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MOLECULAR DIVERSITY AND COAT PROTEIN EXPRESSION OF *SWEET POTATO LEAF CURL VIRUS*

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
Dina Lida Gutiérrez Reynoso
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DEDICATION

This dissertation is dedicated to my parents, for their love and efforts to provide me the best education, as well as, to my brothers and sister, for their guidance and motivation to excel in life. I also dedicate this work to my extended family. Their prayers and moral support gave me the strength to get through the tough times.

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LIST OF ABBREVIATIONS

bp	Base pairs
CP	Coat protein
ELISA	Enzyme-linked immunosorbent assay
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IYVV	*Ipomoea yellow vein virus*
MBP	Maltose-binding protein
MBP*β-gal	Fusion protein comprising the maltose-binding protein and the β-galactosidase α fragment protein
MBP*CP	Fusion protein comprising the maltose-binding protein and the coat protein of *Sweet potato leaf curl virus*
PCR	Polymerase chain reaction
pMAL-CP	Recombinant plasmid containing the coat protein gene of *Sweet potato leaf curl virus* cloned into the pMAL-c2E expression vector.
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPLCGV	*Sweet potato leaf curl Georgia virus*
SPLCV	*Sweet potato leaf curl virus*
SPLCV CP	*Sweet potato leaf curl virus* coat protein
TYLCV	*Tomato yellow leaf curl virus*
US	The United States of America

ABSTRACT

Leaf curl virus diseases have been reported in sweetpotato throughout the world. One of the causal agents is *Sweet potato leaf curl virus* (SPLCV) which belongs to the genus *Begomovirus* (family *Geminiviridae*). In the United States, SPLCV has been found infecting an ornamental sweetpotato and several breeding lines but not in sweetpotatoes grown for commercial production. SPLCV does not cause symptoms on Beauregard, the predominant sweetpotato cultivar in the US, but it can reduce its yield. Since SPLCV could become an important constraint for sweetpotato production; diagnosis, identification, and characterization are essential steps to develop an effective management program.

The variability among begomoviruses obtained from 11 sweetpotato genotypes was evaluated through the analysis of the nucleotide sequence of a fragment of the replication-associated protein gene (AC1). Ten of these begomoviruses were closely related to SPLCV and one was closely related to *Sweet potato leaf curl Georgia virus* (SPLCGV). These results suggest that in the US, SPLCV may be more common in sweetpotato genotypes than SPLCGV. Phylogenetic analysis using the obtained nucleotide sequences of the AC1 and the full length nucleotide sequences of the coat protein gene (AV1) clustered all sweetpotato begomoviruses together. However, SPLCV and SPLCGV were placed in different groups supporting their status as different species.

Serological detection of SPLCV is not currently available due to the difficulties in obtaining purified virions that can be used as antigen for antiserum production. In attempts to obtain the coat protein (CP) of SPLCV for antibody production, primers were designed to amplify the CP gene. This gene was cloned into the expression vector pMAL-c2E, and transformed into *E. coli* XL1-Blue. After gene induction, a fusion protein of 72 kDa was purified

by amylose affinity chromatography. The yield of the purified fusion protein was approximately 200 µg/liter of bacterial culture. Digestion with enterokinase cleaved the fusion protein into a 42.5 kDa maltose-binding protein and a 29.4 kDa protein. The latter protein was identified by mass spectrometry analysis as the CP of SPLCV.

CHAPTER 1: INTRODUCTION

With more than 126 million metric tons (FAO, 2007) in annual production, sweetpotato (*Ipomoea batatas* (L.) Lam) is one of the most important crops worldwide; it is the third most important root crop after potato and cassava and is ranked seventh in global food crop production (Kays, 2005). Due to its nutritional qualities (rich in carbohydrates, dietary fiber, beta carotene, vitamin C, and vitamin B6), sweetpotato is considered as a crop with great potential not only for human consumption but also for animal feeding and industrial use (Bovell-Benjamin, 2007; Huntrods, 2008).

Although sweetpotato originated in Central or South America, the world production is centered in Asia, with China as the major producer with 109 million metric tons that counts for over 86 % of the sweetpotato world production (FAO, 2007). The United States is one of the few developed countries that produce sweetpotatoes (836,970 metric tons) with less than 1 % of the 2007 world production (FAO, 2007). North Carolina is the leading producer with 38.5 % of the 2007 US sweetpotato production, followed by California, Mississippi, and Louisiana with 23 %, 19 %, and 15.9 %, respectively (USDA, 2008).

Sweetpotato belongs to the family *Convolvulaceae* (the morning glory family) and grows widely in tropical, subtropical, and warm temperate regions. Sweetpotato is especially valued because it is highly adaptable, tolerates high temperatures and low fertility soils, is easy to propagate and maintain, and yields well in adverse conditions (Karyeija *et al*., 1998). Many diseases caused by fungi, bacteria, nematodes, viruses, and mycoplasma have been described to affect sweetpotato production (Clark and Moyer, 1988). Because of its vegetative propagation, sweetpotato is prone to accumulate systemic pathogens in propagating materials (Clark and Hoy, 2006).

Yields of sweetpotato cultivars have appeared to gradually decline over the years. This decline in yield and quality may be caused by a combination of several factors, including mutations, viruses and other pathogens (Clark *et al*., 2002; 2003).

For many years, there was little effort to control viruses in sweetpotato primarily because the specific viruses had not been identified and the effect the viruses had on production had not been determined. During the International Workshop on Sweetpotato Cultivar Decline Study (Miyakonojo, Japan, 2000), it was determined that three virus families, *Potyviridae*, *Closteroviridae*, and *Geminiviridae*, should be given particular attention in relation to sweetpotato cultivar decline (Nakazawa, 2001).

Some members of these three families occur in sweetpotato in the US. Within the family *Potyviridae*, *Sweet potato feathery mottle virus* (SPFMV) commonly occurs in sweetpotato fields (Clark and Moyer, 1988); *Sweet potato virus G* (SPVG) and Sweet potato virus 2 (SPV2, synonymous with Ipomoea vein mosaic virus), are less frequent (Souto *et al*., 2003; Tairo *et al*., 2006). *Sweet potato chlorotic stunt virus* (SPCSV), a crinivirus (family *Closteroviridae*), was reported infecting *in vitro* plants of the cultivar White Bunch (Pio-Ribeiro *et al*., 1996), and recently it was found infecting sweetpotatoes in the field (Abad *et al*., 2007). Two begomoviruses (family *Geminiviridae*), *Sweet potato leaf curl virus* (SPLCV) and *Sweet potato leaf curl Georgia virus* (SPLCGV), have been identified and characterized from field grown sweetpotato breeding lines (Lotrakul *et al*., 1998; 2002; 2003; Lotrakul and Valverde, 1999).

1.1 Justification

Viruses have been suggested to cause significant yield reduction and possibly cultivar decline of sweetpotato; therefore, characterization of viruses that affect this crop could lead to a better understanding of their roles in cultivar decline and their effects on yield (Lotrakul, 2000).

Clark and Hoy (2006) evaluated the effect of viruses on yield and quality of Beauregard sweetpotato, the predominant cultivar in the US. They found that three potyviruses: SPFMV-Russet crack, SPVG and SPV2 had little effect on yield or quality of Beauregard. SPLCV did not cause visible symptoms, but it reduced the yield up to 26 %. In addition, infection with SPLCV also resulted in production of storage roots with darker periderm color.

In the US, SPLCV has been found infecting sweetpotato breeding lines but not in sweetpotato grown for commercial production. The expansion of the geographical range of *Bemisia tabaci* (vector of SPLCV) together with potential synergistic effects in mixed infections could become a constraint for sweetpotato production. Therefore, diagnosis, identification, and characterization are essential for the development of appropriate control strategies.

1.2 Objectives

The objectives of this investigation were:

- To determine the molecular diversity of sweetpotato begomoviruses in the US and their relationship with other sweetpotato begomoviruses by analyzing the nucleotide sequence of a fragment of the replication-associated protein gene (AC1).
- To clone and express the coat protein gene (AV1) of the US isolate of SPLCV in *Escherichia coli* for antiserum production.

CHAPTER 2: LITERATURE REVIEW

2.1 Family *Geminiviridae*

The family *Geminiviridae* comprises a diversity of plant viruses that infect a broad variety of plants and cause significant crop losses throughout the world (Hanley-Bowdoin *et al*., 1999; Briddon and Stanley, 2006). Geminiviruses are made of circular single-stranded DNA genomes (2.5-3.0 kb) encapsidated in quasi-isometric virions of about 20-30 nm in diameter (Stanley *et al*., 2005). Geminiviruses utilize bidirectional transcription and overlapping genes for efficient coding of proteins. The coat protein is conserved in its capacity to form these unique virions, but has diverged in terms of specificity for insect transmission (Briddon *et al*., 1990). A single viral coded protein (or two related proteins in the monocot-infecting geminiviruses) is essential for replication: the replication associated protein that is conserved in sequence, position, and function (Gutierrez, 1999; Hanley-Bowdoin *et al*., 2004). All geminiviruses also carry one or more intergenic regions (IRs), one of which contains the origin of replication and the signature stem-loop structure containing an invariant nonanucleotide motif involved in rolling circle replication (Hanley-Bowdoin *et al*., 1999).

Based on their type of insect vector, host range, and genome organization, geminiviruses are classified into four genera (Hull, 2002; Stanley *et al*., 2005; Fauquet *et al*., 2008). Viruses of the genus *Mastrevirus* (type species: *Maize streak virus*, MSV) have monopartite genomes (2.6-2.8 kb circular ssDNA), generally infect monocotyledonous plants and are transmitted by leafhoppers. The genus *Curtovirus* (type species: *Beet curly top virus*, BCTV) include viruses which infect only dicotyledonous plants, also have monopartite genomes (2.9-3.0 kb circular ssDNA) with a different genomic organization and different leafhopper vectors. With only one member, the genus *Topocuvirus* is represented by *Tomato pseudo-curly top virus* (TPCTV)

which infects dicotyledonous plants, has a monopartite genome (2.8 kb circular ssDNA) with a similar organization of curtovirus genomes, but transmitted by a species of treehopper. The genus *Begomovirus* (type species: *Bean golden yellow mosaic virus*, BGYMV) contains the majority of the identified geminiviruses (117 species, Stanley *et al*., 2005). Begomoviruses have bipartite or monopartite genomes (2.5-2.8 kb circular ssDNA), infect only dicotyledonous plants, and are transmitted by the sweetpotato whitefly (*Bemisia tabaci*) (Briddon, 2002; Stanley *et al*., 2005; Seal *et al*., 2006).

Begomoviruses constitute a major constraint to production of economically important crops including beans, cassava, cotton, cucurbits, and tomato, among others (Polston and Anderson, 1997; Otim-Nape *et al*., 1997; Rybicki and Pietersen, 1999; Briddon and Markham, 2000; Morales and Anderson, 2001; Varma and Malathi, 2003). Symptoms on infected plants typically consist of leaf curling, mosaic, vein yellowing or more generalized leaf yellowing, often accompanied by stunting of plant growth (Harrison and Robinson, 1999).

According to their geographical origins and based on phylogenetic analysis, begomoviruses can be generally divided into two groups, the Old World begomoviruses (Eastern Hemisphere, Africa, Asia, Europe, and the Mediterranean areas) and the New World begomoviruses (Western Hemisphere, the Americas) (Padidam *et al*., 1995; Harrison and Robinson, 2002).

Most begomoviruses have two genomic components (DNA-A and DNA-B, both with an approximate size of 2.5-2.8 kb) although an increasing number of begomoviruses with a single genomic component (equivalent to DNA-A) has been reported (Fauquet *et al*., 2003). Even though the encapsidated form of the genome is ssDNA, replication and gene expression occurs through a super-coiled double stranded intermediate, in the nucleus of infected plant cells, and both strands encode gene products (Briddon, 2002; Stanley *et al*., 2005).

DNA-A encodes products involved in DNA replication, control of gene expression, and insect transmission. DNA-A contains six open reading frames (ORF), two in the virion sense (AV1 and AV2) and four in the complementary sense (AC1, AC2, AC3, and AC4). AV1 encodes the coat protein that encapsidates the virion-sense ssDNA and may be involved in virus movement. AV2 has also been implicated in virus movement, and is required for virus accumulation and symptom development (Padidam *et al*., 1996; Stanley *et al*., 2005). This gene is lacking in New World begomoviruses (Harrison and Robinson, 2002). AC1, AC2 and AC3 encode the replication-associated protein (Rep), transcriptional activator protein (TrAP), and the replication enhancer protein (REn), respectively. AC4 encodes a protein that is involved in host range determination, symptom severity, and virus movement (Stanley *et al*., 2005).

DNA-B contains two ORF, one in the virion sense (BV1) and the other in the complementary sense (BC1). BV1 encodes the nuclear shuttle protein (NSP) which controls the transport of viral DNA between the nucleus and the cytoplasm, and the BC1 encodes the movement protein (MP) which mediates virus cell-to-cell movement (Stanley *et al*., 2005; Seal *et al*., 2006).

Begomoviruses that lack a DNA-B component are called monopartite. The DNA-A component alone has been shown to cause wild-type diseases symptoms, as described for *Tomato yellow leaf curl virus* (TYLCV) and *Tomato leaf curl virus* (TLCV) (Kheyr-Pour *et al*., 1991; Navot *et al*., 1991; Dry *et al*., 1993). For many other begomoviruses only DNA-A components have been found (Xie and Zhou, 2003), but until infectious clones are tested, it is not possible to conclude that such viruses have only a single DNA-A component (Seal *et al*., 2006).

Additional DNA components have been shown to be associated with some monopartite begomoviruses (Briddon and Stanley, 2006; Briddon *et al*., 2008). *Ageratum yellow vein virus*

(AYVV), *Cotton leaf curl Multan virus* (CLCuMV), and *Tomato yellow leaf curl China virus* (TYLCCNV) are monopartite begomoviruses. When cDNA infectious clones of these viruses were inoculated in their respective hosts, they were not able to induce the typical disease symptoms. In addition to the DNA-A, these viruses require a ssDNA satellite molecule (DNA β) to develop full disease symptoms (Briddon *et al*., 2000; Saunders *et al*., 2000; Zhou *et al*., 2003). Satellites are defined as subviral agents composed of nucleic acid that depend on co-infection with a helper virus for their replication. Satellite nucleic acids have substantially distinct nucleotide sequences from those of the genomes of their helper viruses. Despite their small size and the apparent absence of potential gene products, satellites may have a dramatic effect on the symptoms induced by their helper viruses (Simon *et al*., 2004).

DNA β molecules are about half the size of the helper DNA-A component, ranging from 1,247-1,374 nucleotides in length (Briddon *et al*., 2003). They are widespread in the Old World begomoviruses and all contain three conserved regions: an A-rich region, a sequence-conserved region (SCR), and an open reading frame termed βC1 (Briddon *et al.*, 2003; Zhou *et al*., 2003; Briddon *et al*., 2008). The βC1 ORF is conserved in position and size and is involved in symptom induction, host range determination, and accumulation of both helper virus and satellite molecules (Saunders *et al*., 2000; Briddon *et al*., 2001; 2003; Zhou *et al*., 2003; Qian and Zhou, 2005).

DNA 1 constitutes another group of ssDNA satellites associated with begomoviruses. It has been proposed that DNA 1 molecules derive from nanovirus components that have become adapted to whitefly transmission by encapsidation within the begomovirus coat protein (Stanley *et al*., 2005). DNA 1 molecules are about half the size of a DNA-A molecule (Mansoor *et al*., 1999; Saunders and Stanley, 1999; Briddon *et al*., 2004; Stanley, 2004). DNA 1 satellites differ from DNA β in encoding a Rep protein similar to that of nanoviruses, enabling autonomous

replication. DNA 1s are not required for helper virus proliferation or disease symptom induction, but these satellites require a helper begomovirus for their movement in plants and encapsidation enabling insect transmission (Seal *et al*., 2006).

Begomoviruses have the facility to form new genetic variants that can arise through simple mutations, pseudorecombination, and recombination in their ssDNA genomes (Seal *et al*., 2006; Pita and Roossinck, 2008). High mutation frequencies for begomoviruses have been reported to occur in wild and cultivated hosts (Ooi *et al*., 1997; Sanz *et al*., 1999). Pseudorecombination describes the exchange of DNA-A and DNA-B genomic components and has been reported for begomoviruses from both Old and New Worlds (Garrido-Ramirez, *et al*., 2000; Pita *et al*., 2001). Recombination is the process by which segments from one nucleotide strand become incorporated into that of a different individual strand during replication (Seal *et al*., 2006). In geminiviruses, recombination is facilitated by a type of rolling circle replication referred to as recombination-dependent replication, which favors recombination and generates a diversity of viral DNA forms (Preiss and Jeske, 2003). Evidence for recombination events in the genus *Begomovirus* has been reported (Harrison *et al*., 1997; Zhou *et al*., 1997; Padidam *et al*., 1999). For example, in Uganda, a severe form of cassava mosaic disease was caused by a recombinant (EACMV-UG2) between *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) (Zhou *et al*., 1997; Sseruwagi *et al*., 2004). Similarly, it was reported that *Tomato yellow leaf curl Malaga virus* (TYLCMalV) is a recombinant derived from the genetic exchange between TYLCV and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Monci *et al*., 2002). Recombination events have also been reported between DNA-A components and satellite DNAs, and between different satellite DNA β molecules (Briddon *et al*., 2001; 2003; Saunders, *et al*., 2001). The diverse population of begomoviruses coupled with the propensity of these viruses to exchange genetic material by recombination, increases the

probability of the emerging of new virus diseases (Mansoor *et al*., 2003). Seal *et al*. (2006) presented an extensive review of the factors involved in begomovirus evolution, considering the sources of genetic variation, mechanisms of genome rearrangements, as well as, selection of variants by the vector and the plant host.

During the last few decades, many begomovirus species have emerged, and as the number of characterized begomoviruses increases, it is more difficult to differentiate with accuracy, the viruses that are strains of already described viruses from those that represent new virus species. In 2003, the ICTV (International Committee on Taxonomy of Viruses) Geminiviridae Study Group proposed new species demarcation criteria. One of the most important is the establishment of a threshold (89 %) for the nucleotide sequence identity of DNA-A. Virus isolates with a DNA-A nucleotide sequence identity below 89% will be considered as new species (Fauquet *et al*., 2003). In addition, demarcation criteria and guidelines to classify and name begomoviruses below the species level was recently proposed (Fauquet *et al*., 2008) to differentiate between strains and variants. Strains are defined as “viruses belonging to the same species that have stable and heritable biological, serological, and/or molecular differences.” Variants are defined as “something that differs slightly from the norm” and it can be used with begomoviruses with very small differences. Based on pairwise sequence analysis of DNA-A component, a threshold of 93 % nucleotide identity was proposed to distinguish among strains, and viruses with more than 94 % nucleotide identity can be considered as variants (Fauquet *et al*., 2008).

Begomovirus species complexes are common and represent serious agricultural threats. Characterization of species complex diversity has substantially contributed to the understanding of both begomovirus evolution, and the ecological and epidemiological processes involved in the emergence of new viral pathogens (Rojas *et al*., 2005; Seal *et al*., 2006; Lefeuvre *et al*., 2007).

2.2 Sweetpotato Viruses

Virus diseases are an important constraint for sweetpotato (*Ipomoea batatas* (L.) Lam) production. In some countries losses of more than half of the potential yield have been attributed to the effect of viruses (Hanh, 1979; Ngeve and Bouwkamp, 1991; Milgram *et al*., 1996; Di Feo *et al*., 2000; Gutierrez *et al*., 2003; Clark and Hoy, 2006). About 20 distinct viruses (Table 2.1) have been isolated, described, and characterized from sweetpotato (Clark and Moyer, 1988; Moyer and Salazar, 1989; Loebenstein *et al*., 2004; Valverde *et al*., 2007; 2008). The high incidence of viruses in sweetpotato is the result of using infected stem cuttings as planting materials and of the presence of insect vectors (aphids and whiteflies). Since sweetpotato is a vegetatively propagated crop, viruses have an efficient mechanism for their perpetuation and dissemination. Virus diseases not only cause reduction in yields but also affect quality of storage roots (Clark and Moyer, 1988; Clark and Hoy, 2006).

