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Assessment of interactions among viruses infecting sweetpotato

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**ASSESSMENT OF INTERACTIONS AMONG VIRUSES
INFECTING SWEETPOTATO**

A Dissertation

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy**

in

The Department of Plant Pathology and Crop Physiology

by

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B.S., Louisiana State University, 2000
M.S., Louisiana State University, 2002
May 2006**

DEDICATION

To a Greek Legend, Stylianos Kazantzidis.

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ABSTRACT

Viral diseases, especially those caused by mixed infections, are among the economically most important diseases of sweetpotato. Real-time PCR assays were developed for the detection and quantification of the potyviruses *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato virus G* (SPVG), *Ipomoea vein mosaic virus* (IVMV); the crinivirus *Sweet potato chlorotic stunt virus* (SPSCV), and the begomovirus *Sweet potato leaf curl virus* (SPLCV) directly from infected sweetpotato plants. Titers of SPFMV, IVMV, and SPVG were lower in singly-infected sweetpotato plants compared to singly-infected plants of the standard indicator host Brazilian morning-glory (*Ipomoea setosa*) and the standard propagation host *I. nil* cv. ‘Scarlet O’ Hara’ plants. The effect of SPSCV on titers of potyviruses infecting sweetpotato in the U.S. was investigated in a separate study. Titers of all potyviruses evaluated were enhanced in the presence of SPSCV suggesting that a conserved mechanism may underlie the enhancement of different potyviruses. Although titers of the common strain of SPFMV (SPFMV-C) were enhanced similarly to the russet crack strain (SPFMV-RC), SPFMV-C did not cause typical sweet potato virus disease (SPVD) symptoms when co-infecting with SPSCV, whereas SPFMV-RC with SPSCV caused severe SPVD symptoms. Titers of SPSCV were lower when coinfecting with potyviruses compared to plants infected with SPSCV alone. Expression analysis using cDNA microarrays revealed that the number of differentially expressed genes in plants infected with either SPFMV or SPSCV alone compared to virus-tested plants was 3 and 14, respectively. These findings were in stark contrast with SPVD-affected plants where over 200 genes were differentially expressed. SPVD-responsive genes are involved in various cellular processes including several that

were identified as pathogenesis- or stress-induced. Even though titers of the U.S. isolate of SPLCV (SPLCV-US) were greater in the presence of potyviruses compared to titers of SPLCV in single infections, they were statistically different only when co-infecting SPFMV-RC and IVMV. Quantification of SPLCV in sweetpotato cultivars revealed that titers were significantly lower in cultivars known to be tolerant of the effects of SPLCV on yield. Real-time PCR was a more sensitive and specific detection method for the viruses evaluated compared to conventional PCR or ELISA assays.

CHAPTER 1: INTRODUCTION

1.1 Sweetpotato and Its Viral Pathogens

Sweetpotato [*Ipomoea batatas* L. (Lam); Convolvulaceae] is the seventh most important food crop in the world after wheat, rice, maize, potato, barley and cassava (FAO, 1993). With a mean annual world production of 132 million metric tons between 1991 and 2000 (FAO, 2000), it ranks fourth in importance in the developing world after rice, wheat, and maize and is grown in more than 100 countries. Although sweetpotato originated in Central and/or South America, world production is centered in Asia. China, which produces about 102 millions metric tons annually, accounts for over 84% of the world's volume (Rubatzky and Yamaguchi, 1997). Production of sweetpotato in Africa, 7.5 millions metric tons, accounts for 6% of world production. About 75% of Africa's sweetpotato production comes from East Africa, mainly from the region around Lake Victoria (Karyeija et al, 1998). Uganda, which is the largest African producer, is also the third largest producer in the world. It produces 2.2 millions metric tons, which is equivalent to the combined production of the Americas (FAO, 1995). The United States is one of the few developed countries that produce sweetpotatoes. Annual production ranges from 36500 to 40500 hectares, and it has a gross value of about 250 million dollars. Louisiana and North Carolina count for more than half of the total production (<http://usda.mannlib.cornell.edu/data-sets/specialty/03001/>).

Production statistics show a net reduction in land area dedicated to sweetpotato production (Rubatzky and Yamaguchi, 1997). Tonnage produced however, has remained constant, due to higher yielding varieties. Production technology and improved varieties are paramount to maintaining competitiveness of sweetpotato as a major world crop.

Varietal maintenance, i.e. virus-tested stock, and use of elite varietal clones is a key factor. The International Potato Center (CIP) in Lima, Peru, has the international mandate for research on sweetpotatoes in the developing countries. The effort of maintaining this crop as an important food source, especially in the developing areas of the world is well worthwhile. Sweetpotatoes are a good source of carbohydrates, and both storage roots and foliage are nutritious foods. Orange-fleshed sweetpotatoes are particularly nutritious, ranking highest in nutrient content of all vegetables for Vitamins A and C, folate, iron, copper, calcium, and fiber and they are an excellent source of the carotenoid β -carotene, a vitamin A precursor (Woolfe, 1992).

In Africa, sweetpotato plays a vital role in people's ability to sustain themselves and their families. Even though, the production volume may not be as high as in Asia, during periods of hardship, root crops such as cassava and sweetpotato become very important. For example, during famine caused by drought sweetpotatoes can provide food security, even in areas where corn is the predominant crop. This can be attributed to the fact that sweetpotatoes are more resistant to such extreme environmental conditions, compared to corn and other important crops (Karyeija et al, 1998). Furthermore, a new crop of sweetpotato can be available year around every 3 to 4 months and can be harvested piecemeal to provide a daily source of carbohydrates for a family. Overall, sweetpotato is a crop that feeds millions of people and rightfully characterized as the poor-man's food. It requires very few inputs, making it appropriate in situations where resources are limited.

Sweetpotatoes are affected by several diseases. Among all diseases observed in this crop, those that are the most economically important are caused by viruses (CIP,

2000). The cultural practice of vegetative, propagation provides viruses an efficient way to be perpetuated and disseminated between cropping seasons and/or growing areas (Salazar and Fuentes, 2001). To date several virus diseases have been reported in sweetpotato, but only a handful of them have been well studied and characterized (Table 1.1) (Clark and Moyer, 1988; Salazar and Fuentes, 2001). Sweetpotato viruses are difficult to transmit mechanically, they cannot be transmitted through seed, and their host range is often restricted to the Convolvulaceae family (Moyer and Salazar, 1989, Wolters et al., 1990). In addition to the lack of support for sweetpotato research, the lack of progress in sweetpotato virus identification and classification is mainly due to two important factors. One is the inherent difficulty to isolate and purify viruses from sweetpotato, and the other is due to the fact that very frequently these agents are observed in mixed infections and synergistic complexes. (Colinet et al., 1998). Further knowledge and understanding of the biology and functions of the viruses parallel to improved detection methods beyond the existing, are the most important prerequisite to the effective control of virus diseases in plants.

The most common of all sweetpotato viruses is the *Sweet potato feathery mottle virus* (SPFMV). This virus occurs virtually everywhere sweetpotato is grown. It has been reported in most tropical and sub-tropical countries as well as in the warm temperate regions (Salazar and Fuentes, 2001). The universal presence of SPFMV has often overshadowed the presence of other viruses in sweetpotato, especially those belonging to the same family, such as Ipomoea vein mosaic virus (IVMV) and *Sweet potato virus G* (SPVG), making the effort to isolate them very difficult (Souto et al., 2003). SPFMV was first discovered in 1945 by Doolittle and Harter in the United States. Twelve years

Table 1.1. Reported viruses of sweetpotato (adapted from Salazar and Fuentes, 2001).

Virus	Shape and Size	Vector	Distribution
SPFMV Potyvirus	Flexuous 850 nm	Aphid	Worldwide, several strains
SPVMV Potyvirus	Flexuous 760 nm	Aphid	Argentina
SPV-II Potyvirus	Flexuous 750 nm	Aphid	Taiwan
SPMSV Potyvirus	Flexuous 800 nm	Aphid	Argentina, Peru, Indonesia, Philippines
SPLSV Luteovirus	Isometric 30 nm	Aphid	Peru, Cuba
SPVG Potyvirus	Flexuous	Aphid	Uganda, Egypt, India, China, USA
IVMV¹ Potyvirus	Flexuous	Unknown	USA
SPMMV Ipomovirus	Flexuous 950 nm	Whitefly	Africa, Indonesia, Papua New Guinea, Philippines, India, Egypt
SPYDV Ipomovirus	Flexuous 750 nm	Whitefly	Taiwan
SPLCV Geminivirus	Geminate	Whitefly	USA
SPCSV Crinivirus	Flexuous 850-950 nm	Whitefly	Africa, Asia, America
SPCSV? Potyvirus	Flexuous 850-900 nm	Unknown	Caribbean region, Kenya, Puerto Rico, Zimbabwe
SPCFV Potyvirus	Flexuous 750-800 nm	Unknown	Peru, Japan, Brazil, China, Cuba, Panama, Colombia, Bolivia, Indonesia, Philippines
SPCaLV Caulimovirus	Isometric 50 nm	Unknown	Puerto Rico, Madeira, Salomon Islands, Australia, Papua New Guinea
SPRSV Nepovirus	Isometric 25 nm	Unknown	Papua New Guinea
Reo-like	Isometric 70 nm	Unknown	Asia
Iilar-like	Isometric 30 nm	Unknown	Guatemala
C-6 Potyvirus	Flexuous 750-800 nm	Unknown	Uganda, Indonesia, Philippines, Peru

¹IVMV and SPVMV may be synonyms for the same virus

later this virus was also identified in the east African countries of Kenya, Tanzania, and Uganda (Sheffield, 1957). This virus is a member of the *Potyvirus* genus and the *Potyviridae* family, which is the largest family of plant viruses. Like other potyviruses, the virions are elongated flexuous rods, 810 to 865nm long, with a monopartite, single stranded, positive sense RNA molecule (Moyer and Cali, 1985). The RNA genome of SPFMV has a molecular weight of 3.65×10^6 (about 10.6 Kb long, which is 10-15 % larger than other members of Potyviridae), whereas the capsid, containing a single polypeptide species has a molecular weight of about 3.8×10^4 (Moyer and Cali, 1985).

SPFMV, like all potyviruses, encodes a large polyprotein product. After translation, the polyprotein is cleaved by an enzyme called helper component protease (HC-Pro) (HC-Pro is cleaved from the polyprotein through the process of autocatalysis), into independent mature proteins including coat, nuclear inclusion body a (NIa) and b (NIb), 6K₂, cytoplasmic inclusion body (C1), 6K₁, P3, helper component protease (HC-Pro) and P1 proteins (Riechmann et al., 1992; Shukla et al., 1994). When the protein products of the severe strain of SPFMV (SPFMV-S) were compared to the corresponding proteins of other distinct potyviruses, the percentage of similarity was low, with NIb being the most conserved with a percent similarity ranging from 59 to 68% (Mori et al., 1995). In a similar study, Abad et al., (1992) found that the capsid protein cistron from serologically distinct strains of SPFMV is conserved towards the region of the 3' end of its sequence.

SPFMV is sap-transmissible when diluted 10-fold in 0.05M potassium phosphate buffer (pH 7.2) as well as graft-transmissible from one host to another. It can also be transmitted in a non-persistent manner by a large number of aphids, including *Aphis*

gossypii, *Aphis craccivora*, *Lipaphis erysimi*, and *Myzus persicae*, the last being the principal vector (Moyer and Kennedy, 1978). This virus has a host range restricted to the Convolvulaceae (*Ipomoea spp.*) and Chenopodiaceae (*Chenopodium spp.*). However, some strains of SPFMV can infect species from the Solonaceae family such as *Nicotiana benthamiana* Domin. and *Nicotiana clevehandii* Gray (Moyer and Salazar, 1989; Salazar and Fuentes, 2001). Except *I. batatas*, which is the major economic host, other members of the Convolvulaceae family that can be infected by SPFMV include *I. nil*, *I. purpurea*, *I. hederacea*, *I. wrightii*, *I. incarnata* and *I. trichocarpa* (Clark et al., 1986). Clark et al., (1986) also reported that in Louisiana *I. trichocarpa* is a perennial reservoir whereas *I. hederacea* and *I. wrightii* are annual hosts of this virus.

Leaf symptoms in sweetpotato plants, naturally infected with SPFMV, are generally mild and transient. In certain cultivars, more susceptible to SPFMV, symptoms are more pronounced. They include vein clearing, defined chlorotic spots bordered by pigmented tissue forming purple rings, and vein feathering. Arrendell et al. (1986) found that foliar symptoms were significantly influenced by the interaction of light intensity, daylight and temperature. On roots, symptoms are observed either in the form of external cracking (typical symptoms of one of the most serious diseases of sweetpotato caused by SPFMV, russet crack) or in the form of internal necrosis (typical symptoms of internal cork disease) (Salazar and Fuentes, 2001; Karyeija et al., 1998). In other *Ipomoea* species and especially those used as indicator plants, symptoms are more severe and may further include mosaic, leaf distortion and/or stunting (Karyeija et al., 1998).

Based on symptoms in different hosts, SPFMV can be subdivided into different strains. In the United States there two major strains are recognized. The so-called russet

crack strain (SPFMV-RC), which causes lateral bands of discoloration with fine longitudinal cracking on susceptible hosts, and the common strain (SPFMV-C) which does not induce symptoms on the roots. These two strains can also be distinguished based on the symptoms they induce on indicator plants (Cali and Moyer, 1981). In Japan SPFMV strains S (severe) and O (ordinary) were also distinguished based on their ability to cause the russet crack disease, also known there as “Obizyo-Sohi” (Usugi et al., 1994). Karyeija et al., (2000a) found two distinct serotypes of SPFMV in Uganda based on their ability to cause sweet potato virus disease (SPVD), a severe disease caused by the synergistic interaction between SPFMV and a crinivirus, *Sweet potato chlorotic stunt virus* (SPCSV). Colinet and Kummert (1993) found that a strain of SPFMV from China, designated SPFMV-CH, had a strong relationship with SPFMV-RC. In Africa, phylogenetic analysis of the coat protein gene revealed that the East African isolates of SPFMV form a distinct cluster from other African isolates which do not cluster according to geographic location (Kreuze et al., 2000).

Little data have also been reported on yield losses due to SPFMV. In South Africa, virus tested clones of the cultivar Impala yielded three times more than “ordinary” plants (not virus-tested and probable carriers of SPFMV) (Joubert et al., 1979). In a similar study in Nigeria, “ordinary” plants yielded 30% less than virus-tested plants. Gibson et al., (1997) reported that in a greenhouse trial, virus-tested plants yielded twice that of those inoculated with SPFMV. Similar studies, in New Zealand and Venezuela revealed similar results (Over de Linden and Elliott, 1971; Pozzer et al., 1994). In a recent study however, Clark and Hoy (2006) reported no significant yield losses between virus-tested and SPFMV-infected Beauregard plants. Overall these studies suggest that

SPFMV alone can cause significant decline in yields, and therefore economic losses in some cultivars, whereas in others such as Beauregard has no significant effect. The major economic losses however, especially in Africa, are observed when sweetpotato plants are co-infected with SPFMV and SPCSV which synergistically cause SPVD (Karyeija et al., 1998; Salazar and Fuentes, 2001). Other names of this virus are: Sweet potato russet crack, Sweet potato internal cork, Sweet potato chlorotic leaf spot, and Sweet potato virus A.

Another important virus that infects sweetpotato is *Sweet potato chlorotic stunt virus* (SPCSV). SPCSV, a member of the genus *Crinivirus* and the family *Closteroviridae*, is the accepted name designated for the whitefly-transmitted component of the SPVD from Nigeria. Other synonyms for this agent include *SPVD-associated Clostrovirus* (SPVD-aC) (Winter et al., 1992; Pio Ribeiro et al., 1996) and *Sweet potato sunken vein virus* (SPSVV) (Cohen et al., 1992). The name *Sweet potato sunken vein virus* was given to the virus first reported in sweetpotato plants from Israel. SPCSV is transmitted in a semi-persistent way by the whitefly *Bemisia tabaci* (Aleyrodidae). It infects members of the Convolvulaceae family, including *I. batatas*, *I. nil*, *I. hederacea*, *I. setosa*, *I. trichocarpa*, and *I. trifida*. This virus can also infect *Nicotiana benthamiana* and *Nicotiana clevelandii*, which are members of the Solanaceae family. Even though transmission can also be achieved by grafting the virus on susceptible hosts, this virus is not transmitted mechanically or through seeds. SPCSV has flexuous, filamentous particles ranging from 850 to 950nm long and 12nm wide, with a capsid polypeptide of 25-34 KDa. The ssRNA bipartite genome of SPCSV consists of RNA1 (9.4 Kb) and RNA2 (8.2 Kb) containing 5 and 7 putative ORF's respectively (Kreuze, 2002). The

analysis of the genomic sequence of this virus revealed some new features as compared to the type member of the crinivirus genus, *Lettuce infectious yellows virus* (LIYV). The most remarkable feature was the presence of a novel gene encoding a putative RNaseIII-like protein, which in eukaryotic and prokaryotic organisms has essential roles in mRNA maturation as well as RNA silencing. Another unique feature is the presence of a near-identical 3'-sequence on both RNA1 and RNA2 genomes (Kreuze, 2002). Furthermore northern analysis at different infection stages revealed that sgRNAs of RNA1 accumulate earlier than those of RNA2 suggesting that RNA1 products may be required during the early stages of infection (Kreuze, 2002).

Symptoms caused by SPCSV vary considerably depending on the host. In *I. batatas*, which is the economic host of the virus, symptoms are mild. In some cultivars the virus can be symptomless, whereas in others symptoms such as mild vein yellowing, sunken secondary veins on abaxial leaf surfaces, and swollen veins on abaxial leaf surfaces are observed. Very severe symptoms associated with SPVD, such as stunting, leaf distortion, crinkling and blistering are observed in sweetpotato plants when the virus coinfects with SPFMV. Symptoms on *Ipomoea nil*, a plant species used as an indicator plant as well as a propagation host for the virus, include chlorosis and epinasty in younger leaves followed by a general stunting, and dwarfing of the mature plant (Salazar and Fuentes, 2001). *Ipomoea setosa* plants exhibit symptoms such as stunting, smaller brittle leaves, and occasional inward leaf rolling. This ssRNA virus is present in the continents of Asia (Taiwan), South America (Brazil, Argentina, and Peru) and Africa (Uganda, Kenya, Nigeria, and Zaire) (Karyeija et al., 1998; Salazar and Fuentes, 2001). Isolates from Argentina, Brazil, USA, Nigeria, Kenya, Israel and Taiwan were found to

be serologically closely related (Salazar and Fuentes, 2001). This suggests that this virus is widely distributed but has not been found in fields in China, Japan, or the United States). SPCSV can be detected by serological, hybridization, dsRNA analysis and RT-PCR methods (Salazar and Fuentes, 2001).

The whitefly-transmitted *Sweet potato leaf curl virus* (SPLCV) is another distinct virus infecting sweetpotato. Geminiviruses have been reported from sweetpotato only from a small number of locations around the globe including Taiwan (Chung et al., 1985), Japan (Onuki and Hanada, 1998), Israel (Cohen et al., 1997) and the United States (Lotrakul et al., 1998). However, the geographic range of these viruses is largely unknown and they may well be present in regions from which investigative studies have not yet been conducted (Clark et al., 2002). Lotrakul et al. (1998) was the first to report the presence of *Sweet potato leaf curl virus* (SPLCV) in the United States. The isolate, designated as SPLCV-US, was found in a sweetpotato plant collected from a breeding line showing leaf curl symptoms. When scions from the diseased sweetpotato plant were grafted in *Ipomoea nil* cv. Scarlet O'Hara, symptoms such as severe leaf distortion and chlorosis were observed (Lotrakul et al., 1998). SPLCV, as mentioned above, is readily transmitted by graft inoculation (but not mechanically) to several members of the Convolvulaceae family including *I. aquatica*, *I. fistulosa*, *I. cordatotriloba*, *I. alba*, *I. lacunosa*, *I. lobata*, *I. nil*, *I. setosa*, and *I. trifida*. Additionally, this virus infects *Nicotiana benthamiana* of the Solanaceae family (Lotrakul and Valverde, 1999). In nature, SPLCV is transmitted by the sweetpotato whitefly, *Bemisia tabaci* biotype B. However, under experimental conditions, this virus is transmitted by this vector inefficiently (Lotrakul et al., 2002).

Currently SPLCV is placed in the *Begomovirus* genus of the family *Geminiviridae*. This virus has a circular ssDNA genome that is about 2828 nucleotides long. Its genomic organization is very similar to that of other monopartite begomoviruses. Phylogenetic analysis of SPLCV and other *Ipomoea*-infecting geminiviruses revealed that the sweetpotato and *Ipomoea* geminiviruses form a distinct cluster (Lotrakul and Valverde, 1999). Sequence analysis of the virus from infected plants from growing areas within the USA and other countries suggested that SPLCV-like isolates evolved from a common ancestor that originated in the Old World (Lotrakul et al., 2000). Analysis of the coat protein of SPLCV-US indicated that the coat protein is unique when compared to its counterparts from both the New and Old World. Overall SPLCV-US was found to be more closely-related to begomoviruses from the Old World (Lotrakul and Valverde, 1999).

Even though SPLCV is an important agent of disease (can reduce yields of Beauregard, one of the most important commercial cultivars in the United States, by 30% and cause grooving and undesirable changes in color of the periderm of the roots), its importance has been overlooked primarily due to the fact that symptoms are not commonly found on sweetpotato plants. Even if leaf curl symptoms develop, they do not persist and plants quickly recover from symptoms. Several factors, such as infection of the host by the virus, susceptibility of the host cultivar, favorable environmental conditions, and the presence of SPFMV, are needed for development of typical leaf symptoms (upward leaf curling) (Clark and Valverde, 2001). Due to the mild nature of the symptoms caused by SPLCV, even on indicator plants, specific sensitive methods

such as PCR have been implemented for its reliable detection (Lotrakul and Valverde, 1999).

Sweetpotato is affected by several virus disease complexes. These complexes, which have been reported from different countries, have in all cases SPFMV as one of the viral components (Salazar and Fuentes, 2001). SPVD is the most important viral complex infecting this crop and has been characterized by many as the major constraint to increase in sweetpotato productivity. SPVD is caused by the synergistic interaction between SPFMV and SPCSV and has a dramatic effect on yield of fresh storage roots (25-30% incidence in Uganda fields; Aritua et al., 1998). Schaefers and Terry in 1976 were the first to report that SPVD was caused by two viral agents, which when present alone cause at most only mild symptoms. Other viruses such as *Sweet potato chlorotic fleck virus* (SPCFV) and *Sweet potato mild mottle ipomovirus* (SPMMV) are occasionally detected in SPVD-affected plants, while *Sweet potato latent virus* (SPLV) was never detected (Gibson et al., 1998). The very first report of this disease, however, came from Stayaert in 1939 (Sheffield 1957) who reported that within a few years sweetpotato crops of 30t/year, in the republic of Congo, were reduced to 4t/year, resulting in their abandonment. Such severe reduction and decline in yield suggests the presence of SPVD. Since then, SPVD has been reported in a number of African countries, including Rwanda, Burundi, Uganda, Ghana, Nigeria, Kenya, Tanzania and Zimbabwe (Karyeija et al., 1998). Outside Africa, this disease has been reported from Israel, Argentina, Brazil, and Peru. In the United States, SPCSV, one of the two components of SPVD, has only been found in one tissue culture sample but never in the field (Pio-Ribeiro et al., 1996).

During co-infection by SPFMV and SPCSV, sweetpotato plants exhibit severe symptoms such as leaf strapping, vein cleaning, leaf distortion, chlorosis, puckering, and stunting (Salazar and Fuentes, 2001). Foliar symptoms, on SPVD-affected sweetpotato plants, develop first in the newly emerging leaves. The time from inoculation to the appearance of symptoms varies depending on age and size of the plant, taking longer in older, larger plants (Karyeija et al., 2000b). The effect of SPVD on growth and yield of sweetpotato is dramatic. Hahn (1979) reported a reduction of fresh roots of almost 80% in plants showing SPVD symptoms. Yield reductions of 50% or more were also observed in Israel, in field plots infected with SPFMV and SPSVV, when compared with plots planted with virus-free propagation stock. No yield reductions were observed in plots planted with SPFMV-infected cuttings. The same trend was observed with plants inoculated with SPSVV alone, with the exception that the second year of planting there was a 30% reduction in yield compared to virus-free controls (Milgram et al., 1996). Six of eight controls tested for resistance to SPVD showed yield reduction up to 90%, and were judged as highly susceptible (Ngeve, 1990). A year later, Ngeve and Bouwkamp (1991) reported that two of eight cultivars, tested the previous year, although showing symptoms of SPVD were tolerant whereas the other six showed the same trend of yield reduction (56 to 90%). In addition to that, a significant correlation between disease severity and yield reductions among the cultivars tested was observed. The yield reduction observed for SPVD-affected cultivars can be directly associated with smaller photosynthetic organs due to the severe leaf stunting and strapping (Salazar and Fuentes, 2001).

Karyeija et al. (2000b) in an effort to elucidate the location of SPFMV and SPCSV in infected tissues found that the titers of SPFMV were high in all leaf parts in SPVD-affected plants and could be detected both by TAS- and NCM-ELISA (Enzyme Linked Immunosorbent Assay). The highest signals were observed in the palisade, parenchyma, and guard cells. In contrast, SPFMV was detected from plants infected with SPFMV alone only on rare occasion by any of the test methods utilized. In SPVD-affected plants SPFMV was not confined to any leaf parts where as SPCSV was found only in the veins of the leaves tested. Furthermore, immunohistochemical tests confirmed that SPCSV was phloem-limited (Karyeija et al., 2000b). The amounts of SPCSV in the mature leaves were significantly greater than in young expanding leaves. Another observation from this experiment was that the movement of SPFMV from the inoculated leaf to the upper leaves occurred at the same rate regardless of whether or not plants were co-infected with SPCSV, suggesting that SPCSV does not aid in any way the distribution, loading or unloading of SPFMV from the phloem. However, quantification data of the two viruses from SPVD-affected plants, which revealed a 600-fold increase of SPFMV RNA compared to SPFMV titer in single infections, led to the hypothesis that SPCSV is able to enhance the multiplication of SPFMV in tissues that normally would not occur by itself. The results of this study are unusual in the sense that potyviruses have been shown to play the role of the “enhancer” in viral complexes (Goldberg and Brakke, 1987; Vance, 1991; Anjos et al., 1992; Vance et al., 1995), which is exactly the opposite of what Karyeija et al., (2000b) reported. This study however, was conducted using the cultivar Tanzania which is a rare example of a sweetpotato cultivar exhibiting

resistance to SPFMV. To date the exact mechanism of synergism between the two viruses that cause SPVD remains elusive (Karyeija et al. 2000b).

