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Nicole E. Bacheller

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**DETECTING, CLONING, AND SCREENING FOR SUPPRESSORS OF RNA
SILENCING IN *MAIZE CHLOROTIC MOTTLE VIRUS* AND *SUGARCANE
MOSAIC VIRUS***

by

Nicole E. Bacheller

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Agronomy

Under the Supervision of Professor Hernan Garcia-Ruiz

Lincoln, Nebraska

April, 2017

**DETECTING, CLONING, AND SCREENING FOR SUPPRESSORS OF RNA
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Nicole E. Bacheller, M.S.

University of Nebraska, 2017

Advisor: Hernan Garcia-Ruiz

Maize lethal necrosis disease (MLND) is one of the most important viral diseases of maize. MLND occurs when *Maize chlorotic mottle virus* (MCMV) co-infects the same plant with one of several potyviruses, including *Sugarcane mosaic virus*, *Wheat streak mosaic virus* or *Maize dwarf mosaic virus*. Originally prevalent in the Midwest and Peru in the 1970s, the disease was called corn lethal necrosis (CLN) and was controlled through breeding and sanitation. Recently, the disease has re-emerged in East Africa and is rapidly spreading and threatening the food sources of subsistence-farming populations. This re-emergence has raised several questions about the unknown molecular mechanisms of MLND. RNA silencing is a prominent antiviral defense system in plants that may be involved in viral synergism. In single and double infections, MCMV and SCMV activate maize antiviral RNA silencing machinery, resulting in the accumulation of virus-derived small RNAs. Most plant viruses encode proteins called viral suppressors of RNA silencing (VSRs) to inactivate RNA silencing and overcome the host defense system. VSRs have been identified in

several potyviruses but no silencing suppressor has been identified in MCMV. In this project, protocols to detect both SCMV and MCMV in plant tissue were established and optimized. A clone of the MCMV Nebraska isolate (MCMV-NE) and each open reading frame (ORF) of MCMV and SCMV were constructed for *Agrobacterium* infiltration. To identify silencing suppressors in MCMV and SCMV, individual proteins were cloned into binary vectors for transient expression in *Nicotiana benthamiana* and candidate proteins with silencing suppression activity have been identified. Identification and characterization of VSRs in MCMV and SCMV establishes the foundation to further study the molecular mechanisms involved in MLND.

ACKNOWLEDGEMENTS

My gratitude to each of you who have helped me along this journey extends far beyond the reaches of this page. Thank you to The University of Nebraska-Lincoln, the Department of Plant Pathology and the Nebraska Center for Virology for providing the infrastructure and opportunity to work, study, research and grow. In addition to the strong leadership of Dr. Charles Wood and Dr. James Steadman, I wish to recognize the remarkable support from their staff. Thank you to Tiffani, Jan, Margaret and Marlene for having the answers to all of my questions. This gratitude continues to all of the professors, classmates and colleagues who inspired, challenged and expanded my mind, especially my fellow plant-path graduate students and mentors.

My appreciation extends to my advisor Dr. Hernan Garcia-Ruiz for supporting me as his first graduate student and as a technician. We started from square one and we built a laboratory! Thank you to my graduate committee members: to Dr. James Van Etten for providing support and advice, and to the entire Van Etten lab for being good neighbors and for setting an example for scientific excellence and teamwork for our green lab to follow. To Dr. Amit Mitra for going above and beyond his responsibilities as committee member and department graduate student advisor. Thank you for the helping me with all of my questions and concerns and for sharing with me your knowledge and stories of science and life. Your mentorship is greatly appreciated.

Hats off to all of my coworkers for working alongside me and giving me the chance to develop my training and teaching skills, especially to all of the undergrads who brought their energy to the lab. Thank you Tania for showing me that it was possible, and Stella for bringing your enthusiasm and demonstrating a sense of responsibility far beyond the scope of most undergraduates.

I send gratitude to my dear friends in Lincoln and around the world. To my friends, colleagues and mentors from Minneapolis, Madison and Montpelier for cheering me on from afar. To the friends in Lincoln who opened their circles to me and became my Lincoln family: because of you I have been truly blessed in this chapter of my story. To my roommates and all of the ladies of the Holdrege House Sisterhood, especially Georgia, Katya, and Nichole: thank you for your advice and support and for listening and sharing the occasional much needed meal or kitchen dance party. Special praise goes to Suzana and Annabel for motivating me to write with them every weekend and for inspiring me with discussions and laughter. My infinite gratitude to Gayan for your steadfast commitment to helping me over the hurdles and conundrums I faced during my program, for being my mentor and cheerleader in science and in life.

Finally, love and appreciation to my family: to my parents, Thomas and Mary Bacheller, to my sister Emily and to Mary Katherine and Larry. Thank you for the phone calls, advice, support, love, care packages, visits and much-needed holidays. Thank you for believing in me and telling me not to worry so much.

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CHAPTER 1
LITERATURE REVIEW
THE RE-EMERGENCE OF MAIZE LETHAL NECROSIS DISEASE

INTRODUCTION

Maize (*Zea mays*) is the number one cultivated crop in the world (Shiferaw et al., 2011). Nearly one billion metric tons of maize are produced each year (USDA, 2015). It is the primary food source for millions of people around the world. While maize is commonly used for fuel or animal feed in developed countries, 85% of the maize produced in sub-Saharan Africa is used for food (Shiferaw et al., 2011). However, there are several constraints that limit the production of maize. One of the major constraints on yield is disease. Diseases caused by bacteria, fungi, viruses, nematodes and other pathogens are estimated to cause annual crop losses of 60 billion dollars (Hsu, 2002). In 2012, an estimated eight billion dollars of maize was lost to disease (Oerke and Dehne, 2004; Loebenstein and Katis, 2014). In maize alone, over 50 virus species have been identified (Lapierre and Signoret, 2004). Viral infections weaken plants and cause yield loss, quality reduction and in severe cases, plant death. MCMV causes disease and yield loss alone and in synergistic infections with several potyviruses to cause Maize Lethal Necrosis Disease (MLND).

MCMV is the type species and lone member of the genus *Machlomovirus* in the family *Tombusviridae*. MCMV infection in maize results in yield losses of 10 to 15% (Castillo and Herbert, 1974; Nault et al., 1981). However, when MCMV co-infects with a potyvirus, including *Wheat streak mosaic virus* (WSMV), *Maize dwarf mosaic virus* (MDMV) or *Sugarcane mosaic virus* (SCMV), a much more severe disease develops. This synergist disease, known as Maize lethal necrosis disease (MLND) or Corn lethal necrosis disease (CLND), causes yield losses of

50 to 90% (Niblett and Claflin, 1978; Uyemoto et al., 1980; Wangai et al., 2012). MCMV was first discovered in maize in Peru in 1973 (Castillo and Herbert, 1974). MCMV was first reported in the United States in 1976 when it was identified causing MLND in association with *Maize dwarf mosaic virus* (MDMV) and *Wheat streak mosaic virus* (WSMV) in maize in Kansas (Niblett and Claflin, 1978). Other viruses from the *Potyviridae* family have since been found in co-infections with MCMV causing MLND. Subsequent incidences of MCMV were reported in Argentina (Teyssandier et al., 1983), Thailand (Sutabutra and Klinkong, 1983) and Mexico (Gordon et al., 1984) in the 1980's and in Hawaii and Colombia in the 1990's (Jiang et al., 1992; Morales et al., 1999). No reports of MCMV came from new countries for approximately ten years between the late 1990s and 2009 when MCMV was reported in China and Kenya in (Xie et al., 2011; Wangai et al., 2012). It is MCMV's recent re-emergence, global spread and role in MLND that have brought MCMV back into the spotlight. Immediate expedited research into the epidemiology and molecular biology of MCMV is required. This review aims to summarize our current understanding of MCMV and its role in MLND.

HOST RANGE

The host range of Maize *chlorotic mottle virus* is limited to the Poaceae (Graminieae) family (Castillo and Herbert, 1974; Bockelman et al., 1982; Scheets, 2004). Maize is MCMV's natural host and dicotyledonous plant species are not susceptible to natural infection or mechanical inoculation (Castillo and Herbert, 1974; Niblett and Claflin, 1978). Susceptible maize varieties include

sweet corn, hybrids, dent inbreds and popcorn (Gordon et al., 1984). Maize, sugarcane, sorghum, wheat and several species of grasses are the only known hosts for MCMV (Uyemoto et al., 1980; Bockelman et al., 1982; Wang et al., 2014). No species are known to serve as overwintering hosts.

SYMPTOMS

Symptom development is correlated with plant growth stage, the age of plants at the time of infection and environmental conditions (Scheets, 2004). Symptoms of MCMV alone include chlorotic streaks on leaves running parallel to veins early in infection (10 days post infection (dpi)) expanding to chlorotic mottling in later infection (Nelson et al., 2011). Ear development is reduced in quantity and size and ears may be short, malformed and partially filled with grain and exhibit prematurely aged husks (Nelson et al., 2011). When MCMV is co-infected with a potyvirus, MLND symptoms are similar but generally escalate quicker and to a greater severity and are more likely to cause higher yield loss and plant death. Symptoms of MLND include leaf chlorosis and necrosis, stunting from the shortening of internodes, and plant death (Castillo and Herbert, 1974; Uyemoto et al., 1980). Late infections established beyond the 14-leaf stage may not impact ear size but may result in reduced kernel quality (Uyemoto, 1981). Male inflorescences may be short and exhibit hard panicles, short rachis and few spikelets. Mottling typically begins at the base of young leaves and extends upwards. Necrosis begins at the leaf margins and moves towards the ribs to cover the entire leaf. When necrosis destroys young leaves in the whorl before

they fully expand the symptom is called “dead heart” and is usually followed by plant death (Makumbi and Wangai, 2013). These symptoms leave farmers facing low yield and low-quality grains.

VIRAL SYNERGISM

The molecular and genetic interactions between *Maize chlorotic mottle virus*, associated potyviruses and their maize host in MLND are not yet well understood. Many potyviruses are known to be involved in synergistic interactions with unrelated viruses (Pruss et al., 1997; Syller, 2012). The classic example of potyviral synergism is illustrated by the interaction between the type species of the *Potyvirus* genus *Potato virus Y* (PVY) and *Potato virus X* (PVX) in *Nicotiana benthamiana*. Enhanced disease symptoms and a dramatic increase in the titer of PVX with no parallel increase in PVY concentration are hallmarks of this co-infection (Rochow and Ross, 1955; Vance, 1991). In co-infections of MCMV with MDMV-B, WSMV or SCMV, MCMV shows a marked increase in concentration as compared to MCMV concentration in single infections (Goldberg and Brakke, 1987; Scheets, 1998; Xia et al., 2016). In many potyviruses, HC-Pro has been identified as a suppressor of post-transcriptional gene silencing (PTGS) (Pruss et al., 1997; Vance and Vaucheret, 2001). Potyviral HC-Pro can mediate viral synergism in co-infections (Vance et al., 1995; Shi et al., 1997; Stenger et al., 2007). Other potyviruses have other silencing suppressors that are involved in viral synergism (Stenger et al., 2007; Tatineni et al., 2012). A silencing

suppressor in MCMV has not yet been identified, and silencing suppression could be involved in the development of MLND.

MCMV and SCMV co-infection induces deleterious changes in cell structure and organelles (Wang et al., 2017). Chloroplasts of cells co-infected with SCMV and MCMV exhibit smaller starch grains than mock or MCMV infected cells, suggesting that photosynthesis is reduced during co-infection. A measured 7-fold reduction in the mRNA level of the pyruvate orthophosphate dikinase (PPDK) enzyme supports the hypothesis that chloroplasts are damaged and photosynthesis is reduced by MLND. The mitochondria of MCMV-infected cells show disorganized cristae and in co-infected cells, the disruption can be severe enough to cause leaking of mitochondrial content. In co-infections, the damage to mitochondria happens earlier on in infection and is more severe, potentially explaining the accelerated damage to plants affected by MLND (Wang et al., 2017).

INSECT VECOTRS

The number of insect species known to transmit MCMV has dramatically increased from six species of beetles to include several other unrelated insect vectors. The increase in known vectors of MCMV parallels the geographical incidence of the virus as it expanded from the Western hemisphere to the rest of the world. Understanding the role of vectors in the transmission of MCMV is essential for understanding the epidemiology and control of MCMV and MLND.

Six species of beetles from the Chrysomelidae family that are able to transmit MCMV, including the cereal leaf beetle (*Oulema melanopa*), corn flea beetle (*Chaetocnema pulicaria*), flea beetle (*Systema frontalis*), southern corn rootworm (*Diabrotica undecimpunctata*), western corn rootworm (*Diabrotica virgifera*), and northern corn rootworm (*Diabrotica longicornis*) (Nault et al., 1978). Japanese beetle (*Popillia japonica*) and black cutworm (*Agrotis ipsilon*) and six species representing the suborder homopteran are not vectors of MCMV. Five of these six species, including *Rhopalosiphum padi*, *Myzus persicae*, *Schizaphis graminum*, *Peregrinus maidis*, *Graminella nigrifrons* (leafhoppers) but not *Trialeuroides vaporariorum* (whiteflies), are known vectors of other maize viruses. *O. melanopa* is a vector of *Cocksfoot mottle virus* and *Phleum mottle virus* in addition to MCMV (Serjeant, 1967; Nault et al., 1978). *O. melanopa* transmits MCMV more efficiently in the larval stage than in the adult stage. This is unique among the three viruses vectored by *O. melanopa*. There was also no evidence to prove that infectious larvae retained the ability to transmit the virus after molting into the adult phase.