2.2.1 Potyviruses

Sweet potato feathery mottle virus (SPFMV) belongs to the genus *Potyvirus* (family *Potyviridae*) and is found everywhere sweetpotato is grown (Clark and Moyer, 1988; Moyer and Salazar, 1989; Loebenstein *et al*., 2004; Valverde *et al*., 2007). SPFMV has flexuous filamentous particles between 830-850 nm in length. Its genome consists of a single stranded, linear, positive RNA of about 10.6 kb (Sakai *et al*., 1997). SPFMV is transmitted in a non-persistent manner by several aphid species, including *Aphis gossypii*, *A. craccivora*, *Lipaphis erysimi*, and *Myzus persicae*. It can be transmitted mechanically to various *Ipomoea* species, although some strains have been reported to infect *Nicotiana benthamiana* and *Chenopodium* spp. (Loebenstein *et al*., 2004).

Several strains of SPFMV have been identified based on symptoms, host range, serology, and nucleotide sequences (Moyer and Kennedy, 1978; Cali and Moyer, 1981; Kreuze *et al*.,

Table 2.1. Sweetpotato viruses (adapted from Valverde *et al*., 2007)

Virus	Family/Genus	Vector
Cucumber mosaic virus (CMV)	*Bromoviridae / Cucumovirus*	Aphids
Ipomoea yellow vein virus (IYVV)	*Geminiviridae / Begomovirus*	Whiteflies
Sweet potato chlorotic stunt virus (SPCSV)	*Closteroviridae / Crinivirus*	Whiteflies
Sweet potato feathery mottle virus (SPFMV)	*Potyviridae / Potyvirus*	Aphids
Sweet potato latent virus (SwPLV)	*Potyviridae / Potyvirus*	Aphids
Sweet potato virus G (SPVG)	*Potyviridae / Potyvirus*	Aphids
Sweet potato leaf curl virus (SPLCV)	*Geminiviridae / Begomovirus*	Whiteflies
Sweet potato leaf curl Georgia Virus (SPLCGV)	*Geminiviridae / Begomovirus*	Whiteflies
Sweet potato leaf speckling virus (SPLSV)	*Luteoviridae / Enamovirus*	Aphids
Sweet potato mild mottle virus (SPMMV)	*Potyviridae / Ipomovirus*	?
Sweet potato mild speckling (SPMSV)	*Potyviridae / Potyvirus*	Aphids
Tomato spotted wilt virus (TSWV)	*Bunyaviridae / Tospovirus*	Thrips?
Tentative species	**Family / Putative genus**	
Sweet potato C-6 virus	?	?
Sweet potato caulimo- like virus	*Caulimoviridae*	?
Sweet potato chlorotic fleck virus (SPCFV)	*Flexiviridae / Carlavirus*	?
Ipomoea crinkle leaf curl virus (ICLCV)	*Geminiviridae / Begomovirus*	?
Sweet potato ringspot virus	*Comoviridae / Nepovirus*	?
Sweet potato vein mosaic virus	*Potyviridae*	Aphids
Sweet potato virus 2 (SPV2)	*Potyviridae / Potyvirus*	Aphids?
Sweet potato yellow dwarf virus (SPYDV)	*Potyviridae / Ipomovirus*	?

2000; Wang *et al*., 2007). Most sweetpotato cultivars infected with SPFMV alone show only mild symptoms that include vein clearing, irregular chlorotic patterns (feathering) along the leaf mid-rib, and chlorotic spots that sometimes have purple pigmented borders especially in the older leaves. Depending on sweetpotato cultivars, storage roots of infected plants may show external necrosis if infected with the russet crack strain of SPFMV (Moyer and Kennedy, 1978; Clark and Moyer, 1988). Losses due to SPFMV infection are minimal, except in highly susceptible cultivars (Clark and Moyer, 1988; Karyeija *et al*., 1998). The ubiquitous presence of SPFMV has often masked the presence of other potyviruses. It is clear that a potyvirus complex affects sweetpotatoes, but it is not clear how these potyviruses relate to one another (Clark *et al*., 2002). In the US, SPFMV is universal, but two other potyviruses *Sweet potato virus G* (SPVG) and Sweet potato virus 2 (SPV2) are also common (Souto *et al*., 2003; Valverde *et al*., 2007).

2.2.2 Closteroviruses

Sweet potato chlorotic stunt virus (SPCSV) belongs to the genus *Crinivirus* (family *Closteroviridae*) and is widespread in different sweetpotato growing regions of the world (Winter *et al*., 1992; Gibson *et al*., 1998; Kreuze *et al*., 2002; Loebenstein *et al*., 2004; Valverde, *et al*., 2007). SPCSV has flexuous and filamentous particles, with lengths ranging from 850-950 nm. It has a bipartite genome that consists of two single stranded, linear, positive sense RNAs (Kreuze *et al*., 2002). SPCSV is transmitted in a semipersistent, non-circulative manner by whiteflies (*B. tabaci* and *Trialeurodes abutilonea*) (Cohen *et al*., 1992; Sim *et al*., 2000). This virus is graft transmissible, but it is not transmitted by mechanical inoculation. SPCSV infects several *Ipomoea* spp. and can also infect *N. benthamiana*, *N. clevelandii*, and *Amaranthus palmeri* (Loebenstein *et al*., 2004). SPCSV can be divided in two major serotypes: the East African serotype (occurs only in East Africa and Peru) and the West African serotype (occurs in US, Argentina, Brazil, West Africa, and Egypt) (Hoyer *et al*., 1996; Kreuze *et al*., 2002; IsHak *et*

al., 2003; Gutierrez *et al*., 2003; Abad *et al*., 2007). Symptoms caused by SPCSV include mild symptoms as slight stunting, mild interveinal chlorosis, and interveinal purpling of older leaves (Gibson *et al*., 1998; Gutierrez *et al*., 2003). SPCSV alone can cause significant yield reductions in sweetpotato yields (Gutierrez *et al*., 2003; Untiveros *et al*., 2007).

In the US, SPCSV has first been reported infecting in vitro plants of the cultivar White Bunch (Pio-Ribeiro *et al*., 1996), and recently, it was found on sweetpotato fields (Abad *et al*., 2007).

2.2.3 Geminiviruses

Begomoviruses have been reported infecting sweetpotato in Israel (Cohen *et al*., 1997), Taiwan (Chung *et al*., 1985), Japan (Onuki and Hanada, 1998), the US (Lotrakul *et al*., 1998; 2003), Spain (Banks *et al*., 1999; Lozano *et al*., 2004), Italy (Briddon *et al*., 2006), China (Luan *et al*., 2006), Peru (Fuentes and Salazar, 2003) and Kenya (Miano *et al*., 2006). It is evident that begomoviruses are associated with sweetpotato in most of the geographical regions where sweetpotatoes are grown, but the prevalence and the distribution in these regions are still not known (Valverde *et al*., 2007).

In the US, *Sweet potato leaf curl virus* (SPLCV) has been found infecting an ornamental sweetpotato and some breeding lines but not in sweetpotato grown for commercial production (Lotrakul *et al*., 1998; Clark and Valverde, 2001; Clark and Hoy, 2006). SPLCV is transmitted by graft inoculations but not mechanically to several *Ipomoea* species. In nature, it is transmitted by the sweetpotato whitefly (*B. tabaci*). Under experimental conditions, the virus is transmitted by this vector at relatively low rates (Valverde *et al*., 2004b). Various *Ipomoea* species are susceptible to SPLCV infection, causing yellow vein symptoms in some *Ipomoea species (I. aquatica, I. fistulosa*, and *I. cordatotriloba*) and leaf curl symptoms in other *Ipomoea species (I.*

alba, I. batatas W-285*, I. lacunosa, I. lobata, I. nil, I. setosa* and *I. trifida*) and *N. benthamiana* (Figure 2.1) (Lotrakul *et al*., 1998; Lotrakul and Valverde, 1999; Ling *et al*., 2008).

SPLCV has a monopartite genome (DNA-A, 2,828 nucleotides) and its organization is typical of Old World begomoviruses, containing six open reading frames and an intergenic region containing a conserved stem-loop motif (Figure 2.2) (Lotrakul and Valverde, 1999). In Japan, SPLCV was partially purified yielding typical geminate particles, and Western blot analysis revealed serological relationships with *Bean golden mosaic virus* (BGMV) and *Mungbean yellow mosaic virus* (MYMV) (Onuki *et al*., 2000).

Ipomoea yellow vein virus (IYVV), also referred as SPLCV-[Ipo], was found in *I. indica* showing yellow vein symptoms in Spain and Sicily (Banks *et al*., 1999; Briddon *et al*., 2006). Also, IYVV has been reported infecting sweetpotatoes in Spain (Lozano *et al*., 2004).

Sweet potato leaf curl Georgia virus (SPLCGV, previously called Ipomoea leaf curl virus, ILCV) was found in a mixed infection with SPLCV in a breeding line from Georgia (US). SPLCGV causes leaf curl symptoms in several *Ipomoea* species. Unlike SPLCV, SPLCGV does not cause yellow vein symptoms in *I. aquatica* and *I. cordatotriloba* (Lotrakul *et al*., 2003). Based on sequence analysis (76.5 percent DNA-A nucleotide sequence identity) and on differential host range (*I. aquatica* and *I. cordatotriloba*), SPLCGV is considered a distinct species of SPLCV (Lotrakul *et al*., 2003; Fauquet *et al*., 2003).

Recently, SPLCV has been reported in Peru, Kenya, and China. Sequence comparison analysis suggests that the isolates from Peru and Kenya are closely related to the US isolate of SPLCV (Fuentes and Salazar, 2003; Miano *et al*., 2006), while the isolate from China is more closely related to SPLCGV (Luan *et al*., 2007).

SPLCV, IYVV, SPLCGV, and SPLCV-China each has monopartite genome organization which is typical of Old World begomoviruses. DNA-B component or DNA satellites (DNA β

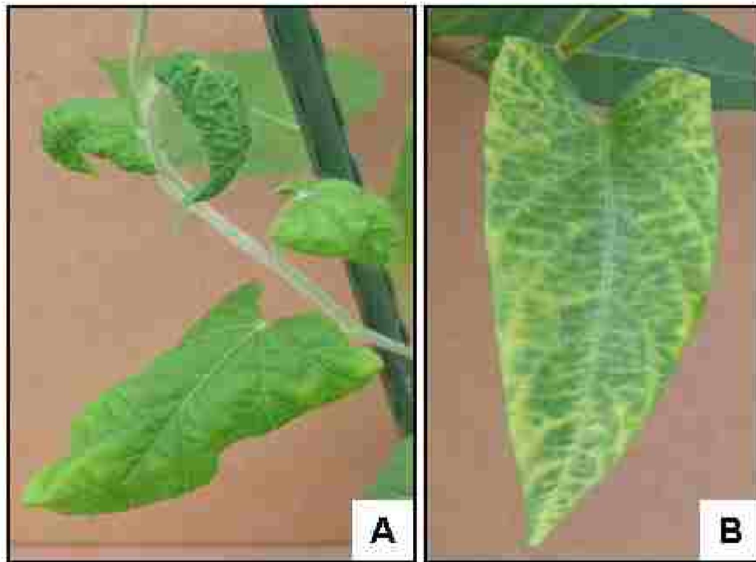


Figure 2.1. Leaf curl and yellow vein symptoms caused by *Sweet potato leaf curl virus* on *Ipomoea nil* (A) and *I. aquatica* (B), respectively.

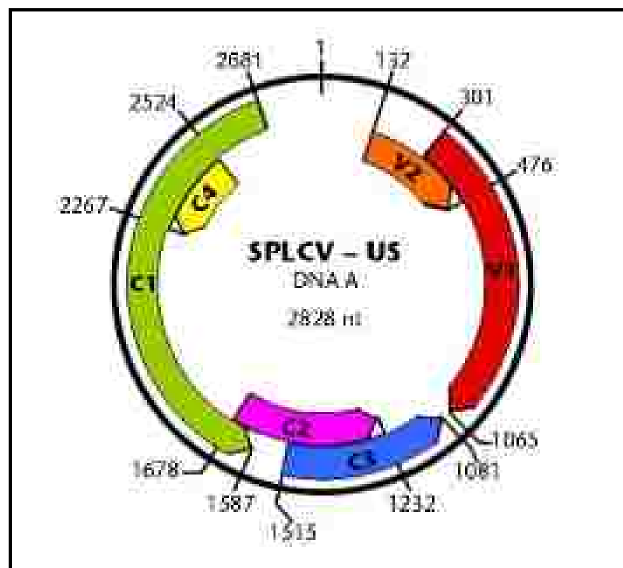


Figure 2.2. Genome organization of the *Sweet potato leaf curl virus* (SPLCV) component A. Arrows represent the orientation of the open reading frames. The numbers represent the position of nucleotides on the genome (Lotrakul and Valverde, 1999).

and DNA 1) have not been found associated with these begomoviruses (Lotrakul and Valverde, 1999; Lotrakul *et al*., 1998; 2003; Briddon *et al*., 2006; Luan *et al*., 2007).

Phylogenetic analysis using the derived amino acid sequences available of the sweetpotato begomoviruses (SPLCV, IYVV, SPLCGV, and SPLCV-China), place these begomoviruses in a distinct cluster apart from begomoviruses that infect other plant species (Lotrakul *et al*., 2003; Luan *et al*., 2007). Among these four sweetpotato begomoviruses, the coat protein was the most conserved; however, it was very different from the coat proteins of other begomoviruses (Lotrakul *et al*., 2003; Luan *et al*., 2007).

Considerable variability among the sweetpotato begomoviruses has been reported in the US (Lotrakul and Valverde, 1999; Lotrakul *et al*., 2002). Some of these viruses either do not induce symptoms or induce very mild, transient symptoms in the standard indicator host, *I. setosa* (Valverde *et al*., 2007). The nucleotide sequence identity (from 87% to nearly 100%) of a fragment of the AC1 of different SPLCV isolates and the phylogenetic analysis of them suggested that there may be more than one begomovirus species (Lotrakul *et al*., 2002).

2.2.4 Sweetpotato Virus Disease Complexes

In many cases, simultaneous infection of sweetpotato plants with more than two different viruses can cause greater damage than infection by each virus separately. This synergism is very clear in sweet potato virus disease (SPVD) which is caused by the synergistic interaction of SPFMV and SPCSV. SPVD has become the major virus constraint for sweetpotato production worldwide causing yield reductions up to 90 % (Schaefers and Terry, 1976; Gibson *et al*., 1998; Carey *et al*., 1999; Karyeija *et al*., 2000; Gutierrez *et al*., 2003; Loebenstein *et al*., 2004; Miano, 2008). Other disease complexes include: sweet potato chlorotic dwarf (SPFMV, SPCSV, and *Sweet potato mild speckling virus*, Di Feo *et al*., 2000), camote kulot (several viruses, Salazar and Fuentes, 2001), and the sweetpotato severe mosaic disease (SPCSV and *Sweet potato mild*

mottle virus, Mukasa *et al*., 2006). Untiveros *et al*., 2007, found synergistic interactions between SPCSV and carla- and cucumoviruses in addition to ipomo- and potyviruses.

The potential importance of sweetpotato begomoviruses has been overlooked, in part, because leaf curl symptoms are not common and do not persist in sweetpotato (Valverde *et al*., 2007). Genotypes that do develop characteristic upward curling symptoms generally do this only during warm periods of the year and may require the presence of other viruses for symptom development (Clark *et al*., 2002). In the US, SPLCV was found in mixed infection with SPFMV and SPLCGV (Lotrakul *et al*., 1998; 2003), while in Peru, SPLCV was found in mixed infection with SPCSV (Fuentes and Salazar, 2003). Therefore, it is likely that SPLCV may interact with these viruses synergistically. Experiments with single and mixed infections with russet crack strain of SPFMV and SPLCV resulted in higher titers of SPLCV in mixed infections, while that of SPFMV remained the same (Kokkinos, 2006). It is possible that higher SPLCV titers could result in a more efficient transmission by whiteflies and therefore natural spread of the virus to uninfected plants. More research is needed to get information on the prevalence, economic impact, and effects of mixed infections with SPLCV.

2.2.5 Diagnosis and Detection

Based on biological, serological, and nucleic acid properties of plant viruses, several diagnostics methods have been developed for sweetpotato virus detection (Valverde *et al*., 2007; 2008). However, detection and identification of sweetpotato viruses is not an easy task due to the low concentration and the uneven distribution of some viruses within the plant (Esbenshade and Moyer, 1982), and the presence of phenolic compounds, latex and inhibitors in sweetpotato tissue (Abad and Moyer, 1992). Also, difficulties in detection have been attributed to the occurrence of mixed infections, and viral strains (Valverde *et al*., 2008). Sweetpotato viruses can be detected by graft-inoculation to susceptible indicator plants, such as *I. setosa* and *I. nil*, among

others. Based on the properties of the coat protein, serological methods such as enzyme-linked immunosorbent assay (ELISA) and serologically specific electron microscopy (SSEM) have been reported (Cadena-Hinojosa and Campbell, 1981; Hoyer *et al*., 1996; Fuentes *et al*., 1996; Souto *et al*., 2003; Gutierrez *et al*., 2003; Untiveros *et al*., 2007). In addition, detection techniques based on nucleic acid properties include: polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR), real-time PCR, and molecular hybridization (Lotrakul, 2000; Li *et al*., 2004; Colinet *et al*., 1998; Sim, 2001; Valverde *et al*., 2004a; Kokkinos and Clark, 2006).

Different diagnostic methods have been developed for SPLCV. Most of them are based on symptomatology of indicator hosts and on the detection of viral DNA. An extensive SPLCV host range study was conducted by Ling *et al*. (2008). Molecular hybridization using a SPLCV-specific probe (coat protein gene) has been used to detect SPLCV from sweetpotato field collected samples (Valverde *et al*., 2004a; 2008). PCR, using specific and degenerate primers, and real-time PCR assays have been successfully used to detect SPLCV from indicator hosts and sweetpotato plants infected with this virus (Lotrakul and Valverde, 1999; Li *et al*., 2004; Kokkinos and Clark, 2006; Valverde *et al*., 2008). Due to the difficulties in obtaining purified SPLCV virions that can be used as antigens for antisera production, serological assays are not currently available.

Diagnosis and identification of sweetpotato viruses are essential for the development of appropriate management programs (Valverde *et al*., 2008). Plant virus diseases cannot be controlled in the same way as fungal or bacterial diseases. Their control relies mainly on preventing the establishment, development, and dispersal of the viruses. Prevention of sweetpotato viral diseases involves a wide variety of measures such as eradication of sources of infection, elimination of alternative hosts, as well as, vectors (Clark and Moyer, 1988). The use of virus-free planting material is the most widely used control strategy, followed by the use of

resistant cultivars (Clark and Moyer, 1988; Loebenstein *et al*., 2004; Valverde *et al*., 2007; 2008).

