harbor thrips or a known virus. Sanitation in general is an effective strategy. In a greenhouse setting, it can be beneficial to remove non-marketable flowers for any crops as well as removing any individual plants which are known to be infected with thrips and the plants surrounding them. Row covers and very fine mesh fabrics can prevent the insect from physically reaching the plant, preventing colonization and feeding. Vented polyethylene and spunbonded polyester are two types of floating row covers which can result in large reductions of thrips depending on the crops grown in the system. Reflective mulch is also a viable option for many scenarios; these mulches reflect light, confusing the insects and repelling them from the plants. Another viable option is using a cultivar which has resistance to either TSWV or one of its thrips vectors if accessible.

Depending on the species of thrips vectoring TSWV, certain greenhouse biological control options may be available. Thrips have many natural enemies; these predators could potentially be released into an area affected by thrips to control their populations. There are species of mites, green lacewings, parasitoid wasps, and even other thrips which feast on larvae and the eggs of certain species. Some of these predators can be extremely efficient, such as *Neoseiulus barkeri,* a mite which acts as a natural predator for many thrips. When *Neoseiulus barkeri* and *Stratiolaelaos scimitus* were introduced to a greenhouse growing cucumbers which hosted tobacco thrips, over six weeks the thrips population was reduced by 76% and 64%, respectively (Wu *et al.*, 2014). Although these results might seem like biological control is the best way to deal with thrips, using a predator is not always this beneficial. When introducing a predator/parasite to control thrips populations, there are a multitude of additional factors which must also be taken into consideration, such as competition between these species.

When thrips are found to already be a pest in a field the use of insecticides is an option that can manage populations, although some insecticides do not kill the thrips quickly enough to prevent infection. There are additional factors and common mistakes which lead to potential ineffectiveness of pesticides during application, such as timing to correctly affect the thrips population and vulnerable life stages as well as the sprays making physical contact with the plants. Chemical control during a season where thrips are already in the field is not as effective at managing tosposviruses as spraying with a chemical before they arrive, to either act as a deterrent or kill the insects when they make contact. Contact insecticides can be an effective tool if thrips are not present before application, specifically those which are not toxic if ingested by people or animals. Some examples would be neem oil (Bonide Neem Oil, Monterey Horticultural Oil) and insecticidal soaps (Safer Pyrethrin Insecticidal Soap).

Tospovirus Proteins and Functions

Although tospoviruses may infect different plant species and cause different symptoms, their morphology and structure are shared. An external view of the virions would expose a quasi-spherical outer viral envelope, ranging in size from 80-120nm in diameter (Adkins, 2000). Inside this envelope are three single stranded RNA segments: L (large), M (medium), and S (small). The L strand is negative sense, and encodes an RNA dependent RNA polymerase protein used for viral replication. The M strand is ambisense, and encodes for two glycoproteins known as Gn and Gc as well as a non-structural movement protein known as NSm. The S strand is also ambisense, it encodes a non-structural silencing protein known as NSs as well as a nucleocapsid protein known as N (Table 1).

When looking at TSWV protein localization, only the proteins found on the M segment and S segment are capable of expression due to the L segment being too large in size for plasmid construction of the entire L protein (8.9kb). These five proteins have been expressed in plant cells, but their localization is unknown in insect cells (Martin *et al.*, in preparation). It is hypothesized that these proteins will localize in insect cells in similar organelles as plant cells - with the exception of insect cells lacking plasmodesmata, a cell wall, and a large central vacuole. NSm is found on the M genome segment, it is 906bp in size and it's function in plant cells is to form tubules for virion/protein movement through the plasmodesmata. Two other proteins can be found on the M segment: the glycoproteins Gn and Gc. Gn has a size of 990bp, while Gc is made of 820bp. The functions of both of these glycoproteins in plant cells is to assist in attaching virions to cell receptors and promote membrane fusion after endocytosis. Gn is always found in the golgi when expressed, while Gc is found in the golgi when infected and the endoplasmic reticulum when infection is not occurring. The S segment encodes two important proteins for viral infection in plant cells, NSs and N. NSs is a non-structural silencing protein with a size of 1401bp. NSs is responsible for acting as a suppressor of RNA-mediated gene silencing to avoid post-transcriptional gene silencing, and is found in the cell periphery when expressed in plant cells. The final protein capable of viral protein expression is the N protein, responsible for encasing genomic RNA. The N protein is 774bp, and when expressed in plant cells it is found throughout the cell.

Summary

The goal of this master of science degree was to further the understanding of the relationship between viruses in Alabama with their insect vectors, as well as to identify protein localization with TSWV when expressed in insect cells. Findings of these experiments can contribute to the success of future soybean growing seasons, as well as furthering the understanding of tospoviruses and their protein localizations when expressed in insect cells.

Table 1: Size, Function, and Expression Location of TSWV Proteins in Plant Cells

Protein	Size (bp)	Function	Expression Location
N	774	Encases genomic RNA	Throughout cell
NSs	1401	Responsible for acting as a suppressor of RNA-mediated gene silencing to avoid post transcriptional gene silencing	Cell periphery
NSm	906	Forms tubules in plant cells for virion/protein movement through plasmodesmata	Plasmodesmata
Gn	990	Used to help attach virion to a cell receptor and promote membrane fusion after endocytosis	Golgi
Gc	820	Used to help attach virion to a cell receptor and promote membrane fusion after endocytosis	Golgi - inf, ER - uninf

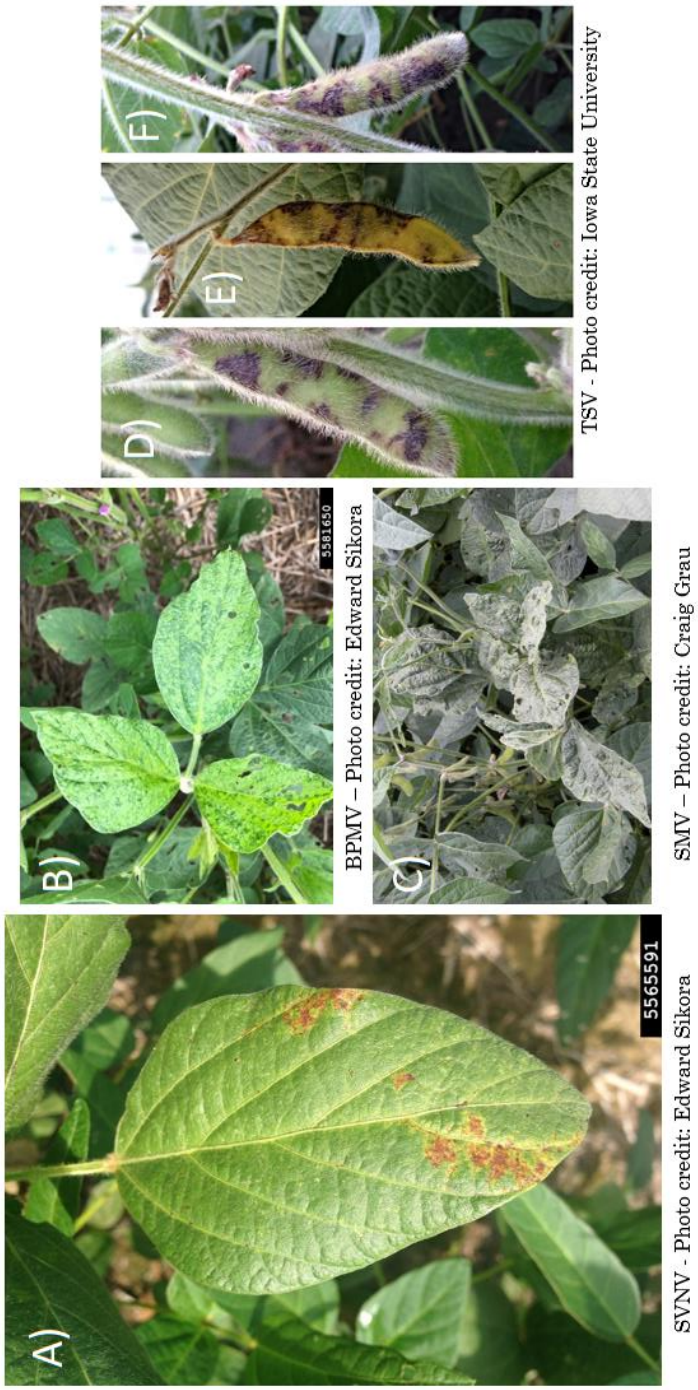

Figure 1 – Symptomatic Soybean Tissue for Viruses Present in Alabama. A) Soybean vein necrosis virus. B) Bean pod mottle virus. C) Soybean mosaic virus. D-F) Tobacco streak virus on pods.