The ability of southern, western and northern corn rootworms to transmit MCMV is not related to age, sex or genotype of *Diabrotica* and no latent period is required before transmission of the virus (Jensen, 1985). MCMV is only present in trace amount in the insects' hemolymph and does not proliferate infection in new hosts, suggesting that the virus is not replicated in or interacting with the circulative system of the vector. However, no inhibitors have been found in the hemolymph or gut. In addition, the virus is not passed transovarially to offspring.

Temperature, 25°C being optimal, has a significant impact on MCMV transmission and is likely correlated to feeding behavior. Adult beetles are active feeders and migrators and may travel several hundred miles within a few days. While larvae are also able to transmit the virus, they do not travel more than a few centimeters in the soil.

Frankliniella williamsi thrips from the order Thysanoptera and family Thripidae were later identified as an aboveground vector of MCMV (Jiang et al., 1992; Nelson et al., 2011). A thrip's ability to transmit MCMV decreases overtime until 6dpi when transmission no longer occurs (Cabanas et al., 2013). Furthermore, virus titers decrease after thrips feed on uninfected tissue. Like *Diabrotica*, adult thrips that emerge from the pupae of infected larvae are not able to transmit MCMV. Another species of thrips, Western Flower Thrips (WFT) (*Frankliniella occidentalis* Pergande), also vectors MCMV and are pests of many agriculturally important crops including over 500 species from 50 different families (Yudin et al., 1986; Zhao et al., 2014). WFT are native to North America but have spread to Europe, Australia and South America. WFT are vectors of several other important plant viruses including the Tospoviruses *Tomato spotted wilt virus* (TSWV) and *Impatiens necrotic spot virus* (INSV). The principal vector responsible for the transmission of MCMV in Africa has not yet been identified. Exposed residues on the viral capsid surface are likely the targets of vectors (Wang et al., 2015). Therefore, it is necessary to further understand the physical surface characteristics of MCMV in order to investigate virus-vector interactions and transmission.

SEED TRANSMISSION

In addition to transmission by insect vectors, MCMV is transmitted at a very low level by seed (Jensen, 1991). Seed transmission was initially considered to explain how MCMV emerged on the isolated islands of Hawaii and can help explain how the virus has spread globally. In a set of 42,000 seeds tested, MCMV was confirmed in 17 seeds (Jensen et al., 1991). This established the rate of MCMV transmission by seed to be between 0.008 and 0.04 percent. Seed transmission varies considerably by seed lot and could be influenced by genotype or other unknown factors. Infection has never been proven from growing clean seeds in infected debris. Seed transmission is an important factor in the epidemiology and spread that could not be explained by insect vectors alone.

EPIDEMIOLOGY AND ECOLOGY

Following the initial discovery of MCMV in Peru and Kansas, MCMV incidences have occurred in various locations around the world. In the United States, MLND was widespread in Kansas and Nebraska but never spread within the continental United States (Uyemoto et al., 1980; Doupnik et al., 1982; Jensen et al., 1991). Over the next four decades, MCMV emerged in maize growing regions around the world. Potyviruses, including MDMV, SCMV and WSMV, were already endemic in many of these regions, and the arrival of MCMV presented an imminent threat of MLND epidemics. MCMV was confirmed in maize fields in Argentina, Thailand, Mexico and Colombia (Sutabutra and

Klinkong, 1983; Teyssandier et al., 1983; Gordon et al., 1984; Morales et al., 1999).

MCMV appeared on the island of Kauai in Hawaii and devastated temperate seed corn production without any associated potyviruses before spreading to the islands of Oahu and Maui, also production sites of susceptible varieties of maize (Jiang et al., 1992; Nelson et al., 2011). The Hawaiian Maize chlorotic mottle disease epidemic is the only event of MCMV causing major damage without an associated potyvirus.

The next published report of MCMV incidence in a new country did not occur for over a decade. MCMV was confirmed in the Yunnan region of China co-infecting maize with SMCV, and a severe case of MLND erupted in maize fields in Taiwan, causing yield losses in sweet corn (Xie et al., 2011; Deng et al., 2014; Wang et al., 2017). To date, MCMV has only been found in the Yunnan and Sichuan regions of China and these isolates cluster with the Taiwan isolate in a group distinct from the MCMV-KS and MCMV-NE isolates (Wang et al., 2017).

The most concerning outbreak of MCMV occurred in the Southern Rift Valley of Kenya in 2011 (Wangai et al., 2012). SCMV had been present in Kenya since at least the 1980s and the population of *Frankliniella williamsi* Hood thrips in symptomatic fields was high (Kulkarni, 1973; Louie, 1980). Maize showed signature MLND symptoms and samples tested positive for both MCMV and SCMV. This confirmed the first incidence of MCMV and MLND in what would become a major epidemic in sub-Saharan Africa (Wangai et al., 2012).

In sub-Saharan Africa, maize is the most important cereal crop and is a major source of calories in the diets of millions of people (IDRC). MLND is a major threat to food security and the livelihoods of over 300 million people in Africa; many are subsistence farmers that depend on maize for both their food and livelihoods, or are otherwise vulnerable to a poor harvest (CIMMYT, July 12, 2013). In Kenya an estimated 77,000 acres of maize were affected by MLND in 2012, resulting in yield losses of 126 million metric tons equal to \$52 million US dollars (Wangai et al., 2012). MCMV rapidly spread to Rwanda and the Democratic Republic of the Congo (Adams et al., 2014; Lukanda et al., 2014). MCMV is predicted to continue to spread to maize production regions across Africa and interact synergistically with established potyviruses to cause MLND (Isabirye and Rwomushana, 2016).

Every severe case of MLND shares several factors that lead from an incident to an epidemic. Monocultures of susceptible maize created an environment where both viruses and vectors thrived. In many cases, year-round cropping of susceptible maize eliminated a temporal gap to quell vector populations. In contrast, fields in the Midwestern United States that were planted with crops other than maize the previous year have predominantly lower rates of MLND (Phillips et al., 1982; Uyemoto, 1983). Several factors, including the year-round, geographically-continuous cropping of monocultures of maize, the lack of infrastructure and education surrounding disease diagnosis and control, and the central role of maize in the diets of millions of people in Sub-Saharan make the re-emergence of MLND an imminent threat to food security.

DETECTION and DIAGNOSIS

Several methods are used to detect MCMV. Symptoms caused by numerous viruses are similar to symptoms caused by MCMV in the same hosts and symptoms vary under different environmental conditions (Mahuku et al., 2015). Therefore, in most cases, symptomatology is not sufficient for diagnosing MCMV. However, MCMV is readily transmissible by sap and the inoculation of specific diagnostic species can aid in the confirmation of MCMV. The sorghum (*Sorghum bicolor*) cultivar Asgrow Bugoff is a diagnostic species used to differentiate between MCMV and MDMV. Asgrow Bugoff is immune to MCMV but shows symptoms, including distinctive red coloration, when inoculated with MDMV strains A and B (Uyemoto et al., 1980). Wheat (*Triticum aestivum*) cultivar 'Parker' is used to distinguish between MCMV, WSMV and MDMV. In Parker, WSMV causes chlorotic streaking, MDMV does not cause infection, and MCMV causes mild mottling (Uyemoto et al., 1980).

Serological methods have proven to be both sensitive and reliable (Uyemoto, 1980; Townsend et al., 1990). Double immunodiffusion (DID) and enzyme-linked immunosorbent assays (ELISA) can effectively detect MCMV (Uyemoto, 1980). ELISA is able to detect virus even in low titers and can differentiate between serotypes (Uyemoto, 1980). MCMV is a moderate to strong immunogen and can be detected by monoclonal and polyclonal antibodies (Niblett and Claflin, 1978; Castillo et al., 1991; Wu et al., 2013). Northern blot hybridization techniques can be used to detect MCMV RNA (Lommel et al., 1991b). Electron microscopy is also used to visualize viral particles in

symptomatic tissue (Xie et al., 2011). RT-PCR with primers specific to viral sequences is effective for detecting MCMV in maize seeds and tissues (Zhang et al., 2011). Real-time RT-PCR with fluorescent probes detects concentrations of MCMV in seeds as low as 4fg/ μ L, lower than any other methods (Zhang et al., 2011). Another rapid and sensitive method for detecting MCMV uses a biosensor based on surface plasmon resonance (SPR) that enables the detection of MCMV at much lower concentrations (> 1 ppb) and much faster (30 minutes) than ELISA detection (Zeng et al., 2013). The SPR method does not require sample preparation beyond crude extractions. These systems are proposed to be more practical and rapid for situations that require immediate detection, such as testing seeds before they cross international borders in customs.

DISEASE MANAGEMENT

The best way to manage MLND and other viral diseases is to prevent the introduction of the virus and vector into an area or field. This can be achieved through quarantine, the use of clean seed, diligent monitoring and accurate detection. Planting certified virus-free seed prevents the introduction of the virus into a field. Once a virus is present in a field, there are several options to control the disease, including sanitation, crop rotation, vector control and the implementation of genetic resistance. Sanitation includes the removal of infected plants, including residues and grassy weed hosts that may serve as reservoirs for the virus and vectors. Maize cropping can be alternated with non-host crops to disrupt the pathogen and vector life cycles. This is especially important in

tropical and subtropical regions where maize is planted year-round because continuous maize monocultures are especially susceptible to epidemics. Incidences of MCMV and MLND have been effectively controlled with crop rotation to non-hosts (Phillips et al., 1982; Uyemoto, 1983).

These methods require proper monitoring and diagnostics to understand when the pathogen is present and needs to be controlled. Effective management of MCMV and MLND will also require an integrated combination of these approaches. Effective disease management in large-scale maize production areas may integrate the use of resistant varieties, chemical control of vectors and cultural practices (Nelson et al., 2011).

The recipe for effective control must be adapted for different regions. Control methods that were effective in commercial settings in the United States, for example, may not be directly translatable to disease control in smallholder farms in sub-Saharan Africa. Education is at the foundation of control in sub-Saharan Africa. Growers must understand the disease, what the symptoms look like and what are the potentially devastating implications of having and spreading the virus. Because saving and sharing seed is an integral part of the culture in many regions, an understanding of the risk of seed transmission is necessary to mitigate spread through infected seed (Mahuku et al., 2015). Access to affordable seed for culturally desirable varieties is essential for growers to be incentivized to purchase clean seed and resistant varieties. The lack of infrastructure and communication to educate farmers and instate quarantine

regulations on seed and pathogens increases the threat of MCMV and MLND spreading trans-continentially across Africa.

The development and implementation of maize varieties genetically resistant to MCMV can effectively alleviate MCMV and MLND in an economically and environmentally viable way. Appropriate use of resistant varieties also reduces the need for insecticides to control vector populations. More is known about genetic resistance to economically important potyviruses than is known about resistance to MCMV (Mahuku et al., 2015). Planting varieties resistant to potyviruses that interact synergistically with MCMV is one way to control MLND. Maize varieties and germplasm ranging from tolerant to resistant to MCMV have been identified (Nelson et al., 2011; Mahuku et al., 2015). Resistant sweet corn and field corn lines are being screened and developed (Nelson et al., 2011). Quantitative trait loci (QTL) mapping and breeding are being implemented in the development of stress-resistant maize for production in sub-Saharan Africa, including maize resistant to MLND (Semagn et al., 2015). CIMMYT and the Kenya Agriculture and Livestock Research Institute (KALRO) began screening maize lines in 2012. Very few lines with resistance were found, but efforts are underway for enhancing resistance and qualities desirable to sub-Saharan African farmers. QTL mapping studies aiming to identify regions associated with MLND-resistance are also in progress and resistant sites are to be used as targets for breeding resistant lines. Screening is underway in both Kenya and Ohio for resistance to MCMV and MLND (Mahuku et al., 2015). At this time, no

transgenic approaches have been reported to manage the disease but this approach has not been ruled out.

MCMV GENOME

The 4.4Kb positive-sense, single-stranded RNA genome of MCMV is singly encapsulated in icosahedron protein shells approximately 30nm in diameter (Nutter et al., 1989; Lommel et al., 1991a; Stenger and French, 2008). Capsids are composed of 180 identical subunits each weighing approximately 38kDa (Wang et al., 2015). MCMV lacks a 5' cap, a polyA tail and a viral genome-linked protein (Nutter et al., 1989; Hull, 2002). The Nebraska isolate of MCMV (MCMV-NE) is 4436 nucleotides while the Kansas isolate (MCMV-KS) is 4437 nucleotides in length (Nutter et al., 1989; Stenger and French, 2008). These isolates share 99.5% nucleotide sequence identity, suggesting a very recent common ancestor (Stenger and French, 2008). The isolates differ at 22 nucleotides, 20 of which are within coding regions, resulting in a total of nine amino acid substitutions in coding regions (Stenger and French, 2008).