1.2 Real-time Quantitative PCR

The invention, more than two decades ago, of a revolutionary technique in molecular biology, called the polymerase chain reaction (PCR), made it possible to detect, amplify and analyze even trace amounts of nucleic acids faster and easier. In 1996, a new technology, the “successor” of PCR as it was characterized by some, was for the first time available to scientists (Zubritsky, 1999). This molecular technique, termed Real-time quantitative PCR, enabled researchers not only to detect and amplify a specific nucleic acid sequence but most importantly to quantify the starting amount of it. Since then Real-time quantitative PCR has been embraced by researchers and clinicians for gene quantitation (Leutenegger et al., 1999) e.g., in cancer research, pathogen detection (Zhang et al., 1999; Belgrader et al., 1999) e.g., in *Human immunodeficiency virus* (HIV) therapy (Lewin et al., 1999), and even for pharmaceuticals applications such as process validation of different products (Zubritsky, 1999).

The development of Real-time quantitative PCR has also eliminated the variability in yield traditionally associated with PCR, by quantifying a PCR product during the cycles when it is first detected rather than the amount of the PCR product accumulated after a fixed number of cycles. This “real-time” system includes a fluorogenic probe in each amplification reaction, an adapted thermal cycler to irradiate the samples with ultraviolet light, and a computer-controlled camera for the detection of the resulting fluorescence (AppliedBiosystems, Foster City, CA). As the amplification process produces increasing amounts of double-stranded DNA, the probe binds to the DNA strands resulting in an

increase in fluorescence signal. Figure 1.1 shows what happens to the TaqMan® fluorogenic probe during the extension phase of PCR. If the target sequence is present, the probe, which has a reporter and a quencher dye on its 5' and 3' ends respectively, anneals downstream from one of the primer sites. The 5' nuclease activity of *Taq* DNA polymerase cleaves the probe as the primer is extended. The cleavage and displacement of the probe from the target strand disrupts the proximity between the two dyes resulting in a fluorescence signal from the reporter dye (Fig. 1.1). Additional reporter dye molecules are cleaved from their respective probes with each PCR cycle, resulting in an increase in fluorescence intensity proportional to the amount of amplicon (target) produced (AppliedBiosystems, Foster City, CA). By plotting the increase in fluorescence (ΔR_n) versus the PCR cycle number (C_T), the system produces plots that provide a more complete picture of the PCR process. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. Finally, the use of a pre-designed internal positive control reagent (i.e., 18S rRNA “household” gene) in parallel reactions to the target reactions allows the normalization of DNA/RNA extraction variations between samples (AppliedBiosystems, Foster City, CA).

Real-time quantitative PCR has been an extremely useful tool for studying various agents of infectious diseases in human and veterinary pathology (Mackay et al., 2002). In plant pathology, recent publications indicate that this technology is increasingly implemented for the study of various causal agents of plant diseases. Several Real-time quantitative PCR assays have been developed to detect bacteria, including *Clavibacter michiganensis subsp. sepedonicus* (Schaad et al., 1999) and *Ralstonia solanacearum* (Weller et al., 2000) in diseased potato tubers, *Agrobacterium*

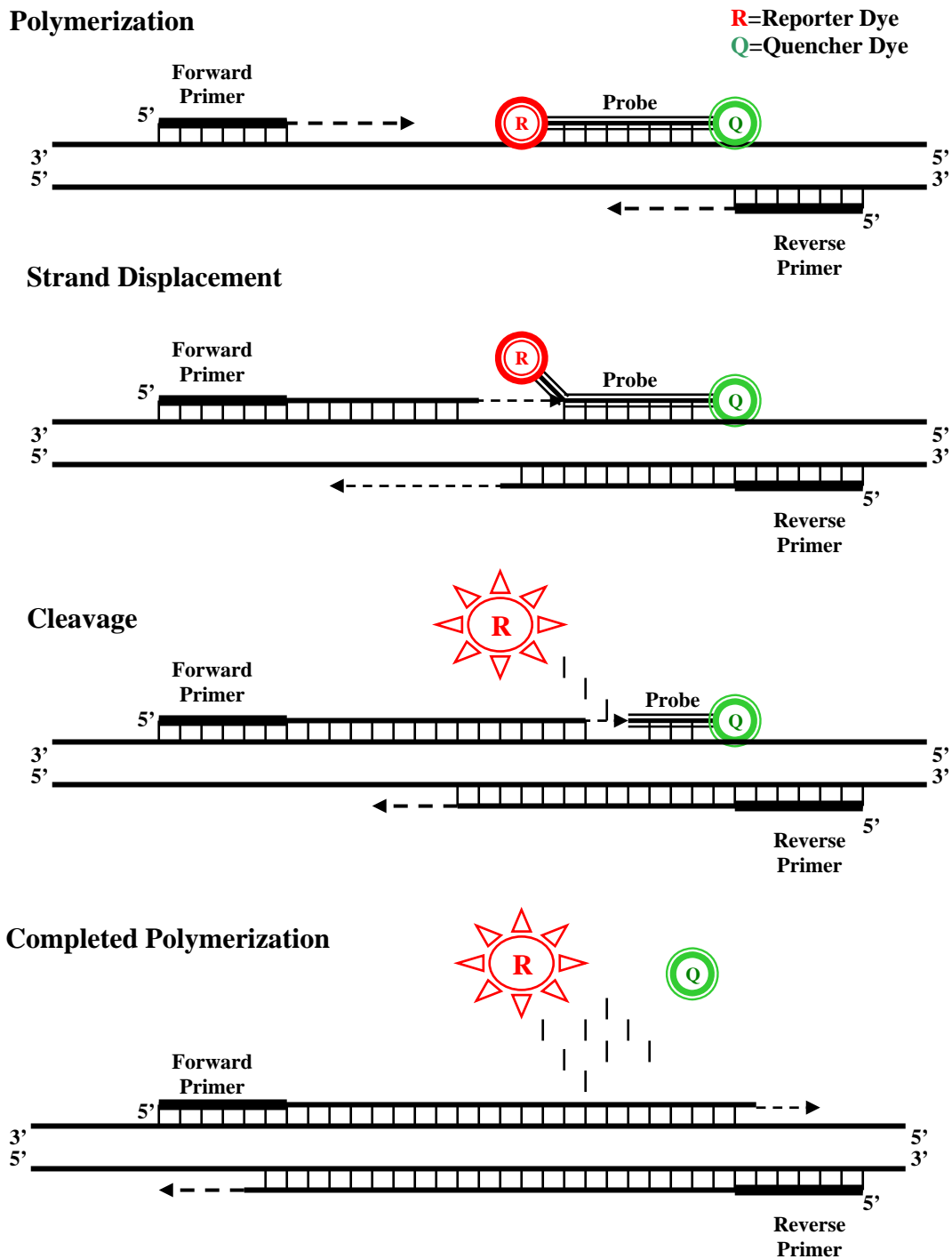


Figure 1.1. The 5'Nuclease activity and TaqMan[®] fluorogenic probe chemistry (AppliedBiosystems, Foster City, CA).

spp. from different plant species (Weller and Stead, 2002), *Acidovorax avenae* subsp. *citrulli* (cause of watermelon fruit blotch) from infected seedlings (Schaad et al., 2001b), and *Xylella fastidiosa* in asymptomatic grape vines (Schaad et al., 2002) and citrus trees (Oliveira et al., 2002). Weller et al. (2000) detected 100 CFU/mL of a pure culture of *R. solanacearum* using TaqMan® chemistry and the ABI 7700® Sequence Detector System. However, when potato tuber extracts were spiked in the assay, for the assessment of inhibitor presence and significance, the threshold for the detection dropped significantly. Specifically, they were only able to detect 10⁵ to 10⁶ CFU/mL in tuber extracts diluted by 1:10. In the undiluted extract the sensitivity was lower, with detection levels at about 10⁷ CFU/mL. Severe inhibition was also observed with *Clavibacter michiganensis* subsp. *sepedonicus* assays, but this problem was easily overcome when BIO-PCR was used (incubation of diseased samples in enriched media for a short time before extraction of nucleic acids is performed) (Schaad et al., 1999).

TaqMan® chemistry has also been utilized for the development of assays for the detection and identification of fungal plant pathogens, including *Tilletia indica*, the causal agent of Karnal bunt disease of wheat, and *Phakospora pachyrhizi*, which causes rust on soybean (Frederic et al., 2000). Similar assays using TaqMan® probes were described for other important fungal plant pathogens. Such assays, some of which have been adapted for use with both the AppliedBiosystem Sequence Detection Systems and “mobile” or “on-site” Real-time quantitative PCR instruments (RAPID® and Smart Cycler®), include the detection and quantification of *Phytophthora infestans* and *Phytophthora citricola* from potatoes and citrus plants respectively (Bohm et al., 1999), *Diaporthe phaseolorum* and *Phomopsis longicola* from soybean seeds (Zhang et al.,

1999), *Magnaporthe grisea*, the causal agent of rice blast, a serious fungal disease of rice worldwide (Qi and Yang, 2002), *Aphanomyces euteiches* in alfalfa (Vandemark et al., 2002), *Phaeocryptopus gaeumanni*, a foliar parasite of Douglas-fir (Winton et al., 2002), and *Helminthosporium solani* from soil and diseased potato samples (Cullen et al., 2001). Taylor et al. (2001) reported the development of a real-time assay for *Pyrenophora graminea* on barley seeds, using a slightly different chemistry, involving SYBR green dyes which are considerably less expensive, but also less sensitive when compared to TaqMan® probes. This is especially true in assays involving quantification of a target, since the SYBR green dye chemistry is not recommended for detection assays (may bind to non-specific double-stranded DNA and/or secondary RNA structures).

Detection of plant viruses, using TaqMan® assays, has also been described in recent publications. The first assay developed involved the detection of *Potato leaf roll virus* (PLRV) (Schoen et al., 1996). Since then several TaqMan® assays have been developed including the detection of *Tomato spotted wilt virus* in thrip vectors (Boonham et al., 2002) and in plants (Roberts et al., 2000), *Sugarcane yellow leaf virus* in sugarcane plants (Korimbocus et al., 2002), *Potato mop-top virus*, and *Tobacco rattle virus* in potato tubers (Mumford et al., 2000), and two orchid viruses, *Cymbidium mosaic potyvirus* (CymMV) and *Odontoglossum ringspot tobamo virus* (ORSV) (Eun et al., 2000). Probes designed to anneal either to the RNA-dependent RNA polymerase gene or to the coat protein gene were efficient enough to detect as little as 5 fg of the ORSV and CymMV in diseased flower tissues (Eun et al., 2000). As in bacterial and fungal Real-time quantitative PCR assays, the use of an efficient nucleic acid extraction method for plant virus isolation ensures sensitivity of target detection and therefore reliable results.

1.3 Objectives

The objectives of this study were:

1. To develop Real-time quantitative PCR assays for the detection and quantification of important sweetpotato viruses, including SPFMV, SPCSV, SPLCV, SPVG, and IVMV.
2. To compare Real-time quantitative PCR with ELISA as tools for the quantification of viruses in infected sweetpotato plants by testing the hypothesis that Real-time quantitative PCR is more sensitive and accurate assay.
3. To assess changes of titers of important sweetpotato viruses in singly-infected sweetpotato plants over time, by testing the hypothesis that viral titers increase but decline with time and age of plants.
4. To study viral synergistic interactions through quantification of titers over time, and gene expression profiles in SPVD-affected plants by testing the hypotheses:
 - a. That the “enhancer” virus (usually the potyvirus), whose titers remain unchanged compared to single infections, promotes the replication and movement of the other viral component in plant tissues that would not normally occur.
 - b. That gene expression profile of SPVD-affected plants is significantly different than virus-tested or singly-infected plants.
5. To determine viral replication rates of SPLCV in cultivars Beauregard, Bienville, Xushu-18, Jonathan, NC-262, Picadito, and Tanzania by testing the hypothesis that viral replication rates are significantly lower in cultivars known to be resistant or tolerant to viruses based on yield data.

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CHAPTER 2: REAL-TIME PCR ASSAYS FOR DETECTION AND QUANTIFICATION OF SWEETPOTATO VIRUSES*

2.1 Introduction

Sweetpotato (*Ipomoea batatas* (L.) Lam.) is the third most important root crop in the world, ranking seventh among all crops in global production and fourth in developing countries after rice, wheat, and corn (FAO, 2000). In Africa, sweetpotato plays a vital role in peoples' ability to sustain their families, especially during periods of hardship. However, sweetpotatoes like other crops are significantly affected by viral diseases. The cultural practice of vegetative propagation provides an efficient way for viruses to be perpetuated and disseminated between cropping seasons and/or growing areas (Salazar and Fuentes, 2001). Several viral diseases have been reported in sweetpotato, but only a few have been well studied and characterized (Clark and Moyer, 1998; Salazar and Fuentes, 2001). Sweetpotato viruses are difficult to transmit mechanically, they are not transmitted through seed, and their host range is often restricted to the family of *Convolvulaceae* (Moyer and Salazar, 1989; Wolters et al., 1990).

The lack of progress in sweetpotato virus identification and classification is due to two important factors. One is the difficulty in isolating and purifying viruses from sweetpotato. The other is the high frequency of mixed infections and synergistic complexes (Colinet et al., 1998). The most common sweetpotato virus is *Sweet potato feathery mottle virus* (SPFMV), a member of the *Potyvirus* genus and *Potyviridae* family. It occurs virtually everywhere sweetpotato is grown, including countries of the tropical and sub-tropical areas as well as in temperate regions (Salazar and Fuentes, 2001). The universal presence of SPFMV has often masked the presence of other viruses in

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sweetpotato, especially those belonging to the same family such as *Sweet potato virus G* (SPVG) and *Ipomoea vein mosaic virus* (IVMV), making the effort to specifically detect or isolate them very difficult (Souto et al., 2003). *Sweet potato chlorotic stunt virus* (SPCSV), a whitefly-transmitted member of the genus *Crinivirus* and the family *Closteroviridae*, interacts synergistically with SPFMV to cause sweet potato virus disease (SPVD), the most serious disease of sweetpotato in Africa, where it was first reported, or in South America, where it has been found more recently (Cohen et al., 1992; Winter et al., 1992; Pio-Ribeiro et al., 1996). *Sweet potato leaf curl virus* (SPLCV), a whitefly-transmitted geminivirus, and related begomoviruses, have been reported from a handful of locations around the globe including Taiwan (Chung et al., 1985), Japan (Onuki and Hanada, 1998), Israel (Cohen et al., 1997) and the United States (Lotrakul et al., 1998). However, the geographic range of this virus is largely unknown and it may well be present in regions from which it has not yet been reported (Clark et al., 2002). Further understanding of the biology and effects of these viruses and improved detection methods, are the most important prerequisites to their proper and effective control.

The objective of this work was to develop real-time PCR assays for the detection and quantification of selected sweetpotato viruses directly from sweetpotato plants, to quantify and compare titer levels of SPFMV, IVMV, and SPVG in different host plants, and to evaluate this method as an alternative detection assay for SPCSV and SPLCV.

2.2 Material and Methods

2.2.1 Plant Material for Potyvirus Quantification

Ipomoea setosa seedlings were mechanically inoculated separately with the russet crack strain of SPFMV (SPFMV-RC, isolate 95-2), IVMV (isolate LSU-2), and SPVG

(isolate LSU-1) (Souto et al., 2003) to generate the scions that were used to graft-inoculate the virus-tested, clonally propagated *I. batatas* cv. Beauregard, and the seed-propagated *I. setosa* Ker., and *I. nil* ‘Scarlet O’Hara’ (SOH) test plants. Test plants were graft-inoculated 3 wk after planting. Two wedge grafts were made per plant by inserting a single node from the source plant into a slit in the stock plant, and only those on which scions survived for 3 wk were used. Each treatment was replicated six times. Plants were grown under standard greenhouse conditions in 15-cm-diameter clay pots containing autoclaved soil mix consisting of 1 part river silt, 1 part sand, 1 part Jiffy-Mix[®] Plus (Jiffy Products of America Inc., Norwalk, OH) and 3.5g per pot of Osmocote[®] 14-14-14 (Scotts-Sierra Horticultural Products Company, Marysville, OH). A weekly insecticide spray program was applied to control aphids and whiteflies. At 3 and 6 wk after inoculation three consecutive leaves collected from the middle of each test plant were combined and immediately frozen in liquid nitrogen and stored at -80°C until extraction.

2.2.2 Total Nucleic Acid Extractions

Frozen leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle and total RNA and DNA were extracted using Qiagen’s RNeasy Plant Mini Kit[®] (Qiagen Inc, Valencia, CA) and GenElute[™] Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO), respectively, according to the manufacturers’ directions. To eliminate residual DNA contamination, all total RNA samples were treated on-column with DNase I using the RNase-Free DNase Set (Qiagen Inc). The DNase treatment was performed as an optional step during the RNA extraction based on manufacturer’s directions.

2.2.3 Primer and Minor Groove Binding (MGB) Probe Development

For each of the five viruses (SPFMV, SPVG, IVMV, SPCSV, and SPLCV) a set of primers, including a forward and a reverse primer, and a fluorogenic probe (TaqMan[®] FAM/ MGB probe) were designed using the Primer Express[™] software according to the manufacturer's guidelines for primer/MGB probe design (AppliedBiosystems, Foster City, CA). Before a potential primer/probe set was used, its putative amplicon sequence (between 63 and 71bp long) was compared to available sequences in the GenBank using the BLAST sequence alignment search tool, available online from the National Center for Biotechnology Information (NCBI), as an initial step in eliminating non-specific primer/probe candidate sets. The second step involved the actual testing of the selected primer/probe sets against templates of either total RNA or DNA extracted from leaves of sweetpotato plants graft-inoculated separately with SPFMV-RC, IVMV, SPVG, SPCSV (isolate BWFT-3), and the United States strain of SPLCV, SPLCV-US (isolate SWFT-1). In addition, the SPFMV primer/MGB probe set was tested against preparations of SPFMV-RC and SPFMV-C (common strain of SPFMV) particles purified following the procedures of DiFeo et al. (Di Feo et al., 2000) and adjusted to a concentration of 25µg/ml based on absorption at 260 and 280 nm. Total RNA and DNA extracts from virus-tested plants were included in each test. The third and final step involved the construction of standard curves of at least five points that could be used for relative quantitative assays. RNA or DNA samples that contained high amounts of a specific target were identified through preliminary real-time PCR assays and serially 10-fold diluted to generate the actual standard curve samples. To obtain relative values, test samples were compared to this standard curve.

2.2.4 Real-time PCR Assays for RNA and DNA Viruses

Real-time PCR assays for RNA viruses were performed in 50µl reaction volume mixtures with 5µl template RNA, 900nM of each primer, 200nM of the MGB TaqMan[®] probe, 25µl of the 2x Master Mix without UNG (AppliedBiosystems), and 1.25µl of the 40x MultiScribe[™] and RNase inhibitor mix (AppliedBiosystems). The 2x and 40x mixes above are the components of the TaqMan[®] One Step PCR Master Mix Reagents kit (AppliedBiosystems). The same protocol was used for the endogenous control reactions (for normalization between samples in quantitative assays) except for the substitution of the primers and probe designed for the target virus with 2.5µl of the eukaryotic 18S rRNA pre-developed primer/probe mix (VIC/ MGB Probe). The following real-time PCR thermal cycler conditions were used: 48°C for 30 minutes (cDNA synthesis), 95°C for 10 minutes (AmpliTaq Gold[®] activation), followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.

Real-time PCR assays for the DNA virus SPLCV were also performed in 50µl reaction volume mixtures with 5µl template DNA, 900nM of each primer, 200nM of the MGB TaqMan[®] probe, and 25µl of the TaqMan[®] Universal Master Mix without UNG (AppliedBiosystems). The following real-time PCR thermal cycler conditions were used: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. The same protocol was used for the amplification of the endogenous control, except for the substitution of the primers and probe designed for the target virus with 2.5µl of the eukaryotic 18S rRNA pre-developed primer/probe mix (VIC/ MGB Probe, AppliedBiosystems).

All real-time PCR reactions were performed on an ABI PRISM[®] 7000 Sequence Detection System using MicroAmp[®] optical 96-well reaction plates that were sealed with optical adhesive covers (AppliedBiosystems). The thermal cycling parameters described above were optimized for use with the TaqMan[®] Universal Master Mix and TaqMan[®] One Step PCR Master Mix Reagents kits in singleplex reactions (AppliedBiosystems). To minimize any errors due to pipetting differences, duplicates of each sample were performed on each plate, and their threshold cycle (Ct) values were averaged during data analysis. In addition, every plate included non-template water controls (NTC) as well as positive (total RNA or DNA from virus-infected tissue) and negative (total RNA or DNA from healthy tissue) controls. The $\Delta\Delta C_t$ quantification method (AppliedBiosystems), which eliminates the use of standard curves on every plate, was implemented for the normalization of samples.

2.2.5 Test for Inhibitors in Total RNA Extracts

A sample of total RNA, extracted from leaf tissue of a virus-tested sweetpotato plant, was serially 10-fold diluted five times to generate a total of six dilutions. Five microliters from each of the six dilution samples was spiked with a 5- μ l aliquot from purified particles of SPFMV-RC and used as template in a real-time PCR assay. The threshold cycle values were compared among the six samples. In addition, two aliquots from purified SPFMV-RC were 10-fold diluted twice to generate two identical groups of three dilutions. One set was spiked with 5 μ l from a total RNA extract from leaves of a virus-tested sweetpotato plant (spiked) and the other had 5 μ l of molecular-grade water added (nonspiked). Both groups of samples were used as templates in the same real-time PCR assay and their Ct values were compared.

2.2.6 Comparison of Real-time and Conventional PCR Assays for SPLCV

Virus-tested roots from the sweetpotato cultivars Beauregard, Bienville, Centennial, Jewel, and Xushu-18, grown by the LSU AgCenter sweetpotato foundation seed program at the Sweet Potato Research Station at Chase, LA, were core-grafted with SPLCV-US (SWFT-1), and planted in field beds to generate the plant material for this experiment. Beds were initially covered with black plastic mulch followed by an agricultural fabric (Agribon+ AG-19, PGI Nonwovens, Dayton, NJ) on hoops to exclude any potential virus vectors. Vine cuttings from these beds were transplanted in an isolated field plot and arranged in a randomized complete block design that included four replications of 5 plants each. Leaves from each replication were collected 22 and 35 days after planting and total DNA was extracted using the method described above. Aliquots of each DNA preparation were used as templates both in a DNA real-time assay as described above and in a conventional PCR assay developed by Lotrakul and Valverde (1999). The two methods were compared for their efficiency to detect SPLCV-RC.

2.3 Results

The sequences for the primer/probe sets, the fluorescent dyes chosen for each probe, and the GenBank accession numbers of the original sequences from which these sets were designed are listed in Table 2.1. The primer/probe SPFMV set amplifies a 63-base pair (bp) fragment from the coat protein gene of SPFMV-RC (AF439637) and SPFMV-C (AF439638). The primer/probe SPCSV set generates an amplicon of 71bp from the heat shock 70 homologue (hHsp70) gene of both West African (AJ278653) and Egyptian (AJ515381) isolates. The primer/probe SPLCV set amplifies a 66-bp fragment from the AC1 gene of the SPLCV-US isolate (AF288227). The SPVG amplicon (67bp)

Table 2.1. Primer/MGB probe sets used for the detection and quantification of *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato leaf curl virus* (SPLCV), *Sweet potato virus G* (SPVG), and *Ipomoea vein mosaic virus* (IVMV).

Name ^a	Sequence	R/Q ^b dyes
SPFMV(AF439637)	Forward Primer: GCCGATTTTAAACACCAGATGGA Reverse Primer: GGTTGCCCACTGTTATTTTCCTT TaqMan Probe: CCATAGTGAAGAAGTTC	FAM/NFQ
SPCSV(AF260321)	Forward Primer: CGAATCAACGGATCGGAATT Reverse Primer: CCACCGACTATTACATCACCCTCT TaqMan Probe: ATCCCAACGTGTTTATCTA	FAM/NFQ
SPLCV(AF288227)	Forward Primer: GGCGCCTAAGTATGGCTGAA Reverse Primer: AACCGTATAAAGTATCTGGGAGTGTGT TaqMan Probe: TGTGGGACCCTTTGC	FAM/NFQ
SPVG(AY178991)	Forward Primer: GAATCAAAGGTGAGGAGCAAGAC Reverse Primer: GCTATGAGCAAATCGTCACCATT TaqMan Probe: AGGTTTGCCTACTTC	FAM/NFQ
IVMV(AY178992)	Forward Primer: GAGACAGCACTGAAAGCTCTGTACA Reverse Primer: CACGAACATACTCGGACAAATCTT TaqMan Probe: TGTGTTGAACCATCAGC	FAM/NFQ

^a The name indicates the virus that the primer/probe set targets. In parenthesis is the GenBank sequence accession number from which the primer/probe sets were developed.

^b Type of 3' Reporter/5' Quencher dye set attached to each probe; NFQ = non fluorescent quencher.

is generated from the coat protein gene of both LSU-1 (AY178991) and LSU-3 (AY178990) isolates whereas the IVMV amplicon (70bp) is homologous to the nuclear inclusion b (NIB) gene of isolate LSU-2 (AY178992) and Sweet potato virus Y (SPVY AY459608).