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Chapter Two: Identification of Soybean Viruses and their Insect Vectors During the 2020 Soybean Growing Season

Abstract

Soybean grown in Alabama is at risk of viral infection that may affect the crop yield each growing season. Viruses in Alabama include Tobacco streak virus, Soybean vein necrosis virus, Soybean mosaic virus, and Bean pod mottle virus; spread by a variety of insects, including soybean thrips, aphids, bean leaf beetles, and sharpshooters, respectively. It was hypothesized that a soybean viral survey across the state of Alabama would reveal viruses in the soybean fields as well as the insects responsible for transmitting the viruses. It was discovered that the only virus positively identified was Soybean vein necrosis virus at the Old Rotation in Auburn, vectored by soybean thrips. A hypothesis explaining minimal viral sampling is that high temperatures and significant rainfall during the 2020 soybean growing season allowed soybean rust to rise to high infection in the fields which prevented widespread virus infection.

Introduction

Soybean is a crop with a rich history, the first recordings of cultivation date back to 1100 BC by Chinese farmers in Southeast Asia. Soybean is used both for its meal as well as oil; data from the united soybean board in 2019 revealed that 80% of the soybean grown in the United States is used for meal purposes, while the other 20% is consumed or used as soybean oil (USB Market View Database). Due to the warm growing climate in Alabama, soybean is a favorable crop amongst many farmers and constitutes a large portion of the state's agriculture economy.

In 2019, 9.36 million bushels were grown across 265,000 acres resulting in an estimated value of 135.5 million dollars (USDA National Agricultural Statistics Service).

One problem which may arise during the soybean growing season is the potential for pathogens to infect a field and cause widespread damage to the crop, specifically viruses (Liu *et al.*, 2016). During the Alabama 2020 soybean growing season, four viruses were predicted to occur in the field due to their prevalence in previous growing seasons (Sikora *et al.*, 2017); these viruses include Tobacco Streak Virus (TSV), Soybean Vein Necrosis Virus (SVNV), Soybean Mosaic Virus (SMV), and Bean Pod Mottle Virus (BPMV) (Sikora *et al.*, 2015). These four viruses are all transmitted by insect vectors found in Alabama during the soybean growing season. These insect vectors include soybean thrips (*Neohydatothrips variablilis*), aphids (*Aphididae*), bean leaf beetles (*Ceratoma trifurcate*), and sharpshooters (*Cicadellidae*). Populations of these insects vary across the state of Alabama in abundance and distribution while also being dependent on a variety of factors including: rainfall, heat, humidity, soil temperature, presence of other insects, and food availability amongst many others. These insects have been observed in previous soybean growing seasons, leading to the hypothesis that these insects will be found in soybean fields during subsequent growing seasons and therefore spread viruses which infect soybean.

Our goal was to identify viruses present during the 2020 Alabama soybean growing season, as well as their insect vectors responsible for transmitting the pathogens. Seven nontreated sentinel soybean plots were surveyed in Alabama, specifically grown at locations that are representative of climate and growing seasons across the state. Each site was visited twice during the soybean growing season to survey insects and plants/tissue which appeared

symptomatic for viruses. Insects collected were identified and recorded, while symptomatic tissue was subjected to DAS-ELISA to identify viruses

Methods

Selection of sites

Seven nontreated soybean plots at Alabama agricultural experiment stations were chosen as collection sites for this experiment, Tennessee Valley, Sand Mountain, Clanton, Old Rotation (Auburn campus), Cullar's Rotation (Auburn), EV Smith, and Brewton. Untreated soybean was preferred to allow higher sampling of insects as well as give an idea of what insects were currently in Alabama soybean. The collection sites were strategically selected across the state of Alabama to determine if changes in climate across the state would affect the collection results across the growing season.

Collection of Insects

Collection of insects was performed by sampling random rows of soybean to eliminate bias and sampling error. Sweep collection was used to capture insects populating the soybean fields at the specific time of sampling. Starting at the beginning of two parallel rows of soybean, 100 sweeps were performed while simultaneously walking 100 steps (Figure 1 – A). At the end of the 100 sweeps net contents were emptied into large plastic Ziploc bags and placed on ice until identification (Figure 1 – B). Ten bags were collected during each survey, with one replication taking place at each location across the season.

Identification of insects

Upon arrival at the lab, each collection bag was removed from the container of ice as identification began. Contents of the bags were emptied into large covered petri dishes where insects were checked to see if they were of interest to the study; insects which could not transmit the viruses of interest were not recorded and subsequently disposed of. Insects which were of interest (soybean thrips – *Neohydatothrips variabilis*, aphids, bean-leaf beetles, and sharpshooters) had their populations recorded onto an Excel document.

Collection of plant tissue

Upon arrival at the collection site, each field was searched for plants which appeared symptomatic for any of the four viruses studied. Tissue which had necrosis, chlorosis, vein-browning, mosaic patterning, mottling patterning, or signs of insect feeding were the criteria for tissue suspected to be infected (Figure 1 – C and D). Once an individual plant was identified as symptomatic, tissue was collected from the plant and placed in a large plastic bag. Bags were transported to a -80°C freezer where they were kept until use in Agdia (Elkhart, IN) DAS-ELISA protocols. Five bags of symptomatic tissue from individual plants were collected at each collection site during each visit, with one replication across the experiment resulting in 10 bags total from each location.

Identification of plant tissue

Collected tissue was retrieved from the storage freezer and ran through a leaf-roller to extract all contents of the tissue. Contamination was prevented by cleaning the equipment with ethanol alcohol after each individual extraction. Identification of this plant tissue as positive/negative for the four viruses studied was performed following the Agdia SVNV, TSV,

SMV, and BPMV DAS-ELISA protocol. Upon completion of the Agdia protocol, the DAS-ELISA plate was exposed to light spectrophotometry to quantify the intensity of the results and confirm if individual samples were positive or negative. Negative controls consisted of healthy soybean grown in a lab setting, while positive controls were lyophilized plant tissue confirmed positive for the four viruses of interest. DAS-ELISA samples were considered positive if they were three times higher than the negative control.

Results

Insect Collection

Results from the first collection trip show that at all collection sites, aphids (*Aphididae*) and sharpshooters (*Cicadellidae*) were considered sparse (no more than 35 of either insect at any collection site). Soybean thrips (*Sericothrips variablilis*) were not collected in Brewton, less than 4 total were identified in Auburn, and less than 35 were identified in Clanton and EV Smith (Table 1). Sand Mountain had the highest first soybean thrips collection on July 8th until the first collection at Tennessee Valley just one week later on July 16th. No higher than 50 bean leaf beetles (*Cerotoma trifurcate*) were identified at any location during the first collection, with Clanton having the most identified at 47 (Table 1).