The nucleotide sequences of the Kansas, Nebraska, and several Chinese isolates have been determined (Nutter et al., 1989; Stenger and French, 2008; Xie et al., 2011; Wang et al., 2017). Since the publication of genome sequences, the functions of some MCMV proteins have been determined. Homology to genes with similar sequences and positions in other viruses in the *Tombusviridae* family, including members of the Carmovirus genus, *Carnation mottle virus* and *Turnip crinkle virus*, has aided in predicting the functions of genes in MCMV

(Nutter et al., 1989; Scheets, 2016). The genome of MCMV has six open reading frames encoding six proteins distributed across one genomic and two subgenomic RNAs. All member of the family *Tombusviridae* produce sgRNAs (Scheets, 2000). The genomic RNA is 4436 nucleotides in length and encodes three proteins. Three more proteins are expressed from a subgenomic RNA which is 1467 or 1468 nucleotides in length, beginning at nucleotide 2970 or 2971 (Scheets, 2000). A second subgenomic RNA of 337 nucleotides is produced starting at nucleotide 4101 but does not encode any proteins and has no known function (Scheets, 2000). The genomic RNA encodes proteins P32, P50, and P111, named by their weights in kilodaltons. P111 results from a read-through of the stop codon between P51 and P60. P32, P50 and P111 are expressed early in infection. P50 and P111 are the only proteins required for viral replication and they function at low levels in *trans* (Scheets, 2016). SgRNA1 is homologous to the 3' terminus of the genomic RNA and encodes four proteins: P7a, P7b, the coat protein (CP) and P31. P31 results from a read-through of the stop codon between P7 and P24 (Scheets, 2000). The coding region is preceded at the 5' end by 24 non-coding nucleotides (Scheets, 2000). None of the proteins produced from sgRNA1 are required for replication (Scheets, 2016).

Putative functions of these genes have been studied using homology to closely related viruses in the *Tombusvirus* genera and through mutagenesis studies (Scheets, 2016). Based on sequence homology to related Tombusviruses, P50 is inferred to be the small subunit of the RNA-dependent RNA polymerase while P111 is the large subunit (Uniprot; Scheets, 2000). P7 is

involved in cell-to-cell movement (Scheets, 2016). The function of P31 is unknown but the protein is expressed late in infection and may enhance systemic viral movement (Scheets, 2000; Scheets, 2016). The CP of MCMV is 25.4kDa and was identified by immunoprecipitation with CP antisera (Nutter et al., 1989; Lommel et al., 1991b). The CP is also required for cell-to-cell movement (Scheets, 2016). P32, a 32kDa protein at the 5' proximal end of the genomic RNA is unique to MCMV. P32 is not required for viral replication but its presence increases the accumulation of viral proteins, while its absence decreases viral accumulation and disease symptoms (Scheets, 2016). MCMV infectious clones have been made for *in vitro* transcription studies (Scheets et al., 1993) and for Agro-infiltration (Wang et al., 2017).

FUTURE DIRECTIONS

Despite the 40 years since the discovery of MCMV, much remains unknown about the biology of the virus and how to control it. Although MCMV causes disease alone, it is more of a problem in co-infections with a potyvirus causing MLND, which is major threat to maize production. The recent dissemination of MCMV across the globe and to Eastern Africa presents a major epidemiological puzzle and a major threat to food security. It demands the immediate attention and collaboration of farmers, breeders, scientists and communities to understand and combat the disease with effective education, control and the deployment of resistant maize varieties. The immediate steps require continued mapping and breeding efforts and a release of effective and

appropriate resistant lines, an advanced understanding of the molecular biology of MCMV, communication with farmers about preventing and the management of the disease and appropriate quarantine and infrastructure regulations to stop the spread across Africa and to new regions.

CHAPTER 2

DETECTION OF *MAIZE CHLOROTIC MOTTLE VIRUS*

AND

SUGARCANE MOSAIC VIRUS

IN MAIZE

INTRODUCTION

Accurate diagnosis of viruses from field samples is essential for management, experimental and breeding purposes. Commercial tests are available to screen for a wide range of plant viruses, including those involved in MLND. We developed highly specific tests to confirm the presence of MCMV and SCMV in samples used in our lab. The objective of these experiments was to develop and refine inoculation protocols for MCMV and SCMV and rapid and reliable protocols to detect one or both viruses from symptomatic maize tissue for experimental purposes. Viral protein and RNA were extracted from mock, single or double infected maize plants and used to establish standard molecular detection protocols using Western and Northern blotting and PCR-based methods.

MATERIALS AND METHODS

Maize inoculation

SDp2 hybrid maize was selected as the preferred line for MLND inoculations because it is susceptible to and shows defined symptoms of both MCMV and SCMV alone and in co-infections. Single seeds were planted one inch deep in potting mix in four-inch square pots. Plants were grown under greenhouse conditions and were inoculated at the V2 leaf stage when two true leaves were present, approximately 14 days after seeding.

MCMV-NE was re-activated from an infected maize leaf dried in 1985 that was provided by Dr. Satyanarayana Tatineni (USDA, ARS, Lincoln, Nebraska).

Subsequent inoculum was prepared from fresh or frozen, virus-infected tissue. To prepare MCMV inoculum, one gram of SDp2 maize tissue showing MCMV symptoms was ground in liquid nitrogen at room temperature with a clean mortar and pestle. Four mL of 50mM potassium phosphate buffer, pH 7.5 were added and the samples were homogenized. The suspension was centrifuged at 4°C for ten minutes at 4,800 RPM and the supernatant was collected and kept on ice. The same protocol was repeated with one gram of SCMV-infected Sart sorghum tissue. The supernatant from each preparation was mixed with potassium phosphate buffer in a one-to-one ratio (Table 2.1). A double inoculation was established by mixing both viruses in a one-to one ratio. All virus preps were kept on ice at all times. SDp2 maize and Sart sorghum were selected as hosts for greenhouse inoculations because they are susceptible and show defined symptoms of MCMV and SCMV, respectively.

Table 2.1. Preparation of inoculum for single and double infections of MCMV and SCMV

Treatment	Virus	Volume (μ L)			
		Buffer	SCMV	MCMV	Total
T1	Mock	1000	0	0	1000
T2	SCMV	500	500	0	1000
T3	MCMV	500	0	500	1000
T4	SCMV + MCMV	0	500	500	1000

For each treatment, ten plants were inoculated. All plants were dusted with carborundum and rub-inoculated with a cotton swab soaked in buffer or the corresponding virus treatment. Two leaves were inoculated per plant. A third leaf

was inoculated if present. The flag leaf was omitted. All leaves were rinsed after the inoculum dried to remove the carborundum. Plants were monitored for the onset of symptoms and tissue was collected 14 dpi (days post inoculation), frozen in liquid nitrogen and stored at -80°C.

Protein extraction

One gram of fresh or frozen maize tissue was ground in liquid nitrogen in clean, chilled mortars and pestles at room temperature in 4mL of Glycine Grinding Buffer (0.1M Glycine-NaOH, pH9.0, 0.1M NaCl, 10mM EDTA, 2% sodium dodecyl sulfate, 1% sodium lauroylsarcosine, (Várallyay et al., 2010)). Samples were centrifuged at 10,000 RPM for five minutes at 4°C and the supernatant was collected. Protein samples were obtained by mixing 200µL of the supernatant with 200µL of 2X Protein Dissociation Buffer (0.0625M Tris pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 10% 2-mercaptoethanol, saturated bromophenol blue) and boiled for three minutes at 100°C. The remaining supernatant was used for RNA extraction. Samples were stored at -80°C.

Western blotting

A commercial antibody, PVAS-52 anti-SCMV Strain D CP (ATCC, Manassas, VA) is available for the detection of SCMV coat protein (CP). Five µg of X/2 dilutions of protein samples were run on 12% acrylamide TGX Stain-Free™ FastCast™ Acrylamide gels (Bio-Rad, Hercules, CA) following the

manufacturer's instructions in 1X Tris-Glycine/SDS buffer. The gels were transferred to Amersham protran 45µm nitrocellulose protein membranes (GE Healthcare, Little Chalfont, UK) for 90 minutes at 95 volts in Mini Trans-Blot® Electrophoretic Transfer Cells (Bio-Rad, Hercules, CA) in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). Gels were stained with Ponceau solution (Sigma-Aldrich, St. Louis, MO) to visualize Rubisco and confirm equal loading of the samples. The blots were de-stained with potassium buffered saline with 0.1% Tween (1X PBS-T) and incubated for one hour at room temperature in 5% milk. The blots were incubated in a 1:1250 dilution of PVAS-52 anti-SCMV strain D CP in 1% milk overnight at 4°C with shaking followed by rinsing and a one-hour incubation in a 1:5000 dilution of secondary antibody Anti-rabbit IgG NA934-1 (GE Healthcare, Little Chalfont, UK) in 1X PBS-T. The blots were developed with Clarity™ Western ECL Blotting Substrate following the manufacturer's guidelines and exposed in a ChemiDoc Imager for ten seconds (Bio-Rad, Hercules, CA).

RNA extraction

To extract RNA, 1mL aliquots of the remaining supernatant were mixed with 800µL of TRIzol Reagent (Invitrogen, Carlsbad, CA) and centrifuged at 8,300xg for three minutes at 4°C. The supernatant was transferred to a tube with 500 µL of Chloroform, vortexed and centrifuged at 14,000 rpm for ten minutes at 4°C. The supernatant was transferred and the chloroform step repeated two times. To precipitate RNA, the supernatant was transferred to a tube with 1mL of

chilled isopropanol, mixed gently and incubated at room temperature for fifteen minutes. After centrifugation at 14,000 RPM for ten minutes at 4°C, the resulting pellet was washed in 70% ethanol and incubated at -80°C overnight. The ethanol was removed and the pellets re-suspended in 50µL of 0.1X TE and normalized to 1µg/µL. One µg of each sample was mixed with 5µL RNA loading dye (95% formamide, 0.025% SDS, 0.025% Bromophenol blue, 0.025% xylene cyanol, 0.5mM EDTA) and run for 40 minutes on a 2% agarose gel at 85V to confirm the presence of total RNA (Figure 2C).

Northern blotting

Four µg of each sample were electrophoresed in a 1% agarose, 2.7% formaldehyde high molecular RNA gels at 90V for one hour. Gels were wet capillary transferred overnight to a nylon membrane (Roche, Basel, Germany) and auto-crosslinked. A template for the MCMV probe was prepared from the partial clone pMCM1067 provided by Dr. Kay Scheets (Oklahoma State University, Stillwater, OK). A 300bp region of the pMCM1067 plasmid was amplified by Taq DNA polymerase and digoxigenin-dUTP (DIG) random labeled using a DIG DNA labeling kit (Roche, Basel, Switzerland). The membranes were pre-hybridized for 6 hours at 55°C in NorthernMax buffer (Ambion, Foster City, CA). Eight µL of the random labeled probe were incubated at 60°C with the blot overnight. The DIG probe was detected by hybridization with Anti-DIG-AP and developed by CDP-Star® Chemiluminescent Substrate solution before imaging (Sigma-Aldrich, St. Louis, MO). An 18sRNA probe was designed and processed

in a parallel hybridization as a loading control. The MCMV probe detected multiple MCMV segments in samples inoculated with MCMV as well as MCMV and SCMV.

Detection by RT-PCR

Reverse transcriptase and sequence-specific primers were used for the detection of gene sequences specific to SCMV and MCMV (Figure 2.1). Four micrograms of total RNA from each mock, SCMV, MCMV and co-infected samples were mixed with 5.8 μ L of nuclease-free water and subjected to DNase treatment using TURBO DNA-free Kit and following the manufacturer's protocol (Ambion, Foster City, CA). The supernatant was removed and saved on ice or at -80°C. Five μ L of each RNA sample was mixed with random primers from the ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA) and cDNA synthesis was performed according to the manufacturer's protocol.

Without dilution, 2 μ L of cDNA from each treatment was subjected to PCR with primers specific for MCMV CP or a fragment of the SCMV N1b gene (Table 2.2). Maize GAPDH was amplified as a loading control (Lin et al., 2014). These amplicons were selected because of the fidelity of the primers and the ease of differentiating among the bands amplified for each viral gene and the maize control. Two μ L of undiluted cDNA were mixed with PCR components to a final volume of 50 μ L with Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA). Reactions were incubated in a thermocycler following

the manufacturer's three-step protocol. Each reaction was denatured at 98°C for 30 seconds followed by 30 cycles of denaturing, annealing, and extension before a final extension at 72°C for five minutes. The 30 cycles were as follows: 10 seconds at 98°C, 20 seconds at 61°C, 10 seconds at 72°C for Nib, 10 seconds at 98°C, 20 seconds at 54°C and 8 seconds at 72°C for GAPDH, and 10 seconds at 98°C, 20 seconds at 60°C and 30 seconds at 72°C for the MCMV CP. PCR products (50µL) were cleaned using the E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek, Norcross, GA). The GAPDH product was eluted in 22µL of water, and SCMV Nib and MCMV CP products in 40µL. Ten µL of GAPDH, 7.5µL of Nib and 5µL of CP PCR product were mixed with 5µL of xylene DNA loading dye and loaded into a 2% agarose gel and electrophoresed for 20 to 30 minutes at 80V.