2.3.1 Standard Curves

Standard curves of at least five duplicated sample dilutions were generated for all five viral targets as well as for the endogenous 18S rRNA reference control (Fig. 2.1 to 2.6). The correlation between Ct values and log relative amounts was very high with R-squared values (R^2) above 0.995 for all standard curves. Validation experiments, done as described in User Bulletin #2 (AppliedBiosystems, Foster City, CA), revealed that the efficiency of amplification between any of the virus targets and the endogenous 18S

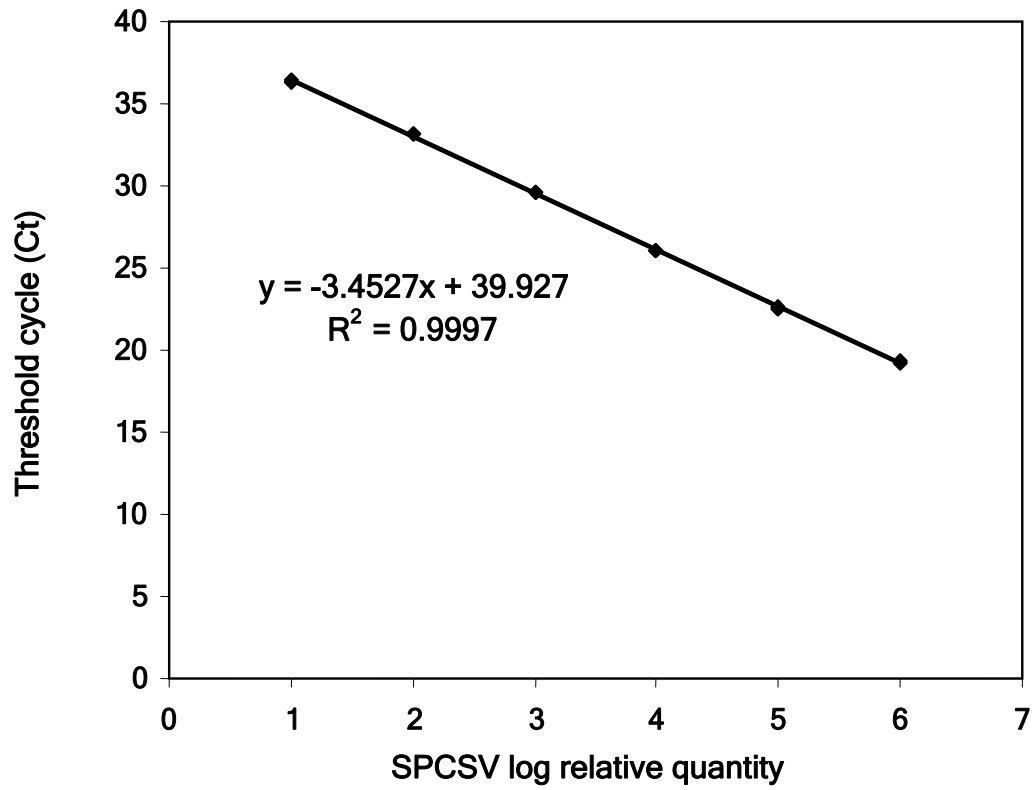


Figure 2.1. Standard curve generated by plotting the log of relative quantity of a concentrated source of virus against critical threshold values from real-time PCR assays for *Sweet potato chlorotic stunt virus* (SPCSV).

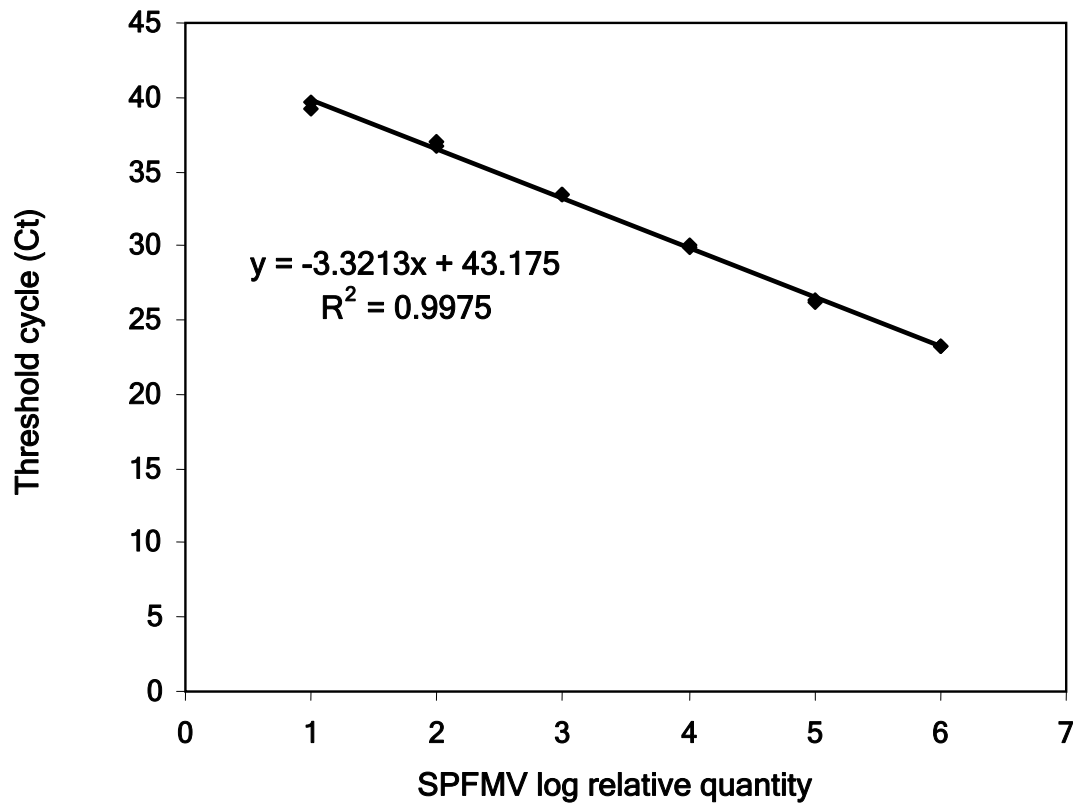


Figure 2.2. Standard curve generated by plotting the log of relative quantity of a concentrated source of virus against critical threshold values from real-time PCR assays for *Sweet potato feathery mottle virus* (SPFMV).

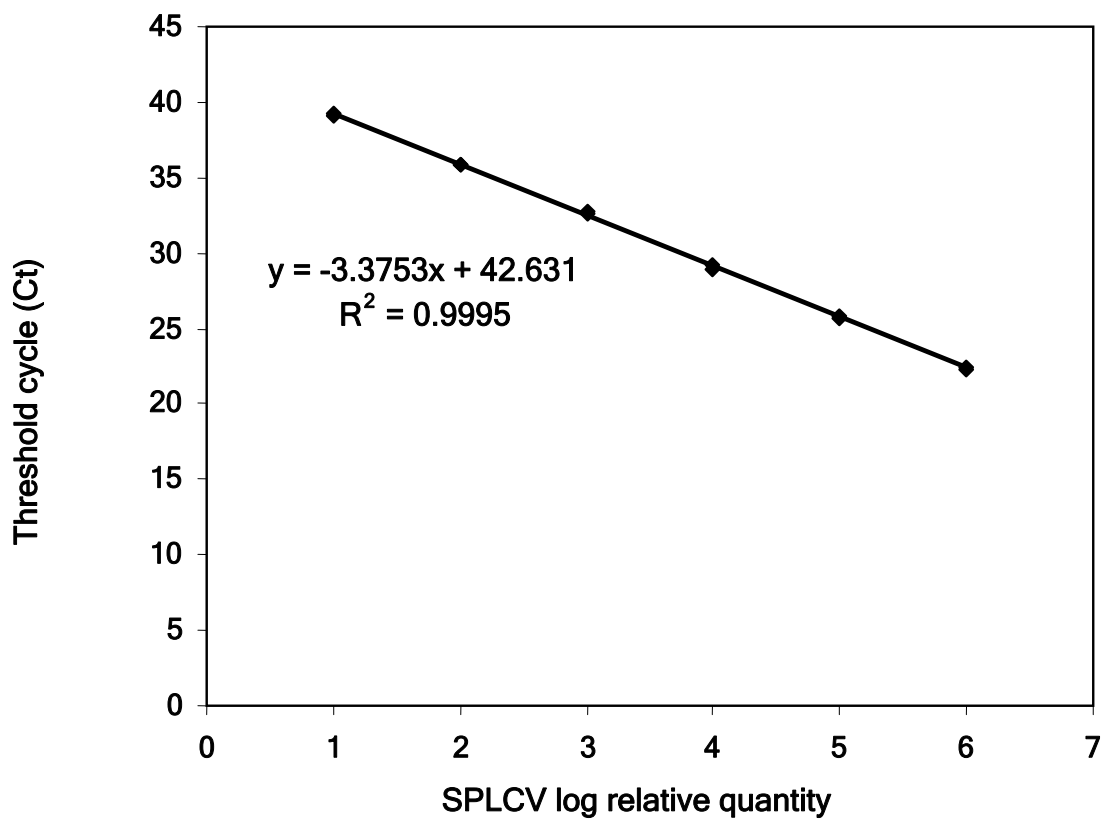


Figure 2.3. Standard curve generated by plotting the log of relative quantity of a concentrated source of virus against critical threshold values from real-time PCR assays for *Sweet potato leaf curl virus* (SPLCV).

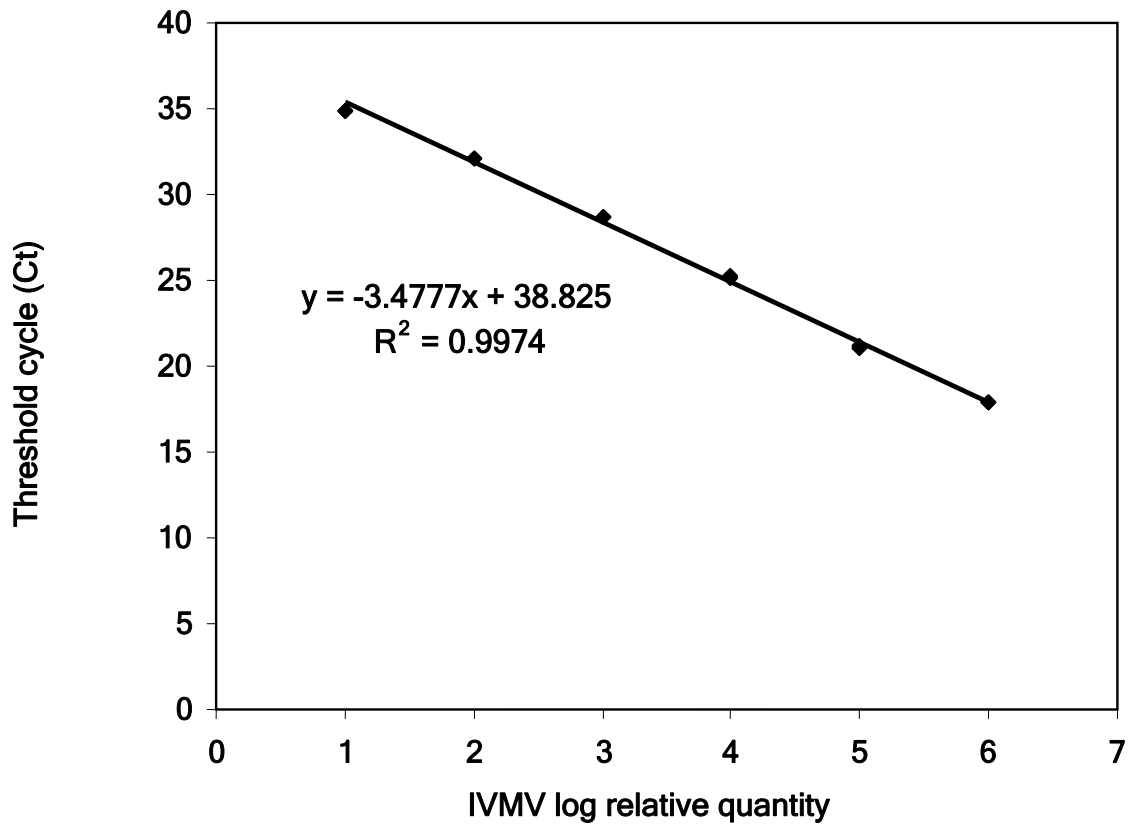


Figure 2.4. Standard curve generated by plotting the log of relative quantity of a concentrated source of virus against critical threshold values from real-time PCR assays for *Ipomoea vein mosaic virus* (IVMV).

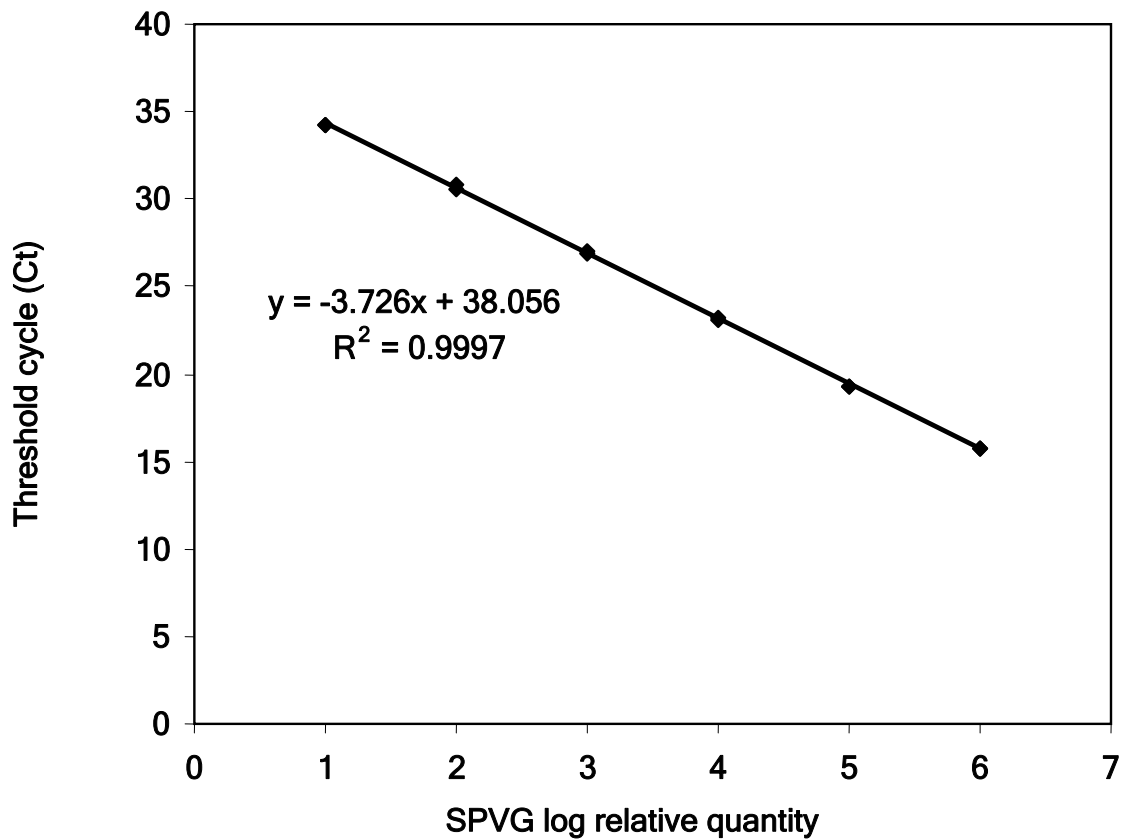


Figure 2.5. Standard curve generated by plotting the log of relative quantity of a concentrated source of virus against critical threshold values from real-time PCR assays for *Sweet potato virus G* (SPVG).

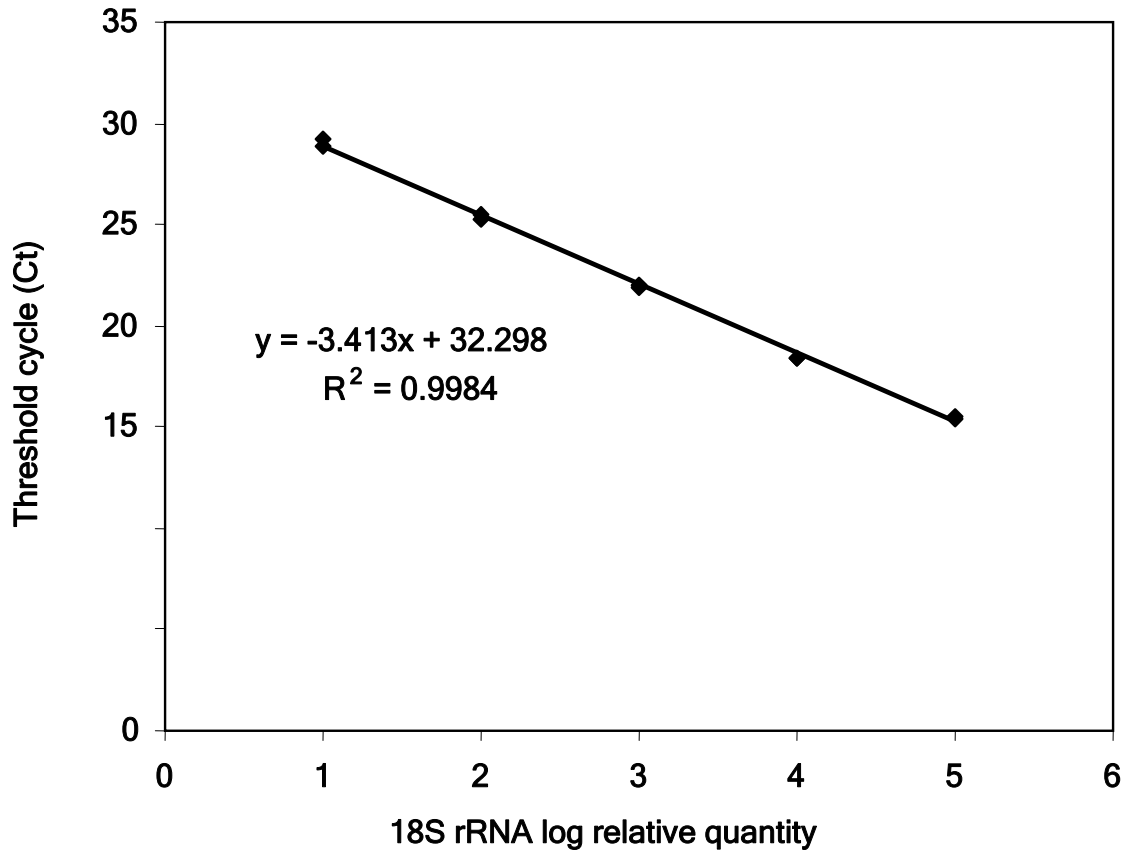


Figure 2.6. Standard curve generated by plotting the log of relative quantity of a concentrated source of virus against critical threshold values from real-time PCR assays for the eukaryotic endogenous control 18S rRNA.

rRNA control was very similar (the slope of log relative amount vs. ΔC_t was < 0.1), thus allowing the use of the $\Delta\Delta C_t$ quantification method which eliminates the need to repeat standard curves each time an assay is conducted. From standard curves generated using purified preparations of SPFMV-C and SPFMV-RC it was estimated that the SPFMV primer/probe set can detect 0.25 pg/ μ l of the virus.

2.3.2 Test for Inhibitors of Real-time PCR Assays

The threshold cycles of the six purified SPFMV-RC samples spiked with different dilutions of the total RNA extract from virus-tested sweetpotato leaves remained unchanged, clearly indicating that potential inhibitors were either not present or did not have any effect on the performance of the real-time PCR assay (Table 2.2). Comparison of threshold cycles between samples of “spiked” and “nonspiked” groups indicates that this is true even when the template used was diluted 10-fold or 100-fold (Fig. 2.7).

2.3.3 Specificity of Probe/primers Sets

The primer/probe sets for SPFMV, SPCSV, and SPLCV were specific for the target viruses for which they were designed. Amplification only occurred with homologous combinations when samples of total RNA from healthy sweetpotato, *I. setosa*, and *I. nil* plants or *I. setosa* plants infected with SPFMV-RC, IVMV or SPVG were tested. Likewise, when total DNA preparations from SPLCV-US infected sweetpotato leaf tissue were used as template only the SPLCV primer/probe set amplified. As expected, the SPFMV primer/probe set amplified with the same efficiency both SPFMV-RC (russet crack) and SPFMV-C (common) strains. The SPLCV primer/probe set amplified with the same efficiency SPLCV from other locations, including Taiwan, Puerto Rico, and Guyana, and therefore could be potentially used to

Table 2.2. Real-time PCR threshold cycles (Ct) of aliquots of purified preparations of particles of the russet crack strain of *Sweet potato feathery mottle virus*, SPFMV-RC (isolate 95-2), each spiked with a different concentration of total RNA extract from healthy sweetpotato leaf tissue (RHT).

Template ^a	Average Ct ^b	Standard deviation Ct
SPFMV-RC + RHT ^d (undiluted)	27.64	0.114
SPFMV-RC + RHT(10 ¹ -fold dilution)	27.16	0.016
SPFMV-RC + RHT(10 ² -fold dilution)	27.78	0.156
SPFMV-RC + RHT(10 ³ -fold dilution)	28.36	0.158
SPFMV-RC + RHT(10 ⁴ -fold dilution)	27.25	0.269
SPFMV-RC + RHT(10 ⁵ -fold dilution)	27.38	0.041

^a Template used in a real-time PCR reaction consisting of 5µl of purified virus and 5µl of total RNA from healthy tissue.

^b Mean threshold cycle of duplicate samples.

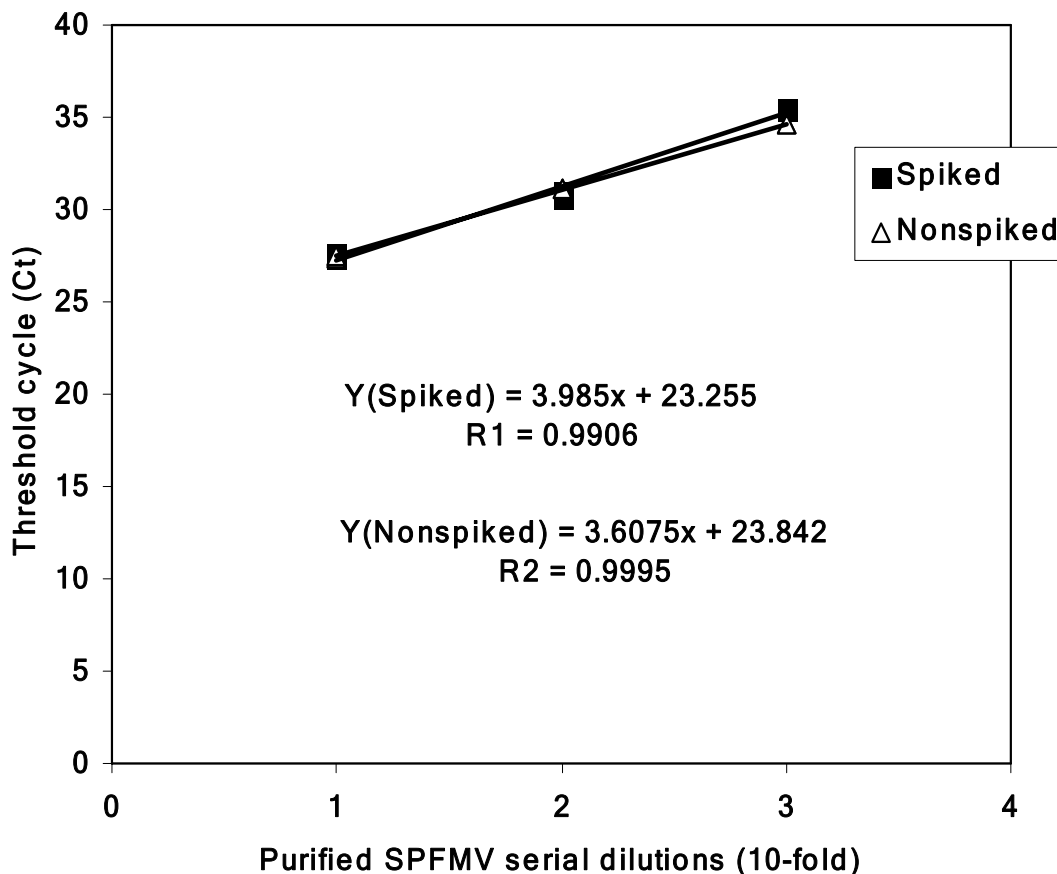


Figure 2.7. Comparison of standard curves of purified *Sweet potato feathery mottle virus* (SPFMV-RC) spiked with 5µl of total RNA extract from healthy sweetpotato tissue (spiked) and purified SPFMV-RC with 5µl of molecular grade water added (nonspiked).

monitor the presence of this virus in plant material coming into the US. Specificity tests involving the SPVG and IVMV primer/probe sets revealed some cross amplification when SPFMV-RC was used as a template. The SPVG primer/probe set amplified SPFMV-RC at threshold cycles ranging between 33 and 38 and the IVMV primer/probe amplified SPFMV-RC at 31-33 cycles, respectively. However, these represent differences of about 10^7 and 10^5 in estimates of virus concentration compared to estimates for the homologous SPVG and IVMV, respectively.

2.3.4 Quantification of SPFMV, IVMV, and SPVG in Different Hosts

Quantitative assays revealed that in most sweetpotato plants the titers of SPFMV-RC were near the threshold of detection. This was true at both 3 and 6 wk after inoculation. In *I. setosa* and *I. nil* the SPFMV-RC titers were greater and the virus was detected in all replicates [mean relative titers (MRT) were 0.27 and 0.46 in *I. setosa*, 0 and 6.5×10^{-5} in *I. batatas*, and 0.58 and 2.21 in *I. nil*, at 3 and 6 wk], but statistical analysis, revealed that at 3 wk after inoculation there was no significant difference ($P = 0.0789$) in titers between the three hosts. At 6 wk however, titers of SPFMV-RC were higher in *I. nil* ($P = 0.009$) as compared to the other two hosts. In the case of SPVG, titers in all three hosts reached levels that were consistently above the threshold of detection. At 3 and 6 wk after inoculation titers were shown to be greater ($P = 0.02$ and $P = 0.03$ respectively) in *I. setosa* (MRT of 424.8 and 450.8) and *I. nil* plants (MRT of 502.5 and 640.7) compared to sweetpotato plants (MRT of 0.005 and 0.001). IVMV titers reached detectable levels in all three hosts and were greatest in *I. nil* (MRT 85.6 and 144). However, statistical analysis revealed that titers of IVMV were not different among the three hosts for either assessment date ($P = 0.09$ and $P = 0.07$ respectively).

2.3.5 Comparison between Real-time and Conventional PCR Assays for SPLCV

Table 2.3 summarizes the findings of this experiment for both assessment dates. Overall the real-time PCR assay detected SPLCV-US in a greater proportion of test plants than the conventional PCR assay. This was especially true during the first assessment date when the real-time PCR assay detected the virus in 17 of 20 samples tested (total number of samples tested which may include inoculation escapes) compared to 5 of 20 for conventional PCR. For the second assessment date, real-time PCR assay detected the virus in 18 of 20 samples compared to 9 of 20 detected with the conventional PCR assay. By obtaining the relative titer levels of SPLCV-US in samples that were positive by both conventional and real-time PCR, we were able to estimate that the difference between the minimum amounts of virus that each method detected was a 1000-fold.

2.4 Discussion

Real-Time PCR is an extremely useful tool for studying various agents of infectious diseases in human and veterinary pathology (Mackay et al., 2002). In plant pathology, this technology is increasingly used for studying various causal agents of plant diseases (Frederick et al., 2000; Mumford et al., 2000; Boonham, 2002; Schaad et al., 2002). This is the first report of the use of real-time PCR technology and TaqMan[®] fluorogenic chemistry for the detection and quantification of sweetpotato viruses. The results obtained in this study show that real-time PCR is not only a faster and safer method (eliminates more hazardous material such as ethidium bromide used in conventional post-PCR analysis) but most importantly a more sensitive method for the detection and quantification of sweetpotato viruses directly from sweetpotato plants

Table 2.3. Comparison of real-time and conventional PCR assays for the detection of the United States strain of *Sweet potato leaf curl virus*, SPLCV-US (isolate SWFT-1), in five sweetpotato cultivars.