Results from the second collection trip show that at each collection site, aphid and sharpshooters were not identified more than three times (Table 1). Soybean thrips numbers from the second collection are relatively similar to the first collection with two exceptions: the large population increase found in both the Old rotation as well as Cullar's rotation (from 1-260 and 3-101, respectively) just two weeks after the first collection date (Figure 2). Bean leaf

beetle numbers decreased at every site, with the exception of the numbers almost doubling in Tennessee Valley (increase from 26-51) just three weeks after the first collection date (Figure 3).

DAS-ELISA Virus Identification

Results from DAS-ELISAs revealed that only one sample of tissue collected tested as positive for any virus. The positive sample was collected from the Old Rotation, during the second collection trip on August 12th (Figure 4). The sample tested positive for SVNV, which is transmitted by soybean thrips. Negative results were received for SMV, TSV, and BPMV from tissue collected during both the first and second collection (Table 2).

Discussion

Insect Collection and DAS-ELISA

Results from the insect collections revealed that no insects were detected in numbers higher than 51 with the exception of soybean thrips. Soybean thrips were found to decrease in some areas over the time from collection 1 to collection 2 as well as increase at other research sites as expected throughout the growing season. The most noticeable change in collections occurs in Auburn; both Cullar's Rotation and the Old Rotation population numbers increased over 33 times and 260 times, respectively. Soybean thrips are the vector for SVNV, the virus detected in samples at the Old rotation from the second collection. Looking at the rise in soybean thrips from the first and second collection in Auburn, the higher numbers of thrips allowed for higher distribution of the virus.

The only virus which was detected by DAS-ELISA from tissue which was believed to be symptomatic was SVNV. The other viruses – TSV, BPMV, SMV were not detected during this Alabama 2020 soybean viral survey. SMV is transmitted by aphids, which were virtually undetected at every collection site during both the first and second collection with the exception of Clanton's first collection date. With low numbers of aphids found on soybean, SMV potentially had lower chances of causing widespread infection during 2020 in Alabama. Bean leaf beetles were found at almost all sites during both collections, with the highest recorded number being 51. The low amounts of bean leaf beetles found throughout all collections potentially caused BPMV to have difficulty distributing throughout Alabama in 2020 as well.

Although it was not initially part of this experiment, tissue that was collected during this soybean viral survey was later used by another student to mechanically inoculate plants as a lab experiment. These inoculated plants were given four weeks to develop symptoms before they were subjected to DAS-ELISAs for SVNV, TSV, BPMV, and SMV. DAS-ELISAs revealed that one of the mechanically inoculated plants tested positive for TSV, despite the same tissue sample being negative in the initial DAS-ELISA survey. An explanation for false-negative results is that with longer time post collection, tissues might accumulate higher viral titer. With four additional weeks before sampling, the mechanically inoculated plants potentially had more time to accumulate viral titer for detection.

Possible reasons for low virus during 2020 soybean growing season

Although SVNV was detected during the Alabama 2020 soybean growing season, viruses overall were not as prevalent as they had been in recent years. Insects capable of spreading

soybean viruses such as aphids, bean leaf beetles, and sharpshooters were detected in relatively low populations over the course of this survey. The only insect to be found in populations higher than 100 during this experiment were soybean thrips, and these two instances were only located in Auburn and Tennessee valley.

Alabama experienced it's third wettest year in documented history, with several counties reporting record-breaking precipitation in 2020 (National Centers for Environmental Information) (Figure 5). High amounts of precipitation can alter insect behaviors and patterns through negative effects, such as limiting ariel travel and shifting to a more shelter-oriented focus (reference needed). Precipitation can also lead to death for insects, specifically thrips which spend two instar stages in the soil before metamorphosizing into an adult and emerging (reference needed). Alabama not only experienced a wet soybean growing season, but a remarkably warm one as well. According to the National Centers for Environmental Information, approximately half of Alabama experienced above average temperatures while the other half experienced much above average from January to July 2020 (National Centers for Environmental Information).

Climate effects of the 2020 season fostered Soybean rust infections. These infections apparently masked or dominated effects from viruses. Soybean rust symptoms are small brown dots/specks on the plant as well as lesions and yellow areas which can develop on the leaves (Goellner *et al.,* 2010). These symptoms could be mistaken at the time of collection as symptoms for any of the four viruses.

<u>Importance and future studies</u>

Findings from this soybean viral survey can be used to develop new management strategies which would reduce the amounts of pathogens in the field during each growing season. One example could be the decision to change planting dates so that weather conditions select against vectors that transmit pathogens. Waiting until a later or earlier date in the year to plant could result in increased exposure to rain and heat and a reduced population of insects found in the field. Post-harvest identification of soybean rust during the 2020 growing season allows farmers to prepare fungicides and other control methods ahead of time in anticipation of later growing seasons experiencing soybean rust. Results from this 2020 survey can also help towards predictions for viruses and their vectors found in future growing seasons by informing the growers of what to be expecting under certain conditions.

This study will be repeated over the Alabama 2021 soybean growing season to establish what viruses are found across a span of multiple years. Future surveys will allow for recorded data collection of interannual variation of both viruses and insects acting as a vector. This experiment also has the potential to be modified, such as including new viruses/vectors for Alabama soybean as they are introduced.

Table 1 – Insects Collected During 2020 Alabama Soybean Viral Survey

Collection 1	Location						
	Clanton - 7/8	Sand Mtn - 7/8	EV Smith - 7/15	TVA - 7/16	Brewton - 7/22	OR - 7/29	Cullar's - 7/29
Insect							
Sericothrips variabilis	32	78	19	168	0	1	3
Aphididae	32	1	0	0	0	0	0
Cicadellidae	22	31	24	18	8	3	9
Cerotoma trifurcata	47	34	42	26	17	8	11
Collection 2	**Location**						
	Clanton - 8/5	Sand Mtn - 8/5	EV Smith - 7/29	TVA - 8/5	Brewton - 10/15	OR - 8/12	Cullar's - 8/12
Insect							
Sericothrips variabilis	31	18	24	176	0	260	101
Aphididae	0	0	0	0	0	2	0
Cicadellidae	0	3	1	0	0	3	2
Cerotoma trifurcata	2	0	8	51	0	0	3