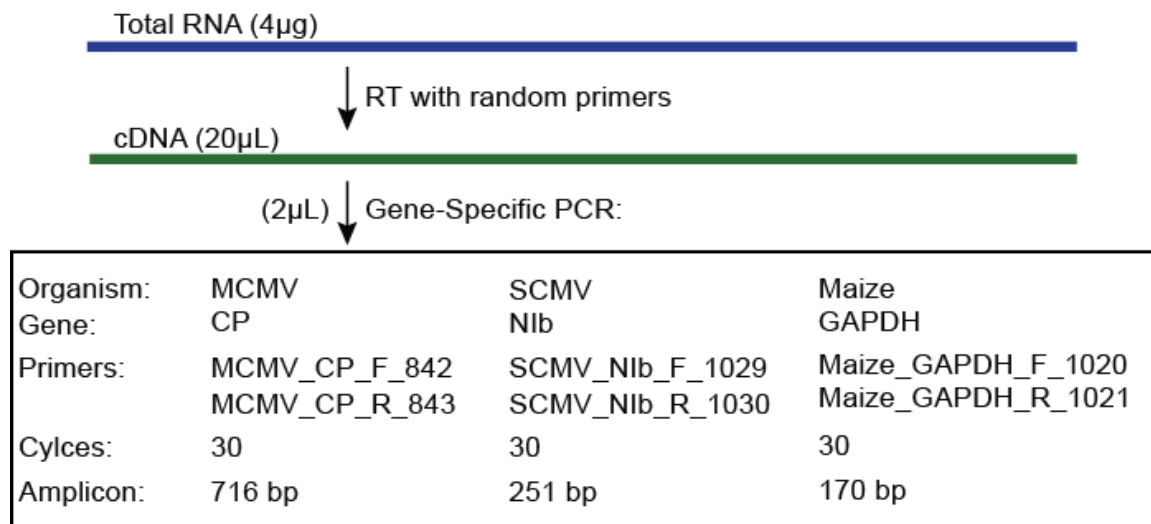


Figure 2.1. RT-PCR and gene-specific PCR strategy for the detection of MCMV and SCMV. Using the NEB ProtoScript First Strand cDNA Synthesis Kit (#E6300S), total RNA was treated with reverse transcriptase and random primers to generate cDNA. cDNA was used as a template for the amplification of sequences specific to each virus following the cycling condition listed. Maize GAPDH was amplified as a loading control.

Table 2.2. Primers used for the amplification of sequences to detect MCMV and SCMV and maize GAPDH.

Organism	Gene	Primers	Primer Sequence
MCMV	CP	MCMV_CP_F	CACCatggcggcaagtagccgg
		MCMV_CP_R	AGATCtcaatgattgccagccctgg
SCMV	Nlb	SCMV_Nlb_F	attgccgagacagcactccgc
		SCMV_Nlb_R	gtgctactaccagatcctgcccc
Maize	GAPDH	Maize_GAPDH_F	ccatcactgccacacagaaaac
		Maize_GAPDH_R	aggaacacggaaggacataccag

RESULTS

Greenhouse inoculation methods using infected tissue as inoculum consistently reproduced single viral infections and MLND in SDp2 maize (Figure 2A). Beyond visual symptoms, SCMV, MCMV and MLND were confirmed in inoculated maize samples using Western blotting, Northern blotting and gene-specific PCR methods. SCMV was detected by probing for the SCMV coat protein in protein samples by Western blotting with SCMV-CP strain D specific antibody. Samples infected with SCMV, either with or without MCMV, tested positive for the approximately 40kD SCMV coat protein (Figure 2B). MCMV RNA was detected by Northern blotting. The DIG labeled MCMV probe hybridizes to multiple segments of the MCMV genome (Figure 2D).

Both viruses were detected by RT-PCR followed by gene-specific PCR. MCMV CP migrated to 716bp, SCMV Nlb to 251bp and maize GAPDH to 170bp (Figure 2E). Gel electrophoresis results confirmed that mock, single and double infected samples could be clearly differentiated by gene-specific PCR. These

protocols and samples are foundational for MLND-related studies conducted in our lab.

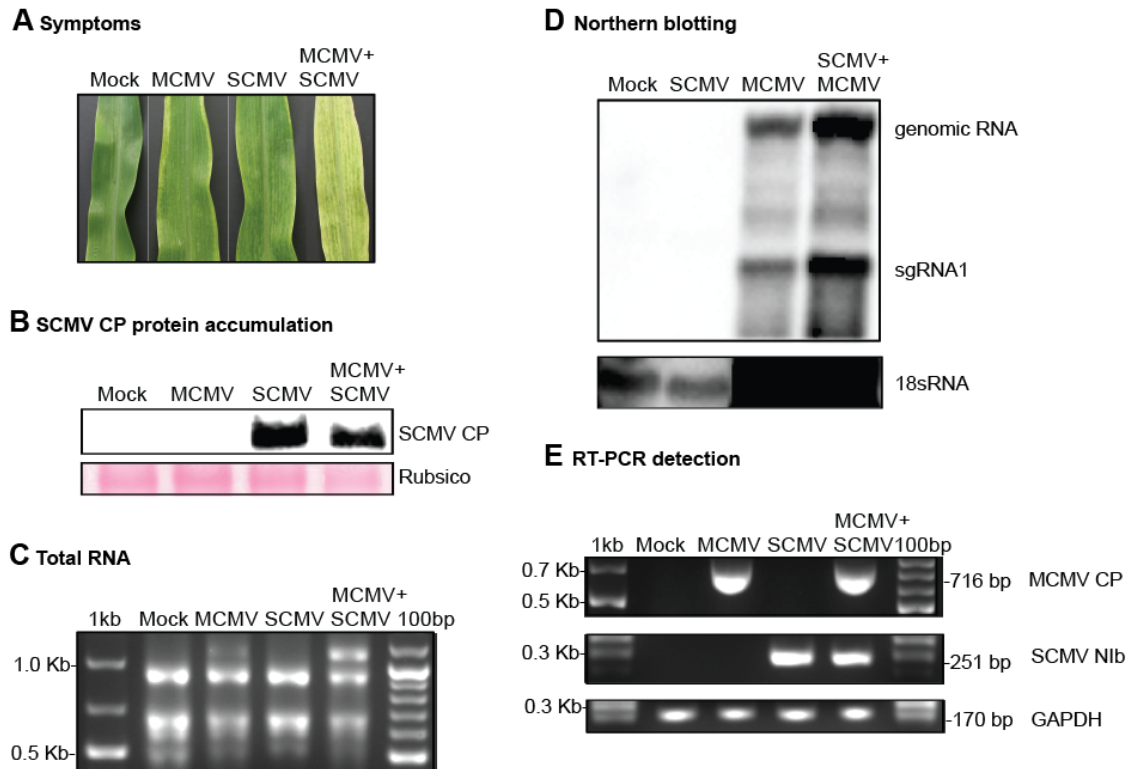


Figure 2.2. Detecting *Maize chlorotic mottle virus* and *Sugarcane mosaic virus* from maize tissue samples. A) Symptoms of single or double virus infection in inoculated SDp2 maize leaves, 15 dpi. B) Western blot showing protein accumulation of SCMV Coat Protein (CP) from total protein samples extracted from infected maize tissue. Rubsico is shown as a loading control. C) Total RNA extracted from leaf samples. D) Northern blot hybridized with a DIG-labeled probe to detect MCMV RNA. Multiple fragments of the genome are detected. MCMV accumulates to higher levels when co-infected with SCMV as compared to a single infection. 18sRNA is shown as a loading control. E) cDNA derived from total RNA was amplified with gene-specific primers for MCMV CP and SCMV Nib amplicons. Maize cellular GAPDH is shown as a loading control.

DISCUSSION

The development of these detection protocols established specific and reliable methods to confirm the presence of MCMV and SCMV in maize tissue samples. Consistent inoculation results are necessary as our research moves into more advanced studies on MLND and its associated viruses, because infected maize plants and tissue are the foundation for future experiments on MLND. The inoculation method described here provides reliable and reproducible results. Infection can then be confirmed by the detection methods previously described.

Currently, the most simple and streamlined of these methods is the gene-specific PCR method because both SCMV and MCMV can be detected in a single assay. Several adjustments to the standard protocols were made to ensure the visualization of the GAPDH and SCMV Nib bands by gel electrophoresis. The GAPDH PCR product needed to be cleaned and concentrated during the elution from binding columns in order for the short 170bp band to be clearly visible on the gel. 2% agarose stained with extra ethidium bromide (0.15 μ L/mL) also enhanced the visibility of the band. Xylene loading dye was used to avoid dye residue migration shadowing the band on the gel. The band was not clearly visible on gels run longer than 20 minutes at 85V. Electrophoresing a larger volume (8 μ L as compared to 5 μ L) of SCMV Nib PCR product under these conditions also produced a sharper, clearer band. The visibility of the MCMV CP band was not dependent upon these adjustments.

Western blotting can also detect both MCMV and SCMV in a single assay from protein samples. This method provides an alternative to PCR-based methods and bypasses the need for time-consuming RNA extractions and handling. Currently, blotting for SCMV CP is not sensitive enough to quantify or compare differences in SCMV protein accumulation in single versus double infections.

In the absence of an MCMV antibody, Northern blotting is available to probe for MCMV and 18sRNA as a control with DIG-labeled probes. This allows for the reliable detection of MCMV without the use of radioactivity. The probes hybridize to multiple MCMV segment representing the genomic and subgenomic RNA. In this assay, it is clear that MCMV accumulates to higher levels in co-infections with SCMV than it does in single infections. All of these methods can be used to detect MCMV and SCMV in samples received from farmers, if the quality of the sample is maintained between sample harvest and analysis. For lab purposes, these methods are superior to commercial tests such as ELISA kits because they are highly specific and will only detect SCMV and MCMV.

CHAPTER 3

CLONING *MAIZE CHLOROTIC MOTTLE VIRUS*

AND

SCREENING FOR SILENCING SUPPRESSOR ACTIVITY

INTRODUCTION

Despite the importance of *Maize chlorotic mottle virus* (MCMV) as a plant pathogen and a component of Maize Lethal Necrosis Disease (MLND), little is known about the genetic functions of MCMV proteins or the mechanisms responsible for disease development. The genomes of other isolates of MCMV, including MCMV-KS and MCMV-YN2, have been cloned but not the MCMV-NE isolate (Scheets et al., 1993; Wang et al., 2017). The first objective of this project was to build a set of molecular tools to be used to conduct meaningful experiments on MCMV and MLND. Therefore, clones of the complete MCMV-NE genome and each individual MCMV-NE open reading frame (ORF) were constructed. These clones were used as foundational tools for studies aimed at answering questions about the molecular mechanisms of MLND.

In some cases, viral pathogenicity and viral synergism have been explained by silencing suppression (Vance and Vaucheret, 2001). Most plant viruses encode at least one protein that functions in suppressing host RNA silencing activity. A silencing suppressor has not been identified in MCMV. The second objective of this project was to screen each MCMV protein for silencing suppression activity in transient assays with single-stranded green fluorescent protein (GFP) in *Nicotiana benthamiana* to identify candidate silencing suppressor proteins.