Cultivar	Rep	PCR ^a		Real-time PCR Ct ^b			
		22 DAP ^c	35 DAP	22 DAP		35 DAP	
Beauregard	A	-	+	18.22	+	18.55	+
Beauregard	B	-	-	35.29	-	38.75	-
Beauregard	C	-	-	20.42	+	19.42	+
Beauregard	D	-	-	19.66	+	19.22	+
Bienville	A	+	+	17.19	+	18.79	+
Bienville	B	+	+	17.23	+	19.10	+
Bienville	C	-	+	18.66	+	18.06	+
Bienville	D	-	+	17.72	+	19.12	+
Centennial	A	-	+	18.15	+	22.08	+
Centennial	B	+	+	18.66	+	22.04	+
Centennial	C	+	-	17.02	+	20.03	+
Centennial	D	-	-	35.49	-	37.77	-
Jewel	A	-	-	17.10	+	20.97	+
Jewel	B	+	+	18.42	+	19.99	+
Jewel	C	-	+	18.93	+	19.16	+
Jewel	D	-	-	18.72	+	21.09	+
Xushu	A	-	-	25.34	+	34.64	+
Xushu	B	-	-	35.02	-	32.74	+
Xushu	C	-	-	25.19	+	30.11	+
Xushu	D	-	-	28.95	+	27.75	+
NTC ^d		-	-	>35	-	>35	-
Healthy ^e		-	-	>35	-	>35	-
Positive ^f		+	+	~23	+	~23	+

^a Detection by agarose gel electrophoresis; + and - indicate presence and absence of the expected size band on the gel.

^b Samples with threshold cycles above 35 are considered negative for the presence of this virus.

^c DAP = days after transplanting SPLCV infected vine cuttings in the field.

^d NTC = no template water control

^e Healthy = a total DNA preparation from a virus-tested sweetpotato plant used as negative control throughout the experiment.

^f Positive = a total DNA preparation from a virus-infected sweetpotato plant used as positive control throughout the experiment.

compared to existing assays such as traditional PCR for SPLCV. However, this technology requires the use of special instruments and reagents which are relatively expensive at the present time.

Previous reports suggested that SPFMV is unevenly distributed within a sweetpotato plant (Green et al., 1988; Gibb and Padovan, 1993). Real-time PCR data presented here support this observation and suggests that titers of SPFMV are at very low levels, in some cases even below the threshold of detection in plants inoculated with individual potyviruses. The worldwide distribution of this virus, and the fact that yield reductions of singly-infected plants are insignificant, (Salazar and Fuentes, 2001; Clark et al., 2002) suggests that today's high yielding sweetpotato cultivars, which were bred in the presence of this virus, might have been unintentionally selected for resistance to this virus. The ability of this method to provide accurate quantitative data on sweetpotato viruses in different hosts, as illustrated by our experiments, will aid future studies to identify hosts from which vectors may be more likely to acquire viruses.

It has not been clear whether difficulty in detecting SPFMV in sweetpotato by other procedures has been due to inhibitors in sweetpotato that interfere with other types of assays, or low virus titers, or both. Serological reactions may be influenced by inhibitors in sweetpotato such as latex, polyphenols, and polysaccharides, and may explain the difficulty of detecting these viruses in symptomless tissues by ELISA (Gibb and Padovan, 1993). This is further supported by McLaughlin et al., (1984) who specifically showed that sap from red clover added in the sample buffer significantly decreased the overall sensitivity of their ELISA detection assay of viruses infecting forage legumes. However, no effect was observed when sap from white clover was used

suggesting that inhibitor levels vary greatly even within cultivars of the same species. The inherent difficulty in isolating and detecting SPFMV as well as other viruses directly from sweetpotato is reflected in the fact that the majority of studies used indicator plants such as *I. setosa*, *I. nil* and others for the indirect isolation of these viruses (Cohen et al., 1997; Lotrakul et al., 1998; Di Feo et al., 2000; Moyer et al., 2002; Souto et al., 2003). Even though at 3 wk after inoculation the titers of SPFMV were not different among the three hosts, the titer levels of potyviruses in *I. setosa* and *I. nil* were clearly and consistently above the threshold of detection while in sweetpotato plants they were often near or below the threshold. The initial virus titers present in each scion at the time of graft-inoculation, the rate of graft union formation, and variability in growth rates of each host, may account for sample to sample variability. However, high level of variability was not observed at 6 wk after inoculation. Potyvirus titers were higher in related species than in sweetpotato and there was no evidence of inhibitors of the potyvirus PCR reactions in sweetpotato, suggesting that difficulty in detecting these viruses in sweetpotato is due to low titers rather than inhibitors or problems with the assay. The fact that the titers of all three potyviruses were higher in *I. nil* may also explain the higher efficiency of aphid transmissions of these viruses from this host compared to sweetpotato (unpublished data).

Even though SPLCV is an important agent of disease [can reduce yields of Beauregard by 30% and reduce quality (Clark et al., 2002)], its importance has been overlooked because symptoms are not commonly found on sweetpotato plants. Symptoms can be mild and/or transient and may require in addition to SPLCV, a susceptible cultivar, favorable environment, and co-infection with SPFMV (Clark et al.,

2002). Due to the mild nature of the symptoms caused by SPLCV, even on indicator plants, specific and sensitive methods are a prerequisite for its reliable detection (Lotrakul and Valverde, 1999; Clark et al., 2002). Real-time PCR efficiently detected SPLCV in the majority of the total DNA preparations (from SPLCV-inoculated sweetpotato) tested. The sensitivity of detection of SPLCV in addition to the quantitative capabilities of this method will aid future studies of the distribution and interactions of this virus with unrelated viruses.

SPVD has not been reported in the US and SPCSV has been found only in an isolated tissue culture sample (Pio-Ribeiro et al., 1996), therefore a sensitive detection assay such as the one described here is of major importance in screening germplasm introduced from countries where this virus is present. The real-time PCR assay reported here provided a highly sensitive assay that allowed unequivocal detection of SPCSV as opposed to the often ambiguous results obtained with conventional PCR and ELISA assays (Clark, unpublished data).

Viral diseases of sweetpotato, especially those caused by commonly occurring mixed infections, are among the most economically important diseases of this crop. The sensitive quantitative capabilities of the real-time PCR assays, provided for the first time important information on replication rates of three potyviruses in different hosts. Future experiments will utilize the quantitative capabilities of real-time PCR to study known synergistic interactions as well as identify novel ones through titer quantification.

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CHAPTER 3: INTERACTIONS AMONG SWEET POTATO CHLOROTIC STUNT VIRUS AND DIFFERENT POTYVIRUSES AND POTYVIRUS STRAINS INFECTING SWEETPOTATO IN THE UNITED STATES

3.1 Introduction

Sweetpotato is affected by several virus disease complexes. These complexes, reported in different countries around the globe, generally include *Sweet potato feathery mottle virus* (SPFMV), a member of the genus *Potyvirus* in the family *Potyviridae*, as one component (Salazar and Fuentes, 2001). Sweet potato virus disease (SPVD), which has been characterized by many as the major constraint in increasing sweetpotato productivity worldwide (Aritua et al., 1998; Karyeija et al., 1998; Salazar and Fuentes, 2001), is caused by the synergistic interaction between SPFMV and *Sweet potato chlorotic stunt virus* (SPCSV), a member of the genus *Crinivirus* in the family *Closteroviridae*. Schaefers and Terry (1976) first reported that alone, SPFMV and SPCSV induce only very mild symptoms, but the synergistic interaction in dual infections causes the severe symptoms observed in the field. Since then, other viruses such as Sweet potato chlorotic fleck virus (SPCFV), *Sweet potato latent virus* (SPLV), Sweet potato mild speckling virus (SPMSV), and *Sweet potato mild mottle virus* (SPMMV) have been detected occasionally in sweetpotato plants exhibiting severe SPVD-like symptoms but never proven to play an important role in this disease complex (Gibson et al., 1998; Gutierrez et al., 2003; Tairo et al., 2004).

Two potyviruses recently described infecting sweetpotato in the United States, Ipomoea vein mosaic virus (IVMV) and *Sweet potato virus G* (SPVG) (Souto et al., 2003) enhanced symptoms in sweetpotato plants when co-infecting with SPCSV. For that reason, IVMV and SPVG have been suspected to contribute to the variation of

SPVD-like symptoms observed in naturally infected plants from different geographic locations (Hurt, personal communication). Their importance however, has often been overshadowed by the universal presence of SPFMV which is found wherever sweetpotatoes are grown (Brunt et al., 1996). Two strains of SPFMV have been recognized in the US, the common strain (SPFMV-C) and the russet crack strain (SPFMV-RC) (Moyer et al., 1980).

To date SPVD has been reported in a number of African countries, including Rwanda, Burundi, Uganda, Ghana, Nigeria, Kenya, Tanzania and Zimbabwe (reviewed by Karyeija et al., 1998), as well as other countries such as Israel (Loebenstein and Harpaz, 1960), Spain (Valverde et al., 2004), and Peru (Gutierrez et al., 2003). In the US, SPCSV, one of the two components of SPVD, has only been found in one sweetpotato tissue culture accession but has not been reported from the field (Pio-Ribeiro et al., 1996).

During co-infections of SPFMV with SPCSV, sweetpotato plants exhibit severe symptoms such as leaf strapping, vein cleaning, leaf distortion, chlorosis, puckering, and stunting (Salazar and Fuentes, 2001). Symptoms on SPVD-affected sweetpotato plants develop first in the newly emerging leaves. The time from inoculation to the appearance of symptoms takes longer in older and larger plants (Karyeija et al., 2000). In the late 1930's, Stayaert reported that within a few years in the republic of Congo, sweetpotato crops of 30t/year, were reduced to 4t/year resulting in their abandonment (Sheffield 1957). Since then, several cases of extreme yield loss (up to 90%) have been reported from different sweetpotato cultivars affected with SPVD (Hahn, 1976; Ngeve, 1990; Gutiérrez et al., 2003), including Beauregard (Aritua et al., 2000) which is the

predominant cultivar in U.S. sweetpotato production. Such dramatic yield reductions are correlated with the severity of leaf stunting and strapping symptoms observed (Salazar and Fuentes, 2001).

Karyeija et al. (2000) found that the titers of SPFMV were relatively high in all leaf parts in SPVD-affected plants and could be detected both by TAS- and NCM-ELISA. In contrast, SPFMV in singly infected sweetpotato plants was detected only on rare occasions by any of the test methods utilized. In SPVD-affected plants SPFMV was not confined to any leaf parts where as SPCSV was found only in the veins of the leaves tested. Furthermore, immunohistochemical tests confirmed that SPCSV was phloem-limited (Karyeija et al. 2000). Quantification of the two viruses using a RNA dot-blot hybridization method revealed a 600-fold increase of SPFMV RNA in SPVD affected plants compared to single infections with SPFMV and led to the hypothesis that SPCSV is able to enhance the multiplication of SPFMV in tissues even though titers of SPCSV remain relatively unchanged. Such results are unusual in that potyviruses have been shown to play the role of the “titer enhancer” in viral complexes (Goldberg and Brakke, 1987; Vance, 1991; Anjos et al., 1992; Vance et al., 1995), exactly the opposite of what Karyeija et al., (2000) reported.

To date the exact mechanism of synergism between the two viruses that cause SPVD is unknown. In this paper we report for the first time the use of real-time PCR, a sensitive quantitative assay, to study the effect of SPCSV on titers of other potyviruses and potyvirus strains infecting sweetpotato. Additionally, three of the most commonly occurring potyviruses in the US, SPFMV-RC, IVMV, and SPVG were further evaluated for their ability to interact with each other in pairwise and three-way combinations.

3.2 Material and Methods

3.2.1 Plant Material and Virus Inoculations

I. batatas plants infected with SPCSV (isolate BWFT-3) alone and *I. setosa* seedlings mechanically inoculated separately with isolate 95-2 of the russet crack strain of SPFMV (SPFMV-RC), the common strain of SPFMV (SPFMV-C), isolate 95-6 of SPFMV (phylogenetically related to SPFMV-C), IVMV (isolate LSU-2), and SPVG (isolate LSU-1) were grown in the greenhouse to generate the scions used to graft-inoculate the virus-tested, clonally propagated *I. batatas* cv. Beauregard. Eleven treatments were evaluated in a randomized complete block design, consisting of each potyvirus inoculated alone and in pairwise combination with SPCSV.

In a separate experiment, scions from *I. setosa* plants mechanically inoculated with SPFMV-RC, IVMV, and SPVG alone were used to graft-inoculate clonally propagated virus-tested Beauregard. In this experiment seven treatments consisting of each potyvirus inoculated alone, in pairwise and three-way combinations with each other were evaluated in a randomized complete block design.

Three weeks after planting (early Fall) vines were cut down to approximately 5 internodes, and a single wedge graft per virus was made. Only plants on which the scion(s) survived for at least 3 wk were used in these studies. All treatments were replicated six times. Plants were grown under standard greenhouse conditions in 15-cm-diameter clay pots containing autoclaved soil mix consisting of 1 part river silt, 1 part sand, 1 part Jiffy-Mix[®] Plus (Jiffy Products of America Inc., Norwalk, OH) and 3.5g per pot of Osmocote[®] 14-14-14 (Scotts-Sierra Horticultural Products Company, Marysville, OH). A weekly insecticide spray program was followed to control aphids and whiteflies.

At 3, 6 and 9 wk after inoculation the first four consecutive fully opened leaves from the top of each test plant were collected, combined and immediately frozen in liquid nitrogen and stored at -80°C until extraction. At each collection date, plants were cut back to an approximate length of 5 nodes. Since plants infected with single potyviruses are typically symptomless, nodes randomly selected after the final leaf collection were grafted on *I. setosa* indicator plants to confirm the presence of the appropriate potyvirus.

3.2.2 Total Nucleic Acid Extractions

Frozen leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle and total RNA and DNA were extracted using Qiagen's RNeasy Plant Mini Kit[®] (Qiagen Inc, Valencia, CA), according to the manufacturers' directions. To eliminate residual DNA contamination, all total RNA samples were treated on-column with DNase I using the RNase-Free DNase Set (Qiagen Inc), following the manufacturer's directions.

3.2.3 Real-time PCR Assays

Real-time PCR assays for relative quantification of these viruses were performed in 50µl reaction volume mixtures containing 900nM of each primer (forward and reverse), 200nM of the MGB TaqMan[®] probe, 25µl or 1.25µl of 2x or 40x master mixes of the TaqMan[®] One Step PCR Master Mix Reagents kit (AppliedBiosystems) respectively, and 5µl template RNA (Kokkinos and Clark, in press). The same protocol was followed for the endogenous control reactions, which enabled normalization between sample extraction variation, except for the substitution of the target virus primer/probe set with 2.5µl of the eukaryotic 18S rRNA pre-developed primer/probe mix (VIC/ MGB Probe) (AppliedBiosystems). The following real-time PCR thermal cycling conditions

were used: 48°C for 30 minutes (cDNA synthesis), 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.

All real-time PCR reactions were performed on ABI PRISM[®] 7000 Sequence Detection System using MicroAmp[®] optical 96-well reaction plates that were sealed with optical adhesive covers (AppliedBiosystems). The thermal cycling parameters were optimized for use with the TaqMan[®] One Step PCR Master Mix Reagent kits in singleplex reactions (AppliedBiosystems). To minimize any errors due to pipetting differences, duplicates of each sample were performed on each plate, and their threshold cycle (Ct) values were averaged during data analysis. In addition, every plate included non-template water controls (NTC) as well as positive (total RNA from virus-infected source plants) and negative (total RNA from virus-tested plants) controls. After validation experiments were performed as described in User Bulletin #2 (AppliedBiosystems), the $\Delta\Delta C_t$ quantification method, which eliminates the use of standard curves on every plate, was implemented for the normalization of samples.

3.3 Results

3.3.1 Virus Symptoms

At the time leaf samples were collected, all test plants co-infected with SPCSV and any of the potyviruses or potyvirus strains (except SPFMV-C) exhibited symptoms such as vein banding, vein necrosis, leaf distortion, chlorosis, puckering, and stunting (Table 3.1). Initial symptoms were observed in plants co-infected with SPFMV-RC and SPCSV at approximately 10 days post inoculation (DPI), followed by plants co-infected with SPFMV-95-6 and SPCSV at 12 DPI. Symptoms of plants co-infected with

Table 3.1. Symptoms observed in treatments involving pairwise infections of *Sweet potato chlorotic stunt virus* (SPCSV) with the russet crack strain of *Sweet potato feathery mottle virus* (SPFMV-RC), the common strain of SPFMV (SPFMV-C), isolate 95-6 of SPFMV (phylogenetically related to SPFMV-C), Ipomoea vein mosaic virus (IVMV), and *Sweet potato virus G* (SPVG).

Treatment	Symptoms ^a		
	3 WPI ^b	6 WPI	9 WPI
SPCSV+SPFMV-RC	CHL, CS, MLD, VB	CHL, LD, MLF, VB	CHL, LD, MLF, VB
SPCSV+SPFMV-C	MIP, TVC	MIP, TVC	MIP, TVC
SPCSV+SPFMV-95-6	CHL, CS, MLD, VB	CHL, LD, VB	CHL, LD, VB
SPCSV+IVMV	CHL, CS, LD, ST, VM	CHL, LD, VM, VN, SP, ST	CHL, LD, VM, VN, SP, ST
SPCSV+SPVG	MCHL, MLD, MVC	CHL, MLD, MVC	CHL, MLD, MVC

^aSymptom abbreviations are CHL=general chlorosis, CS=chlorotic spots, LD=leaf distortion, MCHL=mild general chlorosis, MIP=mild interveinal purpling, MLD=mild leaf distortion, MLF=mild leaf fan, MVC=mild vein clearing, SP=severe puckering, ST=stunting, TVC=transient vein clearing, VB=vein banding, VM=vein mosaic, VN=vein necrosis

^bWPI=weeks post inoculation.

IVMV and SPCSV, which were severe, were also among the latest to develop. Initial symptoms appeared 17 DPI, at a similar time as plants co-infected with SPVG and SPCSV, which however exhibited milder symptoms. Symptoms in plants co-infected with SPFMV-C and SPCSV were mild, including transient vein clearing and mild interveinal purpling, similar to plants singly-infected with SPCSV. Plants infected with any of the potyviruses alone were asymptomatic, as expected. Plants infected with SPFMV-RC, IVMV, and SPVG in pairwise and three-way combinations were also asymptomatic.

3.3.2 Relative Quantification of Potyviruses Alone and in Pairwise Combination with SPCSV

At 3, 6, and 9 wk after inoculation, titers of SPFMV-RC were greater when co-infecting with SPCSV ($P=0.002$, $P<0.001$, $P=0.001$, respectively) compared to titers of

SPFMV-RC in single infections (Fig. 3.1). The same was true for SPFMV-C ($P < 0.001$, $P = 0.003$, $P = 0.001$) (Fig. 3.2), SPFMV-95-6 ($P = 0.003$, $P = 0.001$, $P = 0.007$) (Fig. 3.3), IVMV ($P = 0.031$, $P = 0.041$, $P = 0.003$) (Fig. 3.4), and SPVG ($P = 0.047$, $P = 0.02$, $P = 0.035$) (Fig. 3.5). In the presence of SPCSV, SPFMV-95-6 had the greatest titers of all the potyviruses tested whereas SPFMV-RC was the lowest. In singly-infected plants, titers of these viruses were either very low or below the threshold of detection of the real-time PCR assay (Fig. 3.6) which is consistent with previous findings (Karyeija et al., 2000; Kokkinos and Clark, in press).

3.3.3 Relative Quantification of SPCSV

Titers of SPCSV in single infections were significantly greater than titers of the same virus in co-infections with individual potyviruses (Fig. 3.7) at either 3 ($P = 0.001$) or 6 wk ($P < 0.001$) after inoculation. Even though at 9 wk after inoculation titers of SPCSV in single infections remained significantly greater ($P = 0.006$) compared to titers of SPCSV in pairwise combinations with SPFMV-RC, IVMV, and SPVG, no significant difference was observed between SPCSV alone and SPCSV in pairwise combinations with SPFMV-C and SPFMV-95-6. No significant difference was observed in titers of SPCSV among treatments involving pairwise infections. Unlike any of the potyviruses, SPCSV was detected in all singly-infected plants and its titers were significantly greater ($P < 0.001$) compared to those of any potyvirus in single infections at each assessment date.

3.3.4 Relative Quantification of SPFMV-RC, IVMV, and SPVG in Pairwise and Three-way Combinations

Titers of SPFMV-RC, IVMV, and SPVG were either very low or below the threshold of detection regardless of whether they were infecting alone or with other

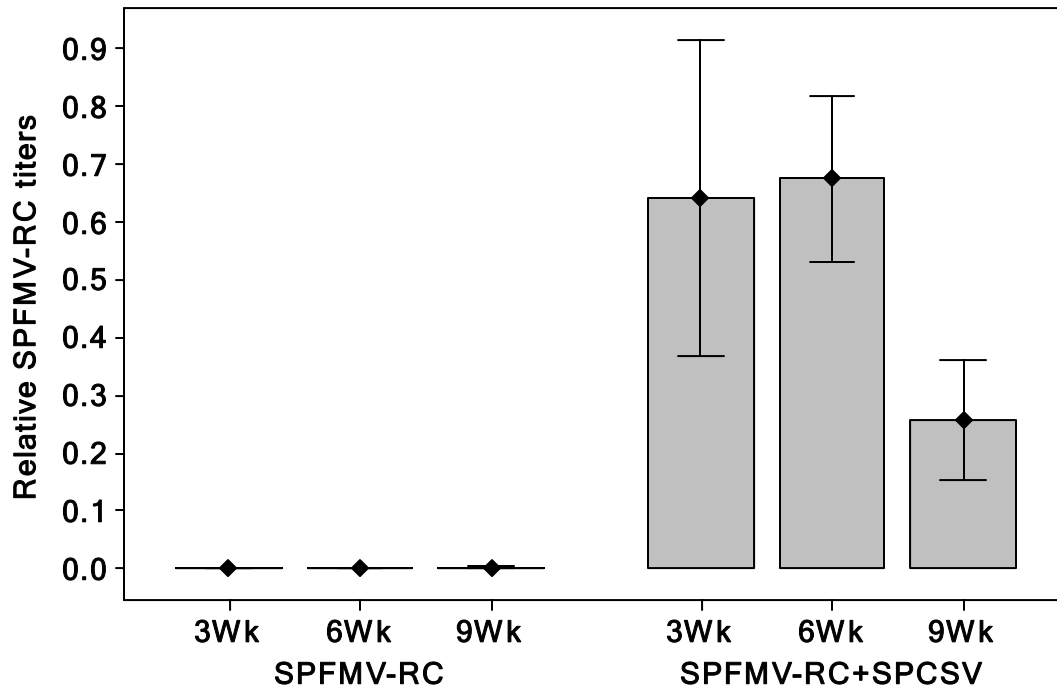


Figure 3.1. Relative virus titers determined by real-time quantitative PCR in single and pairwise infections with SPCSV of the russet crack strain of *Sweet potato feathery mottle virus* (SPFMV-RC) at 3, 6, and 9 wk post inoculation (WPI).

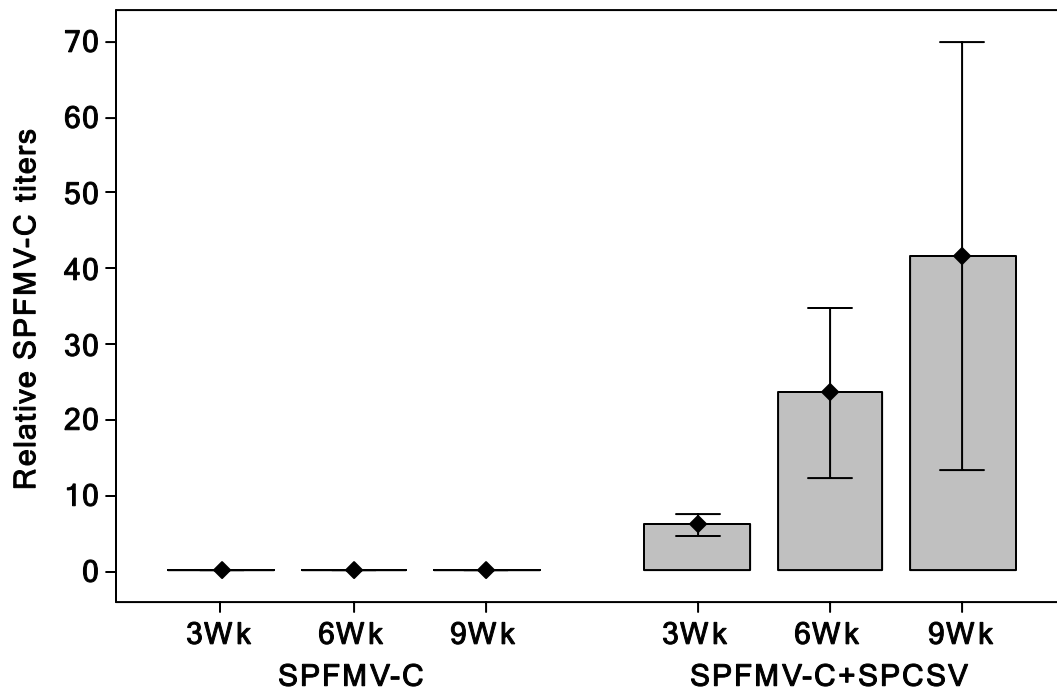


Figure 3.2. Relative virus titers determined by real-time quantitative PCR in single and pairwise infections with SPCSV of the common strain of SPFMV (SPFMV-C) at 3, 6, and 9 wk post inoculation (WPI).