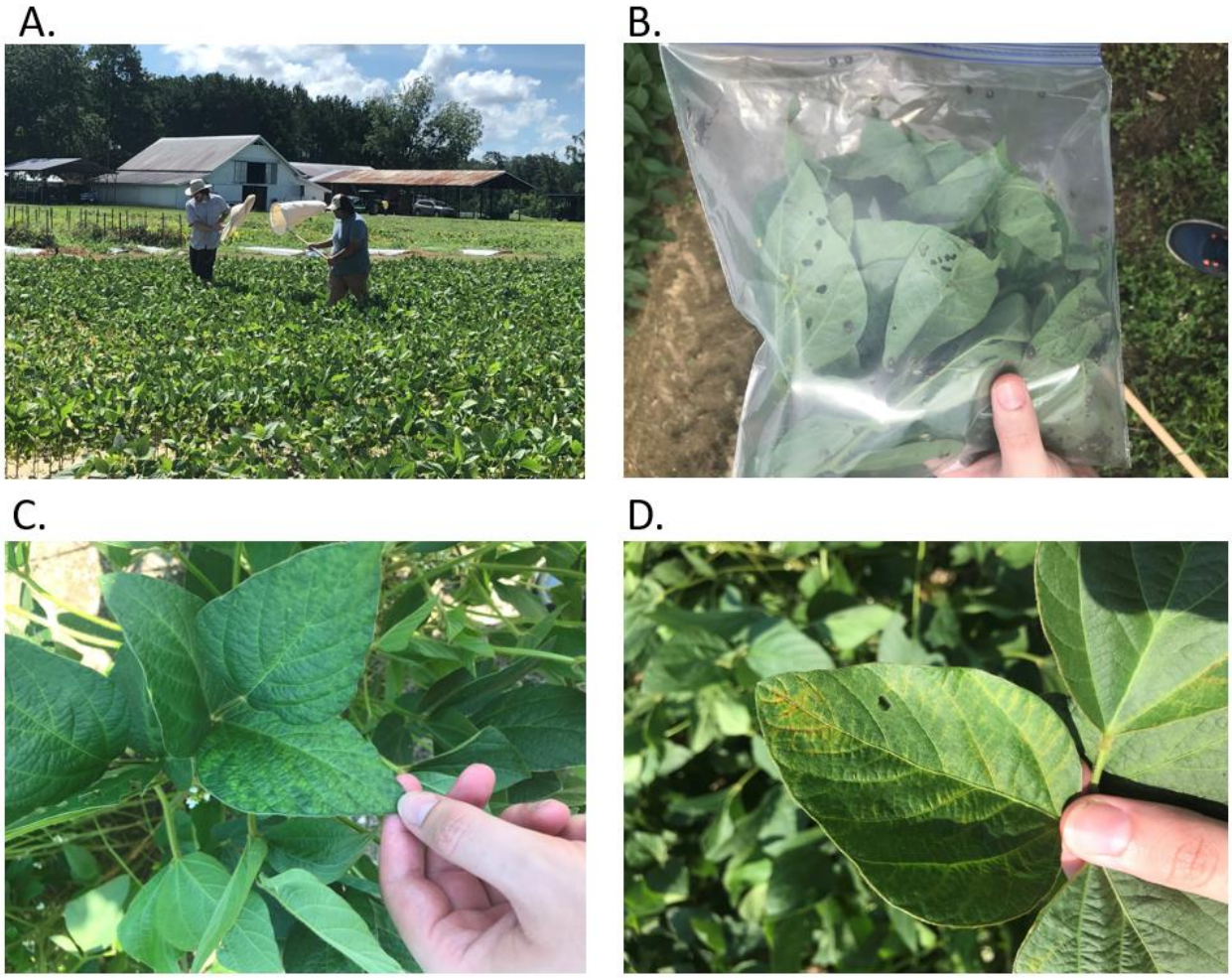

Figure 1 – Collection and Examples of Symptomatic Soybean Tissue. A) Collection of insects using sweep nets. B) Collection bag containing soybean tissue and insects collected from sweep nets. C) Tissue which appeared to be symptomatic for BPMV. D) Tissue which appeared to be symptomatic for SVNV.

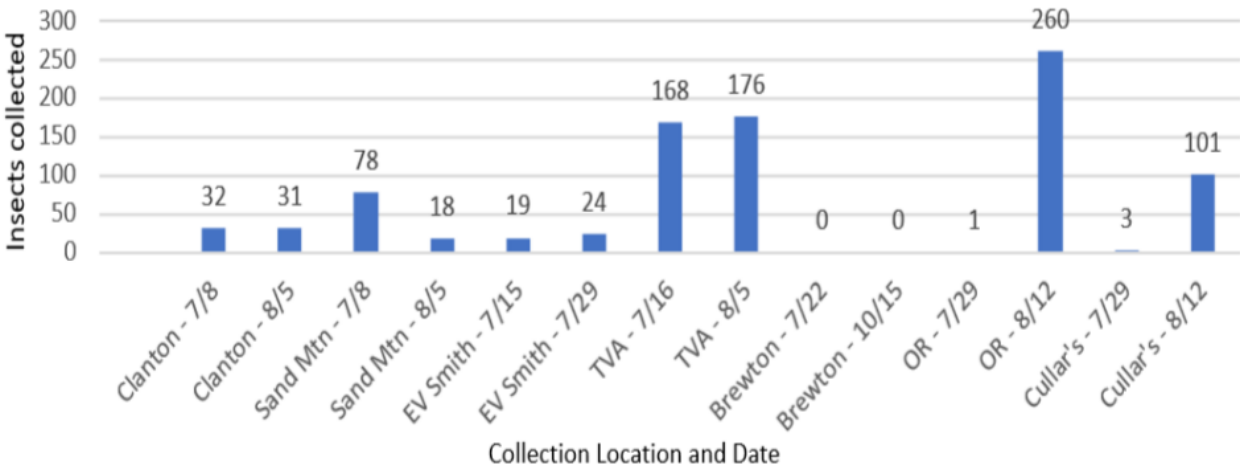

Figure 2 – Soybean Thrips Population Across Collections One and Two. Location and date of collection located on X-axis, number of soybean thrips collected is the Y-axis. Numbers located on the top of each bar represents the number of soybean thrips collected during that individual collection.

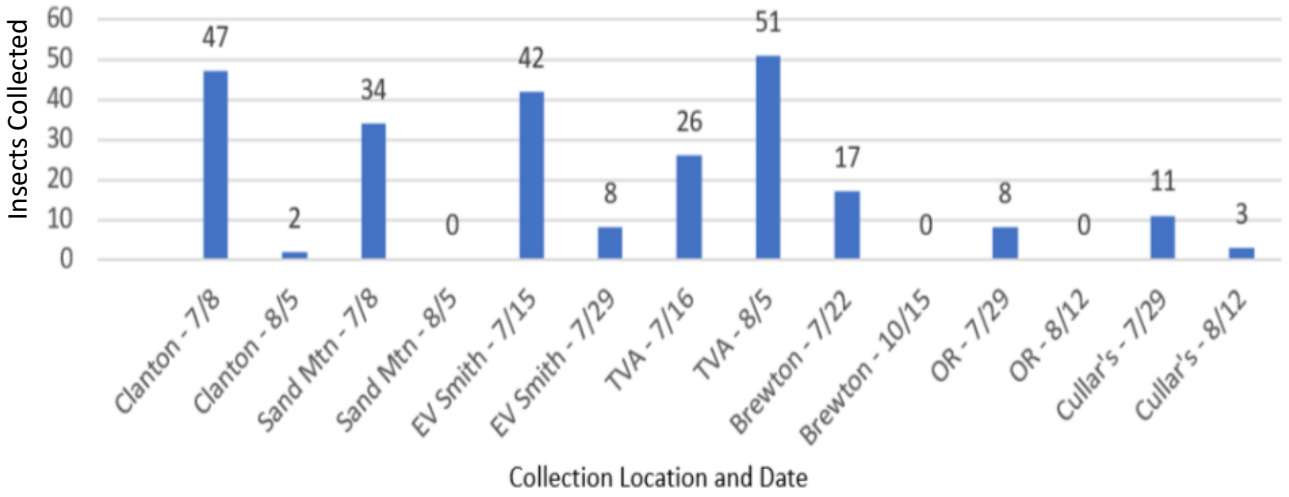

Figure 3 – Bean Leaf Beetle Population Across Collections One and Two. Location and date of collection located on X-axis, number of bean leaf beetles collected is the Y-axis. Numbers located on the top of each bar represents the number of bean leaf beetles collected during that individual collection.