CHAPTER 3: MOLECULAR DIVERSITY OF *SWEET POTATO LEAF CURL VIRUS* IN THE UNITED STATES

3.1 Introduction

Sweet potato leaf curl virus (SPLCV) and *Sweet potato leaf curl Georgia virus* (SPLCGV) are two begomoviruses that have been reported in the United States. SPLCV has been found in an ornamental sweetpotato and some breeding lines, but not in sweetpotato grown for commercial production (Lotrakul *et al*., 1998; Clark and Valverde, 2001), whereas SPLCGV was found in a sweetpotato breeding line, in mixed infection with SPLCV and *Sweet potato feathery mottle virus* (SPFMV) (Lotrakul *et al*., 2003). SPLCV and SPLCGV can be transmitted by graft inoculations but not mechanically to several *Ipomoea* species. In nature, they are likely to be transmitted by the sweetpotato whitefly *Bemisia tabaci* (Lotrakul *et al*., 1998; 2003; Valverde *et al*., 2004b). SPLCV causes yellow vein symptoms in some *Ipomoea* species (*I. aquatica*, *I. fistulosa*, and *I. cordatotriloba*) and leaf curl symptoms in others (*I. alba*, *I. batatas* W-285, *I. lacunosa*, *I. lobata*, *I. nil*, *I. setosa* and *I. trifida*) (Lotrakul *et al*., 2002). SPLCGV causes leaf curl symptoms in several *Ipomoea* species, but unlike SPLCV, SPLCGV does not cause yellow vein symptoms on *I. aquatica* and *I. cordatotriloba* (Lotrakul *et al*., 2003). The genome organization of SPLCV and SPLCGV is similar to that of the monopartite begomoviruses from the Old World. DNA-B component or DNA satellites have not been found associated with these begomoviruses (Lotrakul and Valverde, 1999; Lotrakul *et al*., 2003; Briddon *et al*., 2006).

The potential importance of sweetpotato leaf curl diseases has been overlooked, in part, because leaf curl symptoms are not common and do not persist in sweetpotato (Valverde *et al*., 2007). Genotypes that do develop characteristic upward curling symptoms generally do this only during warm periods of the year and may require the presence of other viruses for symptom

development (Clark *et al*., 2002). Although SPLCV does not cause symptoms on Beauregard, the predominant sweetpotato cultivar in the US, it can reduce its yield up to 26 % (Clark and Hoy, 2006).

Since SPLCV could become an important constraint for sweetpotato production (yield and quality); diagnosis, identification, and characterization are essential for the development of an appropriate management strategy.

Variability among sweetpotato begomoviruses has been reported in the US (Lotrakul and Valverde, 1999; Lotrakul *et al*., 2002). Some of these viruses either do not induce symptoms or induce very mild, transient symptoms in the standard indicator host, *I. setosa* (Valverde *et al*., 2007). Comparisons of the nucleotide sequence of the AC1 fragment of different SPLCV isolates and phylogenetic analysis of them suggest that there may be more than one begomovirus species. Therefore, it is likely that this viral disease might be caused by a species complex (Lotrakul *et al*., 2002).

The aim of this study was to further explore the diversity of begomoviruses infecting sweetpotato genotypes and to determine the frequency of SPLCV and SPLCGV in the US, by analyzing the sequence of a 457 nucleotide fragment from the AC1.

3.2 Materials and Methods

3.2.1 Plant Samples and DNA Extraction

Eleven sweetpotato genotypes (kindly provided by Dr. C.A. Clark, Louisiana State University, Baton Rouge) (Table 3.1) that tested positive for SPLCV and SPFMV were graft-inoculated to indicator plants (*I. setosa*, *I. nil*, and *I. aquatica*). Total DNA was extracted from foliar tissues of these indicator plants (between 4 and 6 weeks after grafting) using Plant DNAzol Reagent (Invitrogen, Carlsbad, CA) and from sweetpotato leaves using DNeasy Plant Mini Kit (Qiagen, Valencia, CA), following the procedure provided by the manufacturers. The DNeasy

Plant Mini Kit (Qiagen) was found to be more efficient to obtain total DNA from sweetpotato leaves compared to Plant DNAzol Reagent (Invitrogen).

Table 3.1. Sweetpotato genotypes tested positive for *Sweet potato leaf curl virus*

Breeding lines	**Source**[a]
W-275	NCSU
Grand Asia	NCSU
NC1554	NCSU
Norton	NCSU
Whitestar	NCSU
98-20	USDA-VL
W-317	USDA-VL
W-287	USDA-VL
W-361	USDA-VL
SC1149-19	USDA-VL
0075	LSU

[a] NCSU: North Carolina State University, USDA-VL: USDA-ARS Vegetable Laboratory, Charleston, South Carolina; LSU: Louisiana State University.

3.2.2 Polymerase Chain Reaction (PCR)

SPLCV-specific primers PW285-1 and PW285-2 (Figure 3.1) were used to amplify a fragment of the AC1 (Lotrakul and Valverde, 1999). PCR reaction mixtures were conducted as described by Lotrakul *et al*. (1998): 50 µl volume containing 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.2 µM of each primer, 2.5 U of Taq DNA polymerase (Promega, Madison, WI) and 1 µl of DNA sample. PCR was performed in a Genius Thermocycler (Techne, Cambridge, UK) with an initial cycle of 94 ˚C for 1 min followed by 45 cycles of 94 ˚C for 1 min, 55 ˚C for 1 min, and 72 ˚C for 3 min, and a final extension cycle of 72 ˚C for 10 min (Lotrakul and Valverde, 1999). To further test the variability of sweetpotato begomoviruses obtained from the analysis of the AC1 fragment, primers SPB-1 and SPB-2 (Figure 3.1) were used to amplify the full length coat protein (CP) gene (AV1). SPB-2 primer sequence was

provided by Dr. P. Lotrakul (Chulalongkorn University, Bangkok, Thailand), while SPB-1 primer was modified from a primer sequence also provided by Dr. P. Lotrakul. PCR reaction mixtures were prepared as described above. PCR was performed in a Genius Thermocycler with 38 cycles of 94 ˚C for 1 min, 58 ˚C for 1 min, and 72 ˚C for 3 min, followed by a final extension cycle of 72 ˚C for 10 min. PCR products were separated by electrophoresis (1.2 % agarose) and stained with ethidium bromide.

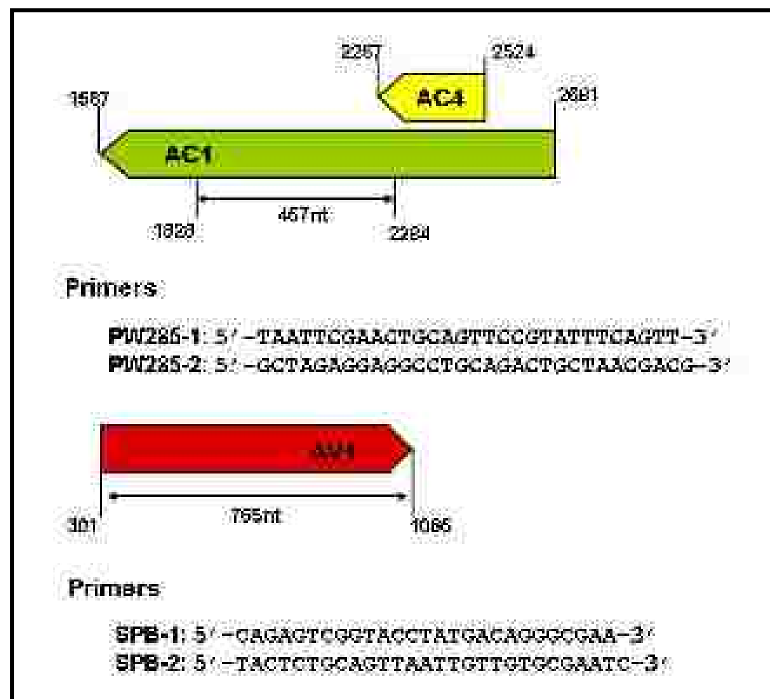


Figure 3.1. Primers used for the amplification of a fragment of the AC1 and the full length coat protein gene (AV1) of sweetpotato begomoviruses.

3.2.3 Cloning and Sequencing

Bands corresponding to the expected PCR products were excised from agarose gels and further purified after electrophoresis using MiniElute (Qiagen) DNA purification kit and ligated into the pGEM-T Easy vector (Promega). Recombinant plasmids were transformed into *Escherichia coli* JM 109 competent cells. Plasmids were isolated from transformant colonies

using the FlexiPrep kit Miniprep procedure (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Successful insertion of PCR products was confirmed by enzyme digestion (*Eco* RI).

Nucleotide sequences were determined by automated sequence analysis at the Research Technology Support Facility of Michigan State University, East Lansing using a Perkin Elmer/Applied Biosystems 3100 capillary sequencer (Perkin Elmer, Foster City, CA).

3.2.4 Sequence Analysis

Nucleotide and derived amino acid sequences of the AC1 fragment and the full length of the CP gene (AV1) were compared to the corresponding sequences of geminiviruses available in the Genbank. Four sequences of SPLCV isolates (GA-00-4, GA-00-3 I, W-328, and LA-Font-4), previously reported in the US (Lotrakul *et al*., 2002), were included in the analysis. Nucleotide sequences of four SPLCV isolates from Peru (CIP 400820, CIP 401212, TB26, and SR 90323) and two from Kenya (Kenya 43 and Kenya 84) were determined from total DNA samples provided by M.S. S. Fuentes (International Potato Center, Lima, Peru) and Dr. D.W. Miano (KARI Biotechnology Centre, Nairobi, Kenya), respectively (Table 3.2). Nucleotide and derived amino acid sequence identities were determined by using pairwise alignments. Pairwise and multiple sequence alignments were conducted using Clustal X version 1.83 (Jeanmougin *et al*., 1998). Neighbor-joining trees with bootstrap analysis (1000 replicates) were constructed from the multiple alignments and drawn with Tree View version 1.6.6 (Page, 1996).

3.3 Results

Only mild leaf curl symptoms on *I. setosa* and *I. nil*, and transient yellow vein symptoms on *I. aquatica* were observed after graft-inoculation experiments using scions from 11 sweetpotato genotypes.

A 512 nucleotide fragment corresponding to the AC1 was amplified with primers PW285-1 and PW285-2. Sequence analyses based on a 457 nucleotide region (selected from the

Table 3.2. Geminivirus sequences used in the analyses of the AC1 fragment and the coat protein gene (AV1) of sweetpotato begomoviruses

Virus names	**Assigned Abbreviation**	**AC1**	**AV1**	**GenBank Accession number**
Sweet potato leaf curl virus	SPLCV	✓	✓	AF104036
Sweet potato leaf curl Georgia virus	SPLCGV	✓	✓	AF326775
SPLCV US-isolates				
AL-Prakash (Alabama)	AL-Prakash	✓		AY679764
GA-00-4 (Georgia)	GA-00-4	●		
GA-00-3 I (Georgia)	GA-00-3 I	●		
W-328 (USDA, SC)	W-328	●		
LA-Font-4 (Louisiana)	LA-Font-4	●		
SPLCV-China	China	✓	✓	DQ512731
SPLCV-Japan (Kyoto)	Japan-Ky	✓	✓	AB433788
SPLCV-Japan (Kunamoto)	Japan-Ku	✓	✓	AB433787
SPLCV-Japan (Miyazaki)	Japan-Mi	✓	✓	AB433786
SPLCV-Puerto Rico I	P.Rico I	✓		AY679766
SPLCV-Puerto Rico II	P.Rico II	✓		AY679767
SPLCV-Korea	Korea	✓		AY679765
SPLCV-Kenya 43	Kenya 43	✓	⅄	DQ361005
SPLCV-Kenya 84	Kenya 84	■	⅄	
SPLCV-Peru 1 (CIP400820)	Peru 1	⅄		
SPLCV-Peru 2 (CIP401212)	Peru 2	⅄	⅄	
SPLCV-Peru 3 (TB26)	Peru 3	⅄	⅄	
SPLCV-Peru 4 (SR90323)	Peru 4	⅄		
Ipomoea yellow vein virus	IYVV	✓	✓	AJ586885
Tomato yellow leaf curl virus – USA	TYLCV-US	✓	✓	EF539831
TYLCV- Dominican Republic	TYLCV-DO	✓	✓	AF024715
Tomato yellow leaf curl China virus	TYLCCNV	✓	✓	NC_004044
Ageratum yellow vein virus	AYVV	✓	✓	X74516
Bean golden yellow mosaic virus	BGYMV	✓	✓	NC_001439
Mungbean yellow mosaic virus	MYMV	✓	✓	NC_001983
Dicliptera yellow mottle virus	DiYMoV	✓	✓	AF139168
Beet curly top virus	BCTV	✓	✓	NC_001412

✓ Sequences available in GenBank, ● Sequences provided by Dr. R.A Valverde, ■ Sequence provided by Dr. D.W. Miano, ⅄ Sequences determined in this study.

amplified fragment) of the begomoviruses obtained from the 11 sweetpotato genotypes indicated that 10 were closely related to SPLCV with nucleotide sequence identities that varied from nearly 100 to 96 %, while one (W-361) was related to SPLCGV with 92 % nucleotide sequence identity (Table 3.3). Nucleotide sequence of the AC1 fragment of W-287 and W-361 isolates were deposited in the GenBank (DQ361002, DQ361003). According to the nucleotide sequence of the AC1 fragments, the isolates from Peru and Kenya were closely related to SPLCV (Table 3.4).

A neighbor-joining tree based on the nucleotide sequence of the AC1 fragments clustered all sweetpotato begomoviruses together. In this cluster, sweetpotato begomoviruses were divided in three major groups (Figure 3.2). Group I included SPLCV and its relatives; this major group can be further divided in eight subgroups (Ia-Ih). Subgroup Ia consisted of SC1149-19, W-275, Grand Asia, W-287, Norton, and NC1554, and two SPLCV isolates from Peru (Peru 1 and Peru 2). Subgroup Ib contained 0075, Whitestar, and W-317, SPLCV, LA-Font-4 and W-328. The isolate 98-20 was clustered with the three SPLCV isolates from Japan (subgroup Ic), Peru 4 was clustered with IYVV (subgroup Id), while the Kenyan isolates (Kenya 43 and 84) were placed together (subgroup Ie). The SPLCV isolates from Korea, Peru (Peru 3), and Puerto Rico (P. Rico I) were placed separately and each one could be considered as subgroup (If-Ih, respectively). Group II included SPLCGV that clustered with W-361, AL-Prakash, GA-00-3 I, GA-00-4, and Puerto Rico II. Group III only contained a SPLCV isolate from China.

Based on the variability obtained from the analysis of the AC1 fragment, isolates W-361, W-287, 0075, and Whitestar were selected for the sequence analysis of the CP gene (AV1). The full length of the CP gene (765 nucleotides) was amplified with primers SPB-1 and SPB-2. Results indicated that these four isolates were closely related to SPLCV with nucleotide sequence identities that ranged from 95 to 99 % (Table 3.5). The CP gene sequence of the

Peruvian isolate, Peru 3, was related to SPLCV; however, Peru 2 was related to SPLCGV. Isolates from Kenya were related to SPLCV (Table 3.4).

A neighbor-joining tree based on the CP gene sequence clustered all sweetpotato begomoviruses together. In this phylogenetic analysis (Figure 3.3), two major groups were observed. Group I with 5 subgroups included W-361, Whitestar, 0075, and SPLCV in subgroup Ia. The isolate from W-287 breeding line was grouped with Japan-Ky (subgroup Ib); whereas, the other two Japanese isolates (Japan-Ku and Japan-Mi) were placed with Peru 3 (subgroup Ic). The SPLCV isolates from Kenya were grouped together, and IYVV was placed alone. In group II, SPLCGV clustered with the SPLCV isolate from China and Peru (Peru 2).

3.4 Discussion

Diseases caused by begomoviruses on sweetpotato have been reported in Israel, Taiwan, Japan, US, Spain, Italy, China, Peru, and Kenya (Cohen *et al*., 1997; Chung *et al*., 1985; Onuki and Hanada, 1998; Lotrakul *et al*., 1998; Banks *et al*., 1999; Briddon *et al*., 2006; Luan *et al*., 2007; Fuentes and Salazar, 2003; Miano *et al*., 2006). Two begomoviruses, SPLCV and SPLCGV, found infecting sweetpotato breeding lines in the US, have been characterized (Lotrakul and Valverde, 1999; Lotrakul *et al*., 2003); however, information about how often these viruses occur in breeding lines and sweetpotato cultivars is not known.

In this study, sequences of 11 begomoviruses infecting sweetpotato genotypes in the US were obtained. Nucleotide sequence identities of the AC1 fragment indicated that ten were closely related to SPLCV, and only one, related to SPLCGV. Therefore, it appears that SPLCV is more common in sweetpotato genotypes than SPLCGV.

Lotrakul and Valverde (1999) have reported that the region of the AC1 that overlaps with the AC4 was the most conserved in the genome of SPLCV. Thus, the percent nucleotide sequence identity obtained from comparing sequences of this region may be higher than that of

Table 3.3. Percent nucleotide (above) and derived amino acid (below) sequence identities of the AC1 fragment of 11 begomoviruses found infecting sweetpotato genotypes in the US

	0075	W-361	SC1149-19	Norton	Whitestar	98-20	W-275	Grand Asia	NC1554	W-317	W-287
SPLCV	100	88	96	97	99	98	97	97	97	98	97
	100	94	99	98	99	99	99	98	98	98	98
SPLCGV	87	92	85	85	87	87	85	85	85	86	85
	94	96	93	92	93	93	93	92	94	92	92
0075		88	96	97	99	98	97	97	97	98	97
		94	99	98	99	99	99	98	98	98	98
W-361			87	87	87	88	87	87	88	87	87
			94	92	94	94	94	93	94	93	93
SC1149-19				98	96	96	99	99	98	96	98
				98	98	98	100	99	99	98	99
Norton					96	96	98	98	98	96	98
					97	97	98	98	98	96	98
Whitestar						98	96	96	96	98	96
						98	98	98	98	98	98
98-20							96	96	96	98	96
							98	98	98	98	98
W-275								99	98	96	99
								99	99	98	99
Grand Asia									98	96	99
									98	97	100
NC1554										96	98
										97	98
W-317											96
											97

Table 3.4. Percent nucleotide (above) and derived amino acid (below) sequence identities of the AC1 fragment and the coat protein gene (AV1) of sweetpotato begomoviruses from Peru and Kenya

	SPLCV[a]		SPLCGV[b]		IYVV[c]	
	AC1	**AV1**	**AC1**	**AV1**	**AC1**	**AV1**
Peru 1 (CIP400820)	97 98		86 92		96 99	
Peru 2 (CIP401212)	96 98	89 95	85 92	94 96	94 97	89 94
Peru 3 (TB26)	94 96	96 97	87 92	90 96	93 95	93 95
Peru 4 (SR90323)	97 99		87 93		97 98	
Kenya 43	96 97	94 95	85 92	89 94	96 96	93 94
Kenya 84	96 98	95 97	84 92	90 95	95 98	94 95

[a] SPLCV: *Sweet potato leaf curl virus*, [b] SPLCGV: *Sweet potato leaf curl Georgia virus*, [c] IYVV: *Ipomoea yellow vein virus*.

Table 3.5. Percent nucleotide (above) and derived amino acid (below) sequence identities of the coat protein gene (AV1) of four selected begomoviruses found infecting sweetpotato genotypes in the US

	W-361	**W-287**	**0075**	**Whitestar**
SPLCV[a]	99 100	95 97	99 99	99 99
SPLCGV[b]	90 94	90 94	89 94	89 94
W-361		95 97	99 99	99 99
W-287			95 97	95 97
0075				99 98

[a] SPLCV: *Sweet potato leaf curl virus*, [b] SPLCGV: *Sweet potato leaf curl Georgia virus*.