MATERIALS AND METHODS

Cloning the MCMV Genome

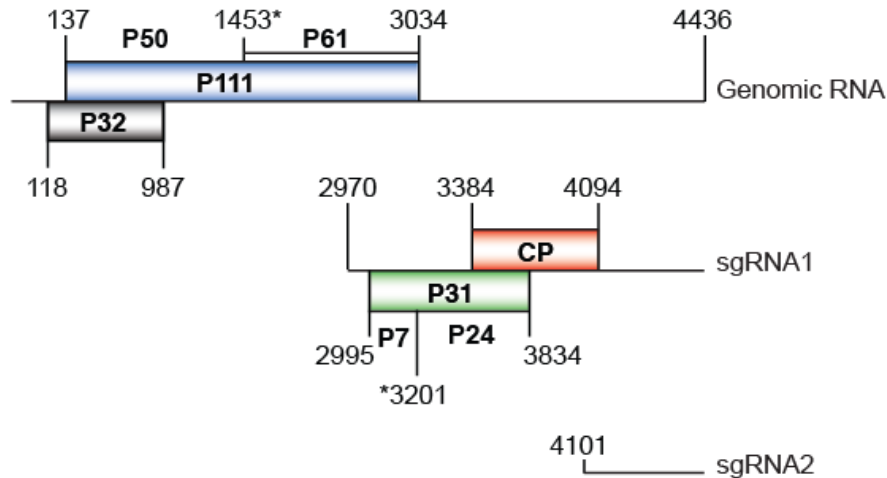


Figure 3.1. *Maize chlorotic mottle virus* genome organization and gene expression

The complete MCMV-NE genome (Figure 3.1) was cloned into a pENTR vector. An overview of the cloning design is illustrated in Figure 3.2A. Following the protocols outlined in chapter 2, total RNA extracted from SDp2 maize tissue infected with MCMV-NE was subjected to reverse transcriptase with primer #800 to make MCMV-NE cDNA (Figure 3.2C). The genome was cloned in two separate overlapping segments, MCMV-A and MCMV-B, because a full-length PCR product was not attainable. MCMV cDNA was amplified by proofreading PfuUltra II Fusion HS DNA polymerase (Agilent, Santa Clara, CA) to make PCR products MCMV-A and MCMV-B (Figure 3.2D). All forward PCR cloning primers

begin with an added CACC sequence to compliment the sequence overhang of the pENTR vector and contain a start codon. The reverse primers were designed to include a stop codon. All cloning primers are listed in Table 3.1 and the nucleotides added for cloning are highlighted in uppercase font. The PCR products were gel extracted using QIAquick Gel Extraction Kits (Qiagen, Hilden, Germany). Blunt-end PCR products for segments MCMV-A and MCMV-B were moved into pENTR vectors behind a T7 promoter using the pENTR-D-TOPO Cloning Kit (Life Technologies, Carlsbad, CA) following the kit's instructions, and incubated for 50 minutes. The TOPO cloning mix was used to transform TOP10 chemically competent *E. coli* cells (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. Cells were plated on LB plates containing 0.1mg/mL of kanamycin and selected colonies were grown individually in 5mL cultures of LB containing 0.1mg/mL kanamycin overnight for minipreps. To determine the preliminary validity of the clones, plasmid DNA was extracted with E.Z.N.A. Plasmid Mini Kit (Omega Bio-Tek, Norcross, GA), normalized to 100ng/ μ L and digested with NcoI and MluI restriction enzymes (New England Biolabs, Ipswich, MA). One MCMV-A and MCMV-B plasmid DNA preparation were selected for ligation to create the full-length MCMV-NE clone.

Insert-vector ligation was performed with StuI, PflMI and Alkaline Phosphatase Calf Intestinal (CIP) to ligate segment B into segment A (New England Biolabs, Ipswich, MA). Only the vector segment, segment A, was treated with CIP to remove phosphorylated ends and prevent re-ligation of the linearized plasmid. After the digestions, reactions were extracted with phenol-

chloroform-isoamyl to inactivate the *Stu*I enzyme. The entire volumes of the reactions were run on a 2% agarose gel and the corresponding vector and insert bands were gel extracted. Preps were diluted to 10ng/μL and 50ng of insert was combined with 20ng of vector and ligated with T4 DNA ligase (Invitrogen, Carlsbad, CA) following the supplied protocol. DH10B *E. coli* cells were heat shock transformed with the ligation reaction. Minipreps were prepared from resulting colonies and restriction digested with *Mlu*I. One sample showing the predicted band pattern was sequenced by Genewiz (South Plainfield, NJ) and confirmed to match the MCMV-NE sequence (accession EU358605.1) in Genbank (NCBI) using Serial Cloner (Softonic International, Barcelona, Spain).

Cloning MCMV ORFs

The first set of MCMV proteins, including all of the proteins encoded on sub-genomic RNA1 (sgRNA1), P7, P31 (p7*p24) and CP, were developed from the partial MCMV clone pMCM1067 provided by Dr. Kay Scheets from Oklahoma State University (Stillwater, OK) (Scheets et al., 1993). One μL of pMCM1067 (50ng/μL) was used as the template for Phusion PCR (Agilent, Santa Clara, CA) following the provided protocol. The second set of ORFs, including P50, P50*61 (P111) and P32 as well as sgRNA2 were cloned from either MCMV-A or the full-length MCMV-NE clone using Phusion High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA). A diagram depicting each pENTR MCMV clone is shown in Figure 3.3 and an overview of the strategy is illustrated in Figure 3.4A.

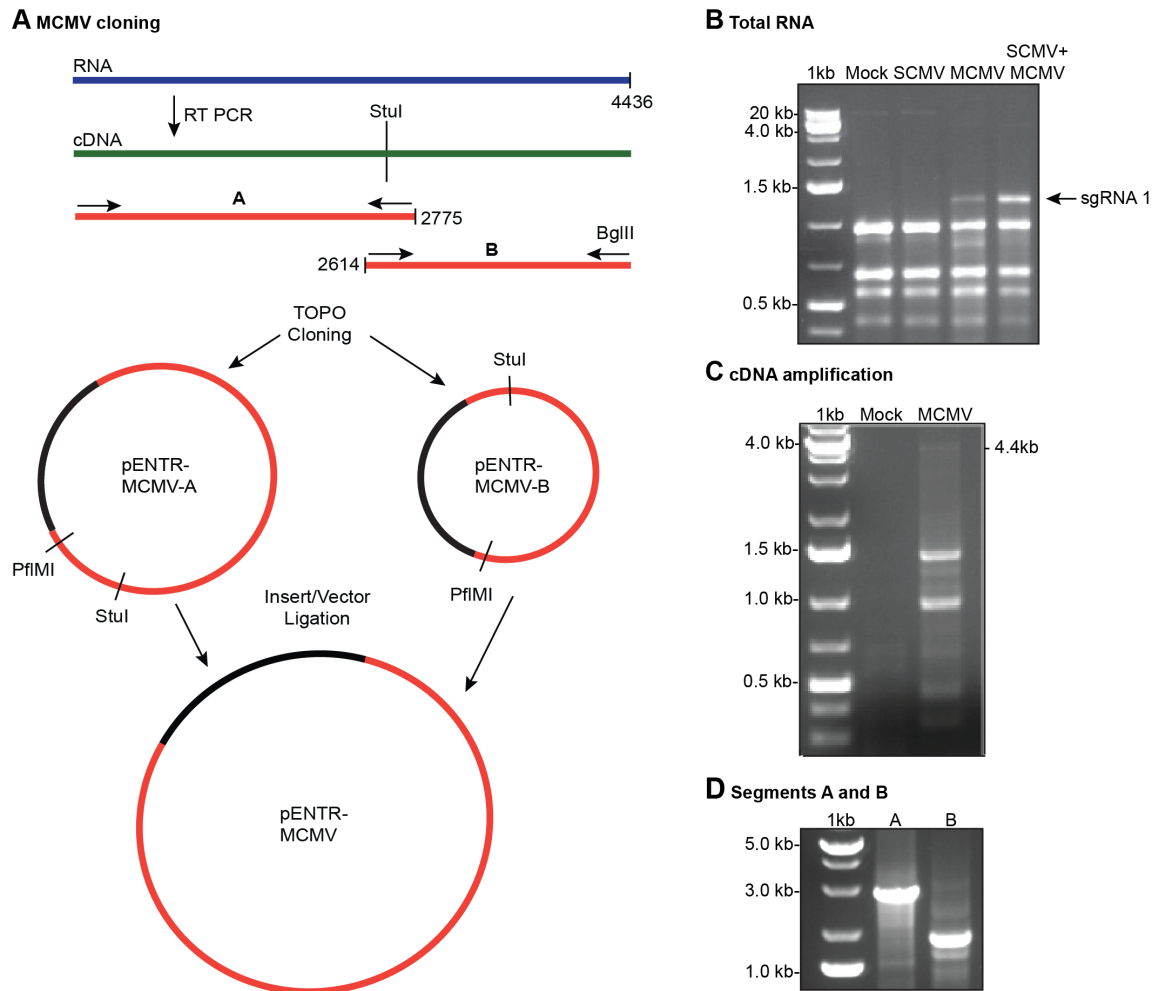


Figure 3.2. Cloning *Maize chlorotic mottle virus*. A) Schematic overview of the strategy used to clone the complete genome of MCMV. Two overlapping segments were cloned by PCR amplification and ligated into plasmids following *StuI*-*BglII* digestion. B) RNA extracted from SDp2 maize tissue inoculated with buffer (mock), MCMV, SCMV, or both MCMV and SCMV. Arrow points to the MCMV sub-genomic RNA1 fragment. C) RT-PCR amplification of MCMV genomic cDNA did not produce a sharp band representing the 4.4kb MCMV genome. D) PCR amplification of segments A and B for cloning into pENTR.

Primers are listed in Table 1 and were designed as previously described for MCMV-A and MCMV-B. BglII restriction enzyme sites were introduced into each clone on the reverse primer for cloning purposes.

Two 50 μ L reactions were performed for each clone and were combined and treated with 20 units of DpnI enzyme to remove vector sequences (New England Biolabs, Ipswich, MA). PCR products were verified by gel electrophoresis and cleaned using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) (Figure 4B). PCR products were cloned into pENTR vectors and screened and sequenced as described for MCMV-A and MCMV-B.

Site-directed mutagenesis was performed to delete the stop codon between p7 and p24 to create the read-through gene product p31 and between p50 and p61 to make p111 (Figure 3A). Primers for mutagenesis are listed in Table 1 and were designed to delete three nucleotides with the forward primer immediately downstream of the TAG stop codon and the reverse primer immediately preceding the codon. Twenty nanograms of plasmid template (P7*P24 or P50*P61) were used for rolling circle amplification with PfuUltra II Agilent HS DNA polymerase. Reactions were denatured at 95°C for two minutes followed by 18 cycles of denaturing at 95°C for 20 seconds, annealing at 49°C for 20 seconds and extension at 68°C for 60 seconds, followed by a final extension at 68°C for five minutes. Twelve μ L of PCR product were treated with 1 μ L of T4 polynucleotide kinase (Thermo Scientific, Waltham, MA) in 4 μ L 5X T4 DNA ligase buffer (Invitrogen, Carlsbad, CA) in a total of 20 μ L and incubated at 37°C for 20 minutes. One μ L of T4 DNA ligase (Invitrogen, Carlsbad, CA) was added

to the reaction and incubated at room temperature for 20 minutes to ligate the linear product back into a circular plasmid. Ten units of DpnI enzyme were added and the reaction was incubated at 37°C for 20 minutes. One microliter of the reaction was used to transform DH10B heat shock cells as previously described. Plasmids were screened and sequenced with the other MCMV ORF clones.

ORF clones in pENTR plasmids were confirmed by Genewiz sequencing and moved to pMDC32 plasmids (Manufacturer's info) by Gateway LR Clonase II (Thermo Fisher Scientific, Waltham, MA) (Figure 3B). The pMDC32 plasmids contain a 35S promoter derived from *Cauliflower mosaic virus* (CaMV) for expression in eukaryotic systems. Colonies resulting from heatshock transformation were screened as previously described and digested with BsaI and XbaI restriction enzymes and sequenced. Confirmed pMDC32 clones were transformed into *Agrobacterium tumefaciens* GV3101 cells in 2mm electroporation cuvettes (Genesee Scientific, San Diego, CA). One μL of plasmid was mixed with 50 μL of cells and shocked at 25 μFD , 400H and 2.5V (Bio-rad, Hercules, CA). Cells were grown for one hour at 28°C in 900mL of LB and 200 μL of X/100 and X/1000 dilutions were plated on LB plates supplemented with 0.1mg/mL of kanamycin and 0.1mg/mL of rifampicin. Plates and glycerol stocks were reserved for *Agrobacterium*-mediated infiltrations.