Table 2 – DAS-ELISA Virus Identification Results

Collection 1										
	Date	7/8/2020	7/8/2020	7/15/2020	7/16/2020	7/22/2020	7/29/2020	7/29/2020		
	Location	Clanton	Sand Mtn	EV Smith	TVA	Brewton	Old Rotation	Cullar's		
Virus										
Soybean vein necrosis virus		-	-	-	-	-	-	-		
Soybean mosaic virus		-	-	-	-	-	-	-		
Tobacco streak virus		-	-	-	-	-	-	-		
Bean pod mottle virus		-	-	-	-	-	-	-		
Collection 2										
	Date	8/5/2020	8/5/2020	7/29/2020	8/5/2020	10/15/2020	8/12/2020	8/12/2020		
	Location	Clanton	Sand Mtn	EV Smith	TVA	Brewton	Old Rotation	Cullar's		
Virus										
Soybean vein necrosis virus		-	-	-	-	-	+	-		
Soybean mosaic virus		-	-	-	-	-	-	-		
Tobacco streak virus		-	-	-	-	-	-	-		
Bean pod mottle virus		-	-	-	-	-	-	-		

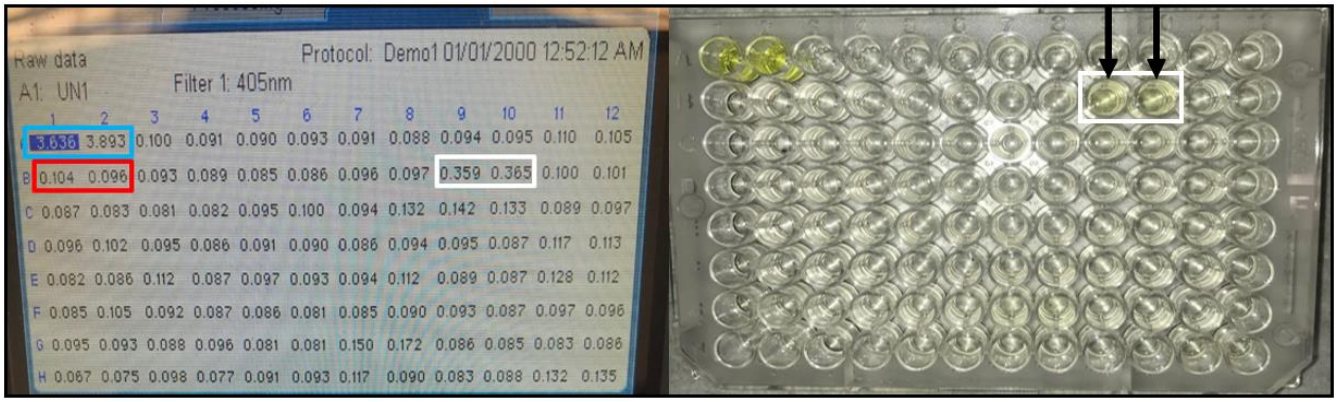

Figure 4 – DAS-ELISA Plate Reader and DAS-ELISA 64 Well Plate. Figure shows the DAS-ELISA plate reader and the 64 well plate which tested positive for SVNV in Auburn during the second collection. Blue box indicates positive control, while the red box indicates negative control (known uninfected tissue). The white box is the sample which tested positive, indicated by both a change in color (inside the white box with black arrows seen on the 64 well plate) and having viral titer values over three times higher than the negative control.

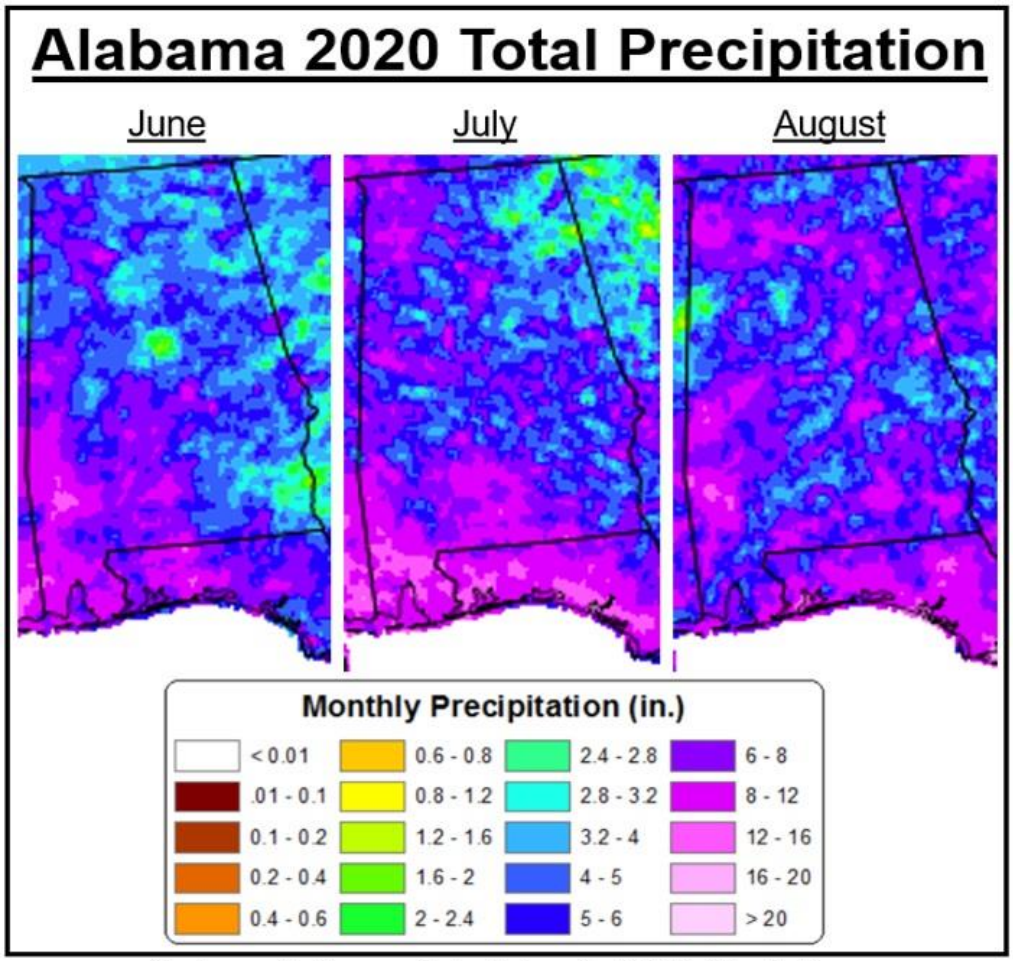

Photo credit: Oregon State University PRISM Climate Group

Figure 5 – Total Precipitation in Alabama from June-August 2020. Photos of Alabama were taken from the Oregon State University PRISM Climate Group across the 2020 soybean growing season (June-August) and compiled together to show monthly precipitation across the state of Alabama.

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Chapter 3: Identification/Expression of Tomato spotted wilt virus Proteins in SF9 Insect Cells

Abstract

Tomato spotted wilt virus (TSWV) protein localization has been identified when expressed in plant cells, while the localization patterns for TSWV proteins when expressed in insect cells has not been examined. It was hypothesized that TSWV proteins would be expressed in similar locations for insect cells as plant cells, with the exception of structural differences between the cell types such as plasmodesmata and cell periphery. Entry clones containing TSWV proteins were transformed with destination vectors containing fluorescent markers. These fluorescent protein constructs were then used to transfect SF9 insect cells for fluorescent microscopy. Localizations for each protein were recorded and have revealed localization patterns for NSm, NSs, N, and Gn proteins, although replicates are still in process.