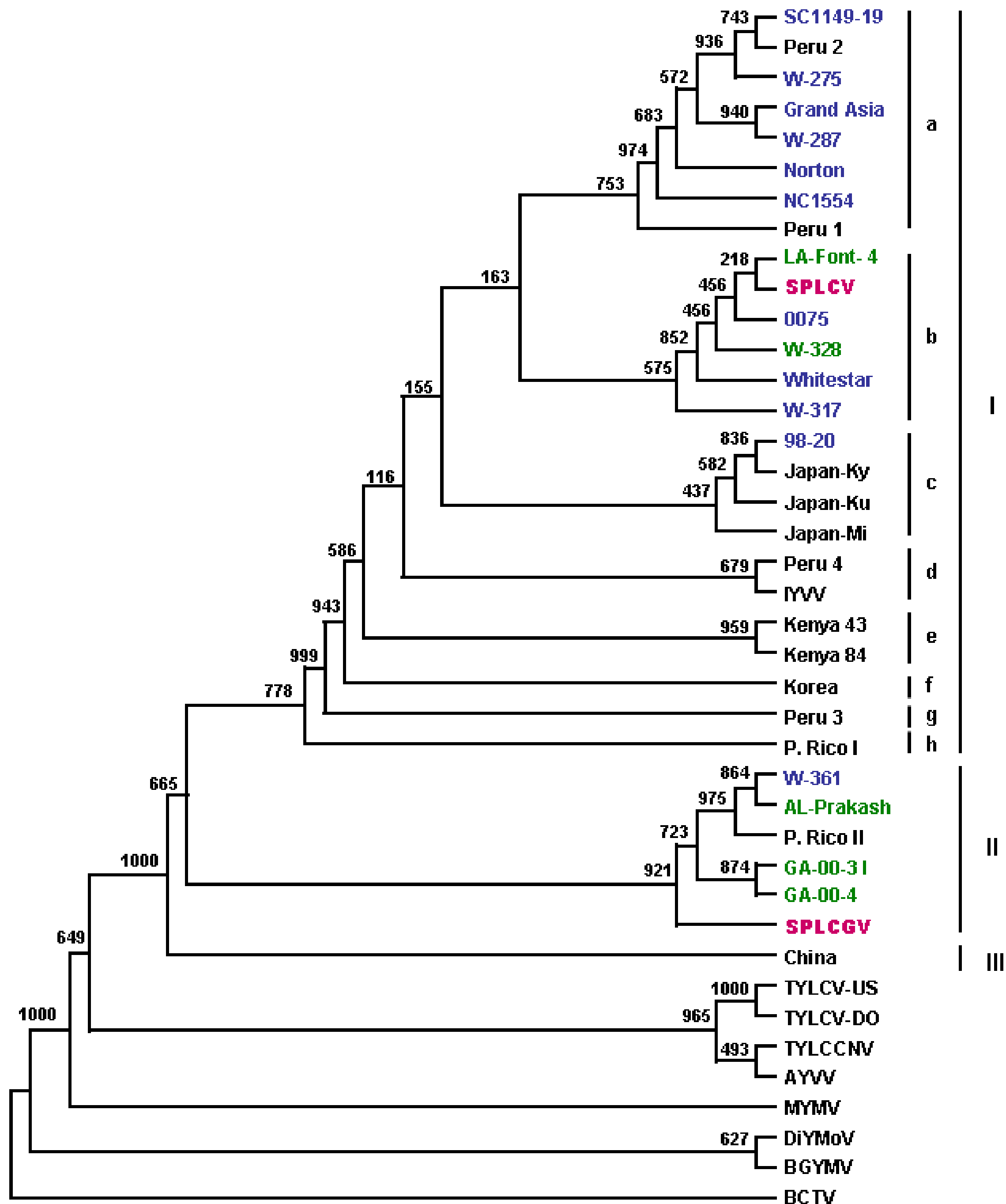


Figure 3.2. Neighbor-joining tree based on the nucleotide sequence of the AC1 fragment shows the relationship among begomoviruses infecting sweetpotato in the US and other begomoviruses. *Beet curly top virus* (BCTV), genus *Curtovirus*, was used as an outgroup. The tree was generated with Clustal X version 1.83 and drawn with Tree View version 1.6.6. Numbers at each branch indicate the bootstrap values. Vertical and horizontal branch lengths are arbitrary. Blue labels indicate US isolates of SPLCV reported in this study, while green labels indicated US isolates previously reported.

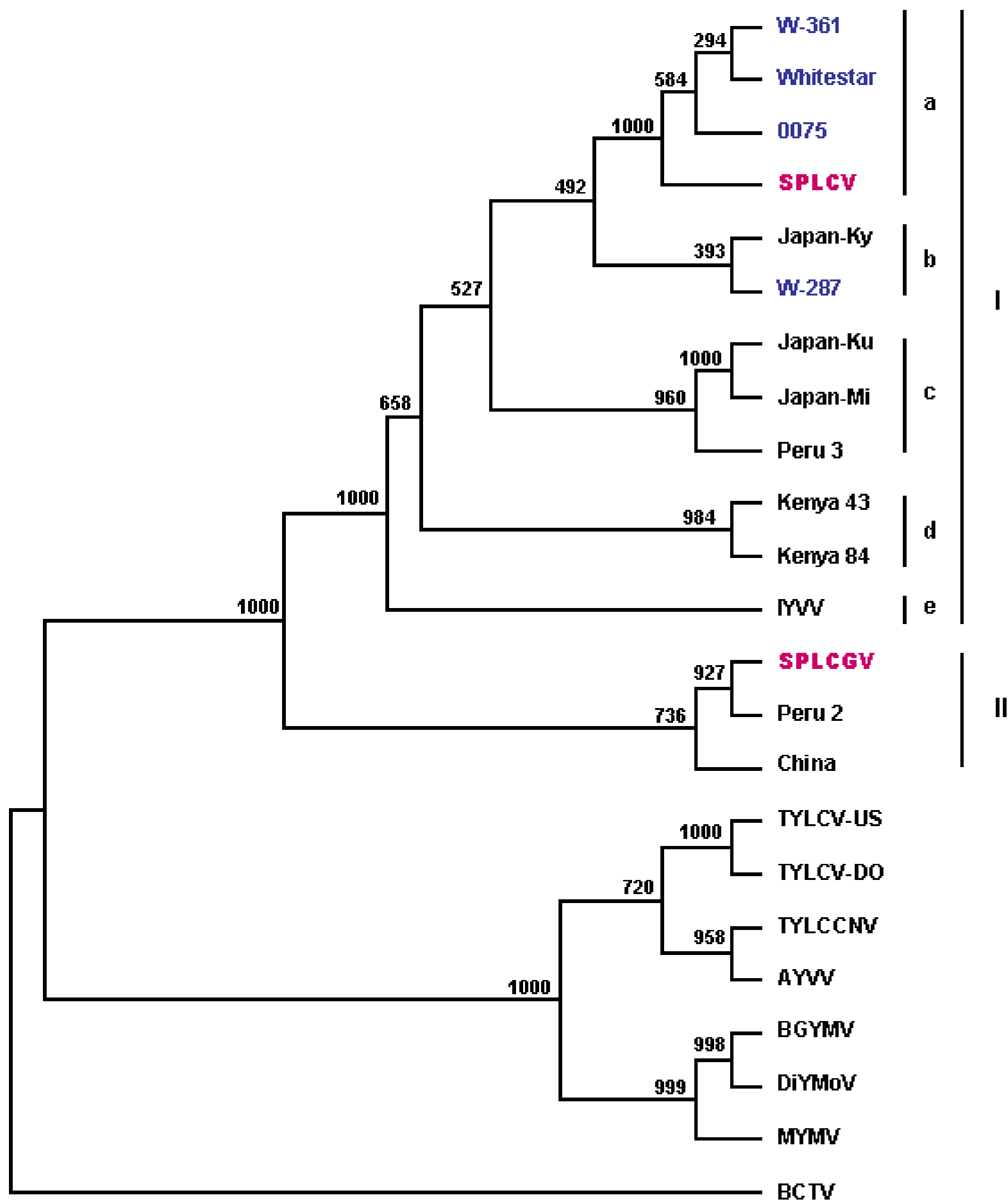


Figure 3.3. Neighbor-joining tree based on the nucleotide sequence of the coat protein gene (AV1) shows the relationship among begomoviruses infecting sweetpotato in the US and other begomoviruses. *Beet curly top virus* (BCTV), genus *Curtovirus*, was used as an outgroup. The tree was generated with Clustal X version 1.83 and drawn with Tree View version 1.6.6. Numbers at each branch indicate the bootstrap values. Vertical and horizontal branch lengths are arbitrary. Blue labels indicate SPLCV isolates reported in this study.

the entire genome (Lotrakul *et al*., 2002). Based on the sequence of a 457 nucleotide fragment of the AC1, SPLCV isolates (analyzed by Lotrakul *et al*., 2002) were clustered into three groups. In this study, SPLCV isolates were also clustered in three major groups that were slightly different from the ones reported by Lotrakul *et al*. (2002).

Most isolates of different geographical regions (Japan, Puerto Rico isolate I, Korea, Peru, and Kenya) were closely related to SPLCV. As was expected, isolates from the same region were more closely related to each other as shown by the isolates from Kenya and Japan. Nevertheless, it was interesting to notice that the isolates from Peru were distributed in different subgroups. The fact that the International Potato Center (Lima, Peru) obtains germplasm from different countries may explain the diversity of SPLCV isolates from Peru. The isolate II from Puerto Rico was closely related to SPLCGV. The isolate from China was placed alone in group III because the AC1 of this particular isolate is shorter than those of other SPLCV strains (Luan *et al*., 2007). It has been reported that the AC1 (Rep) sequences of SPLCV, SPLCGV, IYVV and the SPLCV isolate from China were more conserved than the CP gene (AV1) sequences when they were compared to those of non-sweetpotato begomoviruses (Lotrakul *et al*., 2003; Luan *et al*., 2007).

According to Padidam *et al*. (1995), the 5' end is the most variable region of the begomovirus CP gene, and is representative of the nucleotide sequence variability of the entire viral genome. Therefore, a phylogenetic analysis based on this region is usually sufficient to establish the taxonomic position of a given begomovirus isolate. In order to confirm the variability found in the analysis of the AC1 fragment, sequences of the CP gene (AV1) were obtained from selected SPLCV infected samples. Results of phylogenetic analysis based on CP sequences were slightly different from the AC1. In this case, sweetpotato begomoviruses were split in two groups instead of three. Group I contained SPLCV and its relatives from the US,

Peru (Peru 3), Japan, Kenya, and Spain (IYVV). In Group II, SPLCV isolates from China and Peru (Peru 2) were clustered with SPLCGV.

An unexpected finding was that the nucleotide sequence of the AC1 fragment from the W-361 isolate was more related to SPLCGV (92 %) even though the CP sequence was more similar to the sequence of SPLCV (99 %). Similar results were obtained with one of the isolates from Peru (Peru 2). Based on the AC1 sequence analysis, Peru 2 clustered within the SPLCV group (96 %), while the CP sequence clustered with SPLCGV (94 %). Further studies are needed to determine if these isolates are the result of a recombination.

The sequence analysis of the AC1 fragment and the CP gene (AV1) clustered SPLCV and SPLCGV in two different groups, supporting their status as different species. Also, the results confirm that the CP gene sequence is the most conserved among the sweetpotato begomoviruses but distinct from those of other begomoviruses as previously reported by Lotrakul and Valverde (1999) and Luan *et al*. (2007).

A threshold of 89 % nucleotide sequence identity between the full length genome sequences of the A component has been established to demarcate between distinct species of geminiviruses, and 93 % nucleotide sequence identity to distinguish between strains (Fauquet *et al*., 2003; 2008). According to this rule, the SPLCV isolate from China can be considered as a strain of SPLCGV based on the percentage of the nucleotide sequence identity of its DNA-A, 91 % with SPLCGV and 88 % with SPLCV (Luan *et al*., 2007). Whereas, the viruses from Japan: Kyoto, Kunamoto, and Miyazaki could be considered variants or strains of SPLCV based on their DNA-A nucleotide sequence identities of 96, 93 and 90 %, respectively (Onuki *et al*., unpublished).

The analysis of the AC1 fragment and the CP gene (AV1) confirmed previous suggestions regarding the variability of begomoviruses infecting sweetpotato. However, it should

be emphasized that partial sequences are not enough to distinguish new viral species. A comprehensive characterization on host range, virus transmission, and complete genome sequence needs to be conducted in order to classify a virus strain or a new species as suggested by Fauquet *et al*. (2003).

Evidence for recombination events in the genus *Begomovirus* has been reported (Harrison *et al*., 1997; Zhou *et al*., 1997; Padidam *et al*., 1999). For instance, the virus associated with the cassava pandemic in Uganda (EACMV-UG2), is a recombinant between *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) (Zhou *et al*., 1997). Similarly, it was reported that *Tomato yellow leaf curl Malaga virus* (TYLCMalV) is a recombinant derived from the genetic exchange between *Tomato yellow leaf curl virus* (TYLCV) and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Monci *et al*., 2002).

Mixed infections provide suitable conditions for recombination. In Spain, a high incidence of begomovirus infection on sweetpotato has been reported (Lozano *et al*., 2004). SPLCV and IYVV were found in mixed infections. Moreover, sequence analysis of DNA-A components from various begomovirus isolates suggested the presence of new species (Lozano *et al*., 2004). SPLCV was found in mixed infections with SPFMV and SPLCGV in the US (Lotrakul *et al*., 1998; 2003), whereas with SPCSV in Peru (Fuentes and Salazar, 2003). It is likely that SPLCV may interact with these viruses synergistically. Experiments with single and mixed infections with russet crack strain of SPFMV and SPLCV resulted in higher titers of SPLCV in mixed infections, while that of SPFMV remained the same (Kokkinos, 2006). It is possible that higher SPLCV titers could result in a more efficient acquisition by whiteflies and therefore natural spread of the virus to uninfected plants.

The genetic diversity of sweetpotato begomoviruses may have important implications in host range, disease, and whitefly transmissibility. Some regions of the begomovirus genome are

more prone to variation than others. The part of the genome that shows the greatest variation among different begomoviruses is the intergenic region (IR). Apart from the conserved nonanucleotide sequence and the TATA boxes, the IRs of different viruses may show little similarity to one another (Harrison and Robinson, 1999). According to Seal *et al*. (2006), the main recombination site appears to be in the IR. The AC1 encodes the replication-associated protein (Rep) which binds in a sequence-specific manner to the iterons in the IR. Consequently, variability of the IR coincides with variability of the Rep sequence, especially in the N-terminal half of the Rep, which contains the domain that interacts with the IR (Harrison and Robinson, 1999; Sanz *et al*., 2000).

The AC4 (which is embedded in the AC1) is involved in host range determination, symptom severity, and virus movement (Jupin *et al*., 1994; Laufs *et al*., 1995; Wartig *et al*., 1997). Recombinants in the AC1-AC4 region have been reported (Seal *et al*., 2006); therefore, variability in the AC1-AC4 region may be critical and could affect the fitness of begomoviruses, as well as, their ability to cause disease.

In contrast to the IR, the most conserved region among begomoviruses is the CP gene (AV1). The CP plays a key role in processes that are involved in virus infection, survival and spread (Harrison and Robinson, 1999; Harrison *et al*., 2002). Although the CP is the most conserved protein among sweetpotato begomoviruses, it is very different from the CP of begomoviruses that infect other plant species (Lotrakul and Valverde, 1999; Lotrakul *et al*., 2003; Luan *et al*., 2007). This could be one reason for the low rate of transmission by the sweetpotato whitefly *B. tabaci*.

Genetic diversity may also interfere with the reliability of molecular detection tools, such as PCR and nucleic acid hybridization. Successful amplification of a fragment of the AC1 by SPLCV-specific primers (PW285-1 and PW285-2) required high quality DNA samples from

plant tissues. Any variation in primer recognition sites could affect the amplification. An alternative to virus-specific primers is the use of degenerate primers. Li *et al*. (2004) developed degenerate primers to facilitate the detection of sweetpotato begomoviruses.

The variability among sweetpotato begomoviruses is likely because of genetic recombination. In conclusion, the genetic diversity of sweetpotato begomoviruses must be evaluated and the prevalence of distinct species, strains, or variants must be identified as well. This information will be helpful to breeding programs aimed at development of resistant sweetpotato cultivars.

CHAPTER 4: EXPRESSION OF THE COAT PROTEIN OF *SWEET POTATO LEAF CURL VIRUS* IN *ESCHERICHIA COLI*

4.1 Introduction

Sweet potato leaf curl virus (SPLCV), a whitefly-transmitted geminivirus, has been found in an ornamental sweetpotato and some breeding lines in the United States (Lotrakul *et al*., 1998; 2002; Clark and Valverde, 2001). Leaf curling is a common symptom on infected plants (*I. batatas* W-285, *I. nil*, *I. setosa*), but yellow vein symptoms are observed on some hosts (*I. aquatica*, *I. cordatotriloba*). Although SPLCV does not cause symptoms on Beauregard, the most predominant sweetpotato cultivar in the US, it can reduce the yield up to 26 % (Clark and Hoy, 2006). Since SPLCV could become an important constraint for sweetpotato production; diagnosis, identification, and characterization are essential to develop an appropriate management strategy. Methods available to detect SPLCV are based on symptomatology of indicator hosts (graftings assays) and on the detection of viral DNA (polymerase chain reaction (PCR), and molecular hybridization) (Lotrakul and Valverde, 1999; Li *et al*., 2004).

Despite the availability of sensitive nucleic acid based assays for detecting plant viruses, serological assays are still the methods of choice for screening large numbers of plant materials. Furthermore, these methods are particularly valuable in developing countries since they require few resources.

Serological detection of SPLCV is not currently available due to the difficulties in obtaining purified virions that can be used as antigen for antiserum production. It is possible that either SPLCV virus particles occur in low concentration in plant tissues or viral particles are not stable following standard purification procedures. This problem is not unique to SPLCV. Purification of begomovirus virions can be very difficult; the success of purification methods depends greatly on the virus and host plant (Palmer *et al*., 1998). Using partially purified virions,

serological relationships of SPLCV to *Bean golden mosaic virus* (BGMV) and to *Mungbean yellow mosaic virus* (MYMV) by Western blot was reported by Onuki *et al*. (2000). ELISA on nitrocellulose membranes (NCM-ELISA) and Western blot tests using antisera to *Tomato yellow leaf curl virus* (TYLCV) and leaf extracts and partially purified preparations of SPLCV did not reveal serological relationships between these two viruses (Appendix A).

Cloning and expressing viral coat protein genes in bacteria can overcome the difficulties in obtaining purified plant virus preparations for antiserum production (Nikolaeva *et al*., 1995; Hoyer *et al*., 1996; Meng *et al*., 2003). Polyclonal antisera prepared to four begomovirus coat proteins (BGMV, TYLCV, *Cabbage leaf curl virus*, and *Tomato mottle virus*) expressed in *Escherichia coli* have been prepared and used for begomovirus detection in different immunological assays (ELISA, Western blots, leaf imprint blots, tissue blots, and immunogold labeling in electron miscroscopy) (Abouzid *et al*., 2002).

The availability of the complete genome sequences of most begomoviruses, including SPLCV (Lotrakul and Valverde, 1999), should allow the expression of a recombinant coat protein in bacteria. Therefore, in theory, it should be feasible to use this technology to produce SPLCV antisera for serological based assays.