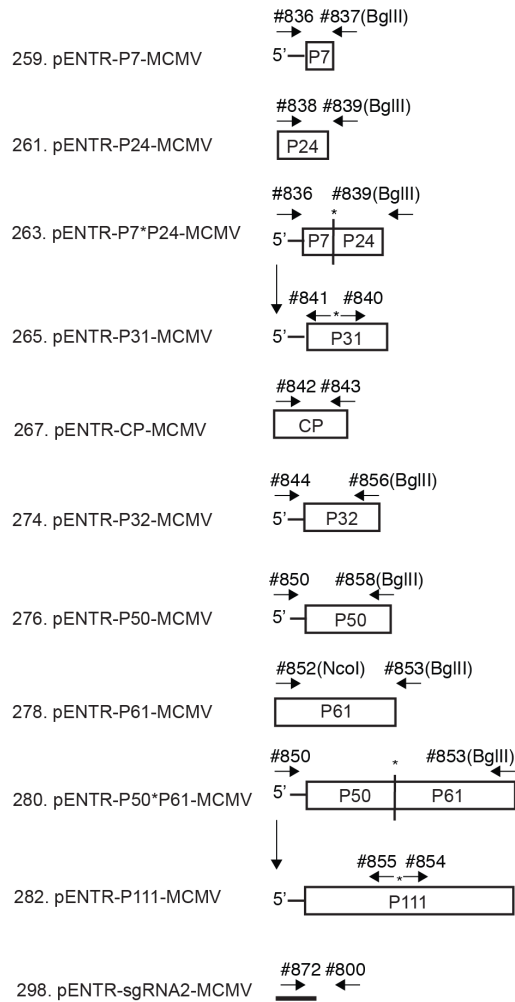
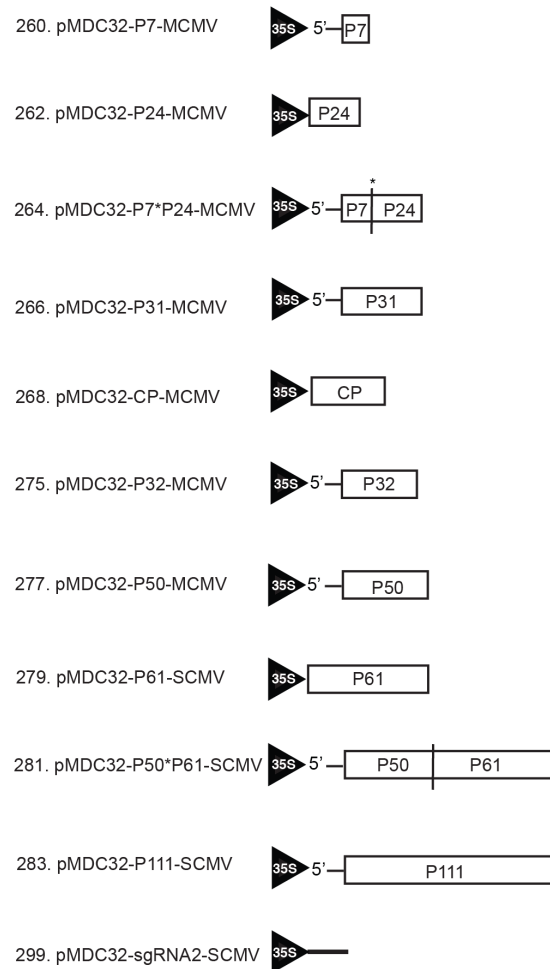
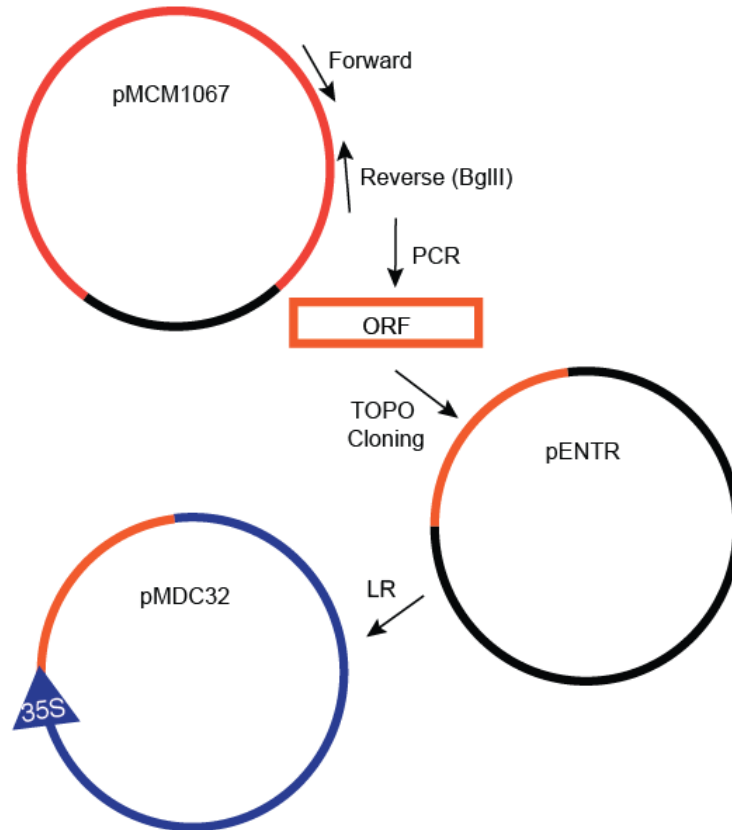
A pENTR clones**B pMDC32 clones**

Figure 3.3. PCR cloning strategy for cloning MCMV genes. A) MCMV genes and primers for PCR amplification and cloning into pENTR plasmids. Mutagenesis was used to remove stop codon to express full-length readthrough proteins P31 and P111 B) pENTR plasmids transformed by LR into pMDC32 plasmids with 35S promoters for expression in *Agrobacterium tumefaciens*.

A Cloning individual open reading frames of MCMV



B PCR of open reading frames

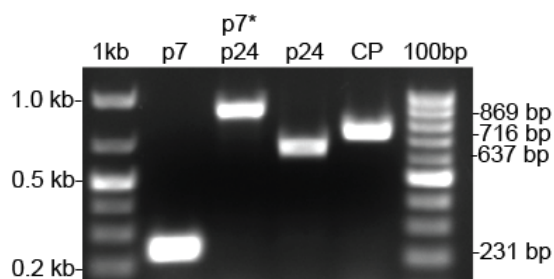


Figure 3.4. PCR cloning of individual MCMV open reading frames from pMCM1067. A) Schematic overview of the ORF subcloning strategy beginning with a plasmid template for PCR followed by TOPO cloning into a pENTR vector and LR Gateway cloning into pMDC32. B) PCR amplification of individual open reading frames from MCMV.

Screening for silencing suppressors

Co-infiltration assays with a plasmid carrying single-stranded green fluorescent protein (ssGFP) and each MCMV ORF clone were modeled after the protocols proposed by Johansen and Carrington (2001) and Powers et al. (2008). The plasmid pPZP-GFP carries the GFP sequence, which triggers the host's silencing machinery. In the absence of a VSR, GFP is targeted as an exogenous foreign gene by the host silencing machinery and GFP expression is silenced. In the presence of a VSR, GFP is expressed and is visualized as bright green signal on infiltrated leaves under UV light. Liquid cultures of *Agrobacterium* carrying this GFP sensor and cultures of *Agrobacterium* carrying a single MCMV gene in the pMDC32 vector were infiltrated simultaneously into *N. benthamiana*. Five colonies from each construct were tested in preliminary experiments to ensure that no gene was overlooked because of an inactive colony being selected. The brightest colony from each construct was selected and further assayed with a complete set of clones representing the entire MCMV genome.

One milliliter *Agrobacterium* cultures of each ORF clone, negative and positive controls and ssGFP were grown for approximately 24 hours in LB broth with 0.1 mg/mL of the corresponding antibiotic for the antibiotic resistance cassette on the vector. Both pPZP and pMDC32 carry cassettes for resistance to kanamycin. Plasmid PZP also carries resistance to spectinomycin and pMDC32 to rifampicin. Beta-glucuronidase (GUS) and *Tomato bushy stunt virus* (TBSV) VSR P19 were used as negative and positive controls, respectively. A second set of controls were later added to the experiment: wild type (WT) and mutant (AS9)

Turnip mosaic virus (TuMV) P1/HC-Pro, a known strong VSR (Kasschau et al., 2003). P1-HC-Pro-WT is as strong of a VSR as TBSV P19 while AS9 shows reduced but not eliminated silencing suppression activity (Garcia-Ruiz et al., 2010). These 1mL cultures were used to inoculate 10mL VIR induction cultures containing 0.1mg/ μ L of the corresponding antibiotics, 10mM MES pH5.2, 100 μ M Acetosyringone to an OD₆₀₀ of 0.002. A larger culture of 25mL was grown for the pPZP-ssGFP sensor. VIR induction cultures were grown at 28°C with shaking and harvested approximately 15 hours later at OD₆₀₀ of approximately 1. Cultures were centrifuged for ten minutes at 6,000 RPM and resuspended in infiltration solution (10mM MgCl₂, 10mM MES, 150 μ M Acetosyringone). Ten mL infiltration cultures were prepared for each treatment by mixing each suppressor candidate culture with the ssGFP culture to a final suppressor concentration of OD₆₀₀=0.5 and ssGFP sensor concentration of OD₆₀₀=0.125. Wild type *N. benthamiana* plants approximately four weeks old were infiltrated. Four plants were infiltrated for each treatment for a total of eight leaves per treatment. Infiltrated plants were kept in highly controlled growth chambers with 16-hour 27°C days and 24°C nights. The intensity of the GFP fluorescence signal was monitored from two to four days post infiltration (dpi) using a handheld long wavelength UV light. GFP fluoresces green while green plant tissue autofluoresces red. Intensity data, pictures and samples were collected for analysis 3dpi. Preliminary intensity data was based on visual observation of the brightness of GFP expression under UV light. The rating scale is based on a true negative expressing no GFP rated as zero and a positive control P19 rated as one. One 0.15-gram sample was

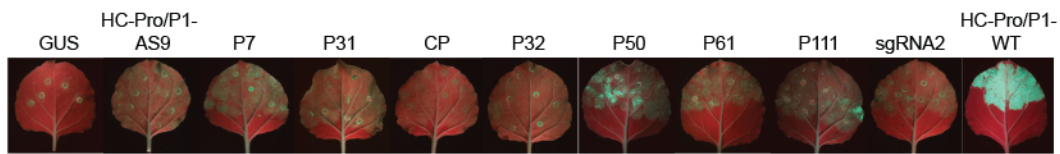
collected from the infiltrated region of each leaf in each treatment. Following the methods described in chapter two, the eight samples representing each treatment were processed and protein samples were obtained. Five μg of X/4 protein samples were run on protein gels and an anti-GFP antibody (Merck Millipore, Darmstadt, Germany) was used to probe for the 28kDa GFP protein. Heatshock protein 70 (HSP70) was used as a loading control. The intensity of each HSP70 (70kDa) and 28kDa GFP band was measured in ImageJ. GFP signal was normalized to the loading control band and GUS control. Means of GFP expression were compared using Tukey's honest significant difference (HSD) test a p value of < 0.05 . All statistical analysis was done using RStudio version 3.2.3 (Team, 2015).

RESULTS

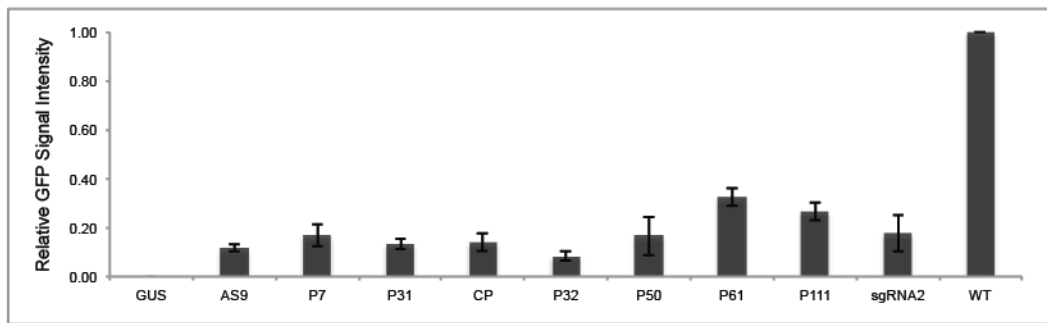
The first complete full-length clone of the MCMV-NE isolate genome was constructed. An infectious version of the MCMV-NE clone has not been derived and tested in maize plants. This clone for agro-infiltrations and an infectious clone for in-vitro transcription are the foundation for further studies on MCMV, RNA silencing and MLND.

A clone for each MCMV ORF encoding P32, P50, P111, P7, P31, CP and sgRNA2 were constructed in both pENTR and pMDC32 vectors. Each clone has been tested for anti-viral silencing suppression activity. However, the functionality of each protein in vivo has not been tested.

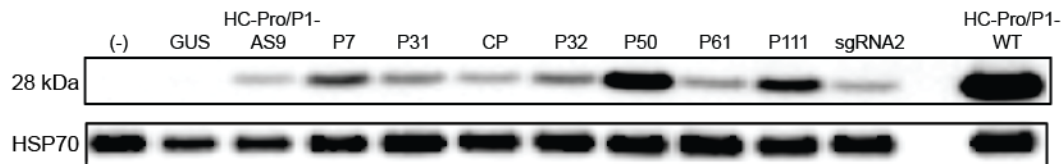
The preliminary screens of five colonies from each construct revealed that all MCMV ORF clones had some silencing suppression activity when infiltrated in plants grown under greenhouse conditions (Data not shown). Observed GFP signal intensity ranged from 10% to 60% of P19 signal intensity 3dpi. The brightest colony from each set of five colonies was selected and was tested together with each ORF in several repeats. Differences in signal between each MCMV construct were highly variable between leaves, plants and individual repeats of the experiments. Adjusting the experimental design to include only plants grown in a growth chamber with 24°C long day conditions under high humidity greatly decreased variability and increased the repeatability of the experiment. GFP was first judged based on visual signal in infiltrated leaves (Figure 3.5A) and rated on a zero to one scale. The ratings of signal from each leaf in each treatment were averaged (Figure 3.5B). Protein samples collected from these leaves were run on gels (Figure 3.5C) and normalized to GUS and HSP70. Normalized GFP signal from four samples revealed candidate genes with silencing suppression activity (Figure 3.5D). Proteins P7, P50, P61 and P111 showed the highest level of GFP expression. P61 is not expressed as a separate protein in vivo but supports signal shown by P111. P7, P50 and its read-through product P111 are the best candidates for VSRs in MCMV.