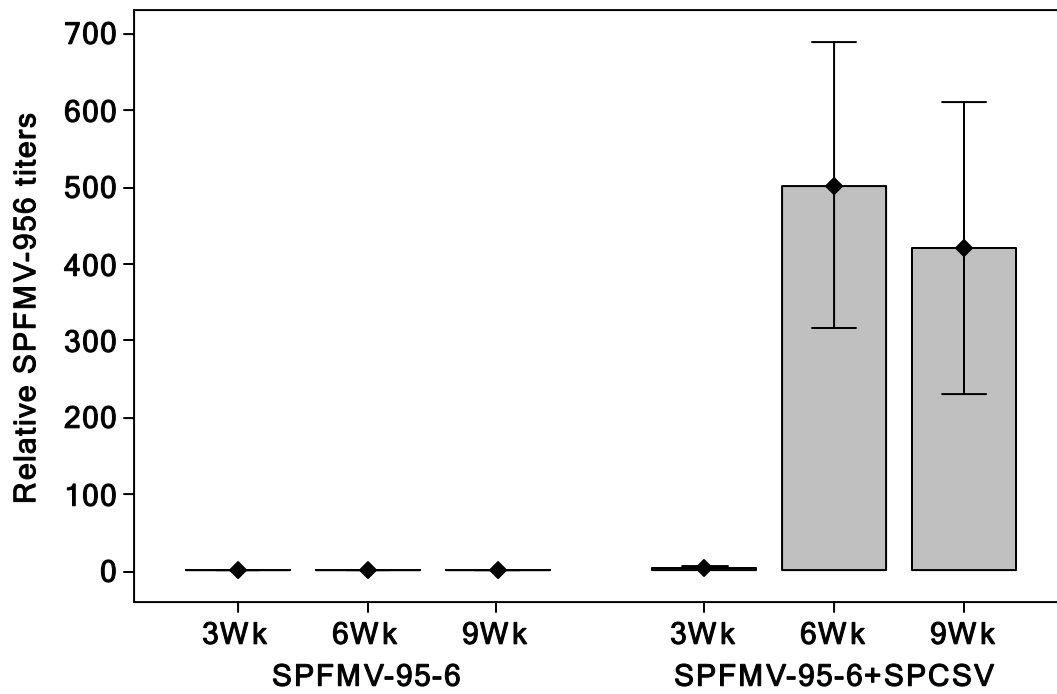


Figure 3.3. Relative virus titers determined by real-time quantitative PCR in single and pairwise infections with SPCSV of isolate 95-6 of SPFMV (SPFMV-95-6) at 3, 6, and 9 wk post inoculation (WPI).

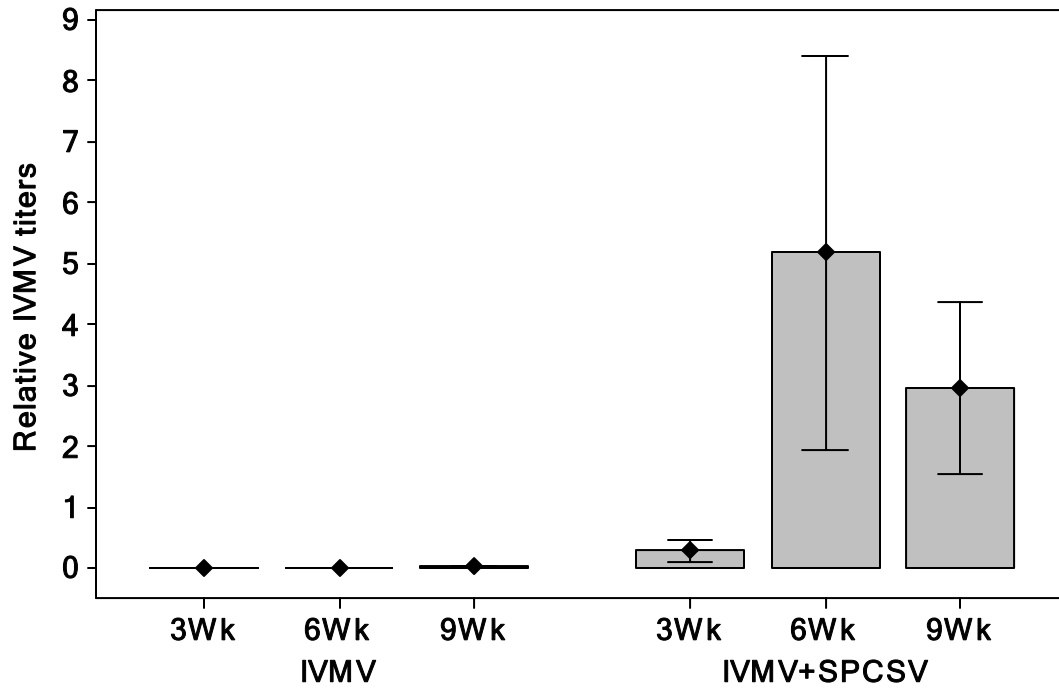


Figure 3.4. Relative virus titers determined by real-time quantitative PCR in single and pairwise infections with SPCSV of Ipomoea vein mosaic virus (IVMV) at 3, 6, and 9 wk post inoculation (WPI).

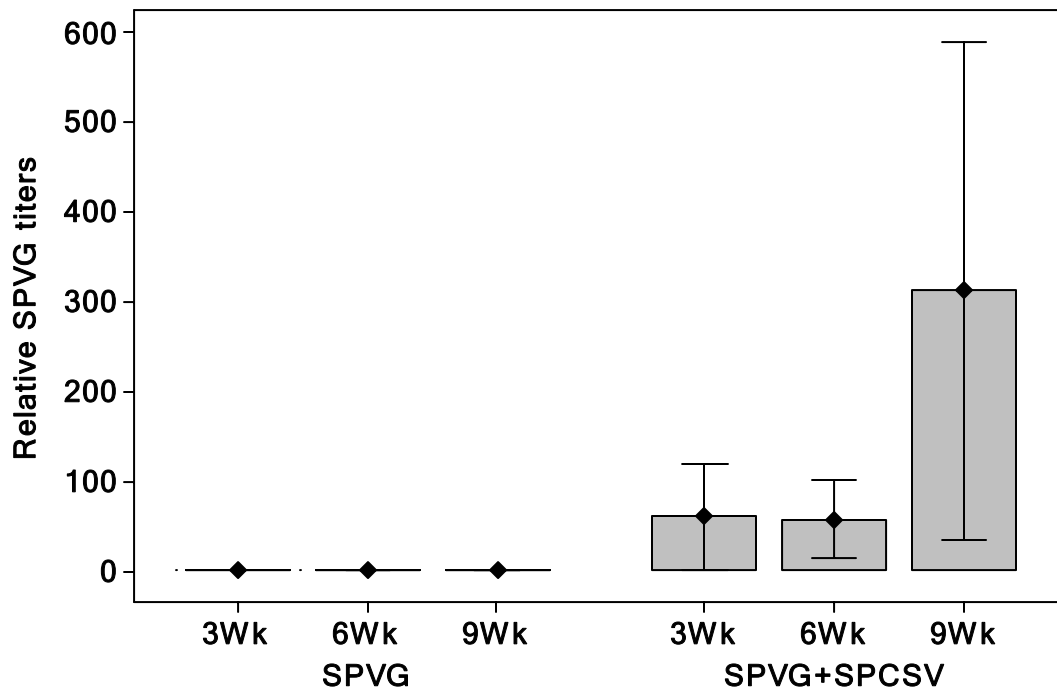


Figure 3.5. Relative virus titers determined by real-time quantitative PCR in single and pairwise infections with SPCSV of *Sweet potato virus G* (SPVG) at 3, 6, and 9 wk post inoculation (WPI).

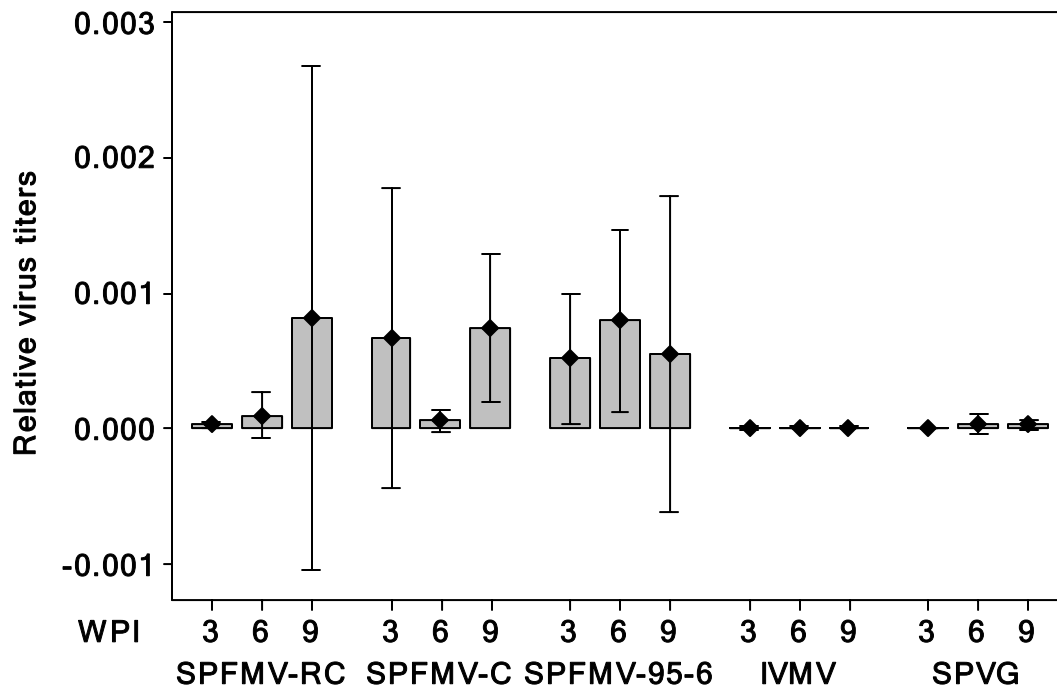


Figure 3.6. Relative virus titers determined by real-time quantitative PCR of the russet crack strain of *Sweet potato feathery mottle virus* (SPFMV-RC), the common strain of SPFMV (SPFMV-C), isolate 95-6 of SPFMV (SPFMV-95-6), *Ipomoea vein mosaic virus* (IVMV), and *Sweet potato virus G* (SPVG), in singly-infected sweetpotato plants at 3, 6, and 9 wk post inoculation (WPI).

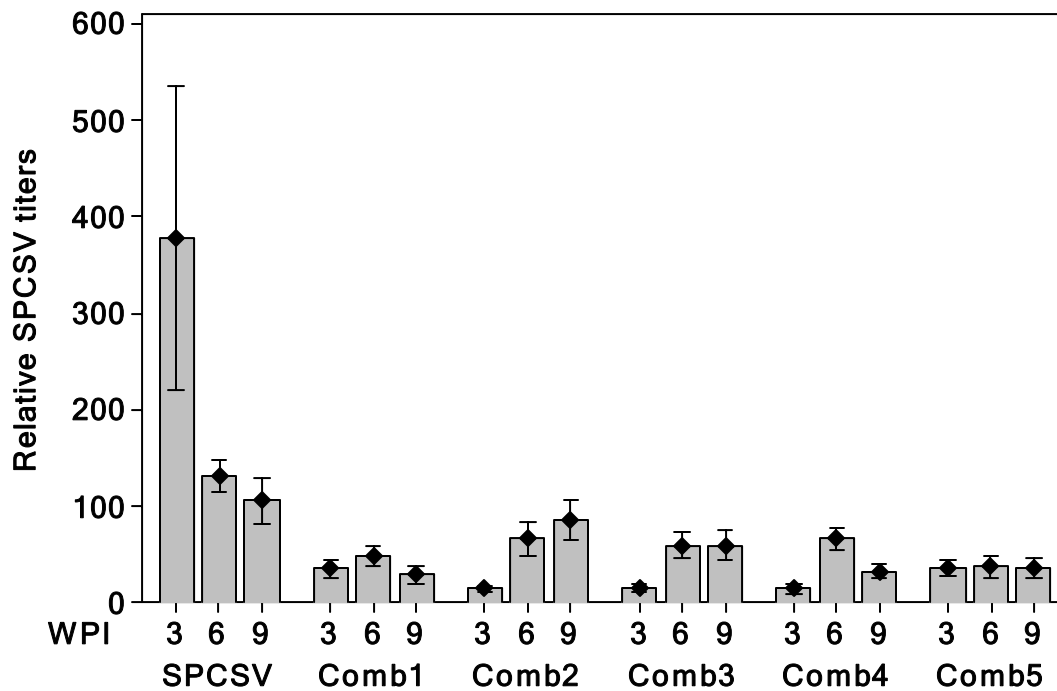


Figure 3.7. Relative virus titers determined by real-time quantitative PCR of *Sweet potato chlorotic stunt virus* (SPCSV) in single and pairwise infections with the russet crack strain of *Sweet potato feathery mottle virus* (SPFMV-RC) (Comb1), the common strain of SPFMV (SPFMV-C) (Comb2), isolate 95-6 of SPFMV (Comb3), Ipomoea vein mosaic virus (IVMV) (Comb4), and *Sweet potato virus G* (SPVG) (Comb5) at 3, 6, and 9 wk post inoculation (WPI)

potyviruses. No significant difference was observed in titers of SPFMV-RC at 3 ($P=0.47$), 6 ($P=0.43$), and 9 wk ($P=0.50$) post inoculation (Fig. 3.8), or for IVMV ($P=0.15$ $P=0.59$ $P=0.60$) (Fig. 3.9) or SPVG ($P=0.16$ $P=0.18$ $P=0.09$) (Fig. 3.10).

3.4 Discussion

SPVD, the most economically important viral disease of sweetpotato, is caused by the synergistic interaction between SPFMV and SPSCV. Many cultivars including Beauregard, the predominant cultivar grown in the U.S., are highly susceptible to SPVD with reported yield losses as great as 90% (Hahn, 1976; Ngeve, 1990; Aritua et al., 2000; Gutierrez et al., 2003). Other potyviruses such as IVMV and SPVG, which when co-infecting with SPCSV also cause severe symptoms, may play an important role in the type and severity of symptoms observed in naturally infected plants (Hurt, personal communication). In this study, all potyviruses evaluated were significantly enhanced in the presence of SPCSV suggesting the existence of a common mechanism underlying the enhancement of potyvirus replication. The degree of titer enhancement however, did not correspond to the intensity of symptoms observed. This was true even among the two strains of SPFMV. Even though titers of SPFMV-C were overall higher than titers of SPFMV-RC or IVMV in the presence of SPCSV, symptoms in plants co-infected with SPFMV-C and SPCSV were significantly milder compared to plants co-infected with either SPFMV-RC or IVMV and SPCSV. This suggests that the enhancement of replication of one virus is not sufficient by itself to induce the severity of symptoms associated with SPVD.

Using TAS-ELISA and RNA hybridization methods, Karyeija et al. (2000) found that titers of an East African isolate of SPFMV (Nam 1) were on average 600-fold higher

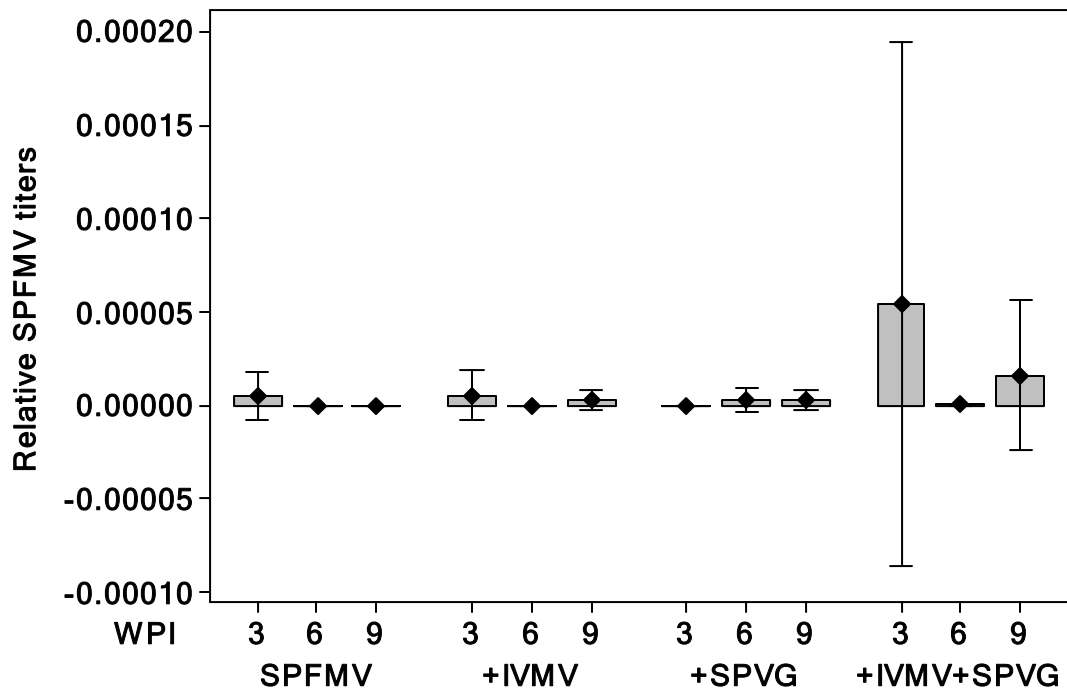


Figure 3.8. Relative titers of *Sweet potato feathery mottle virus* (SPFMV) determined by real-time quantitative PCR in single, pairwise, and three-way infections with *Ipomoea vein mosaic virus* (IVMV), and *Sweet potato virus G* (SPVG) at 3, 6, and 9 wk post inoculation (WPI).

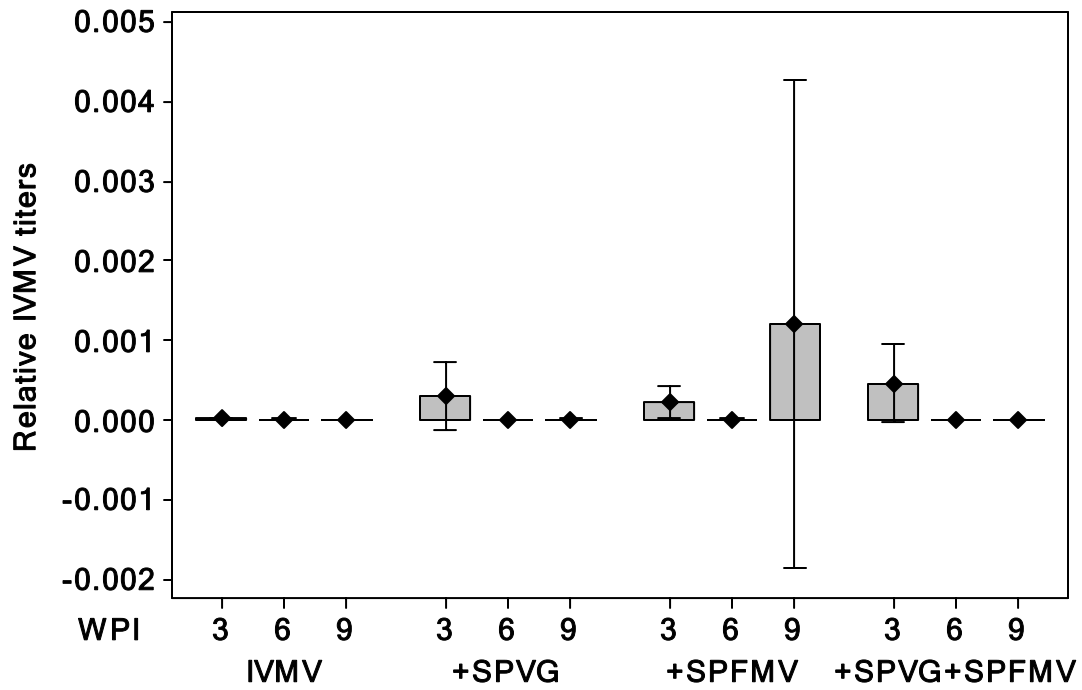


Figure 3.9. Relative titers of Ipomoea vein mosaic virus (IVMV) determined by real-time quantitative PCR in single, pairwise, and three-way infections with *Sweet potato virus G* (SPVG), and *Sweet potato feathery mottle virus* (SPFMV) at 3, 6, and 9 wk post inoculation (WPI).

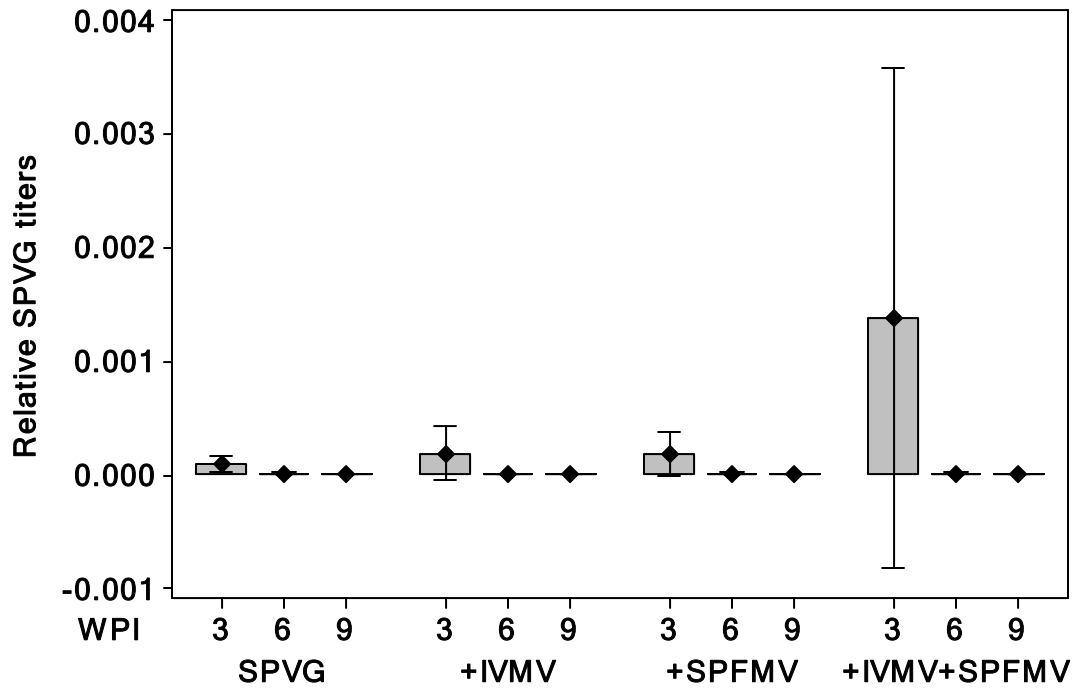


Figure 3.10. Relative titers of *Sweet potato virus G* (SPVG) determined by real-time quantitative PCR in single, pairwise, and three-way infections with *Ipomoea vein mosaic virus* (IVMV), and *Sweet potato feathery mottle virus* (SPFMV) at 3, 6, and 9 wk post inoculation (WPI).

in the presence of SPCSV approximately 5 wk after inoculation. Real-time PCR quantitative data from this study revealed a much greater increase in titer for the particular SPFMV isolates tested. Increases of 10^4 , 4×10^4 , and 3×10^5 -fold were observed for SPFMV-RC, SPFMV-C, and SPFMV-95-6, respectively. These findings can be attributed to the more sensitive quantitative capabilities of the real-time PCR assays, and/or the variability, as observed in this study, in the degree of titer enhancement even between strains of the same virus. In pairwise combination with SPCSV, titers of SPFMV-RC, SPFMV-95-6, and IVMV peaked between 3 and 6 wk post inoculation. In contrast, titers of SPFMV-C and SPVG peaked later, possibly after 9 wk post inoculation. The delay of SPFMV-C and SPVG reaching maximum titers may be associated with the fact that the plants infected with these viruses exhibited milder symptoms compared to plants infected with SPFMV-RC, SPFMV-95-6, and IVMV in pairwise combination with SPCSV.

As opposed to the potyviruses which were enhanced in the presence of SPCSV, titers of SPCSV were reduced in all treatments involving pairwise infections at both 3 and 6 wk after inoculation compared to single infections. Our results are very similar to results obtained by both Gibson et al., (1998) and Karyeija et al., (2000). Karyeija et al., (2000) indicated that SPCSV, which is a phloem-limited virus, remains phloem-limited even when co-infecting with SPFMV. SPCSV replicates well even in single infections and probably encodes a large amount of viral proteins throughout the plant. In pairwise combinations with potyviruses such protein products could be “utilized” for replication by the individual potyvirus, resulting in the reduction of the efficiency of SPCSV replication. A similar hypothesis has been postulated by Poolpol and Inouye (1986) to

explain the reduction of the enhancer virus, *Zucchini yellow mosaic virus* (ZYMV), in dual infections with *Cucumber mosaic virus* (CMV) in cucumber plants.

Titers of SPFMV-RC, IVMV, and SPVG, the commonly occurring potyviruses in the U.S., were neither enhanced nor suppressed in pairwise and three-way combinations with each other as compared to single infections. These results provide evidence that these viruses do not interact synergistically as measured by titer level, and support the work of Clark and Hoy, (2006) who found no significant yield losses between VT controls and plants infected with these viruses alone and only up to 15% reduction in yield with combinations of these potyviruses.

The importance of the potyvirus-encoded helper component proteinase (HC-Pro) in virus replication and accumulation is well documented (Atreya and Pirone, 1993; Kasschau and Carrington, 1995; Kasschau et al., 1997; Pruss et al., 1997). Involvement in viral RNA amplification has been suggested also for the papain-like leader proteinase (L-Pro) of the closteroviruses (Peremyslov et al., 1998). In fact both HC-Pro and L-Pro have been grouped in a large class of papain-like leader proteinases that were shown to aid in viral multiplication (Dougherty and Semler, 1993). Additionally, closteroviruses encode a homologue of plant HSP70 which in immature pea embryos infected by the potyvirus *Pea seed-borne mosaic virus* (PSbMV) was associated with virus replication (Aranda et al., 1996). A simple explanation for the titer enhancement of the potyviruses in pairwise combinations with SPCSV is that potyviruses may take advantage of protein products encoded by SPCSV, such as L-Pro and hHSP70 for their own replication. Such a mechanism however, would naturally create a competition between the two viruses,

something that in this study may be reflected in the significant reduction of SPCSV titers, observed in dual infections.

Since Schaefer and Terry (1976) first described SPVD and its viral components, several hypotheses on the mechanism of this synergism have been formulated. To date however, the exact mechanism of the viral interaction leading to SPVD remains elusive. The fact that the titers of all potyviruses and potyvirus strains tested here were increased in all treatments involving pairwise infections with SPCSV, whereas at the same time titers of SPCSV were reduced regardless of whether symptoms were severe or not, suggests that the conserved mechanism underlying this virus interaction may be independent of the mechanism involved in the development and severity of symptoms of this disease. In a separate study involving cDNA microarrays, most of the sweetpotato genes identified as stress-induced or resistance-related were differentially expressed in plants affected by SPVD but not in plants infected with either SPFMV or SPCSV alone (Kokkinos et al., submitted). Considered together with the results of this study, this suggests that the most probable mechanism resulting in the induction of this severe disease is one involving some form of interaction between the two viruses and the host (viral protein/viral protein/host protein interaction) rather than one involving a virus (SPCSV) suppressing the host's defense mechanism so the other virus (SPFMV) can achieve high titers and cause SPVD. This hypothesis is further strengthened by the inability of the potyviruses evaluated in this study to synergistically interact with each other in the absence of SPCSV.