4.2 Materials and Methods

4.2.1 Cloning of the SPLCV Coat Protein Gene

Total DNA was extracted from *I. setosa* leaf tissue infected with the US isolate of SPLCV using the DNeasy plant mini kit (QIAGEN, Valencia, CA). Based on the known sequence of SPLCV, specific primers SPB-1 (5’-CAGAGTCGGTACCTATGACAGGGCGAA-3’) and SPB-2 (5’-TACTCTGCAGTTAATTGTTGTGCGAATC-3’) were used to amplify the full length coat protein (CP) gene (a *Kpn* I restriction site on SPB-1 and a *Pst* I restriction site on SPB-2 are underlined). SPB-2 primer sequence was kindly provided by Dr. P. Lotrakul

(Chulalongkorn University, Bangkok, Thailand), while SPB-1 primer sequence was modified from a primer sequence (5'-GGTCAAGCTTTATGACAGGGCGAATTCC-3') also provided by Dr. P. Lotrakul. PCR reaction mixtures were prepared as described by Lotrakul *et al*. (1998). Amplification was performed in a Genius Thermocycler (Techne, Cambridge, UK) with 38 cycles of 94 ˚C for 1 min, 58 ˚C for 1 min, and 72 ˚C for 3 min, followed by a final extension cycle of 72 ˚C for 10 min. PCR products were separated by electrophoresis (1.2 % agarose) and stained with ethidium bromide. Bands corresponding to the expected PCR products were excised from agarose gels and further purified using MiniElute (Qiagen) DNA purification kit and ligated into the pGEM-T Easy vector (Promega, Madison, WI). Recombinant plasmids were transformed into *E. coli* JM 109 competent cells following manufacturer's instructions (Promega). Plasmids were isolated from transformant colonies using the FlexiPrep kit Miniprep procedure (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The successful insertion of PCR products were confirmed by enzyme digestions (*Eco* RI, *Kpn* I, and *Pst* I). In order to verify the identity of PCR products as the corresponding SPLCV CP gene, recombinant plasmids were sequenced at the Research Technology Support Facility of Michigan State University, East Lansing using a Perkin Elmer/Applied Biosystems 3100 capillary sequencer (Perkin Elmer, Foster City, CA).

The SPLCV CP gene was excised from the pGEM-T Easy vector by restriction enzymes (*Kpn* I and *Pst* I) and subcloned into the expression vector pMAL-c2E (New England Biolabs, Beverly, MA). The ligation mixture in 10 µl volume consisted of 1.7 µl containing 264 ng of pMAL-c2E vector (*Kpn* I and *Pst* I digested), 0.5 µl containing 90 ng of the CP gene (*Kpn* I and *Pst* I digested), 5 µl of 2X Rapid ligation buffer (Promega), 1 µl of 10 mM ATP, 1 µl of T4 DNA ligase (Promega) and 0.8 µl of nuclease free water. The ligation solutions were mixed and incubated for 1 h at room temperature (24-25 ˚C), and then overnight at 4 ˚C. Recombinant

plasmids were transformed into *E. coli* XL1-Blue competent cells, according to a procedure developed by Dr. N. Murai (Louisiana State University, Baton Rouge). For transformation, 5 µl of the ligation solution was added to a 5 ml sterile capped Falcon tube and diluted with 95 µl of TENC buffer (20 mM Tris HCl, pH 8.0, 30 mM $CaCl_2$, 1 mM EDTA, 20 mM NaCl). Competent cells were prepared as follow: 50 ml of Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter) containing 0.1% glucose was inoculated with 0.5 ml of an overnight culture of XL1-Blue cells and incubated at 37 ˚C, until an OD_{600} value between 0.3 and 0.4 was reached. Cells were harvested by centrifugation in two 30 ml centrifuge bottles (4,000 RPM, 5 min, 4 ˚C in a Beckman Coulter Avanti J-25 Centrifuge, JA 25.5 rotor). Pellets were resuspended in 12.5 ml of 10 mM NaCl, then combined in one tube (final volume: 25 ml) and centrifuged. The pellet was resuspended in 25 ml of 30 mM $CaCl_2$ and incubated on ice for 60 min. The sample was centrifuged again and the pellet resuspended in 5 ml of 30 mM $CaCl_2$ and incubated on ice for another 60 min. After incubation, 200 µl of competent cells were added to the diluted ligation solution and incubated on ice for 60 min. Cells were heat-shocked at 42 ˚C for 2 min, and then kept on ice for at least 2 min. One ml of pre-warmed LB broth was added to the heat-shocked cell suspension, and it was incubated at 37 ˚C with shaking (150-200 RPM) for 60 min. Aliquots of 100, 300, and 600 µl were mixed with 2.5 ml of top agar (LB broth plus 0.75 % agar), and then spread on LB/ampicillin plates. Plasmids were recovered from transformant colonies using the Wizard *Plus* SV Minipreps DNA Purification System (Promega). Recombinant plasmids pMAL-CP were identified by PCR using primers: malE and M13/pUC (New England Biolabs, #S1237S and #S1224S, respectively). PCR reactions were conducted as described previously using the following parameters: 1 cycle of 94 ˚C for 1.5 min, 35 cycles of 94 ˚C for 1 min, 65 ˚C for 1 min, and 72 ˚C for 1.5 min, and 1 cycle of 72 ˚C for 5 min. Also, recombinant plasmids were characterized by restriction enzyme digestions (*Mfe* I, *Nco* I, and

Nhe I). In-frame insertion of the SPLCV CP gene in the pMALc2-E vector (pMAL-CP) was verified by DNA sequencing.

4.2.2 Overexpression Experiments

Bacterial colonies carrying the recombinant plasmid pMAL-CP were grown at 37 ˚C in LB broth containing 0.2 % glucose and 100 µg/ml ampicillin. When cultures reached an OD_{600} value of approximately 0.5, cells were induced with 0.3 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and incubated at 26 and 37 ˚C for 1, 2, 3 and 4 h. For analysis of the total cell protein fraction, 1 ml of both induced and non-induced cells were harvested and resuspended in 25 µl of dH_2O, then 25 µl of 2X loading buffer (0.125 M Tris, pH 6.8, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.2 % bromophenol blue) was added, mixed and kept at -20 ˚C. For samples induced more than 2 h, 1 ml of bacterial cultures were harvested and resuspended in 50 µl of dH_2O, then 50 µl of 2X loading buffer was added, mixed and kept at -20 ˚C. When all samples were collected, proteins were denatured in boiling water for 5 min, and 20 µl of each sample was loaded on SDS-PAGEs (stacking gel 4 % and resolving gel either 8 or 10 %) as described by Laemmli (1970). Proteins were visualized with Coomassie Brilliant Blue staining solution (45 % methanol, 0.1 % Coomassie Brilliant Blue R-250, 10 % acetic acid).

A selected bacterial clone harboring the recombinant plasmid pMAL-CP was chosen for a time course expression experiment. In this assay, bacterial cells were induced with 0.3 mM IPTG and incubated at 26 ˚C for 15 and 30 min, and 1, 2, 4, and 6 h.

Bacterial colonies carrying the pMAL-c2E plasmid (expression vector without insert) were used as controls in every expression experiment.

4.2.3 Expression and Purification of the Fusion Protein

Expression and purification procedures were performed according to Dyer (1993) with some modifications. One liter of LB broth containing 0.2 % glucose and 100 µg/ml ampicillin

was inoculated with 10 ml of an overnight culture of XL1-Blue cells harboring the recombinant plasmid pMAL-CP and incubated at 37 ˚C. When the OD_{600} reached an approximate value of 0.5, IPTG was added to a final concentration of 0.3 mM and incubation was continued at 26 ˚C for 1 h in a gyrotory water bath shaker (at approximately 200 RPM). Cells were harvested by centrifugation (4,800 RPM, 20 min, 4 ˚C, in a Beckman J2-21M Centrifuge, JA 10 rotor). The pellet was kept overnight at -20 ˚C and then resuspended in 50 ml of lysis buffer (10 mM phosphate, 30 mM NaCl, 0.25 % Tween 20, 10 mM EDTA, 10 mM EGTA (ethylene glycol tetraacetic acid), pH 7.0). Freshly prepared lysozyme (1 mg/ml) was added, and the suspension was kept on ice for 20 min. Cells were disrupted by sonication (6 times, 15 s, short pulses, 50% output), and NaCl was added to a final concentration of 0.5 M. After centrifugation (9,000 RPM, 30 min, 4 ˚C, in a Beckman Coulter Avanti J-25 Centrifuge, JA 25.5 rotor), the supernatant was diluted 1:5 with column buffer (10 mM phosphate, 0.5 M NaCl, 1 mM sodium azide, 1 mM EGTA, pH 7.0) plus 0.25 % Tween 20 and kept at 4 ˚C.

The fusion protein MBP*CP comprising the SPLCV CP and the Maltose-binding protein (MBP) was purified using an amylose affinity chromatography. The column was prepared by adding amylose resin (New England Biolabs) to a 30ml-syringe with a final bed volume of approximately 4 ml. The column was washed with 1 volume of column buffer and 3 volumes of column buffer plus 0.25 % Tween 20. The diluted extract was added to the column at a flow rate of approximately 1 ml/min. Then, the column was washed with 3 volumes of column buffer plus 0.25 % Tween 20 and 5 volumes of column buffer. The fusion protein MBP*CP was eluted with column buffer containing 10 mM maltose (14 fractions of 1 ml each). Fractions containing the fusion protein (fractions 5 to 10) were pooled and concentrated by filtration using Amicon® Ultra-4 30,000 NMWL and Microcon YM-30 filter units (Millipore, Billerica, MA), and

reconstituted to about 1 mg/ml in 1X PBS (8 g NaCl, 1.15 g Na_2HPO_4, 0.2 g KCl, 0.2 g KH_2PO_4, per liter, pH 7.4).

4.2.4 Western Blot

Proteins electrophoresed on SDS-PAGE, as previously described, were transferred to nitrocellulose membranes. Polyacrylamide gels were soaked in blotting buffer (0.025 M Tris, 0.192 M glycine, 20 % methanol) before assembling the transfer cassette. Electroblotting was conducted at 4 ˚C, 35 V overnight in a TE 22 Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). Hereafter, all incubations and washings were performed at room temperature in a shaker with gentle agitation. Blotted membranes were blocked for 1.5 h with TBS (0.02 M Tris, 0.5 M NaCl, pH 7.5) containing 3 % nonfat dry milk and 1 % Triton X-100. Blocking solution was discarded, and membranes were rinsed with TBS. Membranes were probed using either Anti MBP polyclonal antibody (1:10,000 dilution) (New England Biolabs) overnight, or with TYLCV polyclonal antiserum #1214 (1:1,000 dilution) (provided by Dr. J. Polston, University of Florida, Gainesville) for 5 h. The TYLCV antiserum #1214 was produced against the TYLCV coat protein expressed in *E. coli* (Abouzid *et al*., 2002). Membranes were washed 4 times, 5 min each with TBS containing 0.05 % Tween 20, and then incubated for 1 h with alkaline phosphatase conjugated goat anti-rabbit (1:10,000 dilution) (Bio-Rad, Hercules, CA). Antisera were diluted in TBS containing 2 % nonfat dry milk. Protein bands were visualized by the addition of BCIP/NBT color development solution (Bio-Rad).

4.2.5 Enterokinase Digestion

The fusion protein MBP*CP was digested with 0.013 % (w/w) enterokinase (New England Biolabs) in a 20.5 µl volume as follows: 10 µl containing 7.5 µg of fusion protein (in 1X PBS), 10 µl of 2X digestion buffer (40 mM Tris-HCl pH 8.0, 100 mM NaCl, 4 mM $CaCl_2$), and

0.5 µl containing 1 ng of enterokinase. The solution was incubated at room temperature for 3 h. Digested proteins were analyzed by 10 % SDS-PAGE.

4.2.6 Mass Spectrometry

A 30 kDa polypeptide obtained after enterokinase digestion was excised from a SDS-PAGE. In gel protein digestion and protein mass spectrometry experiments to facilitate identification and confirmation of this peptide as the SPLCV CP were performed at the Proteomics Core Facility at Pennington Biomedical Research Center, Louisiana State University, Baton Rouge. According to DeLany *et al*. (2005) and Zvonic *et al*. (2007), excised gel plug was deposited into a 96-well plate and transferred to the MassPrep (Waters/Micromass) station. Proteins within the gel plug were automatically destained, reduced, alkylated, dehydrated, rehydrated, and digested with trypsin. The resulting peptides were extracted and separated by capillary liquid chromatography coupled to an ESI MS/MS Micromass Q-TOF mass spectrometer (Waters). MassLynx 4.0 software package (Waters) was used to identify individual mass spectrograms. Database searches for protein identification were done in NCBI database using the ProteinLynx Globalserver 1.1 software (Waters). The number of peptides analyzed and the percentage of coverage of the total amino acid sequence was determined for the protein identified.

4.3 Results

A fragment of about 789 base pairs (bp) containing the SPLCV CP gene was amplified with primers SPB-1 (forward) and SPB-2 (reverse), cloned into pGEM-T Easy vector and subcloned into pMAL-c2E expression vector (Figure 4.1).

After transformation into *E. coli* XL1-Blue, the recombinant plasmid pMAL-CP (SPLCV CP gene cloned into the pMAL-c2E) was identified by PCR. A 937 bp fragment corresponding to the SPLCV CP gene including flanking regions of the recombinant plasmid was amplified

with primers malE and M13/pUC (Figure 4.2, lanes 1-5). While a 196 bp fragment was amplified from the pMAL-c2E (plasmid without insert) using these primers (Figure 4.2, lane 6).

Recombinant plasmids (pMAL-CP) were characterized by restriction enzyme digestions. *Mfe* I, *Nco* I, and *Nhe* I restriction sites are present in the SPLCV CP gene but not in the pMAL-c2E (Figure 4.3). DNA sequencing confirmed that the SPLCV CP gene was in frame for expression in the recombinant plasmid pMAL-CP.

Preliminary expression tests were conducted using two different IPTG concentrations (0.03 and 0.06 mM) and two different temperatures (26 and 37 ˚C) in order to optimize the expression conditions for the fusion protein MBP*CP. However, when non-induced and induced crude extracts were analyzed on SDS-PAGE, expected protein bands were not detected in induced samples (Figure 4.4). Therefore, the predicted amino acid sequence of the fusion protein MBP*CP was analyzed by ProtParam tool (Gasteiger *et al*., 2005) to calculate some of the physico-chemical properties that could give a clue to improve the expression tests (Appendix B). To discard the possibility of bacterial toxicity due to the expression of the fusion protein and a suspected leakage of the fusion protein from the cells, a time course expression experiment was performed using 0.3 mM IPTG, at 26 ˚C, for 15 and 30 min, 1, 2, 4 and 6 h. However, an induced band was not observed (Figure 4.5). To determine if the *E. coli* strain (XL1-Blue) was the factor involved in the lack of expression of the fusion protein due to the presence of some proteases that could degrade the fusion protein, recombinant plasmids pMAL-CP were transformed into *E. coli* TB1 strain, but expression was not observed.

Fusion protein yields of 5 to 6 mg/liter of culture have been reported even when a band was not visible (New England Biolabs, 2004). Therefore, a pilot experiment from 1 liter culture was conducted. After induction of *E. coli* XL1-Blue cells harboring the recombinant plasmid pMAL-CP, a 72 kDa polypeptide was purified from the crude protein extract of *E. coli* by

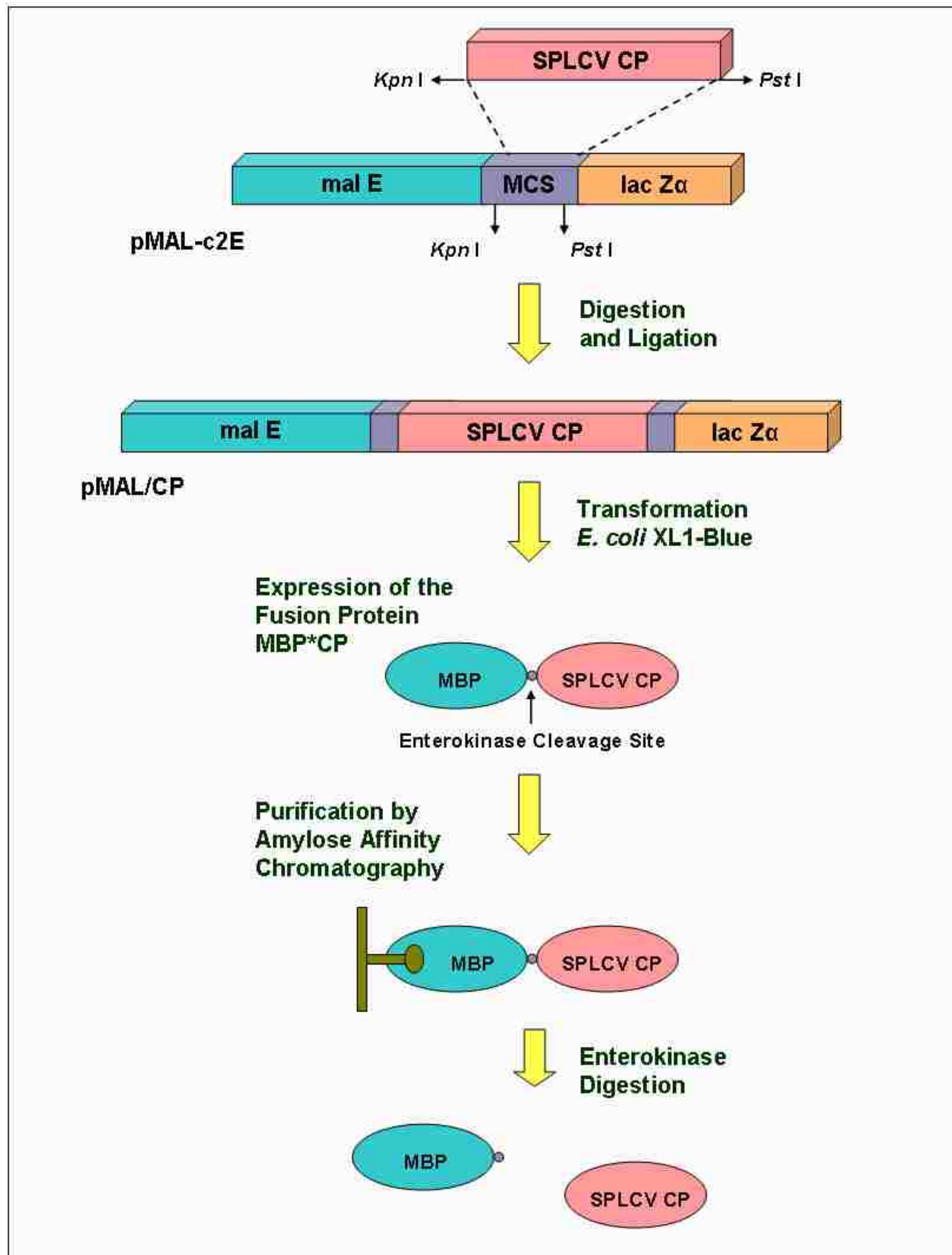


Figure 4.1. Diagram showing the expression of the fusion protein MBP*CP [Maltose-binding protein (MBP) and coat protein (CP) of *Sweet potato leaf curl virus* (SPLCV)]. MCS: Multiple cloning site in pMALc-2E expression vector. mal E: gene of *E. coli* which encodes MBP. lacZα: gene of *E. coli* that encodes β-galactosidase α fragment.

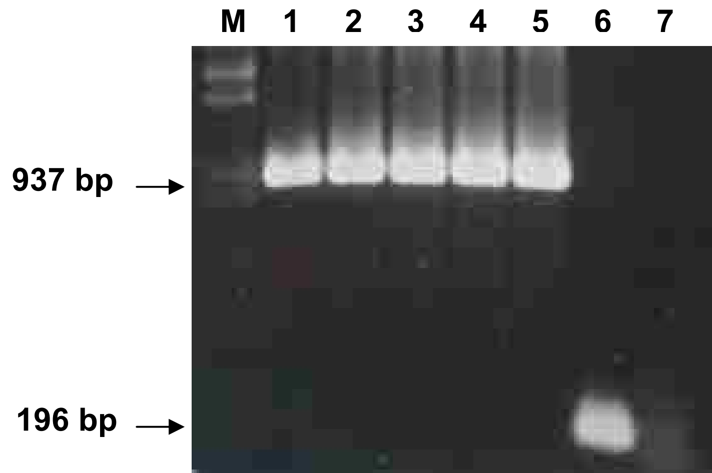


Figure 4.2. PCR amplification of a 937 bp fragment corresponding to the coat protein (CP) gene of *Sweet potato leaf curl virus* (SPLCV) and flanking regions in the recombinant plasmid pMAL-CP using malE and M13/pUC primers. Lanes 1-5, recombinant plasmid pMAL-CP (pMAL-c2E containing the SPLCV CP). Lane 6, pMAL-c2E (without insert). Lane 7, water control. Lane M, DNA ladder.