Introduction

Various legumes are grown throughout the state of Alabama, contributing to the economy through sales and employment. Peanuts are one of the most valuable legumes grown; approximately 400 million pounds of peanuts are harvested annually in Alabama, bringing in over 200 million dollars for the economy. A fraction of the peanuts grown are sold to stores for individual consumption, while large amounts are bought by corporations such as the Mars candy company. The Mars company recently projected that their need for high-quality peanuts will triple by 2025 (CSP Daily News), furthering the need for growers to increase yields. One virus with the capability to lead to high-yield losses with peanuts is Tomato spotted wilt virus

(TSWV) (Sikora *et al.*, 1998). TSWV has an incredibly large host range of over 800 species of plants (Adkins, 2000), however the primary crop of concern in the state of Alabama is peanuts. TSWV has been a concern in Alabama; surveys from previous growing seasons across 288 peanut fields showed that 82% of these fields were infected with TSWV resulting in losses (Hagan, 1990).

TSWV is in the viral family *Tospoviridae*, a family which is capable of infecting plants as well as insects in a circulative, persistent propagative manner – meaning the virus is replicating inside of the insect while crossing tissue/ organ barriers and will stay with the insect throughout it's life. Inside its virion coat there are three segments of viral RNA: L, M, and S. The L segment ("Large") contains 8.9kb and encodes a single polymerase protein (L) (de Haan *et al.,* 1991). The M segment ("Medium") is 5.4kb and encodes a non-structural movement protein (NSm) (Huang *et al.,* 2018) as well as two glycoproteins (Gn and Gc) (Kikkert *et al.,* 2001). The S segment ("Small") is 2.9kb and responsible for encoding a nucleocapsid protein (N) (Lovato *et al.,* 2008) and non-structural silencing protein (NSs) (Margaria *et al.,* 2014). These proteins have various functions: including movement of proteins/virions, capsid formation, suppressing RNA-mediated gene silencing, and helping to attach virions to cell surface after endocytosis (Table 1). All of these proteins along with the L protein in the presence of a suitable host results in successful infection. The Martin lab is interested in the relations between TSWV proteins, and their location of expression during the infection cycle which can give hints towards the function of proteins. TSWV has been studied previously in plants, resulting in the known location for each protein when expressed in plant cells during viral infection (Table 1).

The location of each protein when expressed in insect cells has not been examined, and the results have the potential to increase further understanding of tospoviruses and TSWV. The goal of this experiment was to express TSWV-ORFs fused to sequences encoding fluorescent proteins. pENTR/D-TOPO entry clones containing individual TSWV proteins (obtained from the A. Whitfield lab at NCSU) were recombined with a pIB destination vector containing either GFP or mRFP in two orientations when possible (Figures 1 and 2). Fluorescent microscopy was then performed to reveal the localization of each TSWV protein when expressed in insect cells. It is hypothesized that each protein will be expressed in a similar part of an insect cell correlating to their location in plant cells when possible, such as the cytoplasm or nucleus.

Methods

Construction of plasmids for Transfection

TSWV proteins NSm, NSs, N, Gn, Gc, GcS, and GnS from p-ENTER d-TOPO entry clones were recombined with pIB destination vectors containing either GFP or mRFP using LR gateway cloning. LR Clonase II (Invitrogen) was used following provided protocol to create fusion constructs in one or two orientations when possible (Figures 1 and 2). Plasmids were validated after being sent for sequencing which confirmed constructs.

SF9 Insect Cell Maintenance and Transfection

Lepidopteran 'SF9' cells were transfected at approximately 85-90% confluency with plasmids containing an individual TSWV protein fused to either GFP or mRFP in one or two orientations when possible in a 35 mm^2 dish using Cellfectin II (Gibco) following manufacturer's recommendations. Transfected cells were incubated at 28°C for 72 hours before fluorescent

microscopy was performed. Transfections were performed a total of three times for each construct separately, with a minimum of three fluorescent overlay microscopy photosets taken during each transfection. Each transfection also contained a positive control of pHSP-70-GFP to determine if the transfection itself was successful, as well as a negative control which contained no plasmid DNA to eliminate any internal cell fluorescence.

Results

GFP C1/N1 Fluorescence Comparison

Fluorescent microscopy performed on constructs of GFP fused to NSm, NSs, and N respectively revealed no difference in fluorescence intensity or patterns between the C1 and N1 terminus orientation of the constructs (Table 2). Fluorescence from the constructs was of equal intensity, with the localization of the fluorescent proteins identical when compared to each other (Figure 4). TSWV proteins Gn and Gc, as well as a soluble form of each were not subjected to comparisons between the C1 and N1 terminus due to a signal peptide and transmembrane domain preventing C1 orientation.

GFP Protein Localization

Results of microscopy performed on GFP constructs revealed the localization of NSm when expressed in insect cells. NSm is found throughout the cytoplasm of the cell, forming large aggregates outside of the nucleus (Figure 3).

mRFP C1/N1 Fluorescence Comparison

Fluorescent microscopy performed on constructs of mRFP fused to NSm, NSs, and N respectively revealed no difference in fluorescence between the C1 and N1 terminus constructs. Fluorescence from the constructs was of equal intensity, with the localization of the fluorescent proteins identical when compared (Figure 5). As with mRFP, TSWV proteins Gn and Gc, as well as a soluble form of each were not subjected to comparisons between the C1 and N1 terminus.

mRFP Protein Localization

Results from microscopy performed on mRFP constructs revealed the localization of both N and NSs, respectively when expressed in cells. N forms large congragates which surround the nucleus in the cytoplasm (Figure 5 and 6). During the process of the cell dividing, each nucleus is joined by a congregate of N-expressing cytoplasm (Figure 5). When expressed, NSs was found to be localized throughout the cytoplasm in smaller, scattered agregates surrounding the nucleus (Figure 6).

Discussion

Comparison of N1/C1 Constructs

Fluorescent microscopy was performed on all TSWV proteins capable of being in N1 and C1 orientations (N, NSm, NSs). Fluorescence was of the same intensity for each of the two constructs, on all of the proteins capable of the two orientations (NSm, NSs, and N). Fluorescence between the two constructs did not alter in intensity or location when tested with either mRFP (Figure 5) or GFP (Figure 4). Results from the comparison of N1/C1 constructs show

that there is no difference for protein localization when expressed for NSm, NSs, or N in either the C1 or N1 orientation (Table 2).

Localization of TSWV Proteins in SF9 Cells

Fluorescent microscopy performed on TSWV proteins fused to GFP has revealed the localization for NSm, N, NSs, and Gn when expressed in SF9 insect cells (Figure 3). Of the photographed proteins, NSm-GFP is the only construct to currently have three successful fluorescent overlay photosets complete. When expressed in insect cells NSm is found surrounding the nucleus, while NSm is found in the plasmodesmata in plant cells (reference needed). Plasmodesmata are a structure unique to plant cells, making the cytoplasmic localization for insect cells the closest cellular comparison. NSm uses the plasmodesmata in plant cells for protein/virion transportation (Zhao *et al.,* 2014); it would make sense that when these specialized transport structures are unavailable NSm resorts to localizing in the cytoplasm. N and NSs were also found in the cytoplasm of insect cells, while N is found in the cytoplasm for plants and NSs is found in the cellular periphery of plant cells (Figure 3). N is the nucleocapsid protein, making it necessary to be found throughout both cell types in the cytoplasm to encapsulate proteins (Li *et al.,* 2014). NSs is the non-structural silencing protein, found in the cell periphery in plant cells which is a structure not found in insect cells (Hedil *et al.,* 2015). Cell periphery is the near-outside of plant cells where the cell wall and cytoplasm meet, making the cytoplasm the closest structure in insect cells. Gn was also photographed in one complete photoset, revealing the location of expression in insect cells to be in the cytoplasm directly outside of the nucleus. In plant cells Gn is located in the golgi, meaning the localization in insect cells is completely different since localization appears to occur in the

cytoplasm. Complete photosets in all overlays/wavelengths are in progress for NSs-GFP, N-GFP, Gn-GFP, GnS-GFP, Gc-GFP, and GcS-GFP as well as repeats for Gn to conclude if the current results are anomalies or accurate.