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CHAPTER 4: THE EFFECT OF SWEET POTATO VIRUS DISEASE AND ITS VIRAL COMPONENTS ON GENE EXPRESSION LEVELS IN *IPOMOEA BATATAS* (L.) LAM.[†]

4.1 Introduction

Sweetpotato (*Ipomoea batatas* (L.) Lam.; Convolvulaceae) is the seventh most important food crop in the world (CIP, 1999), with a mean annual world production of 132 million metric tons between 1991-2000 (FAO, 2000). It ranks fourth in importance in the developing world after rice, wheat, and maize. Several diseases affect sweetpotato production. Among them, viral diseases including those caused by mixed infections are of major economical importance in most production areas around the globe. The use of vegetative cuttings as a principal propagation method provides viruses an efficient way to perpetuate and disseminate between growing seasons as well as growing areas (Salazar and Fuentes, 2001). As many as 19 different viruses have been identified in sweetpotato and 11 of these are currently recognized by the International Committee of Taxonomy of Viruses (ICTV) (Kreuze, 2002). The effects of these viruses on production range from minimal, to completely devastating, depending on the infecting virus, virus complexes, and sweetpotato cultivars involved.

The most important and devastating viral disease affecting sweetpotatoes worldwide is sweet potato virus disease (SPVD). Yield losses of up to 90% have been reported in plants affected with SPVD (Hahn, 1976; Ngeve, 1990; Gutiérrez et al., 2003). SPVD is caused by a synergistic interaction between a potyvirus, *Sweet potato feathery mottle virus* (SPFMV), and a crinivirus, *Sweet potato chlorotic stunt virus* (SPCSV). Plants co-infected with SPFMV or other sweetpotato potyviruses and SPCSV exhibit

[†] This chapter is the product of equal participation in both original conceptual input and research execution by the author of this dissertation and C. E. McGregor (PhD candidate, LSU Horticulture)

severe symptoms such as leaf strapping, vein clearing, leaf distortion, chlorosis, puckering, and stunting. The severity of symptoms, which develop first in the newly emerging leaves, can be directly associated with the dramatic yield reductions observed (Salazar and Fuentes, 2001). The time from initial infection to the appearance of symptoms varies depending on age and size of the plant, with symptoms taking longer to develop on older and bigger plants (Karyeija et al., 2000). SPVD has been reported in a number of African countries, including Rwanda, Burundi, Uganda, Ghana, Nigeria, Kenya, Tanzania and Zimbabwe (reviewed by Karyeija et al., 1998a). Outside Africa, this disease has been reported in Israel (Loebenstein and Harpaz, 1960), Spain (Valverde et al., 2004) and Peru (Gutierrez et al., 2003). In Argentina a similar synergism has been reported that also includes a third virus, *Sweet potato mild speckling virus* (Di Feo et al., 2000).

SPFMV, a member of the *Potyviridae* family and the *Potyvirus* genus, is transmitted by a number of aphid species. Plants infected with SPFMV alone, often are symptomless or exhibit mild symptoms and the yield losses are usually minimal (Clark and Hoy, 2005; Gutiérrez et al., 2003). The titers of SPFMV in these plants are similarly low (Kokkinos and Clark, 2006). However, the titers increase dramatically when plants are co-infected with SPCSV (Karyeija et al., 2000; Kreuze, 2002; Kokkinos and Clark, 2004), with a corresponding increase in the severity of disease symptoms and yield loss. SPFMV is common wherever sweetpotatoes are grown (Brunt et al., 1996). In the United States two strains of SPFMV, the common strain (SPFMV-C) and the russet crack strain (SPFMV-RC) are recognized. However, SPFMV-C does not cause typical SPVD

symptoms in the presence of SPCSV. Symptoms are usually mild and transient or typical of single infections with SPCSV (Souto et al., 2003).

Infection of sweetpotatoes with the phloem-limited crinivirus (family of *Closteroviridae*) SPCSV alone can lead to mild to moderate symptoms, with yield losses of up to 43% (Gutiérrez et al., 2003). The titers of this whitefly-transmitted virus are relatively high in infected plants. Interestingly, the titers do not change significantly after co-infection with SPFMV (Kreuze 2002). To date SPCSV has only been found in the US in a tissue culture accession and not in the field (Pio-Ribeiro et al., 1996).

Efforts to breed for resistance to SPVD have until now focused mainly on breeding for resistance to SPFMV and many sweetpotato varieties are reasonably resistant to SPFMV (Gibson et al., 1998). Efforts to use SPFMV resistance to breed for SPVD resistance have been unsuccessful because the SPFMV resistance is broken when plants are co-infected with SPCSV (Karyeija, et al., 1998b). The mechanism underlying the synergistic interaction between SPFMV and SPCSV and its effect on the host's response to infection are not known. It is possible that other molecular interactions in the dual infection process may provide better opportunities for resistance to SPVD than narrowly focusing on resistance to SPFMV. Understanding this phenomenon is essential if breeding for resistance to SPVD is going to be successful. An understanding of host-pathogen interactions on the molecular level can provide new insights into the effect of the synergism between SPFMV and SPCSV on the host, and can lead to new approaches in breeding for resistance to SPVD.

Microarray technology (Schena et al., 1995) makes possible the assessment of relative gene expression levels of thousands of genes simultaneously. Genes from the

organism under investigation (sweetpotato in this case) are spotted on a glass slide, which is then hybridized with mRNA from different treatments. The use of 2 different fluorescent dyes makes it possible to hybridize two treatments (or a treatment and control) on a single array. After hybridization the array is scanned using a fluorescent scanner and computer software is used to extract intensity values from the image. Statistical analysis of the data makes it possible to determine which genes are differentially expressed between treatments. Microarrays have already been used to investigate host-pathogen interactions in plants (Gibly et al., 2004; Dowd et al., 2004; De Vos et al., 2005; Moy et al., 2004) and other organisms (for review see Kato-Maeda et al., 2001). Virus associated host-pathogen interactions have been studied in a range of organisms, from humans (Zhu et al., 1998) to *Arabidopsis* (Whitham et al., 2003; Golem and Culver, 2003). In this paper we report the use of sweetpotato cDNA microarray technology in an effort to better understand the effect of the synergistic interaction between SPFMV and SPCSV on the host's response to infection. To our knowledge this study represents the first effort to investigate the effect of SPVD and its viral components on gene expression of sweetpotato.

4.2 Material and Methods

4.2.1 Plant Material and Inoculations

I. setosa seedlings mechanically inoculated with the russet crack strain of SPFMV (SPFMV-RC, isolate 95-2), and *I. batatas* plants infected with SPCSV (isolate BWFT-3) alone were grown in the greenhouse to generate the scions that were used to graft-inoculate clonally propagated plants of virus-tested *I. batatas* cv. Beauregard. Test plants were graft-inoculated 3 wk after planting. A single wedge graft per virus was performed

and individuals on which the scion(s) survived for at least 3 wk were selected and used in this study. The experiment consisted of the following four treatments in a randomized complete block design: Healthy (virus-tested plants), SPFMV-RC (virus-tested plants graft inoculated with SPFMV-RC alone), SPCSV (virus-tested plants graft inoculated with SPCSV alone) and SPVD (virus-tested plants graft inoculated with SPFMV-RC and SPCSV simultaneously). Each treatment was replicated six times. Plants were grown under standard greenhouse conditions in 15-cm-diameter clay pots containing autoclaved soil mix consisting of 1 part river silt, 1 part sand, 1 part Jiffy-Mix[®] Plus (Jiffy Products of America Inc., Norwalk, OH) and 3.5g per pot of Osmocote[®] 14-14-14 (Scotts-Sierra Horticultural Products Company, Marysville, OH). A weekly insecticide spray program was used to control aphids and whiteflies. At 9 wk after inoculation the first four fully opened leaves from the top of each test plant were collected, combined and immediately frozen in liquid nitrogen and stored at -80°C until extraction.

4.2.2 RNA Isolation, Labeling and Array Hybridization

Total RNA was extracted from 6 plants of each treatment. After leaf material were ground with a mortar and pestle in liquid nitrogen, approximately 0.8g were used to extract total RNA using the RNeasy Maxi Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RNA was further cleaned and concentrated by using the clean-up procedure as described in the RNeasy Mini Kit Manual (Qiagen). During both steps, DNase I digestion was carried out on the column as recommended by the manufacturer. RNA concentrations and quality were determined by measuring absorption at 260nm and 280nm on a spectrophotometer (GeneQuant, Pharmacia).

For each sample 10µg of total RNA was labeled using the SuperScript Indirect cDNA Labeling System for DNA Microarrays (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Samples were labeled with Cy3 or Cy5 fluorescent labels (Amersham Biosciences, Piscataway, NJ) and hybridized onto arrays in a connected loop design (Rosa et al., 2005) using the Pronto hybridization kit (Corning, NY). To limit dye effects the order of the treatments in the loops, as well as the direction of labeling were varied.

4.2.3 Array ARCS_SP02/2

The sweetpotato ARCS_SP02/02 array is a new version of the ARCS_SP02 array. It contains 3600 features, spotted in triplicate with a Genemachines Omnigrid microarray printer (GeneMachines, San Carlos, CA) on Corning GAPSII slides (Corning, NY). The arrays were printed and supplied by Silvia Fluch at ARC Seibersdorf research GmbH (Biogenetics/Natural resources, 2444 Seibersdorf, Austria). The array contains 2765 features from sweetpotato leaf and sweetpotato storage root libraries as well as control features, including non-plant features, spotting buffer features and blanks. The sequence information for the sweetpotato cDNAs features spotted on the array is available online in GenBank.

4.2.4 Array Scanning, Image Quantification and Statistical Analysis

Arrays were scanned with an AlphaArray Reader (Alpha Innotech, San Leandro, CA) and spots were detected and quantified using UCSF Spot (Jian et al. 2002). After comparing the effects of different normalization methods using MA- and spatial image plots, data were normalized within (print-tip loess) (Smyth and Speed, 2003) and between slides (scaled). Linear models (Smyth, 2004) were fitted for comparisons between

treatments and genes were considered differentially expressed if $P < 0.05$ after applying the Holm (1979) multiple testing correction. All normalizations and statistical analyses were carried out using limmaGUI software (Wettenhall and Smyth, 2004).

4.2.5 Real-time Quantitative PCR (Q-RT-PCR)

Two-step real-time quantitative PCR was carried out for 7 genes using RNA from the 6 VT and 6 SPVD affected plants. First-strand cDNA synthesis was carried out using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) and the resulting product was diluted by adding 40 μ l water. One microliter of the dilution was used for Q-RT-PCR on the ABI PRISM® 7000 Sequence Detection System using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 600nM of each primer (Table 4.1) in a final volume of 25 μ l. The following PCR protocol was followed: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 55°C for 1 minute. Amplifications from 18S ribosomal RNA specific primers (Applied Biosystems) were used to normalize data and dissociation curves were used to detect nonspecific amplification. Significant differences ($p = 0.05$) between treatments were determined using a t-test (variances not assumed equal) of normalized values.

4.3 Results

In order to identify differentially expressed genes between treatments, leaf material was collected from 6 individual plants (6 biological replicates) for each treatment (and control). The RNA samples were hybridized in a connected loop design consisting of 6 individual loops. This design ensures that samples are labeled an equal number of times with Cy3 and Cy5. The order of samples in the loops and the direction of the labeling were different for different loops to ensure that a specific comparison in

Table 4.1. Q-RT-PCR primers used for validation of microarray results. The primers were designed using Primer Express (version 2.0) (Applied Biosystems, Foster City, CA).

Gene	GenBank Accession	Primer Name	Sequence 5'-3'
Cat2	DV036659	Fwd	GGGCCAATTCTGTTGGAAGA
		Rev	TCTGGGATCCTTTCACGAGTG
ERD15	CB330921	Fwd	CCAGCAGCAGGGAACAGAAT
		Rev	CATCGAGATCAATGGTATCAGGC
TIR-NBS-LRR	DV036322	Fwd	TCACCTCTTTGCAGCGTTGT
		Rev	GTCCTTTACGGAGCTCTTCTTCAT
HSP70-1	DV037387	Fwd	CTTGGTCTTGAAACTGCCGG
		Rev	TTCTTGGTGGGAATGGTGGT
LHCB3	CB330249	Fwd	TTTTCTGCCCAAACCTCCTTCAT
		Rev	AAACCAGCAGTGTCCCATCC
Ankyrin	DV036499	Fwd	CATGTCCACCATGCTTGAGAGT
		Rev	TGCGTGCCATTCGTTCTTC
MT2A	CB330120	Fwd	CGGGTGCAAGATGTACCCAG
		Rev	CGCCAAGAACAAGGGTCTCA

the loop is not always labeled with the same dye and hybridized together on the same array (and by implication, that some comparisons are never hybridized on the same array).

LimmaGUI (Wettenhall and Smyth, 2004) was used to analyze the data specifically because it can handle this type of experimental design with two-color microarrays. Print-tip loess normalization was used within slides and scaled normalization between slides. These normalization methods were chosen after evaluating MA-plots before and after different normalizations. A p-value cutoff ($P < 0.05$ after multiple testing correction) was used instead of a fold change cut-off because previous data (C. McGregor unpublished data) showed that this is more appropriate when a large number of replicates are used. Also, various studies (Iyer et al., 1999; Yang et al., 2002; Yuen et al., 2002; Brinker et al., 2004; Czechowski et al., 2004; Dowd et al., 2004; Larkin et al. 2004) have shown that fold changes obtained from microarray analysis often

underestimate fold changes when compared with real-time PCR. In this study, the output from limmaGUI is in the form of M-values (\log_2 fold change) (Wettenhall and Smyth, 2004) (Table 4.2).

Gene descriptions were obtained by comparison of sequences to GenBank and *Arabidopsis thaliana* protein sequences (TIGR) (BLASTX E-value < 1E-5). Functional classification of genes in Table 4.2 was based on information from the Munich Information Center for Protein Sequences (MIPS) (Schoof et al., 2002). Several genes characterized as “Unclassified” have been shown in the literature to be related to plant defense or stress. Further information regarding some of these genes is presented in the discussion section.

The number of genes differentially expressed between virus tested (VT) plants and the 3 treatments varied enormously. Between VT and SPFMV-RC alone, and VT and SPCSV alone, only 3 and 14 genes were differentially expressed, respectively, compared to 216 between VT and SPVD (Table 4.2). It is noted that the number of differentially expressed genes was analogous to the severity of symptoms observed in the three viral treatments. At the time leaf samples were collected from SPFMV-RC-infected plants, and throughout the time period between inoculation and sample collection, no symptoms were observed, typical of single potyvirus infections (presence of the virus was confirmed by grafting of scions from test plants to indicator plants). Symptoms of SPCSV-infected plants at the time of collection however, were distinct and characteristic of SPCSV single infections and included interveinal chlorosis and mild purpling. As expected, the most severe symptoms were observed with SPVD-affected plants, which exhibited vein clearing, leaf distortion, chlorosis, puckering, and overall stunting. Nine

Table 4.2. Selected genes in sweetpotato differentially expressed ($P < 0.05$) between virus-tested (VT), *Sweet potato feathery mottle virus* russet crack strain-infected (SPFMV), *Sweet potato chlorotic stunt virus*-infected (SPCSV), and plants infected with SPFMV and SPCSV (SPVD).

GenBank Accession number	Gene Description	M-values ^a					
		VT-SPFMV	VT-SPCSV	VT-SPVD	SPFMV - SPCSV	SPFMV -SPVD	SPCSV -SPVD
CELL RESCUE, DEFENSE AND VIRULENCE							
CB330627	Bet v I allergen family protein			0.68		0.63	0.50
DV036659	catalase 2			-0.62			
DV035471	disease resistance protein (CC-NBS-LRR class)			-0.40			-0.43
DV036322	disease resistance protein (TIR-NBS-LRR class)			-0.47		-0.44	-0.48
CB330666	metallothionein-like type 1 protein	-0.46	-0.76	-0.89		-0.42	-0.46
CB330120	metallothionein protein, putative (MT2A)			0.43			
CB330891	NDR1/HIN1-like protein			-1.02		-0.97	-0.89
CB330630	peroxidase 42 (PER42) (P42) (PRXR1)			0.55		0.48	
CB330206	Rac-like GTP-binding protein (ARAC10)			0.72		0.71	0.52
CB330564	trigger factor type chaperone family protein			0.72		0.77	
PROTEIN SYNTHESIS & PROTEIN FATE							
DV035469	20S proteasome beta subunit E, putative			-0.61		-0.68	
DV034935	30S ribosomal protein S13, chloroplast (CS13) ribosomal protein S13 precursor			-0.56			
CB330853	30S ribosomal protein S18 family			0.80		1.06	0.68
DV034886	40S ribosomal protein S3 (RPS3C)			-0.79		-0.69	-0.71
DV037420	40S ribosomal protein S10 (RPS10C)			-0.50		-0.52	-0.44
CB330693	40S ribosomal protein S12 (RPS12C)			0.40		0.43	
CB330148	40S ribosomal protein S30 (RPS30C)			-0.92		-0.94	-0.81

(Table 4.2 cont'd.)

DV036725	60S ribosomal protein L6 (RPL6A)	-0.29			
DV036214	60S ribosomal protein L11	-0.46		-0.44	
DV037150	60S ribosomal protein L12 (RPL12C)	-0.52		-0.56	
DV037214	60S ribosomal protein L13A (RPL13aB)	-1.08		-1.14	
CB330735	60S ribosomal protein L26 (RPL26A)	-0.74		-0.73	-0.68
DV036489	60S ribosomal protein L31 (RPL31A)	0.37		0.39	0.41
CB330088	60S ribosomal protein L36a/L44	-0.80		-0.73	-0.74
CB330146	elongation factor 1B-gamma, putative / eEF-1B gamma, putative	0.65		0.59	0.48
CB329890	eukaryotic translation initiation factor 2B family protein / eIF-2B family protein	0.54		0.48	
CB330048	cyclophilin-type family protein	-0.61		-0.66	-0.63
CB330102	polyubiquitin (UBQ10) (SEN3)	-0.53		-0.77	-0.52
CB330070	senescence-associated protein subtilase family protein	0.77		0.63	0.54
METABOLISM					
CB329937	5-methyltetrahydropteroyltriglutamate- homocysteine methyltransferase / vitamin- B12-independent methionine synthase / cobalamin-independent methionine synthase (CIMS)	0.67		0.51	
CB330699	adenine phosphoribosyltransferase, putative	0.36		0.35	
DV037724	adenosine kinase 2 (ADK2)	-0.42			
CB330272	carbonic anhydrase 1, chloroplast / carbonate dehydratase 1 (CA1)	0.95		1.07	0.91
CB330084	cinnamyl-alcohol dehydrogenase (CAD), putative	0.33			0.36
CB330293	coproporphyrinogen III oxidase, putative	1.03		0.73	0.67

(Table 4.2 cont'd.)

DV037506	eukaryotic translation initiation factor 5A-1 (eIF-5A 1)	-0.50	-0.57	-0.46
CB330285	ferredoxin-thioredoxin reductase, putative	0.66	0.75	0.67
CB330640	fructose-bisphosphate aldolase, putative	0.90	0.96	1.23
CB329981	glucose-6-phosphate isomerase, putative	0.55		
CB330405	glutamate:glyoxylate aminotransferase 2 (GGT2)	0.49		
CB330166	glyceraldehyde-3-phosphate dehydrogenase, cytosolic (GAPC)	0.48		
CB330355	glycine cleavage system H protein, mitochondrial, putative	0.60	0.59	
CB330829	glycine dehydrogenase [decarboxylating], putative / glycine decarboxylase, putative / glycine cleavage system P-protein, putative	0.44	0.47	0.59
CB329974	glycine hydroxymethyltransferase, putative / serine hydroxymethyltransferase, putative / serine/threonine aldolase, putative	0.99	0.79	0.74
CB330544	phosphoglycolate phosphatase, putative	0.68	0.55	0.55
CB330622	ribulose bisphosphate carboxylase small chain 2B / RuBisCO small subunit 2B (RBCS-2B) (ATS2B)	1.15	1.05	1.34
DV035761	shaggy-related protein kinase kappa / ASK-kappa (ASK10)	0.76	0.59	0.56
DV037227	sterol desaturase family protein	-0.55	-0.56	-0.54
CB330375	terpene synthase/cyclase family protein	0.81	0.67	0.81
TRANSCRIPTION				
CB329931	CBS domain-containing protein	0.42		

(Table 4.2 cont'd.)

DV035417	CCR4-NOT transcription complex protein, putative	-0.53		-0.49
CB330050	pentatricopeptide (PPR) repeat-containing protein	1.04	0.89	0.81
CB330261	PHD finger family protein	0.63		
CB330874	RNA polymerase II mediator complex protein-related	0.55	0.50	
ENERGY				
DV035668	ATPase alpha subunit	0.70	0.66	0.66
CB330656	chlorophyll A-B binding protein / LHCI type I (CAB)	0.80	0.71	0.62
CB330553	chlorophyll A-B binding protein / LHCI type III (LHCA3.1)	0.95	0.84	0.85
CB329932	chlorophyll A-B binding protein / LHCII type I (LHB1B2)	0.68	0.63	0.54
CB330898	chlorophyll A-B binding protein / LHCII type II (LHCB2.2)	1.29	0.94	0.79
CB330249	chlorophyll A-B binding protein / LHCII type III (LHCB3)	0.67	0.64	0.64
CB330265	cytochrome B6-F complex iron-sulfur subunit, chloroplast / Rieske iron-sulfur protein / plastoquinol-plastocyanin reductase (petC)	0.66	0.71	0.67
CB330941	cytochrome c oxidase subunit Vc family protein / COX5C family protein	0.54	0.50	
CB330771	oxygen-evolving enhancer protein 3, chloroplast, putative (PSBQ1) (PSBQ)	0.64	0.64	0.53
CB330449	photosystem I reaction center subunit XI, chloroplast (PSI-L) / PSI subunit V	0.89	0.79	0.79

(Table 4.2 cont'd.)

SUBCELLULAR LOCALIZATION

CB330038	calmodulin			-0.88		-0.79	-0.79
DV035218	transducin family protein / WD-40 repeat family protein			-0.30			
DV037573	tubulin alpha-6 chain (TUA6)				0.50		
DV035184	vacuolar ATP synthase subunit G 1			-0.56		-0.46	-0.44

TRANSPORT

CB330313	acyl carrier family protein / ACP family protein					0.59	
DV037387	heat shock protein 70 (HSP70-1)			-0.51		-0.50	-0.45
CB330259	ferredoxin, chloroplast (PETF)			0.66		0.75	0.67
CB330095	lipid transfer protein 3 (LTP3)			-0.80		-0.82	-1.09
CB330457	lipid transfer protein (LTP) family protein		0.48				

INTERACTION WITH THE ENVIRONMENT

DV034646	gibberellin-regulated protein 5 (GASA5)			-0.57		-0.61	-0.54
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SIGNAL TRANSDUCTION

CB330823	mitogen-activated protein kinase / MAPK, putative			-1.31		-1.42	
DV035511	receptor-like protein kinase, putative			-0.60		-0.64	-0.58

UNCLASSIFIED PROTEINS

CB330182	16kDa membrane protein				0.99		
DV035493	26S proteasome regulatory subunit S2 (RPN1)	-0.51	-0.78	-0.40			
DV037499	60S ribosomal protein L29 (RPL29B)			-0.38			
DV036499	ankyrin repeat family protein			0.44			0.43
DV036039	Armadillo/beta-catenin repeat family protein		-0.67				
CB330237	ATP synthase D chain-related			0.48			
CB329999	ATP synthase g subunit family protein (mitochondrial)			0.57		0.45	

(Table 4.2 cont'd.)

CB329954	BTB/POZ domain-containing protein		0.51		0.46	
CB330400	bundle-sheath defective protein 2 family / bsd2 family				0.48	
DV037490	casein kinase, putative		-0.45		-0.52	
DV035142	chloroplast nucleoid DNA-binding protein-related		-0.58		-0.55	-0.61
CB330921	dehydration-induced protein (ERD15)		-0.45		-0.52	
DV034827	DNA-binding storekeeper protein-related		-0.33			
CB330447	DNAJ heat shock N-terminal domain- containing protein			-0.83		-0.66
DV036723	dormancy-associated protein, putative (DRM1)		-0.55		-0.55	
AF146691.1	ELI3		-0.75			
CB330810	emp24/gp25L/p24 protein-related		0.59			
DV037327	epoxide hydrolase, putative	-0.49		-0.43		
CB330021	fructosamine kinase family protein		0.62			
CB330841	Ferredoxin I, chloroplast precursor		-0.63		-0.65	-0.58
CB330388	hevein-like protein (HEL)		0.56			
DV036087	hydroxyproline-rich glycoprotein family protein (HRGP)		-0.50		-0.46	
CB329970	membrane protein, putative		1.01		0.84	0.74
DV037229	methyl-CpG-binding domain-containing protein				-0.69	
DV035503	methyltransferase MT-A70 family protein	-0.66	-0.45			
DV036783	myb family transcription factor					-0.52
DV035732	pentatricopeptide (PPR) repeat-containing protein		-0.40		-0.42	-0.35
CB330263	photosystem II 10 kDa polypeptide	0.76	0.97		0.97	0.63

(Table 4.2 cont'd.)

CB330912	photosystem II core complex proteins psbY, chloroplast (PSBY) / L-arginine metabolising enzyme			1.13	0.87	0.90
CB330616	pit2 (which expression is induced by Pi starvation),			-0.52	-0.56	-0.59
CB330154	plastocyanin	0.50	0.55	0.83		
DV037560	polygalacturonase inhibiting protein 2 (PGIP2)			-0.38	-0.42	-0.34
DV034758	protein kinase family protein			-0.66	-0.58	-0.52
DV036718	Reticulon family protein (RTNLB3)			-0.74	-0.71	-0.68
DV034984	Riboflavin synthase			-0.37		
CB330073	senescence-associated family protein			-0.70	-0.73	-0.75
DV035136	senescence-associated protein-related			-0.68	-0.64	-0.64
DV037482	Sporamin (Kunitz type trypsin inhibitor family)		-0.72		-0.79	0.65
CB330386	SWIB complex BAF60b domain- containing protein			0.71	0.65	0.51
DV037510	TATA-binding protein-associated factor TAFII55 family protein			-0.53	-0.54	-0.44
CB330112	wound-responsive family protein			-0.67	-0.46	-0.44
DV034644	yippee family protein			-0.81	-0.46	-0.44
DV035849	zinc finger (C2H2 type) family protein		-0.50			

^a M-values = \log_2 (fold change)

weeks after inoculation was selected as the collection date to ensure better uniformity in virus titers (Kokkinos and Clark, in press) and symptom development between biological replicates.

When comparing VT plants and plants infected with SPCSV alone, only 3 of the 14 genes were suppressed by SPCSV. One of these genes, plastocyanin, was in fact suppressed in all virus-infected treatments. Of the 216 genes differentially expressed between VT and SPVD, 93 genes were induced in SPVD and 123 suppressed. Many of the genes suppressed in SPVD are related to photosynthesis and metabolism. Of the induced genes many are involved in protein synthesis and protein fate. Among the group of SPVD-responsive genes that are specifically associated with plant defense some were induced whereas others were suppressed.