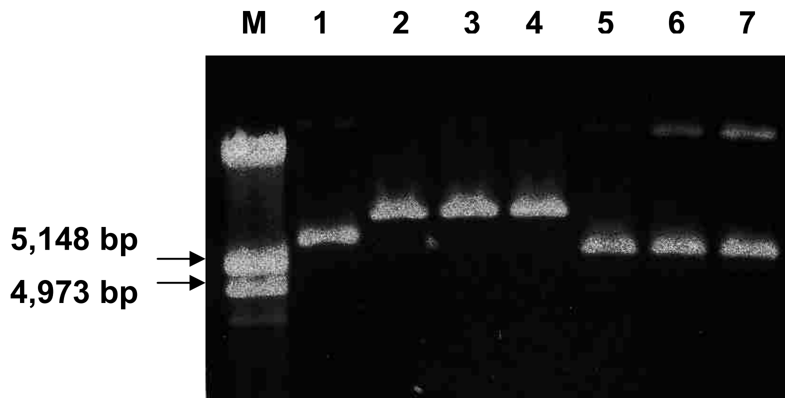


Figure 4.3. Restriction enzyme analysis of the recombinant plasmid pMAL-CP containing the coat protein (CP) gene of *Sweet potato leaf curl virus* cloned into the pMAL-c2E expression vector. Lane 1, pMAL-CP (7,392 bp). Lane 2, pMAL-CP digested with *Mfe* I. Lane 3, pMAL-CP digested with *Nco* I. Lane 4, pMAL-CP digested with *Nhe* I. Lane 5, pMAL-c2E digested with *Mfe* I. Lane 6, pMAL-c2E digested with *Nco* I. Lane 7, pMAL-c2E (6,651 bp). Lane M, DNA ladder.

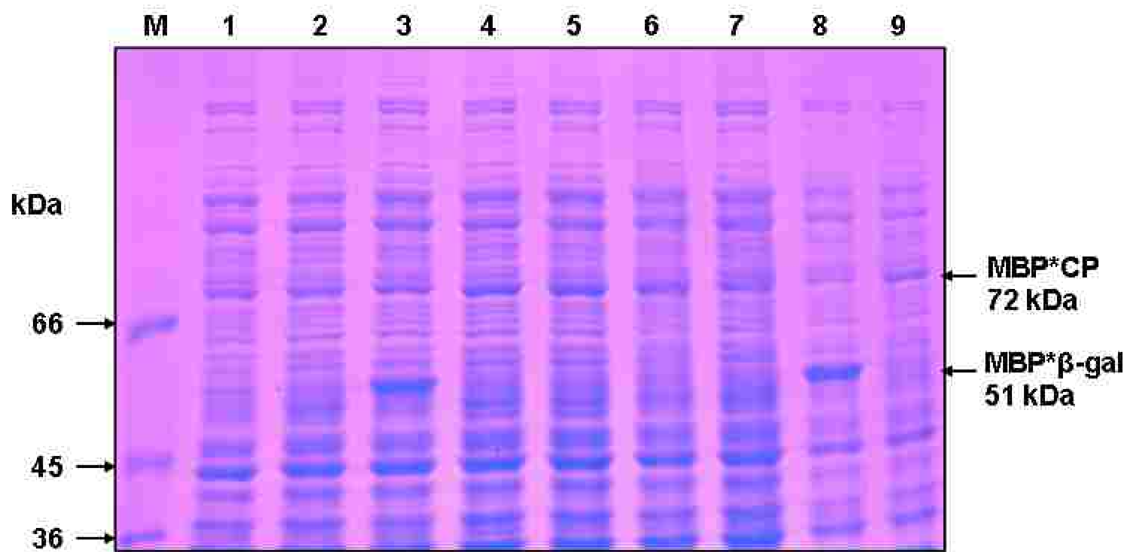


Figure 4.4. Expression of the fusion protein MBP*CP [Maltose-binding protein (MBP) and coat protein (CP) of *Sweet potato leaf curl virus*] in *E. coli* XL1-Blue. Cells were induced with 0.3 mM IPTG, at 26 ˚C, for 1 and 3 h. Induced cells harboring the recombinant plasmid pMAL-CP expressed the fusion protein MBP*CP, while induced cells harboring the pMAL-c2E vector expressed the MBP fused with the β-galactosidase α fragment protein (MBP*β-gal). Total proteins were separated by 8 % SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, non-induced cells harboring pMAL-c2E. Lane 2, non-induced cells harboring pMAL-CP. Lane 3, induced cells (1 h) harboring pMAL-c2E. Lanes 4-7, induced cells (1 h) harboring pMAL-CP. Lane 8, induced cells (3 h) harboring pMAL-c2E. Lane 9, induced cells (3 h) harboring pMAL-CP. Lane M, molecular weight marker.

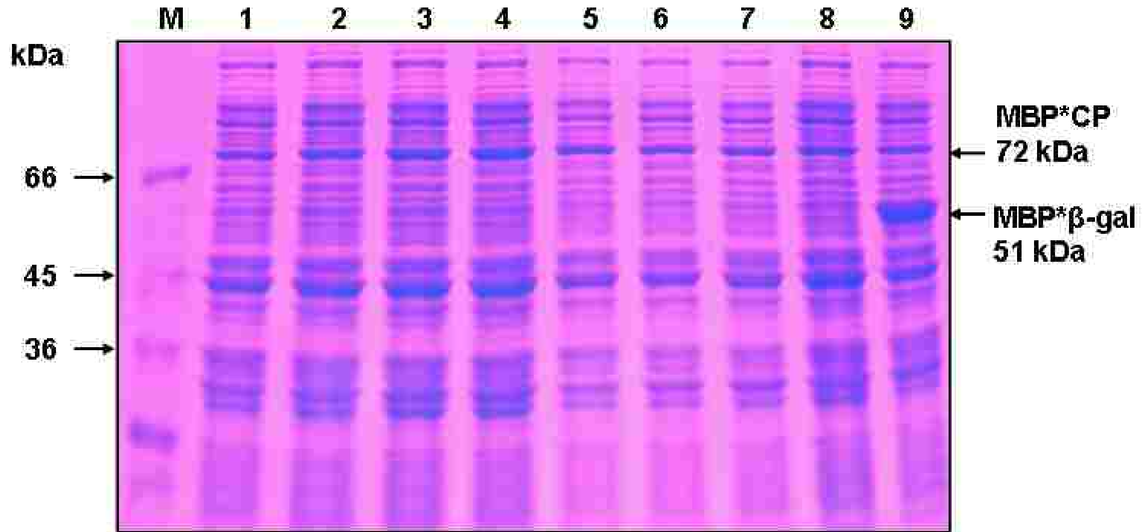


Figure 4.5. Time course expression of the fusion protein MBP*CP [Maltose-binding protein (MBP) and coat protein (CP) of *Sweet potato leaf curl virus*] in *E. coli* XL1-Blue. Cells were induced with 0.3 mM IPTG at 26 ˚C. Induced cells harboring the recombinant plasmid pMAL-CP expressed the fusion protein MBP*CP, while induced cells harboring the pMAL-c2E vector expressed the MBP fused with the β-galactosidase α fragment protein (MBP*β-gal). Total proteins were separated by 10 % SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, non-induced cells harboring pMAL-CP. Lanes 2-7, induced cells (15 min, 30 min, 1 h, 2 h, 4 h, and 6 h, respectively) harboring pMAL-CP. Lane 8, non-induced cells harboring pMALc-2E. Lane 9, induced cells (2 h) harboring pMAL-c2E. Line M, molecular weight marker.

affinity chromatography on an amylose column. The 72 kDa polypeptide was eluted from the amylose column with 10 mM maltose (14 fractions of 1 ml each) (Figure 4.6, Figure 4.7, lanes 6-8). This polypeptide was identified as the expected fusion protein based on its size 72 kDa (42.5 kDa for MBP plus 29.4 kDa for the SPLCV CP) and its reaction with the Anti-MBP polyclonal antiserum by Western blot analysis. Moreover, the 72 kDa polypeptide reacted with the TYLCV polyclonal antiserum #1214 on Western blot analysis. Although this antiserum was not specific (it reacted with other bacterial proteins), it did not react with the MBP. Therefore, it is possible that some antibodies reacted with some epitopes of the SPLCV CP.

Yield of the purified MBP*CP fusion protein was approximately 200 μg/liter of bacterial culture. After the MBP was separated from the fusion protein by enterokinase digestion, a 30 kDa protein band was obtained. The 30 kDa protein is expected to produce 40 peptide fragments when digested with trypsin, using the PeptideCutter software (Appendix D). Trypsin digestion products were separated by capillarity liquid chromatography, and fractionated peptides were analyzed by the ESI MS/MS Micromass Q-TOF mass spectrometer.

The 30 kDa protein from the MBP*CP was identified as the SPLCV CP based on the mass spectrometer determination of trypsin digestion products.The peptide sequences of SPLCV CP indicated in Table 4.1 were identified by mass spectrometry analysis (Appendix E).

4.4 Discussion

Serological assays to detect SPLCV are not available because of the difficulties in obtaining purified virions that can be used as antigen for antiserum production. Cloning and expressing the CP gene of SPLCV in bacteria can overcome this limitation.

Many systems for expression of foreign genes in bacteria have been developed. The pMAL-c2E expression vector of the pMAL Protein Fusion and Purification System from New England Biolabs was selected to express the CP gene of SPLCV in *E. coli*. The target gene

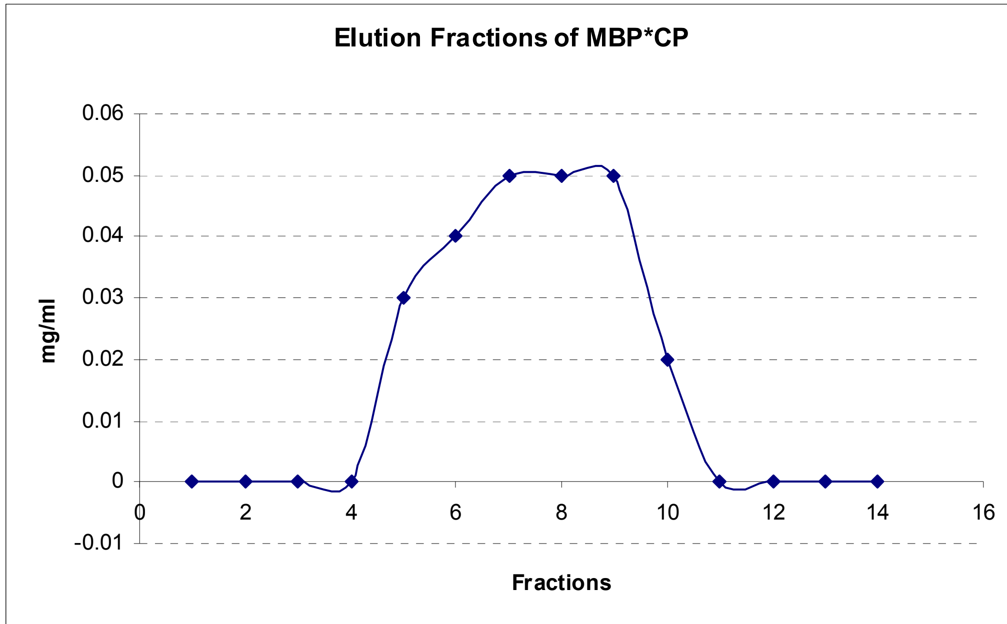


Figure 4.6. Elution fractions of the fusion protein MBP*CP [Maltose-binding protein (MBP) and coat protein (CP) of *Sweet potato leaf curl virus*] after amylose affinity chromatography.

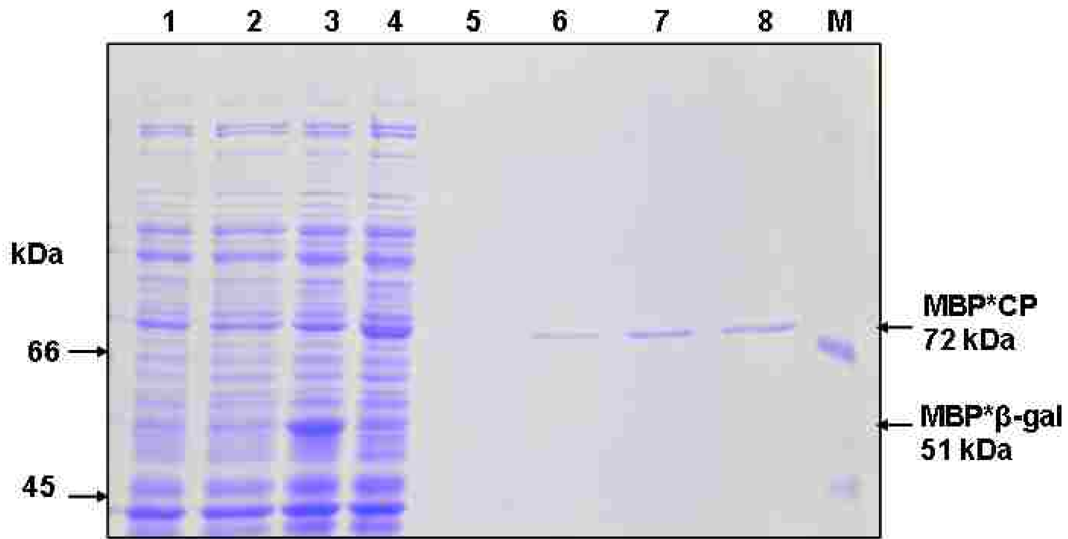


Figure 4.7. Expression of the fusion protein MBP*CP [Maltose-binding protein (MBP) and coat protein (CP) of *Sweet potato leaf curl virus*] in *E. coli* XL1-Blue. Cells were induced with 0.3 mM IPTG, at 26 ˚C, for 1 h. Induced cells harboring the recombinant plasmid pMAL-CP expressed the fusion protein MBP*CP, while induced cells harboring the pMAL-c2E vector expressed the MBP fused with the β-galactosidase α fragment protein (MBP*β-gal). Total proteins were separated by 8 % SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, non-induced cells harboring pMAL-c2E. Lane 2, non-induced cells harboring pMAL-CP. Lane 3, induced cells harboring pMAL-c2E. Lane 4, induced cells harboring pMAL-CP. Lanes 5-8, purified MBP*CP fusion protein, fractions # 6, 7, 8, and 9, respectively. Lane M, molecular weight marker.

Table 4.1. Peptide sequences of *Sweet potato leaf curl virus* coat protein identified by mass spectrometry analysis

Amino acid residues	**Peptide sequence**
13-20	PYGGRPVR
22-42	RLNFETAIVPYTGNAVPIAAR
86-94	FVCVSDFTR
117-125	VWMDDNVAK
127-138	DHTNIITYWLIR
179-191	FSVTVSGGPYSHK
204-216	YNHVTYNHKEEAK
246-254	AYFYDSHNN

(SPLCV CP) was inserted downstream from the mal E gene of *E. coli*, which encodes a maltose-binding protein (MBP), resulting in a cytoplasmic expression of a fusion protein (MBP*CP). This method uses the strong "tac" promoter and the mal E translation initiation signals to express large amounts of fusion protein (New England Biolabs, 2004). The fusion protein was then purified by affinity chromatography using an amylose column, and cleaved from the MBP by enterokinase.

Subcloning SPLCV CP gene into the pMALc-2E was not an easy task considering the size of the insert (766 bp) and the size of the vector (6,626 bp). High quality DNA as well as an appropriate insert:vector ratio (1:3) was required for the ligation solutions.

Overexpression tests with bacterial cells harboring the recombinant plasmid pMAL-CP were disappointing because expected protein bands were not detected in induced samples. Attempts to optimize the expression conditions for the fusion protein MBP*CP using different IPTG concentrations, temperatures, incubation times, and different bacterial strains were not successful.

After induction of *E. coli* XL1-Blue cells harboring the recombinant plasmid pMAL-CP, the 72 kDa fusion protein (42.5 kDa for MBP plus 29.4 kDa for the SPLCV CP) was expressed at low levels. Thus, the fusion protein MBP*CP was probably masked by bacterial proteins of similar size (72 kDa) present in the crude extract, making its detection difficult in initial overexpression experiments.

Yield of the purified MBP*CP fusion protein was approximately 200 μg/liter of bacterial culture. According to the information provided by the manufacturer (New England Biolabs, 2004), the yield of fusion protein from the affinity purification can be up to 200 mg/liter culture, with typical yields in the range of 10-40 mg/liter. However, yields can vary greatly depending upon the sequences fused to malE. Nikolaeva *et al*. (1995) used the pMAL system to produce

polyclonal antisera to the coat protein of *Citrus tristeza virus* obtaining 100 mg/liter culture of purified fusion protein, whereas Meng *et al*. (2003) using the same system to produce a polyclonal antiserum to the coat protein of *Rupestris stem pitting associated virus* obtained only 12 mg/liter culture of purified fusion protein.

Several factors could affect the expression of MBP*CP fusion protein. The efficient expression of foreign genes in *E. coli* can be affected by the physico-chemical properties of the target protein, the stability of the mRNA, degradation of the target protein by *E. coli* proteases, the presence of rare codons, or the toxicity of the expressed target protein (Chen and Texada, 2006). Time course protein expression experiments were performed to determine the possibility of bacterial toxicity due to the expression of the fusion protein and/or a suspected leakage of the fusion protein from the cells. Nevertheless, fusion protein toxicity or leakage was not detected.

Similar expression results were obtained when the fusion protein was expressed in two different *E. coli* strains (XL1-Blue and TB1) suggesting that proteases (which could degrade the fusion protein) could not be the limiting factor for the low expression of the fusion protein MBP*CP.

Bacterial colonies harboring the plasmid pMAL-CP were kept on LB/ampicillin plates, and renewed on fresh LB/ampicillin plates, monthly. To discard the possibility of bacterial stress that could affect protein expression due to continual transfers, new transformations of pMAL-CP on XL2-Blue (Ultracompetent cells, Stratagene) were performed. Overexpression tests were conducted using these new transformants with different IPTG concentrations, temperatures, and incubation times; however, expression of the fusion protein MBP*CP could not be increased.

The solubility of the fusion protein could also be involved in the low expression. It is common for recombinant proteins expressed in *E. coli* to be produced as aggregates (inclusion bodies). Even when inclusion bodies are formed, a proportion of the target protein is usually

soluble within the cell (Novagen, 2006). Growth at 37 ˚C can cause some proteins to accumulate as inclusion bodies, while incubation at 30 ˚C may lead to soluble proteins (Schein and Noteborn, 1988; Schein, 1989). In expression experiments of bacterial cells harboring the pMAL-c2E that were induced with 0.3 mM IPTG, at 37 ˚C, the MBP*β-gal protein (51 kDa) was expressed at high levels as a soluble form. However, when bacterial cells harboring the recombinant plasmid pMAL-CP were induced with 0.3 mM IPTG, at either 26 or 37 ˚C, expression of the fusion protein MBP*CP (72 kDa) was too low to be clearly detected in SDS-PAGEs. The grand average of hydropathicity (GRAVY) index (Kyte and Doolittle, 1982) were estimated for both the MBP*β-gal (-0.483) and MBP*CP (-0.537), indicating that both proteins were soluble (positive GRAVY: hydrophobic, negative GRAVY: hydrophilic) (Appendices B and C). Moreover, the solubility of these proteins was confirmed by another sequence-based protein solubility evaluator (PROSO server, Smialowski *et al*., 2007). Consequently, the solubility of the fusion protein MBP*CP may not be related to the low expression levels of this protein in *E. coli*.