B Average relative intensity of GFP signal



C GFP protein accumulation



D Normalized GFP protein accumulation

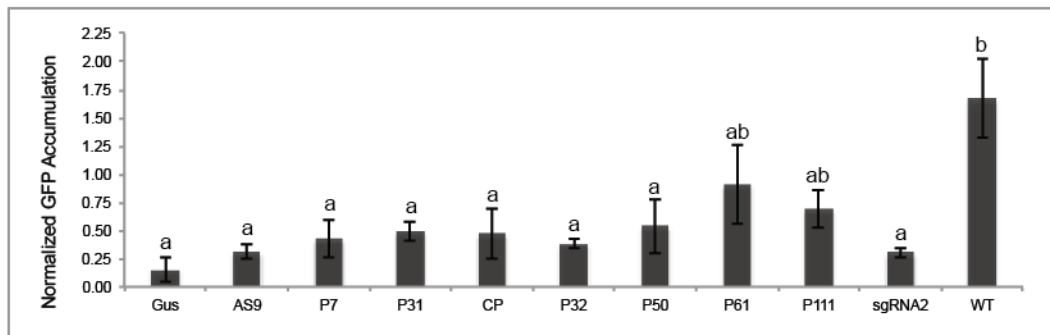


Figure 3.5. Single-stranded GFP complementation with MCMV genes. A) Expression of GFP in *Nicotiana benthamiana* leaves infiltrated with ssGFP and a single MCMV open reading frame, 3 dpi. B) Visualized intensity of GFP expression in infiltrated leaves under UV light. Average of four leaves. C) Western blot showing intensity of GFP expression in leaf samples. D) Normalized GFP accumulation averaged from four samples as compared to GUS. Significant differences evaluated based on ANOVA analysis and Tukey's test with $p > 0.05$.

DISCUSSION

The complete MCMV-NE clone is an essential tool for studies on MCMV and MLND. The 35S promoter version in pMDC32 can be used in complementation and transient assays facilitated by *Agrobacterium* infiltrations. The genome can also be fused with a P7 promoter to create a clone for in vitro inoculations. Clones for the MCMV-NE genome and ORFs are foundational material for future experiments on MCMV and MLND, including elucidating the molecular mechanisms of viral synergism.

Several adjustments were made to the transient assay experiment before true signal and repeatable results were achieved. Preliminary replications of the experiment led us to standardize the OD₆₀₀ values for optimal GFP and ORF construct expression. Once stable expression of GUS and P19 were achieved, several other adjustments were made to the protocol to ensure that weaker VSRs would still be distinguishable in this system. Several issues occurred with plants grown under greenhouse conditions. Although a highly regulated environment, the greenhouse experienced fluctuations in temperature and light throughout the year and throughout the day due to changes in season, day length and cloud cover. Plants grown in the greenhouse were easy to infiltrate but did not produce consistent or repeatable results. High amounts of background were seen in all constructs with signal ranging somewhere above GUS but far below P19. To address this problem, young plants were transferred from the greenhouse to growth chambers approximately two weeks after

seeding. Chamber conditions were set to 24°C with long day length and high humidity to encourage the development of stomata to ease infiltration. Plants grown in the chambers were physiologically different from those grown in the greenhouse and produced thinner leaves that were highly sensitive to wilting at room temperature but were able to capture true differences in GFP signal among different MCMV ORFs. Different leaves and different aged plants were assayed until the ideal leaves for infiltration were determined. The oldest and youngest leaves were bypassed in favor of intermediate leaves. Typically leaves four and five were infiltrated. Covering large, continuous areas of a single leaf is ideal for visualizing GFP signal and collecting tissues samples. Immediately after infiltration, plants were transferred to a different growth chamber with 27°C 10 hours days and 24°C nights. All plants in a single experiment were kept on the same shelf of the same incubator and each replication was stored on this same shelf to reduce and chances of variation as a result of temperature or light. Plants were randomized on the shelf and rotated daily.

Once all of these conditions were standardized, differences in GFP expression began to arise amongst treatments. P31, CP, P32 and sgRNA2 consistently showed little to no GFP expression that faded by day four or five. P7, P50, P61 and P111 showed the strongest signal of all of the MCMV ORFs and are candidate VSRs for MCMV. The GFP signal was not comparable to TuMV-P1/HC-Pro but was clearly above the other constructs and negative controls. TuMV-P1/HC-Pro and TBSV P19 are especially strong VSRs and weaker VSRs are not uncommon. P50 and P111 are involved in genome replication. Other

RNA viruses, such as *Tobacco mosaic virus* (TMV) are known to have multifunctional replicase genes that also function in silencing suppression (Csorba et al., 2007). P7 is the movement protein of MCMV and could also be involved in the suppression of RNA silencing. There may be more than one VSR in a single viral genome and more than one mode of action represented by different VSRs. It is also possible that one VSR may function locally while other functions systemically.

Running statistical analysis including ANOVA and Tukey's HSD test in R software revealed few significant differences. Only P1/HC-Pro varied significantly from GUS and AS9. P61 and P111 are not significantly different from either the negative or positive controls. One of the main issues with this analysis comes from the small sample size. For this analysis, only four sets of samples and western blots were analyzed. Although the variation within treatments (i.e. among leaves in the same treatment) has been greatly reduced through optimizing and standardizing experimental controls, enough variation still exists to affect the statistical results. In some repeats, strong GFP signal was seen, but only in one or two leaves among numerous infiltrated leaves. When the averages of several leaves are taken, the differences are less apparent. Despite the issues with variation and averaging, P50, P61 and P111 showed the most consistently elevated signal, such as is show in figure 3.5A.

Even GUS, although consistently near zero, shows slight variation. Normalizing each sample to the GUS control on individual Western blots and then averaging the replicates from each blot results in high error. Ideally,

repeating the experiment and collecting a large number of higher quality samples will decrease the effects of variability and increase the significant differences between treatments.

This experiment reveals VSR candidates but is not conclusive to confirm the function and mechanism of these proteins. True VSR activity requires confirmation by more precise and quantitative experiments involving RNA analysis. The accumulation of small RNAs (sRNAs) is one method to examine if a protein is functioning in RNA silencing suppression. The accumulation of GFP mRNA is also a more precise quantitative measurement of GFP expression beyond rough protein accumulation estimations made through Western blot analysis. In addition, VSRs can act in local or systemic silencing. Systemic silencing is best observed in transgenic 16C *N. benthamiana* plants that endogenously express GFP (Brigneti et al., 1998). Systemic silencing is visualized by the silencing of GFP in leaves younger than the infiltrated leaves. The experimental design for our experiment is designed only for identifying local silencing suppressors. The candidate VSRs P7, P50 and P111 will require further analysis with these methods before their identities and later mechanisms can be concluded.

An additional important consideration for this experiment is that MCMV is a virus that naturally infections monocots and *N. benthamiana* is a dicot. Complex interactions exist between viruses and their hosts. There could be interactions between MCMV and maize that are not captured or considered in this artificial system, as well as interactions between MCMV proteins. This could

change the behavior of the virus or viral proteins. Once final VSR candidates are identified in this experimental set up, the results can be confirmed in the natural host. This can be achieved by using the full-length clone fused to a T7 promoter for mechanical inoculation on maize. Mutant viruses lacking the presumed VSR will not be able to move and proliferate in the natural host as compared to the wild type virus.

Table 3.1. Primers used for RT-PCR and gene amplification of MCMV open reading frames

Gene	Primer Number	Primer Name	Primer Sequence
MCMV	800	MCMV_Rev	gggccggaagagaggggcattacc
Segment A	844	MCMVclone_F	CACCaggtaatctgcggcaacagacccaacg
	847	MCMVnt2774_Rev	Agatctagtccgtgttttacgagagc
Segment B	846	MCMVnt2614_F	caccacatgtccaagatgcc
	845	MCMV_Rev	AGATCTgggccggaagagaggggc
P7	836	MCMV_sgRNA1_F	CACCgggtattttggcagaaattcccg
	837	MCMV_p7_Rev	AGATCTTAtcagttgaaattgaagtgg
P24	838	MCMV_p24_F	CACCgctggagtgtgtgtgtgtag
	839	MCMV_p31_R	AGATCTctattgtagctgagggcacg
P7*P24	836	MCMV_sgRNA1_F	CACCgggtattttggcagaaattcccg
	839	MCMV_p31_R	AGATCTctattgtagctgagggcacg
P31	840	MCMV_p31mut_R	gctggagtgtgtgtgtgtagattcg
	841	MCMV_R	gttgaattgaagtggttattgataacc
CP	842	MCMV_CP_F	CACCatggcggcaagtagccgg
	843	MCMV_CP_R	AGATCTcaatgattgccagccctgg
P32	844	MCMVclone_F	CACCaggtaatctgcggcaacagacccaacg
	856	MCMVp32_R	AGATCTAttagtcagacagtctgaaggatag
P50	850	MCMVp50_F	Caccctccctgcacttatggcgac
	858	MCMVp50_R	AGATCTTActatttcaactcctggaatagggctgg
P61	852	MCMVp61_F	CACCATGgggtgtcttgaagagtgg
	853	MCMVp61_R	AGATCTTActacgtcggtagggagggg
P50*P61	850	MCMVp50_F	Caccctccctgcacttatggcgac
	859	MCMVp61_R	AGATCTTActacgtcggtagggaggggattg
P111	854	MCMV_P111_F	gggtgtcttgaagagtggcttgggg
	855	MCMV_P111_R	tttcaactcctggaatagggctgg
sgRNA 2	872	sgRNA2MCMV_F	CACCtggcaatcattgaacacaaggtgagcc
	800	MCMV_Rev	gggccggaagagaggggcattacc

CHAPTER 4

CLONING *SUGARCANE MOSAIC VIRUS*

AND

SCREENING FOR SILENCING SUPPRESSOR ACTIVITY

INTRODUCTION

Sugarcane mosaic virus (SCMV) is a member of a large group of plant viruses known as potyviruses (family *Potyviridae*, genus *Potyvirus*). SCMV causes devastating disease and yield loss in Gramineae (Poaceae) plants worldwide. While limited to Gramineae hosts, the SCMV host range includes several economically important grasses such as sugarcane (*Saccharum*), Maize (*Zea mays*), and Sorghum (*Sorghum bicolor*), as well as other wild and cultivated grasses (Gonçalves et al.; Tosić et al., 1990; Yuan et al., 2003). In these hosts SCMV causes mosaic, chlorosis and necrosis on leaves and stems. Symptoms often occur as streaking of healthy dark green and chlorotic yellow tissue. Infected plants are often stunted and have compromised yields. SCMV is transmitted among host plants non-persistently by many species of aphids (Brault et al., 2010). Virions are long, flexuous, filamentous particles ranging in length up to 750nm with a diameter of 13nm and lack an envelope. The single-stranded positive-sense RNA genome of potyviruses is highly conserved among potyvirus species and consists of a single open reading frame that translates the entire 10Kb genome into a single polyprotein (Figure 4.1). The polyprotein is then cleaved into eleven proteins (Urcuqui-Inchima et al., 2001).

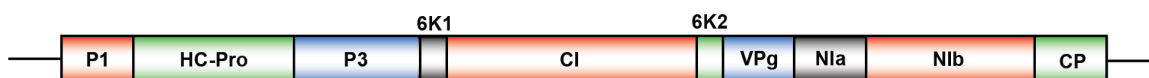


Figure 4.1 *Sugarcane mosaic virus* genome organization.

A major characteristic of SCMV and other potyvirus pathogens that makes them so devastating to crops is their ability to synergistically aid infection caused by a secondary virus. In some cases, synergistic effects have been attributed to the virus's highly effective and non-specific silencing suppressor, HC-Pro (Pruss et al., 1997; Shi et al., 1997). HC-Pro is known to be a silencing suppressor in some potyviruses (Anandalakshmi et al., 1998; Kasschau et al., 2003). HC-Pro suppresses host RNA silencing mechanisms by binding and sequestering small RNAs, thus preventing them from being identified and taken to the argonaut-associated RISC complex for degradation (Zhang et al., 2008). Other viruses may take advantage of the strong silencing suppressors in potyviruses and cause severe secondary infections. This phenomenon could explain the severity of MLND. The non-specific binding of small RNAs by HC-Pro could prevent the small RNAs of MCMV from being degraded.

Compared to MCMV, more is understood about the biology and epidemiology of SCMV and other potyviruses. Despite the dramatic advances in research surrounding the mechanisms and control of SCMV, much remains to be discovered in order to fully understand this virus and its synergistic interactions. HC-Pro is a silencing suppressor in SCMV and hypotheses can be drawn from other potyviruses to predict that HC-Pro and P1 are silencing suppressor candidates (Zhang et al., 2008; Tatineni et al., 2012). The objectives of this experiment were to clone each individual gene in the SCMV polyprotein genome and screen each gene for silencing suppressor activity. This was achieved following the experimental design explained for MCMV in chapter three.