Future Studies

Once localization of individual TSWV proteins has been recorded when expressed in insect cells, co-localization studies can be performed. These co-localization studies would reveal if TSWV proteins change localization patterns when expressed in pairs. Future studies could also be performed on other tospoviruses present in Alabama, such as Soybean vein necrosis virus. SVNV protein localization is currently unknown in both plant and insect cells, allowing for individual protein expression in both systems to be explored.

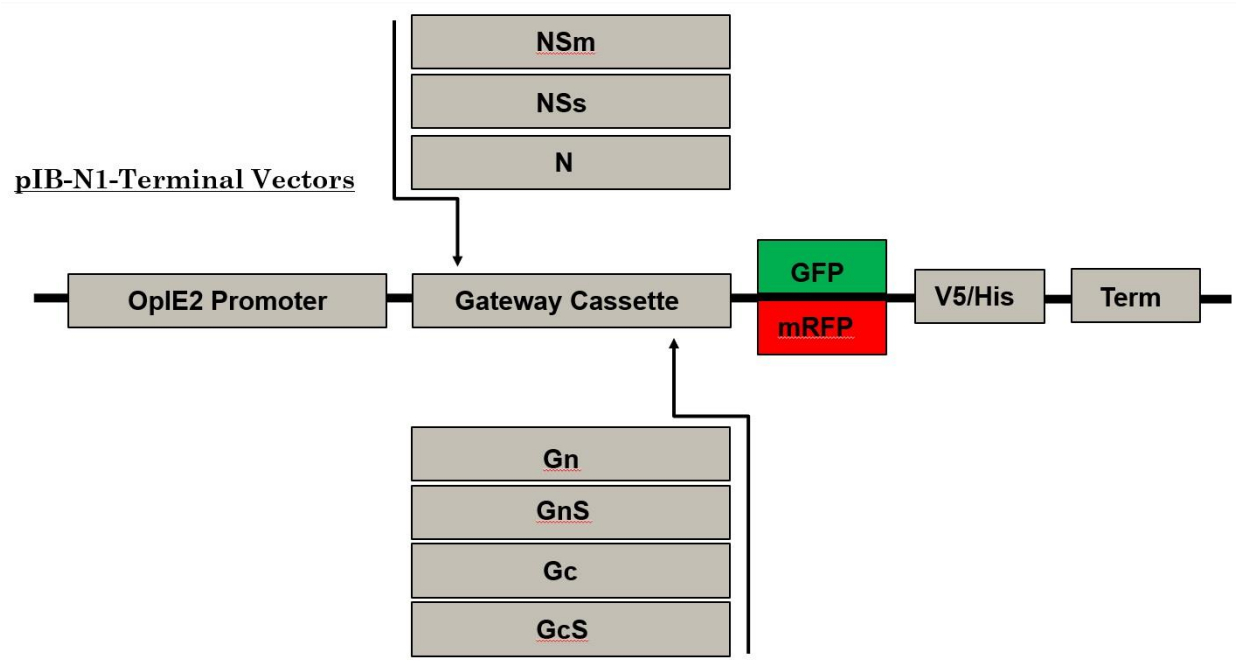

Figure 1: pIB-N1-Terminal Vectors. Linear view of constructs used for transfection of TSWV proteins. OpIE2 promoter is located before the gateway cassette, which in this orientation is located before the fluorescent tags. Arrows indicate individual TSWV proteins which replace the gateway cassette during transfection.

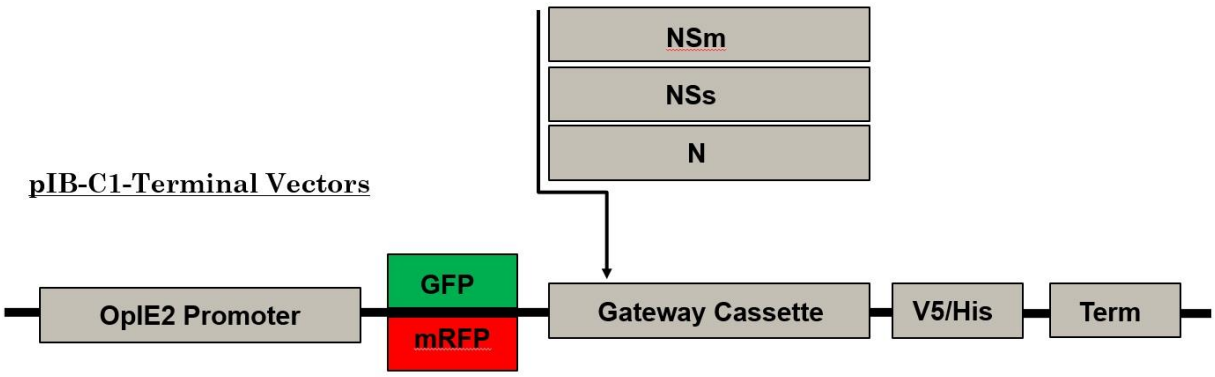

Figure 2: pIB-C1-Terminal Vectors. Linear view of constructs used for transfection of TSWV proteins. OpIE2 promoter is located before the fluorescent tags, which in this orientation is located before the gateway cassette. Arrows indicate individual TSWV proteins which replace the gateway cassette during transfection.

Table 1: Size, Function, and Expression Location of TSWV Proteins in Plant Cells

Protein	Size (bp)	Function	Expression Location
N	774	Encases genomic RNA	All throughout cell
NSs	1401	Responsible for acting as a suppressor of RNA-mediated gene silencing to avoid post transcriptional gene silencing	Cell periphery
NSm	906	Forms tubules in plant cells for virion/protein movement through plasmodesmata	Plasmodesmata
Gn	990	Used to help attach virion to a cell receptor and promote membrane fusion after endocytosis	Golgi
Gc	820	Used to help attach virion to a cell receptor and promote membrane fusion after endocytosis	Golgi - inf, ER - uninf

GFP
DAPI
BRIGHT
OVERLAY 1
OVERLAY 2
NSm-GFP-C1
NSs-GFP-C1
N-GFP-C1
Gn-GFP-C1
GnS-GFP-C1
Gc-GFP-C1
GcS-GFP-C1

Figure 3 – GFP Localization of TSWV Proteins in SF9 Insect Cells. Column 1, localization of TSWV proteins fused to GFP. Column 2, localization of nuclear stain. Column 3, brightfield view of cell. Column 4, overlay of columns 1,2, and 3. Column 5, overlay of columns 1 and 2. Rows indicate TSWV protein fused to GFP. Rows from top to bottom are as follows: Row 1, TSWV-NSm-GFP-C1. Row 2, TSWV-NSs-GFP-C1. Row 3, TSWV-N-GFP-C1. Row 4, TSWV-Gn-GFP-C1. Row 5, TSWV-GnS-GFP-C1. Row 6, TSWV-Gc-GFP-C1. Row 7, TSWV-GcS-GFP-C1.