It has been reported that proteins that contain highly charged domains may associate with other cellular components, for example, basic proteins may bind to DNA. If this is the case, the protein may partition with cellular debris (Novagen, 2006). This could be a reason for the low expression of MBP*CP which is a basic protein (theoretical pI 8.88) that contains 77 negatively charged residues (Asp + Glu) and 86 positive charged residues (Arg + Lys). In contrast, the MBP*β-gal protein with a theoretical pI of 5.04 contains 63 negatively charged residues and 50 positive charged residues.

Another factor affecting protein expression is the presence of rare codons which are defined as low-usage codons that are not only used rarely or infrequently in a genome but also decoded by a low-abundant tRNA (Chen and Texada, 2006). Analysis of *E. coli* codon usage

reveals that several codons are underrepresented; therefore, when the mRNA of heterologous target gene is overexpressed in *E. coli*, differences in codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the population. A number of studies have indicated that high usage of the arginine codons AGA and AGG can have severe effects on protein yield (Novagen, 2006). Eight rare codons (argnine codon: AGG) were found in the sequence corresponding to the SPLCV CP of the fusion protein MBP*CP (Figure 4.8), and they may have affected translation of this fusion protein.

SPLCV CP was expressed as a fusion protein in *E. coli*. Despite the low expression levels of MBP*CP, SPLCV CP can be obtained after enterokinase digestion and could be used as an antigen to produce antibodies to SPLCV. The availability of an antiserum to SPLCV will allow the development of a practical and inexpensive serological method for SPLCV detection. Moreover, an SPLCV specific antiserum may be used to study serological relationships of SPLCV with other begomoviruses.

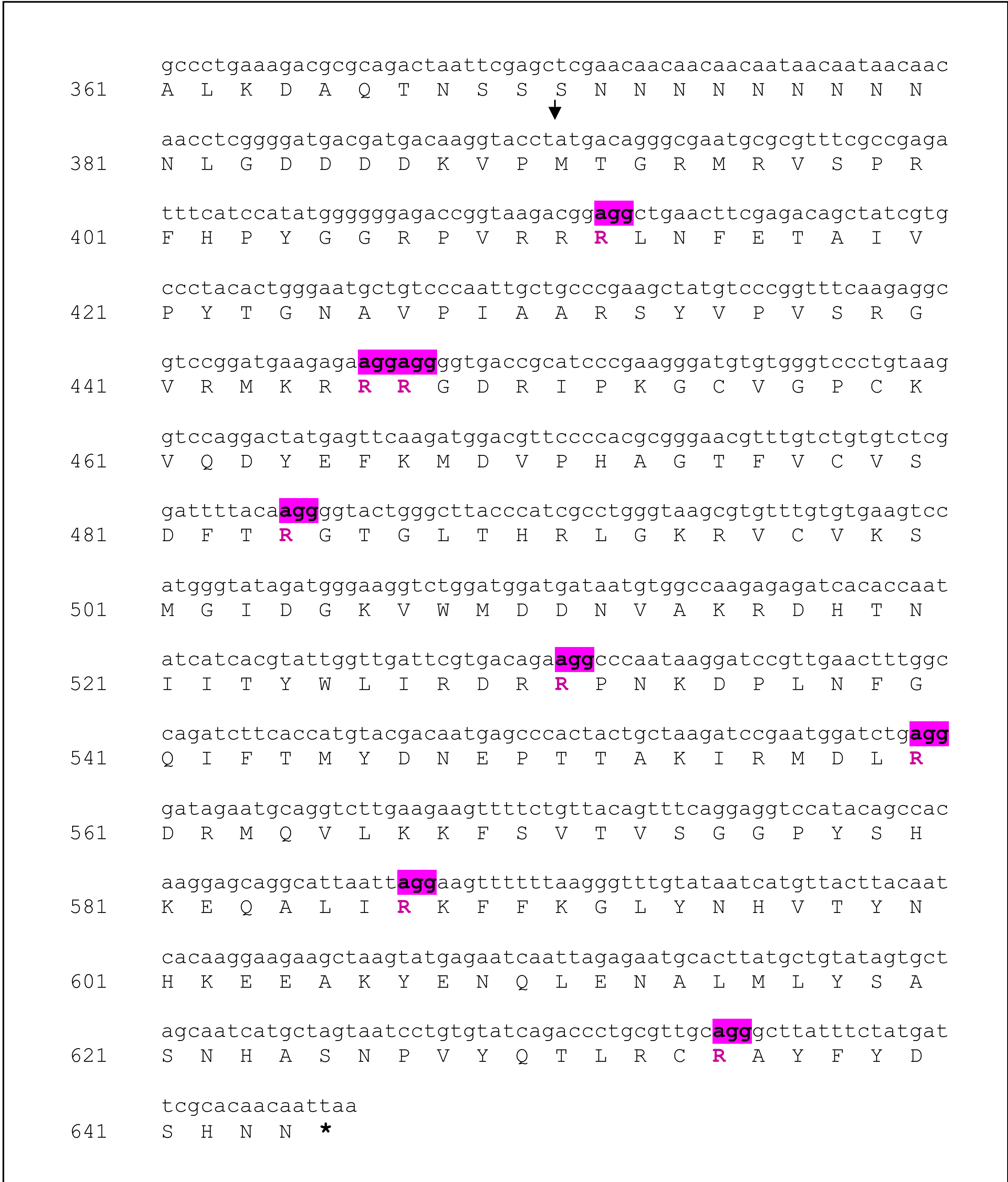


Figure 4.8. Identification of *E. coli* rare codons in the peptide sequence of the fusion protein MBP*CP [Maltose-binding protein (MBP) and *Sweet potato leaf curl virus* (SPLCV) coat protein (CP)]. Rs are Arginine rare codons (AGG) in the positions 412, 446, 447, 484, 531, 560, 587, and 635 of the fusion protein that correspond to the SPLCV CP region. The arrow head indicates the the start codon (391) and the asterisk the stop codon (645) of SPLCV CP.

CHAPTER 5: SUMMARY AND CONCLUSIONS

5.1 Summary and Conclusions

Due to its nutritional qualities, sweetpotato (*Ipomoea batatas* (L.) Lam) is considered as a crop with great potential to alleviate food security concerns. Yields of sweetpotato cultivars have appeared to gradually decline over the years. Several factors including mutations and accumulation of systemic pathogens, especially viruses, may be involved in sweetpotato cultivar decline (Clark *et al*., 2002; 2003). Viral diseases occur everywhere sweetpotato is grown, and they are an important constrain for sweetpotato production.

Leaf curl diseases have been reported in different sweetpotato growing regions, and their importance have been overlooked because leaf curl symptoms are not common and do not persist in sweetpotato (Valverde *et al*., 2007). One of the causal agents of these diseases is *Sweet potato leaf curl virus* (SPLCV) which belongs to the genus *Begomovirus* (family *Geminiviridae*) (Lotrakul and Valverde, 1999). In the United States, SPLCV has been found infecting an ornamental sweetpotato cultivar and several breeding lines but not in sweetpotatoes grown for commercial production (Clark and Valverde, 2001). SPLCV does not cause symptoms, but it can reduce the yields of Beauregard, the predominant sweetpotato cultivar in Louisiana (Clark and Hoy, 2006). Since SPLCV could become an important constraint for sweetpotato production, diagnosis and identification are essential to develop an effective management strategy.

In this study, sequences of 11 begomoviruses infecting sweetpotato genotypes in the US were obtained. Molecular diversity of these begomoviruses was investigated by analyzing the sequence of a nucleotide fragment of the AC1. Data obtained from this analysis suggested that SPLCV is more commonly found infecting sweetpotato genotypes than *Sweet potato leaf curl Georgia virus* (SPLCGV), another begomovirus reported in the US. Moreover, sweetpotato

begomoviruses from different geographical regions that were investigated were closely related to SPLCV. Phylogenetic analysis based on the nucleotide sequence of the AC1 fragment and the full length nucleotide sequence of the coat protein (CP) gene (AV1) clustered all sweetpotato begomoviruses together but apart from begomoviruses that infect other plant species. Within the sweetpotato begomovirus cluster, SPLCV and SPLCGV clustered in different groups supporting their status as different species. These results illustrate the variability and the complexity of begomoviruses infecting sweetpotato. However, analysis of partial sequences does not provide sufficient data to establish new species (Fauquet *et al*., 2008). In order to establish a new virus species, a comprehensive characterization that includes host range, virus transmission, and complete genome sequence must be conducted.

The CP gene sequence was highly conserved among the sweetpotato begomoviruses analyzed in this study. However, it was quite distinct from that of other begomoviruses. This may explain the low rate of transmission of SPLCV by the sweetpotato whitefly *Bemisia tabaci*. In most whitefly transmission experiments, indicator plants (*I. setosa*, *I. nil*, and *I. aquatica*) infected with SPLCV have been used as acquisition and transmission hosts. Preliminary whitefly transmission experiments were conducted using Beauregard sweetpotato infected with SPLCV as the acquisition host, and *I. nil* as the transmission host. But whitefly transmissions were not successful. When *I. nil* was used as acquisition and transmission hosts, and the number of whiteflies per transmission was increased, SPLCV was transmited at low rates (Appendix F).

Mixed infections of different begomoviruses, SPLCV and SPLCGV in the US and SPLCV and *Ipomoea yellow vein virus* (IYVV) in Spain (Lotrakul *et al*., 2003; Lozano *et al*., 2004), are likely to provide suitable conditions for recombination that could favor the occurrence of new virus species. Therefore, the high variability found among sweetpotato begomoviruses in

this study is not surprising. This supports the suggestion that this viral group might be another case of a species complex.

The potential of a synergistic interaction between SPLCV and SPFMV has been suggested. SPLCV DNA titers increased in mixed infection with the russet crack strain of SPFMV (Kokkinos, 2006). Higher SPLCV titers could result in a more efficient acquisition by whiteflies, and therefore spread of the virus to uninfected plants.

Sweetpotato begomoviruses could be a problem for quarantine and seed foundation programs because infected plants are symptomless. Therefore, sensitive methods for their detection and identification are very important. Methods available to detect SPLCV include: graft inoculations to indicator hosts (*I. setosa*, *I. nil*, *I. aquatica*), polymerase chain reaction (PCR), and molecular hybridization (Valverde *et al*., 2008). Serological detection of SPLCV from crude sap extracts, which can be practical for diagnosticians in developing countries, is not currently available due to the lack of an antiserum specific for SPLCV. Attempts to purify the virus for antiserum production have not been successful. Antisera to *Tomato yellow leaf curl virus* (TYLCV) were not practical to detect SPLCV by ELISA and Western blot analysis (Appendix A).

An alternative approach to obtain the CP of SPLCV for antibody production is by cloning and expressing the CP gene in bacteria. Thus, the SPLCV CP gene was amplified, cloned into the expression vector pMAL-c2E and transformed into *Escherichia coli* XL1-Blue. A 72 kDa polypeptide was obtained and identified as the expected fusion protein based on its size (42.5 kDa for maltose binding protein (MBP) plus 29.4 kDa for the SPLCV CP) and the reaction with Anti-MBP polyclonal antiserum by Western blot. Digestion with enterokinase cleaved the fusion protein into a 42.5 kDa maltose-binding protein and the 29.4 kDa SPLCV CP. The CP was identified by mass spectrometry analysis. Despite the low expression levels of the fusion protein,

SPLCV CP can be obtained after enterokinase digestion and used as an antigen to produce antibodies to SPLCV.

5.2 Future Research

Future research in the following areas needs to be considered:

- Analysis of complete genome sequences of sweetpotato begomoviruses from different geographical regions will allow for a better understanding of their evolution and variability.
- Production of SPLCV infectious clones will be helpful to determine the presence of either DNA-B component or DNA satellites (DNA β and DNA 1) and to develop resistant cultivars.
- SPLCV is transmitted by the sweetpotato whitefly *B. tabaci* at low rates. Most transmission experiments used SPLCV infected indicator plants (*I. setosa*, *I. nil*, and *I. aquatica*) as acquisition and transmission hosts. In order to better understand epidemiological aspects of SPLCV spread in natural conditions, sweetpotato cultivars need to be used as acquisition and transmission hosts in whitefly transmission experiments.
- DNA titers of SPLCV were increased in mixed infections with SPFMV. Therefore, it is possible that these higher SPLCV titers could result in more efficient transmission by whiteflies. Whitefly transmission using single infections (SPLCV) and mixed infections (SPLCV and SPFMV) needs to be evaluated to test this hypothesis.
- Production of an antiserum to SPLCV may allow the development of a practical and inexpensive serological method for SPLCV detection and the study of serological relationships of SPLCV with other begomoviruses.

LITERATURE CITED

Abad, J.A. and Moyer, J.W. 1992. Detection and distribution of *Sweet potato feathery mottle virus* in sweetpotato by in vitro-transcribed RNA probes (riboprobes), membrane immunobinding assay and direct blotting. Phytopathology 82: 300-305.

Abad, J.A., Parks, E.J., New, S.L., Fuentes, S., Jester, W., and Moyer, J.W. 2007. First Report of *Sweet potato chlorotic stunt virus*, a component of Sweet potato virus disease, in North Carolina. Plant Disease 9: 327.

Abouzid, A.M., Freitas-Astua, J., Purcifull, D.E., Polston, J.E., Beckham, K.A., Crawford, W.E., Petersen, M.A., Peyser, B., Patte, C., and Hiebert, E. 2002. Serological studies using polyclonal antisera prepared against the viral coat protein of four begomoviruses expressed in *Escherichia coli*. Plant Disease 86: 1109-1114.

Banks, G.K., Bedford, I.D., Beitia, F.J., Rodriguez-Cerezo, E., and Markham, P.G. 1999. A novel geminivirus of *Ipomoea indica* (*Convolvulacae*) from Southern Spain. Plant Disease 83: 486.

Bovell-Benjamin, A.C. 2007. Sweet potato: A review of its past, present, and future role in human nutrition. Advances in Food and Nutrition Research 52: 1-59.

Briddon, R.W. 2002. Diversity of European begomoviruses: Identification of a new disease complex. EPPO Bulletin 32: 1-5.

Briddon, R.W., Brown, J.K, Moriones, E., Stanley, J., Zerbini, M., Zhou, X., and Fauquet, C.M. 2008. Recommendations for the classification and nomenclature of the DNA- β satellites of begomoviruses. Archives of Virology 153: 763-781.

Briddon, R.W., Bull, S.E., Amin, I., Idris, A.M., Mansoor, S., Bedford, I.D., Dhawan, P., Rishi, N., Siwatch, S.S., Abdel-Salam, A.M., Brown, J.K., Zafar, Y., and Markham, P.G. 2003. Diversity of DNA-β, a satellite molecule associated with some monopartite begomoviruses. Virology 312: 106-121.

Briddon, R.W., Bull, S.E., Amin, I., Mansoor, S., Bedford, I.D., Rishi, N., Siwatch, S.S., Zafar, Y., Abdel-Salam, A.M., and Markham, P.G. 2004. Diversity of DNA 1: A satellite-like molecule associated with monopartite-DNA-β complexes. Virology 324: 462-474.

Briddon, R.W., Bull, S.E., and Bedford, I.D. 2006. Occurrence of *Sweet potato leaf curl virus* in Sicily. Plant Pathology 55: 286.

Briddon, R.W., Mansoor, S., Bedford, I.D., Pinner, M.S., and Markham, P.G. 2000. Clones of cotton leaf curl geminivirus induce symptoms atypical of cotton leaf curl disease. Virus Genes 20: 17-24.

Briddon, R.W., Mansoor, S., Bedford, I.D., Pinner, M.S., Saunders, K., Stanley, J., Zafar, Y., Malik, K.A., and Markham, P.G. 2001. Identification of DNA components required for induction of cotton leaf curl disease. Virology 285: 234-243.

Briddon, R.W. and Markham, P.G. 2000. Cotton leaf curl disease. Virus Research 71: 151-159.

Briddon, R.W., Pinner, M.S., Stanley, J., and Markham, P.G. 1990. Geminivirus coat protein gene replacement alters insect specificity. Virology 177: 85-94.

Briddon, R.W. and Stanley, J. 2006. Subviral agents associated with plant single-stranded DNA viruses. Virology 344: 198-210.

Cadena-Hinojosa, M.A. and Campbell, R.N. 1981. Characterization of isolates of four aphid-transmitted sweetpotato viruses. Phytopathology 71: 1086-1089.

Cali, B.B. and Moyer, J.W. 1981. Purification, serology, and particle morphology of two russet crack strains of *Sweet potato feathery mottle virus*. Phytopathology 71: 302-305.

Carey, E.E., Gibson, R.W., Fuentes, S., Machmud, M., Mwanga, R.O.M., Turyamureeba, G., Zhang, L., Ma, D., Abo EL-Abbas, F., EL-Bedewy, R., and Salazar, L.F. 1999. The causes and control of virus diseases of sweetpotato in developing countries: Is sweetpotato virus disease the main problem? Pp. 241-248. In: Impact on a Changing World. CIP Program Report 1997-98. CIP, Lima, Peru.

Chen, D. and Texada, D.E. 2006. Low-usage codons and rare codons of *Escherichia coli*. Gene Therapy and Molecular Biology 10: 1-12.

Chung, M.L., Liao, C.H., Chen, M.J., and Chiu, R.J. 1985. The isolation, transmission and host range of sweet potato leaf curl agent in Taiwan. Plant Protection Bulletin (Taiwan) 27: 333-341.

Clark, C.A. and Hoy, M.W. 2006. Effects of common viruses on yield and quality of Beauregard sweetpotato in Louisiana. Plant Disease 90: 83-88.

Clark, C.A., LaBonte, D.R., Valverde, R.A., Hoy, M.W., Lotrakul, P., Kokkinos, C., and McGregor, C. 2003. Technology to improve the quality of sweet potato ‘seed’. Louisiana Agriculture 46: 29-31.

Clark, C.A. and Moyer, J.W. 1988. Compendium of Sweet Potato Diseases. The American Phytopathological Society. St. Paul, Minnesota, USA.

Clark, C.A. and Valverde, R.A. 2001. Viruses and sweetpotato cultivar decline in Louisiana, USA. Pp. 62-69. In: International Workshop on Sweetpotato Cultivar Decline Study, 2000. Eds: Nakazawa, Y. and Ishiguru, K. Miyakonojo, Japan.

Clark, C.A., Valverde, R.A., Fuentes, S., Salazar, L.F., and Moyer, J.W. 2002. Research for improved management of sweetpotato pests and diseases: Cultivar decline. Acta Horticulturae 583: 103-112.

Cohen, J., Franck, A., Vetten, H.J., Lesemann, D.E., and Loebenstein, G. 1992. Purification and properties of closterovirus-like particles associated with a whitefly-transmitted disease of sweetpotato. Annals of Applied Biology 121: 257-268.

Cohen, J., Milgram, M., Antignus, Y., Pearlsman, M., Lachman, O., and Loebenstein, G. 1997. Ipomoea crinkle leaf curl caused by a whitefly-transmitted gemini-like virus. Annals of Applied Biology 131: 273-282.

Colinet, D., Nguyen, M., Kummert, J., and Lepoivre, P. 1998. Differentiation among potyviruses infecting sweetpotato based on genus-and virus-specific reverse transcription polymerase chain reaction. Plant Disease 82: 223-229.

DeLany, J.P., Floyd, Z.E., Zvonic, S., Smith, A., Gravois, A., Reiners, E., Wu, X., Kilroy, G., Lefevre, M., and Gimble, J.M. 2005. Proteomic analysis of primary cultures of human adipose-derived stem cells - Modulation by adipogenesis. Molecular and Cellular Proteomics 4: 731-740.

Di Feo, L., Nome, S.F., Biderbost, E., Fuentes, S., and Salazar, L.F. 2000. Etiology of sweet potato chlorotic dwarf disease in Argentina. Plant Disease 84: 35-39.