MATERIALS AND METHODS

Cloning SCMV ORFs

The SCMV Ohio isolate (SCMV-OH) was provided to us by Dr. Mark Jones (USDA-ARS, Wooster, OH). The inbred maize line OH28 is highly susceptible to SCMV and was chosen as the host for inoculation and RNA extraction. The same methods for inoculation, RNA extraction and cDNA formation described in chapters two and three were used to derive SCMV-OH cDNA for cloning purposes. Total RNA extracted from SCMV-OH infected OH28 maize tissue was treated with reverse transcriptase with an Oligo-dT primer (Table 1) to make SCMV-OH cDNA beginning at the PolyA tail. In the absence of a full-length clone of the SCMV genome, cDNA was used as a template for cloning each SCMV gene. Each pENTR and pMDC32 clone is modeled in Figure 2. Two μ L of undiluted SCMV cDNA were used as a template for each PCR reaction. PCR was performed with Phusion High Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) following the supplied protocol. PCR products were cleaned or gel extracted, moved in to pENTR plasmids, sequenced and moved into pMDC32 plasmids following the procedures used for MCMV cloning in chapter three. Each pMDC32 clone was sequenced. One amino acid change is present in the CP clone whereas two amino acid changes are present in the HC-Pro clone. Each pMDC32 clone was electroporated into *Agrobacterium tumefaciens* and used for transient assays in *N. benthamiana*.

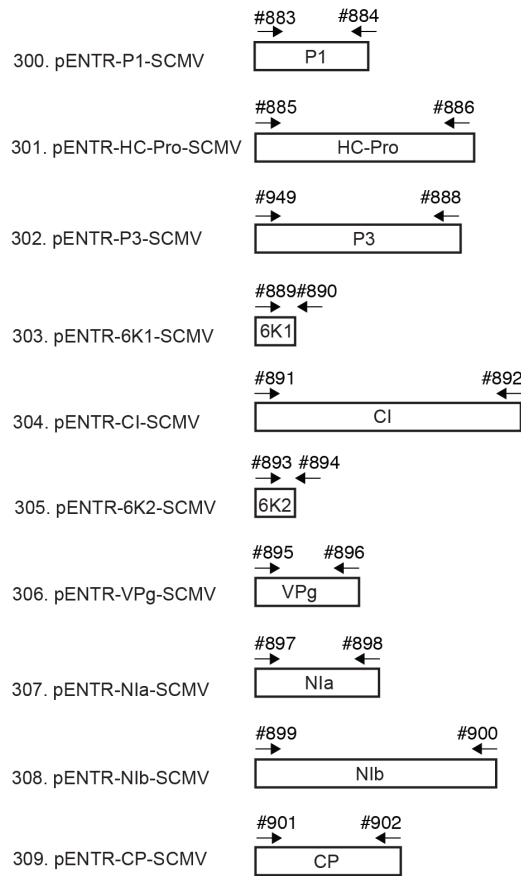
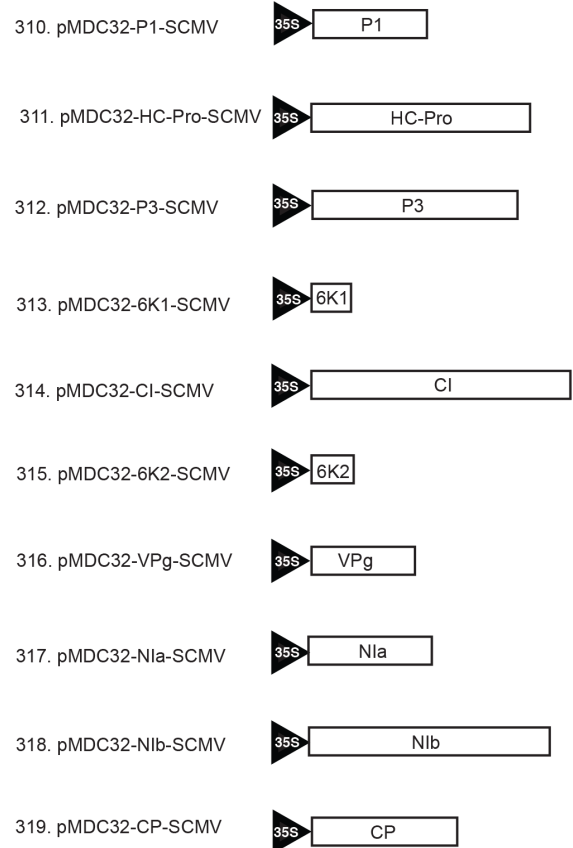
A pENTR clones**B pMDC32 clones**

Figure 4.2. PCR cloning strategy for cloning SCMV genes. A) SCMV genes and primers for PCR amplification and cloning into pENTR plasmids. B) pENTR plasmids transformed by LR Gateway cloning into pMDC32 plasmids with 35S promoters for expression in *Agrobacterium tumefaciens*.

Screening for silencing suppressors

The screening of the SCMV clones representing the ten proteins in the SCMV genome for silencing suppressor activity is identical to the setup described in chapter three for screening the MCMV genome for silencing suppression activity. Five different colonies were screened with single-stranded Green Fluorescent Protein (ssGFP) as a reporter. The brightest colonies from each gene were selected and tested together in assays representing the entire genome. Infiltrated plants were monitored for GFP expression and silencing from two to four days post infiltration. Data was collected and Western blot analysis was used to measure and compare the level of GFP protein expression supported by each SCMV gene.

RESULTS

With the exception of one amino acid change in the CP gene and two in HC-Pro, all of the SCMV genes were cloned into vectors and match the sequence of SCMV-OH available in Genbank (JX188385.1). A complete clone of the full genome has not yet been achieved.

The results of the preliminary screen of five colonies from each construct showed that in this system, all proteins had some level of activity as compared to the negative control at three and 4dpi. The results from these initial screens showed NIa, VPg, P1 and P3 supporting the highest level of GFP expression (Figure 4.3A and B). Samples were collected and run on Western blots as

described in chapter 3 (Figure 4.3C). Blots were normalized to Rubisco and GUS. The data collected from six different samples run on six Western blots was collected and analyzed. The means of each treatment over seven blots were compared using ANOVA and Tukey's HSD test in R. The analysis revealed that at a p value of 0.05, VPg is the only SCMV protein significantly different from the negative control, GUS (Figure 4.3D).

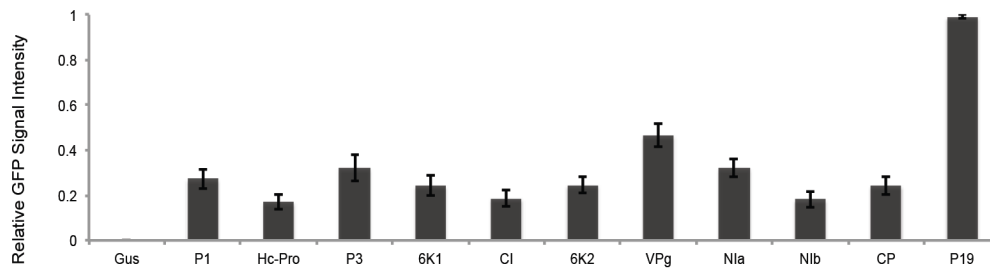
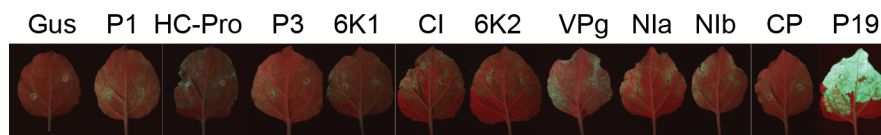
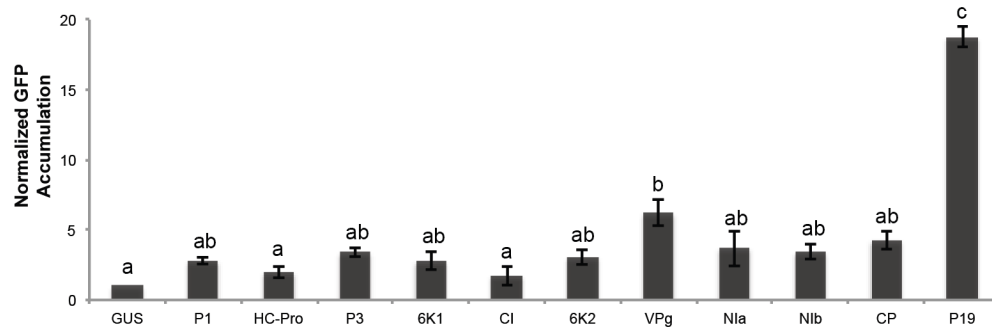
A Average relative intensity of GFP signal**B** GFP expression in *N. benthamiana***C** GFP protein accumulation**D** Normalized GFP protein accumulation

Figure 4.3. Single-stranded GFP complementation with SCMV genes. A) Visualized intensity of GFP expression in infiltrated leaves under UV light on a zero to one scale. B) Expression of GFP in *N. benthamiana* leaves infiltrated with ssGFP and a single SCMV ORF, 3dpi. C) Western blot showing expression of GFP protein in leaf samples. D) Normalized GFP accumulation of seven samples from two independent trials as compared to GUS. Significant differences evaluated based on ANOVA analysis and Tukey's HSD test with $p > 0.05$.

DISCUSSION

The issues described in chapter three relating to the co-infiltration assays in *N. benthamiana* were also faced during SCMV transient assay experiments utilizing this same experimental design. High variation was seen in most initial trials of the experiment. However, after several repeats of the experiment in highly controlled conditions, patterns emerged among the SCMV proteins. VPg shows the highest GFP signal. Although not near to the expression of P19, GFP expression in treatments co-infiltrated with VPg are significantly higher than those infiltrated with GUS. Until an HC-Pro construct without any amino acid changes resulting from mutations can be tested in this system, VPg remains the strongest candidate for a VSR in SCMV.

VPg is a multifunctional potyviral protein involved in replication and movement (Charron et al., 2008). This is not the first evidence of potyviral VPg functioning as a VSR. The VPg is potyvirus *Potato virus A* is a known VSR (Rajamäki et al., 2014). Potyviral VPg has been demonstrated to be involved in anti-viral RNA silencing suppression by interacting with the host gene suppressor of gene silencing 3 (SGS3) (Cheng and Wang, 2017). This recent evidence supports our results and the presence of additional silencing suppressors beyond HC-Pro in potyviruses. However, other genes cannot be ruled out as candidate suppressors at this stage. Further experiments, including RNA analysis, will be required before VSR(s) are confirmed.

Table 4.1. Primers used for RT-PCR and gene amplification of SCMV

Gene	Primer Number	Primer Name	Primer Sequence
SCMV	831	Adaptor_dT	agtactagtcgacgcgtggcccgatcgTTTTTTTTTTTTTTT
P1	883	P1-SCMV-F	CACCAatggcgggaacgtggacctacg
	884	P1-SCMV-R	TCACTAgtagtgctcaatatccaaaaccg
HC-Pro	885	HC-Pro-SCMV-F	CACCAatggcagatcccaggctaag
	886	HC-Pro-SCMV-R	TCACTAatcctactatgtattcgcgcatctac
P3	949	P3-SCMV-F2	CACCAatgggaactctcacgcaacagacattcaacacac
	888	P3-SCMV-R	TCACTAatcgtgtatgacgcctgtgtgag
6K1	889	6K1-SCMV-F	CACCAatgggaaaatctaactctcg
	890	6K1-SCMV-R	TCACTActgtgtgtcactggagg
CI	891	CI-SCMV-F	CACCAatgagtgtagatgttgatgagcc
	892	6K1-SCMV-R	TCACTAatgggtgaataactgtgtaagagc
6K2	893	6K2-SCMV-F	CACCAatgggaatggacgcaactg
	894	6K1-SCMV-R	TCACTActgatgtgagacattgtg
VPg	895	VPg-SCMV-F	CACCAatggggaaaaaacaacgc
	896	VPg-SCMV-R	TCACTActcgtgtgcaaccctg
NIa	897	NIa-SCMV-F	CACCAatgtcaaaatccatgatgaatggg
	898	NIa-SCMV-R	TCACTAatgctcctcaacgctcgcg
NIb	899	NIb-SCMV-F	CACCAatgtgcaagatcactgaaacatgg
	900	NIb-SCMV-R	TCACTAatgacttcagttacaccag
CP	901	CP-SCMV-F	CACCAatggcaggggctggcggtag
	902	CP-SCMV-R	TCACTAgtggtgctgctgactccaac
P3N-PIPO	887	P3-SCMV-F	CACCAatgggaactctcacgcaacagac
	1082	SCMV_PIPO_R	TCAatcatatagaaaccgttgcggagttgg

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