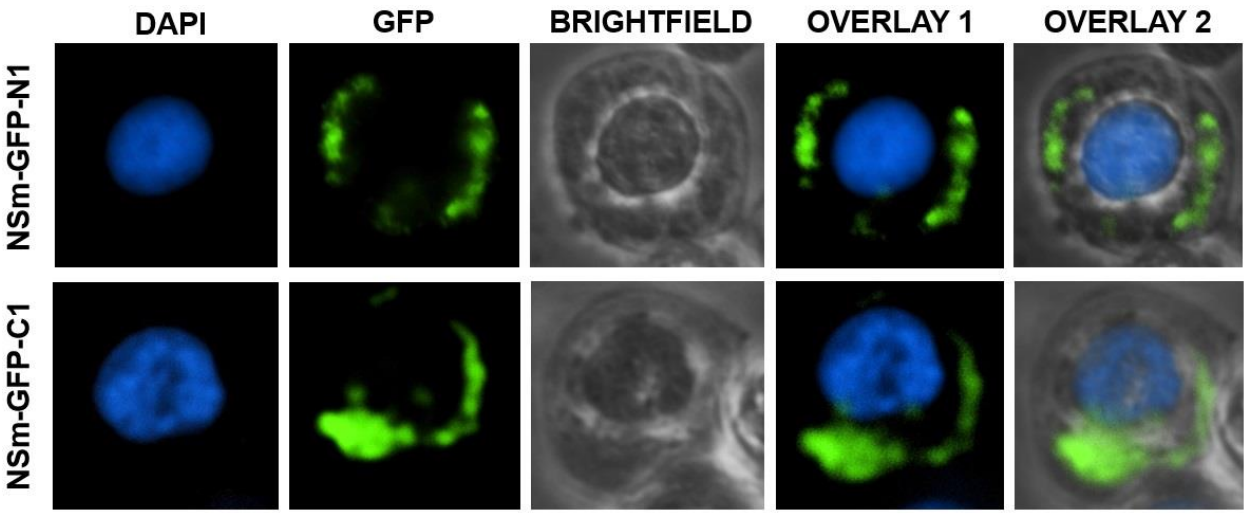

Figure 4: GFP C1/N1 Fluorescence Comparison. Column 1, localization of nuclear stain. Column 2, localization of TSWV NSm protein fused to GFP. Column 3, brightfield view of cell. Column 4, overlay of columns 1 and 2. Column 5, overlay of columns 1, 2, and 3. Rows indicate TSWV protein fused to GFP. Rows from top to bottom are as follows: Row 1, TSWV-NSm-GFP-N1. Row 2, TSWV-NSm-GFP-C1.

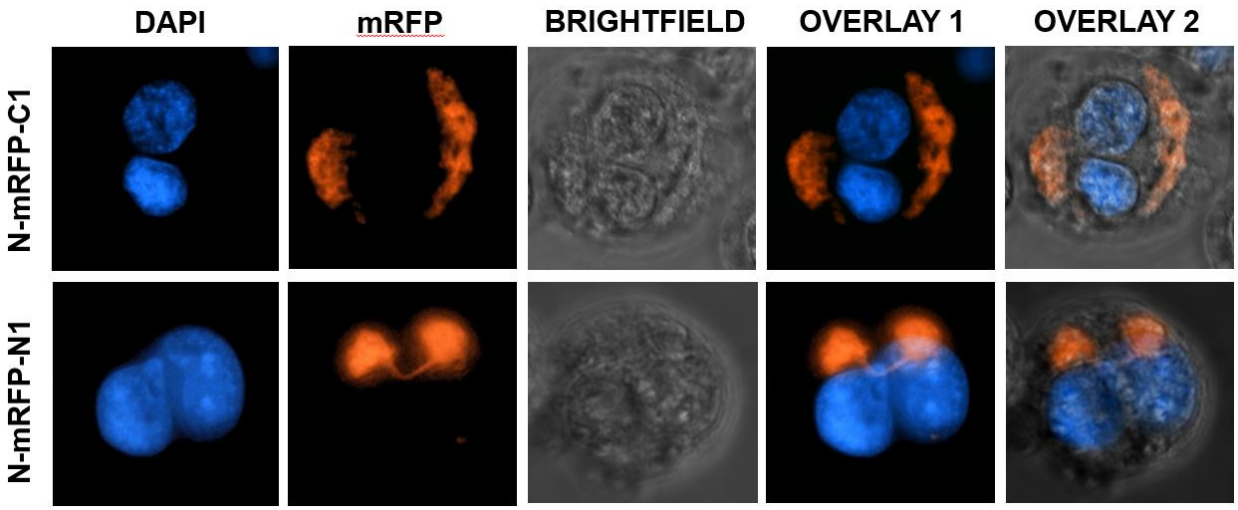

Figure 5: mRFP C1/N1 Fluorescence Comparison. Column 1, localization of nuclear stain. Column 2, localization of TSWV N protein fused to mRFP. Column 3, brightfield view of cell. Column 4, overlay of columns 1 and 2. Column 5, overlay of columns 1, 2, and 3. Rows indicate TSWV protein fused to mRFP. Rows from top to bottom are as follows: Row 1, TSWV-N-mRFP-C1. Row 2, TSWV-N-mRFP-N1.

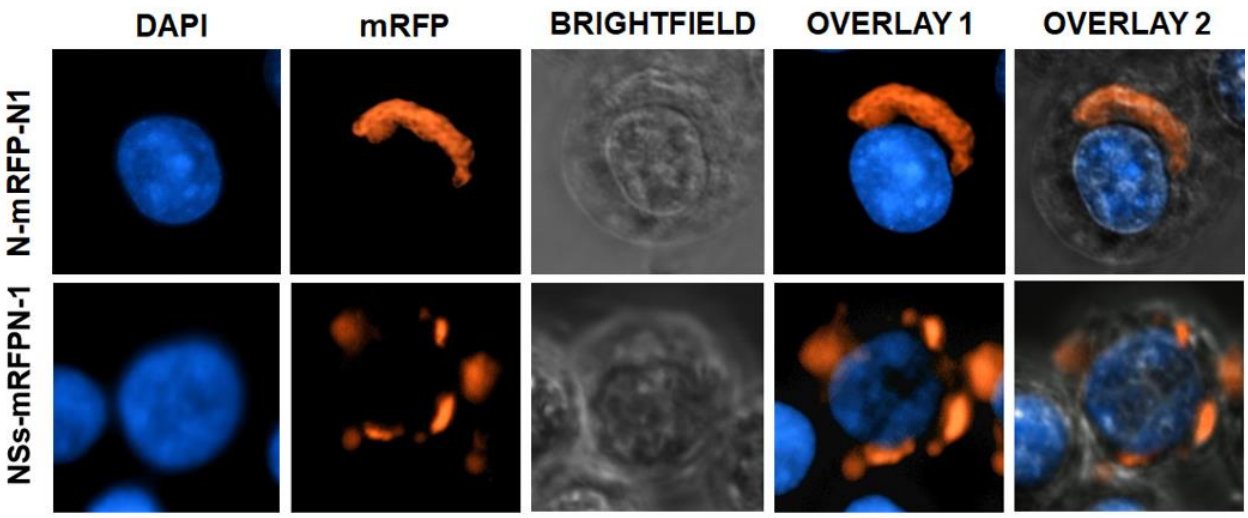

Figure 6: mRFP Localization of TSWV NSs and N Protein in SF9 Insect Cells. Column 1, localization of nuclear stain. Column 2, localization of TSWV proteins fused to mRFP. Column 3, brightfield view of cell. Column 4, overlay of columns 1 and 2. Column 5, overlay of columns 1, 2, and 3. Rows indicate TSWV protein fused to mRFP. Rows from top to bottom are as follows: Row 1, TSWV-N-mRFP-N1. Row 2, TSWV-NSs-mRFP-N1.

Table 2: TSWV Proteins N1/C1 Comparison and Localization

Name	Initial Insect Results	Plant Expression	Do N1/C1 agree?
N	Cytoplasm	Cytoplasm	Yes
NSm	Surrounding nucleus	Plasmodesmata	Yes
NSs	Cytoplasm	Cell periphery	Yes

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