Q-RT-PCR analysis was carried out for 7 genes determined to be differentially expressed between VT and SPVD affected plants by microarray analysis. The results indicated that all 7 genes were also significantly differentially expressed ($P \leq 0.05$) using Q-RT-PCR with comparable fold changes (Table 4.3).

4.4 Discussion

During their life cycle, viruses need plant proteins for accumulation and movement. Gene expression in the host is affected by virus infection. The host plant can respond to an infection by activating specific or general resistance pathways (Whitham et al., 2003). By determining which genes are differentially expressed in the host during infection, we hope to elucidate how the response of sweetpotato plants to dual infections of SPFMV and SPCSV differs from response to single infections.

Table 4.3. Comparison of average fold-change values between real-time quantitative PCR (Q-RT-PCR) and microarray assays of randomly selected genes differentially expressed in SPVD-affected plants compared to virus-tested controls.

Gene Name	GenBank Accession	Fold Change ^a	
		Q-RT-PCR	Microarray
catalase 2	DV036659	-1.47	-1.54
dehydration-induced protein (ERD15)	CB330921	-2.13	-1.37
disease resistance protein (TIR-NBS-LRR)	DV036322	-2.35	-1.85
heat shock protein 70 (HSP70-1)	DV037387	-1.73	-1.42
chlorophyll A-B binding protein/LHCII type III (LHCB3)	CB330249	3.74	1.59
ankyrin repeat family protein	DV036499	1.38	1.36
metallothionein protein, putative (MT2A)	CB330120	1.40	1.35

^a Positive fold changes denote down-regulation, while negative values represent induction in SPVD affected plants. All fold changes were statistically significant using a p-value cutoff of 0.05 (after Holm multiple testing correction for microarray data).

The reduction of expression levels of genes that are directly or indirectly involved in the overall photosynthetic pathway, clearly observed in the SPVD-affected plants in this study, is a phenomenon commonly observed in yellows diseases and leaves of plants showing typical chlorotic or mosaic symptoms as a result of virus infection (Hull, 2002). Our data support previous reports, which indicate that the reduction in photosynthesis, observed in virus infected plants, is correlated with the reduction of photosynthetic pigments, rubisco, and specific proteins associated with photosystem II (Naidu et al., 1986). Reduction in photosynthetic activity can also be attributed to irreversible damage to photosystem II (van Kooten et al., 1990) and reduced activity of the crassulacean acid metabolism (CAM) (Izaguirre-Mayoral et al., 1993) observed in TMV-infected tobacco and orchids, respectively. As expected, the effect on expression levels of “photosynthetic” genes in plants infected with either SPFMV or SPCSV alone was minimal since these viruses, when infecting this particular sweetpotato cultivar alone, cause mild and transient symptoms.

Plant resistance genes (R genes) are able to recognize pathogens carrying the corresponding avirulence genes (gene-for-gene resistance). This recognition triggers the hypersensitive response (HR), which includes programmed cell death (PCD). The HR is often preceded by the accumulation and production of reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂). Several genes, which were differentially expressed only in plants affected by SPVD, were identified as resistance-related or stress-induced genes. Interestingly, some of these genes were down-regulated whereas others were up-regulated. Two putative R genes, one belonging to the TIR-NBS-LRR class (DV036322) and the other belonging to the CC-NBS-LRR class (DV035471) were induced in SPVD affected plants. A NDR1/HIN1-like (CB330891) gene, known to be required by most CC-NBS-LRR class resistance genes in *Arabidopsis* (Aarts et al., 1998) was also induced in SPVD. DV036322 shows homology to At5g17680.1 of *A. thaliana*, while DV035471 is homologous to At1g58602.1. These genes are similar to known disease resistance proteins rpp8 and RPP1-WsB respectively. To our knowledge, no R genes have been reported, nor is there previous evidence for gene-for-gene resistance in sweetpotato. Therefore, it is probable that these two genes play some other role in sweetpotato, possibly in apoptosis or ATP-binding.

One of the genes found to be down-regulated in SPVD, encodes a product belonging to the ankyrin repeat-containing protein family (DV036499). In *Arabidopsis* an ankyrin repeat-containing protein was found to be directly associated with the oxidative metabolism of the host's resistance to disease and stress response (Yan et al., 2002). Using the antisense technique, Yan et al., (2002) generated transgenic plants in which the gene encoding this protein was down regulated. The down-regulation of

ankyrin was accompanied by increased levels of ROS such as H₂O₂, leading to the activation of pathogen- and stress-related protein-encoding genes, which ultimately caused transgenic plants to exhibit necrotic lesions similar to those observed in hypersensitive response (Yan et al., 2002). The down regulation of the ankyrin gene in SPVD affected plants may be indirectly associated with the up-regulation of some of the other stress response genes, reported in this study through the activity of ROS, or the gene may simply be repressed by the virus. However, in some cases excessive amounts of these toxic compounds interfere with the efficiency of the host to restrict pathogen infection (Moreno et al., 2005).

A particularly interesting gene that is up-regulated in SPVD compared to all other treatments is eukaryotic translation initiation factor 5A (eIF-5A) (DV037506). This protein factor contains the unique amino acid, hypusine. In *Arabidopsis* there are 3 isoforms of eIF-5A, two of which are involved in senescence and the other one in cell division (Thompson et al., 2004; Gatsukovich, 2004). Transgenic *Arabidopsis* plants with decreased deoxyhypusine synthase (DHS) levels, the enzyme that is required for eIF5A activation, showed increased resistance to lethal drought stress (Wang et al., 2003). In humans it is a crucial co-factor of the Rev pathway (Hoffman et al., 2001) essential for HIV1 replication (Pollard and Malim, 1998). So much so, that suppression of DHS has been suggested as a mechanism for antiretroviral therapies (Hauber et al., 2005). The up-regulation of eIF-5A in SPVD is most likely related to leaf senescence. However the possibility that eIF-5A has an additional role in virus replication (as in humans) cannot be excluded.

Another group of gene products implicated in the responses of plants to pathogens and other stresses are peroxidases (Lagrimini and Rothstein, 1987; Yan et al., 2002). Peroxidases have been shown to be involved in scavenging of H₂O₂ from peroxisomes (Wang et al., 1999). Wounding and TMV infection of tobacco resulted in significant increase of several peroxidase isozymes in leaf and pith tissues (Lagrimini and Rothstein, 1987). The down-regulation of a peroxidase gene (CB330630) only in SPVD-affected plants that may be associated with the prevention of downstream activation of ROS-dependent host defense responses, suggests that the differential expression of this gene is directed by the two interacting viruses.

Many of the pathogen-related (PR) proteins exhibit enzymatic activities. A major group of such pathogenesis related proteins, reported from tomato plants, are proteases. These proteases are involved in specific proteolytic events in the extracellular matrix during infection. (Vera and Conejero, 1988; Tornero et al., 1997). A member of this group (PR-P69), which was later identified as subtilisin-like proteases (Tornero et al., 1996), was induced in plants infected with *Citrus exocortis viroid* (Vera and Conejero, 1988). In this experiment, a subtilase gene (CB330070) was down-regulated only in SPVD affected plants. This suggests that this and other down regulated PR genes play an important role in this host's defense mechanism even though they are "defeated" by this viral interaction.

Epoxide hydrolase (DV037327), induced in SPCSV, is also induced in tobacco leaves infected with TMV (Guo et al., 1998). Catalase II (DV036659), an enzyme that breaks down H₂O₂ and is inhibited by salicylic acid (Conrath et al., 1995), is induced in SPVD affected plants. In tomato plants infected with *Cucumber mosaic virus* (CMV)

and D satellite RNA, the induction of catalase II was associated with accumulation of H₂O₂ (Xu et al., 2003). ERD15 (CB330921), a gene that has been shown to be induced by the addition of external H₂O₂ in *Arabidopsis* (Dunaeva and Adamska, 2001), was also up-regulated in SPVD. ERD15 was first identified as a drought responsive gene (Kiyosue et al., 1994), but was also induced in *Arabidopsis* plants inoculated with plant-growth-promoting rhizobacteria (PGPR) (Timmusk and Wagner, 1999). These plants were more resistant to *Erwinia carotovora* and drought stress. Timmusk and Wagner (1999) speculated that the unexpected induction of ERD15 was a result of stunting of roots of inoculated plants. Our results suggest a probable role for ERD15 in general stress response.

Our results are similar to results obtained in the study by Xu et al. (2003). They found that in spite of the induction of multiple defense responses, tomato plants infected with CMV and D satellite RNA cannot overcome the infection and eventually die. CMV without D satellite RNA does not lead to this severe outcome. Neither SPCSV nor SPFMV are known or expected to contain satellite RNAs. However, it appears that these two phenomena, dual infection with SPCSV and SPFMV, and CSV and D satellite RNA, may trigger similar responses in the host.

The induction of polyubiquitin (CB330102) and heat-shock protein 70 (HSP70) (DV037387) during virus infections have been reported earlier (Aranda et al., 1996; Escaler et al., 2000; Whitham et al., 2003). In fact, Glotzer et al. (2000) reported that induction of HSP70 and HSP40 promote adenovirus infection. Our results indicate that HSP70 was induced in SPVD compared to all other treatments. It is unclear whether this indicates non-transient accumulation of HSP70, or is due to new cells continuously

inducing HSP70 transiently as they become infected (Whitham et al. (2003). It should be noted that HSP70 was not induced in SPCSV. Like other members of *Closteroviridae*, SPCSV encodes its own HSP70 homolog (Kreuze, 2002) that assists with movement through the plasmodesmata (Prokhnevsky et al., 2002). Aparicio et al. (2005) recently showed that induction of HSP70 is a general response to protein accumulation in the cytosol. The induction of HSP70 in SPVD may be due to protein accumulation associated with increased levels of SPFMV during the dual infection. The function of HSP70 for virus families, other than *Closteroviridae* has not been proven, but a similar role in cell-to-cell trafficking seems likely (Aoki et al., 2002; Aparicio et al., 2005).

Since SPVD and its viral components were first described by Schaefer and Terry (1976), several hypotheses on the mechanism underlying this disease have been formulated. Immunohistochemical staining experiments conducted by Karyeija et al., (2000) indicated that SPCSV, a phloem-limited virus, does not exit the phloem even when coinfecting with SPFMV. Furthermore, SPCSV, whose titers are significantly greater than those of SPFMV in single infections, remains relatively unchanged during SPVD (Karyeija et al., 2000; Kokkinos and Clark, 2004) and potentially has the ability to encode and “supply” a significant amount of its protein products throughout the plant. These findings in conjunction with data from our study, which show that most of the genes identified as stress-induced or resistance-related are not differentially expressed in plants infected either by SPFMV or SPCSV alone, suggest that the most probable mechanism of this disease is one involving some form of interaction between the two viruses, leading to enhancement of SPFMV (may not be sufficient for severe disease development), and the host rather than one involving a virus (SPCSV) suppressing the

host's defense mechanism so the other virus (SPFMV) can achieve high titers and cause SPVD. This hypothesis should be further tested at the viral gene expression level in an effort to identify any viral proteins that potentially interact with each other and the host, as well as at the host gene expression level to reveal whether other host responses take place well before this time (9 wk) and during the very early stages of infection.

A caveat of this research is that the genes on the array represent only a small proportion of the total sweetpotato genome. This means that there are certainly many genes that may be differentially expressed that are not detected in this study. However, the fact that the number of differentially expressed genes detected seem to follow the same trend as the severity of the symptoms, indicate that the number of genes on the array is not so small that differences between treatments become meaningless. This is also the first study to our knowledge that aims to investigate gene expression levels in sweetpotato infected with viruses (or any other pathogen), and even though the information gained from this study is limited by the small array, it is still very informative.

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CHAPTER 5: EFFECT OF INTERACTION WITH POTYVIRUSES AND SWEETPOTATO CULTIVAR ON TITERS OF *SWEET POTATO LEAF CURL VIRUS*

5.1 Introduction

Sweetpotato (*Ipomoea batatas* (L.) Lam.), a member of the Convolvulaceae, is affected by several diseases, among the most economically important of which are those caused by viruses. Geminiviruses including *Sweet potato leaf curl virus* (SPLCV), have been reported from sweetpotato from only a few locations around the globe including Taiwan (Chung et al., 1985), Japan (Onuki and Hanada, 1998), Israel (Cohen et al., 1997), and the United States (Lotrakul et al., 1998). However, these viruses may occur in regions where studies have not yet been conducted and thus the geographic range of sweetpotato geminiviruses may be wider than currently recognized (Clark et al., 2002). Even though in the early 1960s several whitefly-transmitted virus-like diseases were reported in the United States, the putative viruses were never isolated and characterized (Girardeau and Ratcliffe, 1960; Hildebrand, 1959; Hildebrand, 1961). Lotrakul et al. (1998) was the first to report the presence of *Sweet potato leaf curl virus* (SPLCV) in the United States. In nature, SPLCV is transmitted by the sweetpotato whitefly, *Bemisia tabaci* biotype B. However, under experimental conditions, this virus is not efficiently transmitted by this vector (Lotrakul et al., 2002).

SPLCV, a member of the *Begomovirus* genus and the *Geminiviridae* family, has a relatively small ssDNA circular genome of 2828 nucleotides in length. The genomic organization of SPLCV is very similar to that of other monopartite begomoviruses. Phylogenetic analysis by Lotrakul and Valverde (1999) using amino acid sequences of SPLCV and other *Ipomoea*-infecting geminiviruses, revealed that the sweetpotato and

Ipomoea geminiviruses form a distinct cluster. When viral sequences from SPLCV-infected sweetpotato samples, obtained from different growing areas within the USA and other countries (Puerto Rico, China, Korea, and Taiwan) were analyzed, results suggested that all SPLCV-like isolates evolved from a common ancestor that originated in the Old World (Lotrakul et al., 2002). Analysis of the coat protein of SPLCV-US indicated that the coat protein is unique when compared to its counterparts from both the New and Old World, but overall, SPLCV-US was found to be more closely-related to begomoviruses from the Old World (Lotrakul and Valverde, 1999).

SPLCV, when infecting alone, reduced yields of Beauregard, the predominant cultivar in U.S sweetpotato production, by an average of 25% compared to virus tested plants, whereas when co-infecting with the russet crack strain of *Sweet potato feathery mottle virus* (SPFMV-RC) yields were reduced by an average of 16% (Clark and Hoy, 2006). In addition to yield reductions, storage roots of plants infected with SPLCV alone or in combination with SPFMV-RC exhibit shallow, longitudinal grooving and undesirable changes in the color of the storage root periderm (Clark and Hoy, 2006). Typical upward leaf curling symptoms develop in infected sweetpotato plants only in susceptible cultivars, when favorable environmental conditions are present, and when plants are also infected with SPFMV (Clark and Valverde, 2001). Leaf curl symptoms are also transient and plants quickly recover. As a result, even though SPLCV can cause significant yield reduction, its importance can be overlooked because its presence is not easily recognized. Due to the mild nature of the symptoms caused by SPLCV even when co-infecting with other potyviruses, sensitive methods are needed for its reliable detection (Lotrakul and Valverde, 1999). Recently, sensitive real-time quantitative PCR assays for

the detection and quantification of sweetpotato viruses, including SPLCV were developed (Kokkinos and Clark, in press).

The role of plant potyviruses as “titer enhancers” in complexes with unrelated viruses is well documented (Goldberg and Brakke, 1987; Vance, 1991; Anjos et al., 1992; Vance et al., 1995). However, no such data exist to document whether sweetpotato potyviruses have a similar effect on SPLCV titers in mixed infections. In this paper we report for the first time the use of real-time quantitative PCR to evaluate the effect of the most commonly occurring sweetpotato potyviruses in the U.S., *Ipomoea vein mosaic virus* (IVMV), the common strain of SPFMV (SPFMV-C), SPFMV-RC, and *Sweet potato virus G* (SPVG) on titers of SPLCV. Additionally, in an effort to evaluate whether replication rates of SPLCV correspond to the different levels of yield reduction observed in cultivars known to be either tolerant or sensitive to this virus, titers of SPLCV were assessed in seven commercial cultivars including one from Africa and another from Asia, at 3 and 6 wk post inoculation. The variability observed in titers of SPLCV between cultivars, is discussed in relation to data from an independent replicated yield reduction study, involving the same cultivars in which SPLCV titers were evaluated.

5.2 Material and Methods

5.2.1 Plant Material and Virus Inoculations for Titer Assessment of SPLCV in Pairwise Combinations with Potyviruses

Ipomoea batatas plants infected with SPLCV-US (isolate SWFT-1) alone and *I. setosa* seedlings mechanically inoculated separately with IVMV (isolate LSU-2), SPFMV-C, SPFMV-RC (isolate 95-2), or SPVG (isolate LSU-1) were grown in the greenhouse to generate the scions used to graft-inoculate virus-tested, clonally

propagated *I. batatas* cv. Beauregard. A single wedge graft per virus was made and only plants on which the scion(s) survived for at least 3 wk were selected and used in this study. All test plants were graft-inoculated 3 wk after planting. Six replications of nine treatments were evaluated in a randomized complete block design, consisting of SPLCV and each potyvirus inoculated alone or in pairwise combinations of each potyvirus with SPLCV. Plants were grown under standard greenhouse conditions in 15-cm-diameter clay pots containing autoclaved soil mix consisting of 1 part river silt, 1 part sand, 1 part Jiffy-Mix[®] Plus (Jiffy Products of America Inc., Norwalk, OH) and 3.5g per pot of Osmocote[®] 14-14-14 (Scotts-Sierra Horticultural Products Company, Marysville, OH). A weekly insecticide spray program was followed to control aphids and whiteflies. At 6 wk after inoculation the first four consecutive fully opened leaves from the top of each test plant were collected, combined and immediately frozen in liquid nitrogen and stored at -80°C until extraction.

Virus-tested Beauregard plants were graft-inoculated as above to test how titers of SPLCV change over time in single infections or combined with SPFMV-RC. Six replications were grown in a randomized complete block design under standard greenhouse conditions (described above). At 3, 6, and 9 wk after inoculation leaves from the first four consecutive fully opened leaves from the top of each test plant were collected, combined and immediately frozen in liquid nitrogen and stored at -80°C until extraction.

5.2.2 Plant Material and Virus Inoculations for Titer Assessment of SPLCV in Different Sweetpotato Cultivars

Ipomoea batatas cv. Beauregard plants infected with SPLCV-US (isolate SWFT-1) alone were grown in the greenhouse to generate the scions used to graft-inoculate

virus-tested, clonally propagated 3-wk-old plants of *I. batatas* cv. Bienville (Bien), Beauregard (Bx), Jonathan (Jon), NC-262, Picadito (Pic), Tanzania (Tanz), and Xushu-18. A single wedge graft was made per plant and plants on which the scion did not survive for 3 wks were eliminated from the study. Seven treatments replicated six times were evaluated in a randomized complete block design. Plants were grown under standard greenhouse conditions as described above. A weekly insecticide spray program was followed to control aphids and whiteflies. At 3 and 6 wk after inoculation the first four consecutive fully opened leaves from the top of each test plant were collected, combined, and immediately frozen in liquid nitrogen and stored at -80°C until extraction.

As part of a separate study on virus resistance, virus-tested roots from several cultivars including Beauregard, Bienville, and Xushu-18 were core-grafted with plugs from storage roots of Beauregard infected with isolate SWFT-1 of SPLCV-US, and planted in field beds to generate the plant material for this experiment. Beds were initially covered with black plastic mulch followed by an agricultural fabric (Agribon+ AG-19, PGI Nonwovens, Dayton, NJ) on hoops to exclude any potential virus vectors. Vine cuttings from these beds were transplanted in an isolated field plot and arranged in a randomized complete block design that included four replications of 5 plants each. Fully open leaves from test plants of cultivars Bienville, Beauregard, and Xushu-18 were collected 22 and 35 days after planting, frozen in liquid nitrogen and stored at -80°C until total DNA was extracted.

5.2.3 Total Nucleic Acid Extractions

Frozen leaf tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle and total RNA and DNA were extracted using Qiagen's RNeasy Plant Mini

Kit[®] (Qiagen Inc, Valencia, CA) and GenElute[™] Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO), respectively, according to the manufacturers' directions. To eliminate residual DNA contamination, all total RNA samples were treated on-column with DNase I using the RNase-Free DNase Set (Qiagen Inc) based on manufacturer's directions.

5.2.4 Real-time PCR Assays

Real-time PCR assays for the relative quantification of the potyviruses SPFMV-RC, IVMV, and SPVG were performed in 50 μ l reaction volume mixtures with 5 μ l template RNA, 900nM of each primer, 200nM of the MGB TaqMan[®] probe, 25 μ l of the 2x Master Mix without UNG, and 1.25 μ l of the 40x MultiScribe[™] and RNase inhibitor mix. The 2x and 40x mixes are components of the TaqMan[®] One Step PCR Master Mix Reagents kit (AppliedBiosystems). The following real-time PCR thermal cycler conditions were used: 48 $^{\circ}$ C for 30 minutes (cDNA synthesis), 95 $^{\circ}$ C for 10 minutes (AmpliTaq Gold[®] activation), followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 15 seconds and annealing/extension at 60 $^{\circ}$ C for 1 minute.

Real-time PCR assays for relative quantification of SPLCV were performed in 50 μ l reaction volume mixtures with 5 μ l template DNA, 900nM of each primer, 200nM of the MGB TaqMan[®] probe, and 25 μ l of the TaqMan[®] Universal Master Mix without UNG (AppliedBiosystems). The following real-time PCR thermal cycler conditions were used: 50 $^{\circ}$ C for 2 minutes, 95 $^{\circ}$ C for 10 minutes, followed by 40 cycles of denaturation at 95 $^{\circ}$ C for 15 seconds and annealing/extension at 60 $^{\circ}$ C for 1 minute.

All endogenous control reaction mixtures were the same as the ones described for the potyvirus quantification, except for the substitution of the virus primers/probe set

with 2.5µl of the eukaryotic 18S rRNA pre-developed primer/probe mix (VIC/ MGB Probe). Real-time PCR reactions were performed on an ABI PRISM[®] 7000 Sequence Detection System using MicroAmp[®] optical 96-well reaction plates that were sealed with optical adhesive covers (AppliedBiosystems). The thermal cycling parameters described above were optimized for use with the TaqMan[®] Universal Master Mix and TaqMan[®] One Step PCR Master Mix Reagents kits in singleplex reactions (AppliedBiosystems). To minimize the effects of any errors due to pipetting differences, duplicates of each sample were run on each plate, and their threshold cycle (Ct) values were averaged. Non-template water controls (NTC) as well as positive (total RNA or DNA from virus-infected tissue) and negative (total RNA or DNA from healthy tissue) controls were included on every plate. The $\Delta\Delta C_t$ quantification method (User Bulletin #2, AppliedBiosystems), which eliminates the use of standard curves on every plate, was implemented for the normalization of samples.

5.3 Results

5.3.1 The Effect of Potyviruses on Titers of SPLCV

At the time leaf samples were collected (6 wk), titers of SPLCV-US in pairwise combination with SPFMV-RC and IVMV were significantly greater ($P<0.001$) compared to titers of SPLCV alone or in any of the other pairwise combinations (Fig. 5.1). On average, titers of SPLCV were greatest in the presence of SPFMV-RC, and least in the presence of SPVG among the pairwise combinations (Table 5.1). No significant difference was observed in titers of SPLCV among treatments involving pairwise combinations with SPFMV-C and SPVG. Titers of all potyviruses in single and pairwise combination with SPLCV were either very low or below the threshold of detection of the

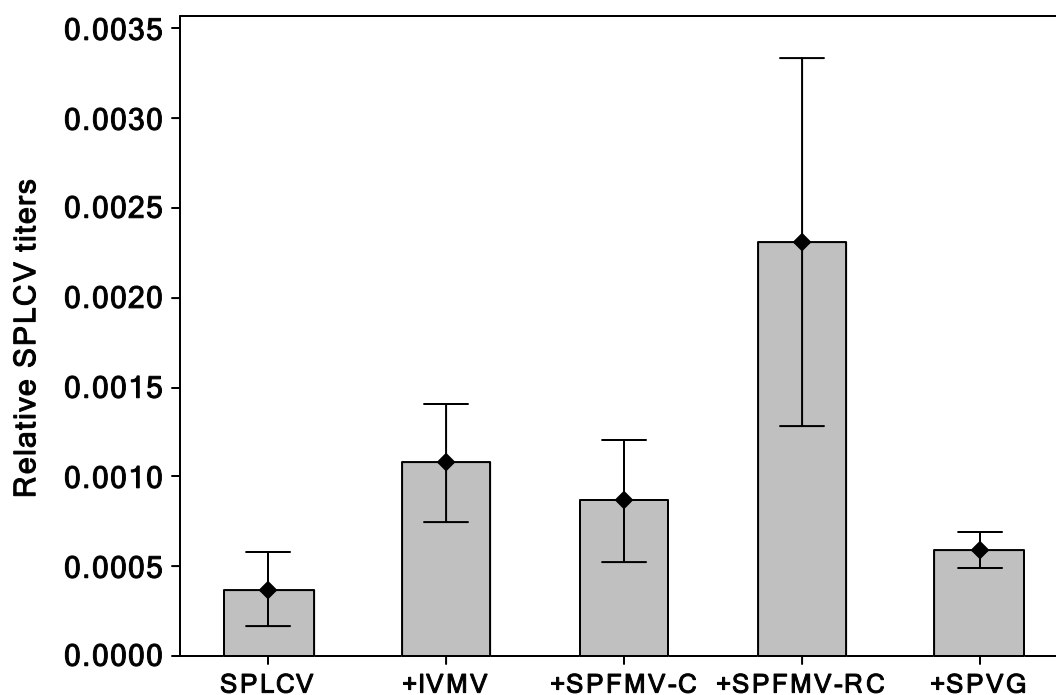


Figure 5.1. Relative titers of *Sweet potato leaf curl virus* (SPLCV) determined by real-time quantitative PCR in single and pairwise infections with *Ipomoea vein mosaic virus* (IVMV), the common strain of *Sweet potato feathery mottle virus* (SPFMV-C), the russet crack strain of SPFMV (SPFMV-RC), and *Sweet potato virus G* (SPVG) at 6 wk post inoculation (WPI).

Table 5.1. Mean relative titers of *Sweet potato leaf curl virus* (SPCLV) alone and in co-infections with *Ipomoea vein mosac virus* (IVMV), the common strain of *Sweet potato feathery mottle virus* (SPFMV-C), the russet crack strain of SPFMV (SPFMV-RC), and *Sweet potato virus G* (SPVG).

Treatment	N^a	Mean^b	StDev^c
SPLCV	6	0.0003740	0.0002015
SPLCV+IVMV	6	0.0010799	0.0003179
SPLCV+SPFMV-C	6	0.0008685	0.0003257
SPLCV+SPFMV-RC	6	0.0023145	0.0009818
SPLCV+SPVG	6	0.0005902	0.0000970

^aNumber of biological replications

^bMean relative titers of SPLCV

^cStandard deviation from the mean

real-time quantitative PCR assays (data not shown), which is consistent with previous findings (Karyeija et al., 2000; Kokkinos and Clark, in press).

At 3, 6, and 9 wk after inoculation, titers of SPLCV were significantly greater ($P=0.034$, $P=0.013$, and $P=0.016$, respectively) in plants co-infected with SPFMV-RC compared to plants inoculated with SPLCV alone. Even though no statistical difference was observed in titers of SPLCV among assessment dates of the same treatment, at 9 wk after inoculation, titers of SPLCV in single and dual infections with SPFMV-RC were numerically less than at 3 and 6 wk (Fig. 5.2). Titers of SPFMV-RC in single infections were very low or below the threshold of detection. Test plants in which SPLCV titers were evaluated were asymptomatic.

5.3.2 Titers of SPLCV in Different Sweetpotato Cultivars

Even though at 3 wk after inoculation SPLCV titers were relatively low in all cultivars, titers in Beauregard and NC-262 were significantly greater compared to Picadito, Tanzania, or Xushu-18 ($P<0.001$, $\alpha=0.05$) (Fig. 5.3). At 6 wk after inoculation, titers of SPLCV in cultivars Picadito, Tanzania, and Xushu-18 were significantly less than for all other cultivars ($P<0.001$, $\alpha=0.05$). Titers in Bienville were significantly greater than for all other cultivars (Fig. 5.3). Bienville plants were also the only plants that exhibited moderate symptoms of upward leaf curling, which are typical of symptoms observed in certain sweetpotato cultivars infected by SPLCV. No significant difference in titers of SPLCV was observed between cultivars Beauregard, Jonathan, and NC-262.

Results of quantification of SPLCV in field-grown Bienville, Beauregard, and Xushu-18 were similar to the same cultivars evaluated in the greenhouse (Fig. 5.4). At 22 and 35 days after planting, SPLCV titers in Bienville were significantly greater than

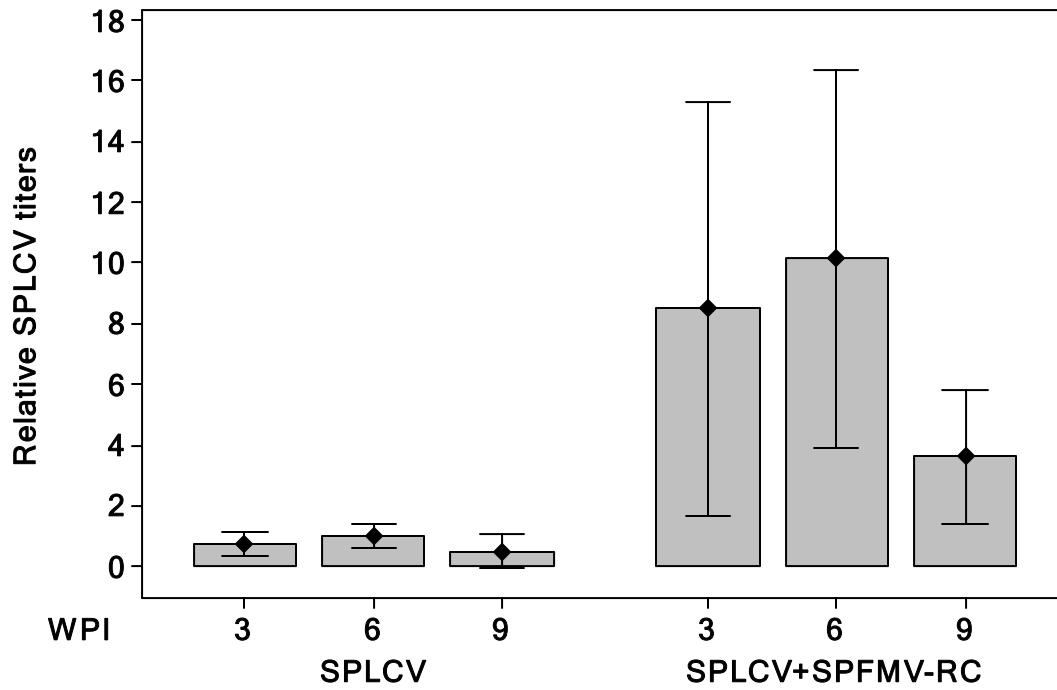


Figure 5.2. Relative titers of *Sweet potato leaf curl virus* (SPLCV) determined by real-time quantitative PCR in single and pairwise infections with the russet crack strain of *Sweet potato feathery mottle virus* (SPFMV-RC) at 3, 6, and 9 wk post inoculation (WPI).

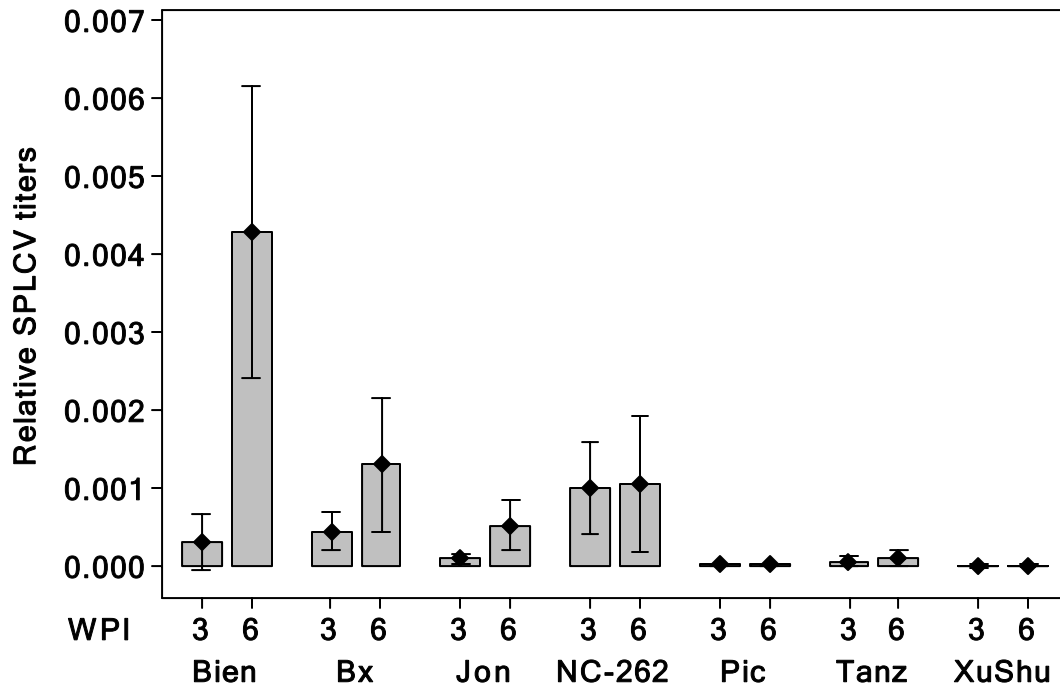


Figure 5.3. Relative titers of *Sweet potato leaf curl virus* (SPLCV) determined by real-time quantitative PCR in singly-infected sweetpotato cultivars Bienville (Bien), Beauregard (Bx), Jonathan (Jon), NC-262, Picadito (Pic), Tanzania (Tanz), and Xushu-18 (Xushu) at 3 and 6 wk post inoculation (WPI).

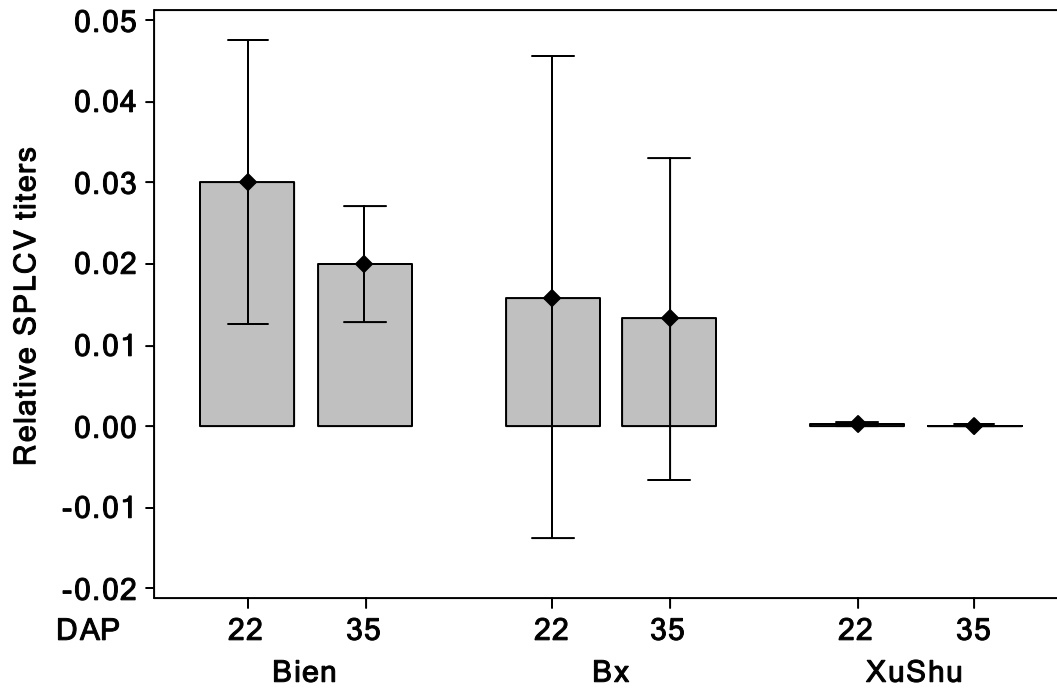


Figure 5.4. Relative titers of *Sweet potato leaf curl virus* (SPLCV) determined by real-time quantitative PCR in singly-infected sweetpotato cultivars Bienville (Bien), Beauregard (Bx), and Xushu-18 (Xushu) at 22 and 35 days after planting (DAP).

titers in Xushu-18 ($P=0.014$ and $P=0.005$ respectively). Titers in Beauregard, were much more variable and although it was intermediate between the other two cultivars, did not differ significantly from either Bienville or Xushu-18 at either sampling date.

5.4 Discussion

SPLCV titers were significantly enhanced in Beauregard plants in the presence of SPFMV-RC and IVMV. This occurred even though the plants were asymptomatic, as has been reported previously for plants infected with SPLCV alone or combined with SPFMV (Lotrakul et al., 1998; Clark and Hoy, 2006). Replication of geminiviral DNA, which occurs entirely in the nucleus, is regulated by specific interactions between host cellular and viral proteins (Xie et al., 1999; Gutierrez, 2000). SINAC1, a member of the functionally diverse NAC-domain protein family (Aida et al., 1997), was reported to be upregulated in response to *Tomato leaf curl virus* (TLCV) (Selth et. al., 2005). The induction of *SINAC1*, which is mediated by the viral replication enhancer protein (REn), led to the significant enhancement of the accumulation of TLCV ssDNA (Selth et. al., 2005). Interestingly, other members of the NAC-domain protein family (CUC1 and CUC2) have been known to accumulate to higher levels in potyviral P1/HC-Pro-expressing inflorescence tissues (Kasschau et al., 2003). These findings may explain the enhancement of SPLCV DNA accumulation in plants co-infected with potyviruses, where P1/HC-Pro may induce other NAC-domain proteins, which in turn interact with the SPLCV REn resulting in the enhancement of the virus titer.

Clark and Hoy (2006) reported significant yield losses of 26 and 16% in asymptomatic Beauregard plants due to infection by SPLCV alone or when with SPFMV-RC, respectively. This led to the hypothesis that SPLCV, a phloem infecting

virus, may be involved in the reduction of assimilates translocated to the roots, resulting in yield reduction, without causing foliar symptoms. Based on this hypothesis however, the virus quantitative data obtained in this study, which clearly show that the concentration of SPLCV RNA is on average 6-fold higher in plants co-infected with SPFMV-RC, are in contrast with the lower yield reduction percentage reported by Clark and Hoy (2006) in Beauregard plants co-infected with the same virus strains. It is possible that whatever effect the virus has on yield is not necessarily proportional to virus titer, which might explain why levels of yield reduction do not correspond to increased virus concentrations in plants infected with both viruses. Another hypothesis is that the 6-fold enhancement of SPLCV, which is significantly smaller compared to the fold-changes of the enhanced virus reported in sweet potato virus disease (SPVD) (Karyeija et al., 2000; Kokkinos and Clark, submitted), may trigger host responses opposite of those in plants infected with SPLCV alone. Such changes may cause an increase in the production and translocation of assimilates to the roots, resulting in higher yields or as suggested for other members of the *Geminiviridae* family may interfere with the cell's decision to leave a continuous proliferating phase in favor of other pathways such as senescence or differentiation (Gutierrez, 2000). A slight increase in yields of virus-infected compared to virus-tested plants in the field is a phenomenon that has been reported in some sweetpotato cultivars in the past (Clark and Valverde, 2001; Clark and Hoy, 2006). Results from the work of Clark and Hoy (2006) and this study warrant further research into evaluating whether host responses are different in plants infected with SPLCV alone compared to plants co-infected with SPLCV and SPFMV.

Nine weeks post inoculation, titers of SPLCV in single infections and in dual infections with SPFMV-RC were found to be numerically lower compared to 3 and 6 wk post inoculation. Such reduction in titers, even though in this case not statistically significant, may be the result of the host's defense responses to the virus, and more specifically to post transcriptional gene silencing (PTGS) (Vanitharani et al., 2005). To date, very little is known on how geminivirus titers fluctuate in the host plant and what factors may interact with viral regulatory processes to control or change viral replication. However, host factors are more likely to control replication of SPLCV, since the only viral protein detected in the particles of geminiviruses is the coat protein, which is not required for replication of the viral genome (Elmer et al., 1988; Woolston et al., 1989). Physiological changes due to age, especially of herbaceous plants, are significant factors in the course of a viral disease (Matthews, 1992). In a relatively fast growing herbaceous plant, such as sweetpotato, the observation that SPLCV titers decreased at approximately the same time, regardless of the presence or not of SPFMV-RC, is more likely to be attributed to physiological changes occurring in the host over time rather than a relatively late activation of its defense responses.

When titers of SPLCV were measured in field-grown plants and compared to yield of those plants, virus titer was positively related to the level of yield reduction observed in the corresponding cultivar. Titers of SPLCV in cultivars exhibiting high percentage of yield reduction (Bienville and Beauregard) due to SPLCV infection were high, whereas in cultivars with little or no yield reduction (Picadito, Tanzania, and Xushu-18), titers were low. In Bienville, where average yield reduction was estimated at 60%, SPLCV titers 6 wk post inoculation were the highest of all the cultivars evaluated.

The comparison between virus titers and yield reduction clearly indicates that cultivars which are considered to be tolerant to this virus based on yield data have also the capacity to inhibit virus replication. The direct linkage of virus replication with resistance, which was supported by the quantitative studies of SPLCV under greenhouse and field conditions, is an important step towards the effort of efficiently screening breeding material for resistance to this virus. It also provides an argument against the hypothesis that virus titer may not be proportional to effects on yield as stated above.

The greatest concentration of SPLCV, observed in plants of the cultivar Bienville 9 wk post inoculation, was also associated with the development of upward leaf curling symptoms. As with infection of plants with other phloem-infecting viruses, such as the closterovirus *Beet yellows virus* (BYV), damage to the phloem can cause disruption of the translocation of photoassimilates. Such disruption can lead to the development of thickened leaves and leaf rolling symptoms, which are attributed to accumulation of photoassimilates in the leaf lamina (Hull, 2002).

To our knowledge, this is the first report of an interaction at the titer level between a potyvirus and a geminivirus in plants. This interaction differed from that between SPFMV and another phloem-limited virus, *Sweet potato chlorotic stunt virus* (SPCSV), in which SPFMV titers were greatly enhanced and the titers of SPCSV decreased (Karyeija et al., 2000; Kokkinos and Clark, submitted). Enhancement of SPLCV by the two potyviruses, SPFMV and IVMV, is similar to other reports in which potyviruses enhanced replication of other plant viruses (Anjos et al., 1992; Vance, 1991). The enhanced replication of SPLCV did not cause any increase in symptoms or yield reductions compared to plants infected with SPLCV alone (Clark and Hoy, 2006; this

study). However, the enhancement of SPLCV titers may occur commonly since potyviruses such as SPFMV are found anywhere sweetpotato is grown (Brunt et al., 1996), and therefore may play an important role in the ability of this virus to be disseminated in the field. The molecular mechanisms underlying the unique interactions of SPFMV with SPLCV and the host remain poorly understood and therefore further studies addressing these issues are needed.

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CHAPTER 6: SUMMARY AND CONCLUSIONS

Sweetpotato (*Ipomoea batatas* Lam.) is one of the most important and nutritious crops worldwide. Yields of sweetpotato cultivars tend to decline after they are released to farmers. Cultivar decline is a complex phenomenon in sweetpotato that requires intense, multidisciplinary research efforts. It can involve several contributing factors, including accumulation of pathogens, especially viruses, and mutations in vegetative propagating material. Viruses, which are commonly found in sweetpotato plants worldwide, may affect yield and quality directly, but also may have an indirect effect by causing activation of transposable elements that may cause stable mutations. The most devastating example of direct yield decline due to viruses is the sweet potato virus disease (SPVD). This viral complex, which occurs mainly in Africa and is one of the major biological constraints to sweetpotato production in sub-Saharan Africa, is the result of the synergistic interaction between the aphid-transmitted *Sweet potato feathery mottle virus* and the whitefly-transmitted *Sweet potato chlorotic stunt virus*.

Recent discussions at the International Workshop of Sweetpotato Cultivar Decline in Mijakonojo, Japan in 2002 concluded that three major groups of viruses infecting sweetpotato should be given detailed attention in relation to sweetpotato cultivar decline. These viruses include the *Potyviridae* family, the *Closteroviridae* family, and the *Geminiviridae* family. Despite the detection of sweetpotato viruses with serological and other methods, including PCR, there were no reports prior to this study on how titers of these viruses change overtime at the whole plant level under greenhouse or field conditions. Furthermore, there were no reports to link viral replication rates and titer levels with symptom severity, productivity, and host resistance. Data on how the titers of

sweetpotato viruses are influenced by the presence of another virus in specific viral complex situations, commonly occurring in sweetpotato, were minimal or non-existent. The development of the real-time quantitative PCR technology and the related fluorogenic chemistry, which have been successfully employed to study human and animal agents of disease, provided an excellent opportunity to investigate in detail these important biological relations involving the sweetpotato as the host plant and specific viruses and viral complexes previously implicated in the phenomenon of cultivar decline.

Difficulties inherent in detecting, quantifying, and isolating viruses directly from sweetpotato have impeded rapid progress in sweetpotato virus research. In the current research, the development of Real-time quantitative PCR assays for the detection and relative quantification in singleplex reactions of viruses in the *Potyviridae*: SPFMV, *Sweet potato virus G* (SPVG), and Ipomoea vein mosaic virus (IVMV); the *Closteroviridae*: *Sweet potato chlorotic stunt virus* (SPCSV), and the *Geminiviridae*: *Sweet potato leaf curl virus* (SPLCV) directly from infected sweetpotato plants, is reported for the first time. The effect of potential inhibitors in total nucleic acid extracts from sweetpotato leaves on the performance of the real-time PCR assays was evaluated and determined to be not significant. Virus titers of SPFMV, IVMV, and SPVG quantified using real-time PCR were found to be lower in singly-infected sweetpotato plants as compared to singly-infected Brazilian morning-glory (*Ipomoea setosa* Ker.) and *I. nil* (L.) Roth cv. 'Scarlet O' Hara' plants. Overall, the Real-time PCR assays described here were more sensitive and specific detection methods for the viruses evaluated than conventional PCR, hybridization, and serological (DAS-ELISA) methods. However, Real-time PCR assays require the use of special instruments and reagents which at the

present time are relatively expensive and not widely available. The per sample expense of Real-time PCR assays (including the cost of extracting nucleic acids) is a disadvantage for using them for routine or large scale detection of these viruses.

The effect of SPSCV on titers of different potyviruses and potyvirus strains infecting sweetpotato in the U.S. was also investigated using real-time quantitative PCR. Titers of all potyviruses and potyvirus strains evaluated were enhanced in the presence of SPSCV suggesting that a conserved mechanism may underlie these interactions. The degree of titer enhancement did not correspond to the severity of symptoms observed in certain treatments involving pairwise infections. Titers of the common strain of SPFMV (SPFMV-C), which did not cause SPVD-like symptoms when co-infecting with SPSCV, were enhanced in the presence of SPSCV similarly to the russet crack strain, SPFMV-RC, which did cause SPVD symptoms when co-infecting with SPSCV. Furthermore, titers of SPSCV were found to be lower in treatments involving pairwise infections compared to plants infected with SPSCV alone. No significant difference was observed in titers of SPFMV-RC, IVMV, and SPVG between single and mixed infections with each other. These results suggest that the most probable mechanism resulting in the induction of SPVD is one involving some form of interaction between the two viruses and the host (viral protein/viral protein/host protein interaction) rather than a previously proposed hypothesis suggesting that SPSCV suppresses the defense mechanism of sweetpotato allowing SPFMV to achieve high titers and cause SPVD.

cDNA microarrays, containing 2765 features from sweetpotato leaf and storage root libraries, were used in an effort to assess the effects of SPVD and its individual viral components on the gene expression profile of the sweetpotato cultivar Beauregard.

Expression analysis revealed that the number of differentially expressed genes in plants infected with SPFMV alone and SPCSV alone compared to virus-tested plants was only 3 and 14, respectively. However, these findings were in stark contrast with SPVD-affected plants where more than 200 genes were found to be differentially expressed. SPVD-responsive genes were found to be involved in a variety of cellular processes including several that were identified as pathogenesis- or stress-induced. Results from the microarray study suggest that the most probable mechanism underlying SPVD is one involving some form of interaction between the two viruses and the host (SPCSV protein/SPFMV protein/host protein interaction) rather than one involving a virus (SPCSV) suppressing the host's defense mechanism so the other virus (SPFMV) can achieve high titers and cause SPVD. Therefore, further research is warranted into identifying the viral proteins from each virus that potentially interact with each other and the host, as well as identifying responses of the host plant that take place during the very early stages of infection.

Sweet potato leaf curl virus (SPLCV), a geminivirus, can cause yield reductions of up to 30% and is potentially a significant factor in productivity and cultivar decline in sweetpotato. Its importance has been overlooked because symptoms are not commonly found on sweetpotato plants. The relative quantification of the U.S. isolate of SPLCV (SPLCV-US) in sweetpotato plants co-infected with commonly occurring potyviruses in the United States revealed a never before described interaction, at the titer level, between a geminivirus and a potyvirus. Even though titers of SPLCV appeared greater in the presence of *Sweet potato virus G* and the common strain of *Sweet potato feathery mottle virus* compared to titers of SPLCV in single infections, SPLCV titers were significantly

increased only when co-infecting with the russet crack strain of SPFMV or Ipomoea vein mosaic virus. Titers of SPLCV in different commercial sweetpotato cultivars were significantly different ranging from high in Bienville, to low in Picadito and Xushu-18. The variability of SPLCV titers observed among cultivars was negatively related to yield of SPLCV-infected plants of the same cultivars in the field. The correlation of virus replication with resistance (based on yield of fresh storage roots), is an important step towards the effort of efficiently screening breeding material for resistance to this important agent of disease.

The viruses chosen for use in this study represent those that commonly affect productivity of the crop, and in some cases become a major constraint in the production of this crop worldwide. In the United States, several years of field research have shown that virus-infected sweetpotato cultivars experienced 10-30% yield reduction, which translates to millions of dollars in losses. The use of viruses in mixed infections in sweetpotato, lead to the identification of new interactions between members of distinct virus families, which until now were unknown. Furthermore, results obtained in this study strengthened the basic understanding of many aspects of replication of the viruses evaluated but also revealed new insights into mechanisms that may be involved in disease development due to virus infection in sweetpotato.

APPENDIX: PERMISSION LETTERS

To: "c Kokkinos" <ckokkin@lsu.edu>
CC:
Subject: [Fwd: RE: permission for dissertation use]
Date: Mon, 06 Mar 2006 10:26:49 -0600

----- Original Message -----

Subject: RE: permission for dissertation use

Date: Mon, 6 Mar 2006 09:55:48 -0600

From: Karen Cummings <kcummings@scisoc.org>

To: Chris Clark <cclark@agctr.lsu.edu>

Dear Dr. Clark,

Permission is hereby granted for the inclusion of the article cited below in Charlambos Kokkinos's dissertation provided Plant Disease is properly credited.

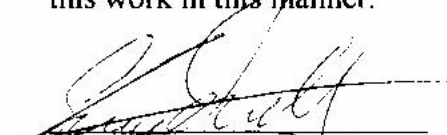
If there is anything else you need, please do not hesitate to contact me.

Sincerely,


Karen Cummings
Director of Publications, Production
The American Phytopathological Society
3340 Pilot Knob Road

To Whom it May Concern,

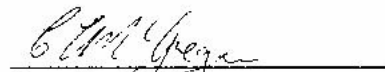
This dissertation includes a chapter entitled: "*The Effect of Sweet Potato Virus Disease and Its Viral Components on Gene Expression Levels in Ipomoea batatas (L.) Lam.*". This chapter is the product of equal participation in both original conceptual input as well as in execution of the actual research by two Ph.D. candidates, Charalambos Kokkinos and Cecelia McGregor, and is therefore included in the dissertation of each candidate in identical form. All other portions of each dissertation are the result of independent scholarly research by the respective candidates. As attested below, all parties involved: each candidate and their respective advisory committee chairman are completely in agreement with presenting this work in this manner.


Charalambos D. Kokkinos

1/6/06
Date


Christopher A. Clark

1/6/06
Date


Cecelia McGregor

1/6/06
Date


Don R. LaBonte

1/6/06
Date

VITA

Charalambos D. Kokkinos was born in Limassol, Cyprus, on November 26th, 1975. After his graduation from the St. Peter and Paul Lyceum in June of 1993, he served for two years as a second lieutenant in the Greek Army's and Cyprus National Guard's Special Forces. After he was honorably discharged from the army, he moved to the United States where he started his undergraduate studies in the Department of Horticulture at the Louisiana State University in Baton Rouge. In May of 2000, shortly after he received the degree of Bachelor of Science, he was offered a graduate assistantship in the same department to study under the direction of Professor Don R. LaBonte the effect of viruses on activation of retrotransposons in sweetpotato. In 2002, after he received his Master of Science degree he initiated his studies towards a doctoral degree in the Department of Plant Pathology and Crop Physiology under the direction of Professor Christopher A. Clark. He is a candidate for the degree of Doctor of Philosophy during the Spring Commencement 2006.