Dry, I.B., Rigden, J.E., Krake, L.R., Mullineax, P.M., and Rezaian, M.A. 1993. Nucleotide sequence and genome organisation of tomato leaf curl geminivirus. Journal of General Virology 74: 147-151.

Dyer, J.M. 1993. Structural analysis and methionine enhancement of the bean seed storage protein phaseolin. Doctoral Dissertation. Louisiana State University, Baton Rouge, USA.

Esbenshade, P.R. and Moyer, J.W. 1982. Indexing system for *Sweet potato feathery mottle virus* in sweetpotato using enzyme-linked immunosorbent assay. Plant Disease 66: 911-913.

FAO. 2007. FAOSTAT database (http://faostat.fao.org/). Food and Agriculture Organization of the United Nations, Rome, Italy.

Fauquet, C.M., Bisaro, D.M., Briddon, R.W., Brown, J.K., Harrison, B.D., Rybicki, E.P., Stenger, D.C., and Stanley, J. 2003. Revision of taxonomic criteria for species demarcation in the family *Geminiviridae*, and updated list of begomovirus species. Archives of Virology 148: 405-421.

Fauquet, C.M., Briddon, R.W., Brown, J.K., Moriones, E., Stanley, J., Zerbini, M., and Zhou, X. 2008. Geminivirus strain demarcation and nomenclature. Archives of Virology 153: 783-821.

Fuentes, S., Mayo, M.A., Jolly, C.A., Nakano, M., Querci, M., and Salazar, L.F. 1996. A novel luteovirus from sweet potato, sweet potato leaf speckling virus. Annals of Applied Biology, 128: 491-504.

Fuentes, S. and Salazar. L.F. 2003. First report of *Sweet potato leaf curl virus* in Peru. Plant Disease 87: 98.

Garrido-Ramirez, E.R., Sudarshana, M.R., and Gilbertson, R.L. 2000. *Bean golden yellow mosaic virus* from Chiapas, Mexico: Characterization, pseudorecombination with other bean-infecting geminiviruses and germ plasm screening. Phytopathology 90: 1224-1232.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., and Bairoch, A. 2005. Protein identification and analysis tools on the ExPASy Server. Pp. 571-607. In: The Proteomics Protocols Handbook. Ed: Walker, J.M. Humana Press. Totowa, New Jersey, USA.

Gibson, R.W., Mpembe, I., Alicai, T., Carey, E.E., Mwanga, R.O.M., Seal, S.E., and Vetten, H.J. 1998. Symptoms, aetiology and serological analysis of sweet potato virus disease in Uganda. Plant Pathology 47: 95-102.

Gutierrez, C. 1999. Geminivirus DNA replication. Cellular and Molecular Life Sciences 56: 313-329.

Gutierrez, D.L., Fuentes, S., and Salazar, L.F. 2003. Sweet potato virus disease (SPVD): Distribution, incidence, and effect on sweetpotato yield in Peru. Plant Disease 87: 297-302.

Hanh, S.K. 1979. Effects of Viruses (SPVD) on growth and yield of sweetpotato. Experimental Agriculture 15: 253-256.

Hanley-Bowdoin, L., Settlage, S.B., Orozco, B.M., Nagar, S., and Robertson, D. 1999. Geminiviruses: Models for plant DNA replication, transcription, and cell cycle regulation. Critical Reviews in Plant Sciences 18: 71-106.

Hanley-Bowdoin, L., Settlage, S.B., and Robertson, D. 2004. Reprogramming plant gene expression: A prerequisite to geminivirus DNA replication. Molecular Plant Pathology 5: 149-156.

Harrison, B.D. and Robinson, D.J. 1999. Natural genomic and antigenic variation in whitefly-transmitted Geminiviruses (Begomoviruses). Annual Review of Phytopathology 37: 369-398.

Harrison, B.D. and Robinson, D.J. 2002. Green shoots of geminivirology. Physiological and Molecular Plant Pathology 60: 215-218.

Harrison, B.D., Swanson, M.M., and Fargette, D. 2002. Begomoviruses coat protein: serology, variation and funtions. Physiological and Molecular Plant Pathology 60: 257-271.

Harrison, B.D., Zhou, X., Otim-Nape, G.W., Liu, Y., and Robinson, D.J. 1997. Role of a novel type of double infection in the geminivirus-induced epidemic of severe cassava mosaic in Uganda. Annals of Applied Biology 131: 437-448.

Hoyer, U., Maiss, E., Jelkman, W., Lesemann, D.E., and Vetten, H.J. 1996. Identification of the coat protein gene of a sweet potato sunken vein closterovirus isolate from Kenya and evidence for a serological relationship among geographically diverse closterovirus isolates from sweetpotato. Phytopathology 86: 744-750.

Hull, R. 2002. Matthews' Plant Virology. Fourth Edition. Academic Press. San Diego, California, USA.

Huntrods, D. 2008. Sweet Potato Profile. Agricultural Marketing Resource Center. Iowa State University. http://www.agmrc.org/agmrc/commodity/vegetables/sweetpotatoes/ sweetpotatoprofile.htm

IsHak, J.A., Kreuze, J.F., Johansson, A., Mukasa, S.B., Tairo, F., Abo El-Abbas, F.M., and Valkonen, J.P.T. 2003. Some molecular characteristics of three viruses from SPVD-affected sweetpotato plants in Egypt. Archives of Virology 148: 2449-2460.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins D.G., and Gibson, T.J. 1998. Multiple sequence alignment with Clustal X. Trends in Biochemical Sciences 23: 403-405.

Jupin, I., De Kouchkovsky, F., Jouanneau, F., and Gronenborn, B. 1994. Movement of tomato yellow leaf curl geminivirus (TYLCV): Involvement of the protein encoded by ORF C4. Virology 204: 82-90.

Karyeija, R.F., Gibson, R.W., and Valkonen, J.P.T. 1998. The significant of *Sweet potato fathery mottle virus* in subsistence sweetpotato production in Africa. Plant Disease 82: 4-15.

Karyeija, R.F., Kreuze, J.F., Gibson, R.W., and Valkonen, J.P.T. 2000. Synergistic interactions of a potyvirus and a phloem-limited crinivirus in sweet potato plants. Virology 269: 26-36.

Kays, S.J. 2005. Sweetpotato production worldwide: assessment, trends and the future. Acta Horticulturae 670: 19-25.

Kheyr-Pour, A., Bendahmane, M., Matzeit, V., Accotto, G.P.M., Crespi, S., and Gronenborn, B. 1991. *Tomato yellow leaf curl virus* from Sardinia is a whitefly-transmitted monopartite geminivirus. Nucleic Acids Research 19: 6763-6769.

Kokkinos, C.D. 2006. Assessment of interactions among viruses infecting sweetpotato. Doctoral Dissertation. Louisiana State University, Baton Rouge, USA.

Kokkinos, C.D. and Clark, C.A. 2006. Real-time PCR assays for detection and quantification of sweetpotato viruses. Plant Disease 90: 783-788.

Kreuze, J.F., Karyeija, R.F., Gibson, R.W., and Valkonen, J.P.T. 2000. Comparisons of coat protein gene sequences show that East African isolates of *Sweet potato feathery mottle virus* form a genetically distinct group. Archives of Virology 145: 567-574.

Kreuze, J.F., Savenkov, E.I., and Valkonen, J.P.T. 2002. Complete genome sequence and analyses of the subgenomic RNAs of *Sweet potato chlorotic stunt virus* reveal several new features for the genus Crinivirus. Journal of Virology 76: 9260-9270.

Kyte, J. and Doolittle, R. 1982. A simple method for displaying the hydropathic character of a protein. Journal of Molecular Biology 157: 105-132.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Laufs, J., Jupin, I., David, C., Schumacher, S., Heyraud-Nitschke, F., and Gronenborn, B. 1995. Geminivirus replication: Genetic and biochemical characterization of Rep protein function, a review. Biochimie 77: 765-773.

Lefeuvre, P., Martin, D.P., Hoareau, M., Naze, F., Delatte, H., Thierry, M., Varsani, A., Becker, N., Reynaud, B., and Lett, J.M. 2007. Begomovirus 'melting pot' in the south-west Indian Ocean islands: molecular diversity and evolution through recombination. Journal of General Virology 88: 3458-3468.

Li, R., Salih, S., and Hurtt, S. 2004. Detection of geminiviruses in sweetpotato by polymerase chain reaction. Plant Disease 88: 1347-1351.

Ling, K., Harrison Jr, H.F., Simmons, A.M., and Jackson, D.M. 2008. Sweet potato leaf curl virus: Virus reservoir in species of wild morning glory. Meeting Abstract. National Sweetpotato Collaborators Group Progress Report 2007.

Loebenstein, G., Fuentes, S., Cohen, J., and Salazar, L.F., 2004. Sweet potato. Pp. 223-248. In: Virus and Virus-like Diseases of Major Crops in Developing Countries. Eds: Loebenstein, G. and Thottappilly, G. Kluwer Academic Publishers. Dordrecht, Netherlands.

Lotrakul, P. 2000. Biological and molecular properties of *Sweet potato leaf curl virus*. Doctoral Dissertation. Louisiana State University, Baton Rouge, USA.

Lotrakul, P. and Valverde, R.A. 1999. Cloning of a DNA-A-like genomic component of sweet potato leaf curl virus: nucleotide sequence and phylogenetic relationships. http://www.bspp.org.uk/mppol/1999/0422lotrakul/paper.htm

Lotrakul, P., Valverde, R.A., Clark, C.A., and Fauquet, C.M. 2003. Properties of a Begomovirus isolated from sweetpotato infected with *Sweet potato leaf curl virus*. Revista Mexicana de Fitopatologia 21: 128-136.

Lotrakul, P., Valverde, R.A., Clark, C.A., Hurtt, S., and Hoy, M.W. 2002. *Sweet potato leaf curl virus* and related geminiviruses in sweetpotato. Acta Horticulturae 583: 135-141.

Lotrakul, P., Valverde, R.A., Clark, C.A., Sim, J., and De La Torre, R. 1998. Detection of a geminivirus infecting sweet potato in the United States. Plant Disease 82: 1253-1257.

Lozano, G., Nunez, A., Valverde, R., and Navas-Castillo, J. 2004. Begomoviruses infecting sweetpotato in Spain (poster). In: Proceedings, 2nd European Whitefly Symposium. October 5-9, Cavtat, Croatia.

Luan, Y.S., Zhang, J., and An, L.J. 2006. First Report of *Sweet potato leaf curl virus* in China. Plant Disease 90: 1111.

Luan, Y.S., Zhang, J., Liu, D.M., and Li, W.L. 2007. Molecular characterization of *Sweet potato leaf curl virus* isolate from China (SPLCV-CN) and its phylogenetic relationship with other members of the Geminiviridae. Virus Genes 35: 379-385.

Mansoor, S., Briddon, R.W., Zafar, Y., and Stanley, J. 2003. Geminivirus disease complexes: an emerging threat. Trends in Plant Science 8: 128-134.

Mansoor, S., Khan, S.H., Bashir, A., Saeed, M., Zafar,Y., Malik, K.A., Briddon, R., Stanley, J., and Markham, P.G. 1999. Identification of a novel circular single stranded DNA associated with cotton leaf curl disease in Pakistan. Virology 259: 190-199.

Meng, B., Credi, R., Petrovic, N., Tomazic, I., and Gonsalves, D. 2003. Antiserum to recombinant virus coat protein detects *Rupestris stem pitting associated virus* in grapevines. Plant Disease 87: 515-522.

Miano, D.W. 2008. Replication of viruses responsible for sweet potato virus disease in resistant and susceptible sweetpotato genotypes and identification of molecular markers linked to resistance. Doctoral Dissertation. Louisiana State University, Baton Rouge, USA.

Miano, D.W., LaBonte, D.R., Clark, C.A., Valverde, R.A., Hoy, M.W., Hurtt, S., and Li, R. 2006. First report of a begomovirus infecting sweetpotato in Kenya. Plant Disease 90: 832.

Milgram, M., Cohen, J., and Loebenstein, G. 1996. Effects of Sweet potato feathery mottle virus and sweet potato sunken vein virus on sweet potato yields and rates of reinfection of virus-free planting material in Israel. Phytoparasitica 24: 189-193.

Monci, F., Sanchez-Campos, S., Navas-Castillo, J., and Moriones, E. 2002. A natural recombinant between the geminiviruses *Tomato yellow leaf curl Sardinia virus* and *Tomato yellow leaf curl virus* exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish population. Virology 303: 317-326.

Morales, F.J. and Anderson, P.K. 2001. The emergence and dissemination of whitefly-transmitted geminiviruses in Latin America. Archives of Virology 146: 415-441.

Moyer, J.W. and Kennedy, G.G. 1978. Purification and properties of *Sweet potato feathery mottle virus*. Phytopathology 68: 998-1004.

Moyer, J.W. and Salazar, L.F. 1989. Viruses and viruslike diseases of sweetpotato. Plant Disease 73: 451-455.

Mukasa, S.B., Rubaihayo, P.R., and Valkonen, J.P.T., 2006. Interactions between a crinivirus, an ipomovirus, and a potyvirus in coinfected sweetpotato plants. Plant Pathology 55: 458-467.

Nakazawa, Y. 2001. Report on the International Workshop on Sweetpotato Cultivar Decline Study. Miyakonojo, Japan, 2000. Sweetpotato Research Front 11: 1-2.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D., and Czosnek, H. 1991. *Tomato yellow leaf curl virus*: A whitefly-transmitted geminivirus with a single genomic component. Virology 185: 151-161.

New England Biolabs. 2004. pMAL protein fusion and purification system. Instruction Manual version 5.1.

Ngeve, J.M. and Bouwkamp, J.C. 1991. Effects of Sweet Potato Virus Disease (SPVD) on the Yield of Sweet Potato Genotypes in Cameroon. Experimental Agriculture 27: 221-225.

Nikolaeva, O.V., Karasev, A.V., Gumpf, D.J., Lee, R.F., and Garnsey, S.M. 1995. Production of polyclonal antisera to the coat protein of citrus tristeza virus expressed in *Escherichia coli*: Application for immunodiagnosis. Phytopathology 85: 691-694.

Novagen. 2006. pET System Manual. Eleventh edition.

Onuki, M. and Hanada, K. 1998. PCR amplification and partial nucleotide sequences of three dicot-infecting geminiviruses ocurring in Japan. Annals of the Phytopathological Society of Japan 64: 116-120.

Onuki, M., Honda, Y., and Hanada, K. 2000. Geminate particle morphology of *Sweet potato leaf curl virus* in partially purified preparation and its serological relationship to two begomoviruses by Western blotting. Journal of General Plant Pathology 66: 182-184.

Ooi, K., Ohshita, S., Ishii, I., and Yahara, T. 1997. Molecular phylogeny of geminivirus infecting wild plants in Japan. Journal of Plant Research 110: 247-257.

Otim-Nape, G.M., Thresh, J.M., and Shaw, M.W. 1997. The effects of cassava mosaic virus disease on yield and compensation in mixed stands of healthy and infected plants. Annals of Applied Biology 130: 503-521.

Padidam, M., Beachy, R.N., and Fauquet, C.M. 1995. Classification and identification of geminiviruses using sequence comparisons. Journal of General Virology 76: 249-263.

Padidam, M., Beachy, R.N., and Fauquet, C.M. 1996. The role of AV2 (“pre-coat”) and coat protein in viral replication and movement in tomato leaf curl geminivirus. Virology 224: 390-404.

Padidam, M., Sawyer, S., and Fauquet, C.M. 1999. Possible emergence of new geminiviruses by frequent recombination. Virology 265: 218-224.

Page, R.D.M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357-358.

Palmer, K.E., Schnippenkoetter, W.H., and Rybicki, E.P. 1998. Geminivirus isolation and DNA extraction. Pp. 41-52. In: Methods in Molecular Biology, vol 81: Plant Virology Protocols. Eds: Foster, G.D. and Taylor, S.C. Humana Press Inc. Totawa, New Jersey, USA.

Pio-Ribeiro, G., Winter, S., Jarret, R.L., Demski, J.W., and Hamilton, R.I. 1996. Detection of sweet potato virus disease-associated closterovirus in a sweet potato accession in the United States. Plant Disease 80: 551-554.

Pita, J.S., Fondong, V.N., Sangare, A., Otim-Nape, G.W., Ogwal, S., and Fauquet, C.M. 2001. Recombination, pseudo-recombination and synergism of geminiviruses are determinant keys to the epidemic of the cassava mosaic disease in Uganda. Journal of General Virology 82: 655-661.

Pita, J.S. and Roossinck, M.J. 2008. Virus populations, mutation rates and frequencies. Pp. 109-121. In: Plant Virus Evolution. Ed: Roossinck, M.J. Springer. Heidelberg, Germany.

Polston, J.E. and Anderson, P.L. 1997. The emergence of whitefly transmitted geminiviruses in tomato in the Western Hemisphere. Plant Disease 81: 1358-1369.

Preiss, W. and Jeske, H. 2003. Multitasking in replication is common among geminiviruses. Journal of Virology 77: 2972-2980.

Qian, Y. and Zhou, X. 2005. Pathogenicity and stability of a truncated DNA β associated with *Tomato yellow leaf curl China virus*. Virus Research 109: 159-163.

Rojas, M.R., Hagen, C., Lucas, W.J., and Gilbertson, R.L. 2005. Exploiting chinks in the plant's armor: Evolution and emergence of geminiviruses. Annual Review of Phytopathology 43: 361-94.

Rybicki, E.P. and Pietersen, G. 1999. Plant virus disease problems in the developing world. Advances in Virus Research 53: 127-175.

Sakai, J., Mori, M., Morishita, T., Tanaka, M., Hanada, K., Usugi, T., and Nishiguchi, M. 1997. Complete nucleotide sequence and genome organization of *Sweet potato feathery mottle virus* (S strain) genomic RNA: the large coding region of the P1 gene. Archives of Virology 142: 1553-1562.

Salazar, L.F. and Fuentes, S. 2001. Current knowledge on major virus diseases of sweetpotatoes. Pp. 14-19. In: International Workshop on Sweetpotato Cultivar Decline Study, 2000. Eds: Nakazawa, Y. and Ishiguru, K. Miyakonojo, Japan.

Sanz, A.I., Fraile, A., Gallego, J.M., Malpica, J.M., and Garcia-Arenal, F. 1999. Genetic variability of natural populations of cotton leaf curl geminivirus, a single-stranded DNA virus. Journal of Molecular Evolution 49: 672-681.

Sanz, A.I., Fraile, A., Garcia-Arenal, F., Zhou, X., Robinson, D.J., Khalid, S., Butt, T., and Harrison, B.D. 2000. Multiple infection, recombination and genome relationships among begomovirus isolates found in cotton and other plants in Pakistan. Journal of General Virology 81: 1839-1849.

Saunders, K., Bedford, I.D., Briddon, R.W., Markham, P.G., Wong, S.M., and Stanley, J. 2000. A unique virus complex causes *Ageratum* yellow vein disease. Proceedings of the National Academy of Sciences USA 97: 6890-6895.

Saunders, K., Bedford, I.D., and Stanley, J. 2001. Pathogenicity of a natural recombinant associated with ageratum yellow vein disease: Implications for begomovirus evolution and disease aetiology. Virology 282: 38-47.

Saunders, K. and Stanley, J. 1999. A nanovirus-like DNA component associated with yellow vein disease of *Ageratum conyzoides*: Evidence for interfamilial recombination between plant DNA viruses. Virology 264: 142-152.

Schaefers, G.A. and Terry, E.R. 1976. Insect transmission of sweet potato disease agents in Nigeria. Phytopathology 66: 642-645.

Schein, C.H. 1989. Production of soluble recombinant proteins in bacteria. Bio/Technology 7: 1141-1149.

Schein, C.H. and Noteborn, M.H.M. 1988. Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperature. Bio/Technology 6: 291-294.

Seal, S.E., vandenBosch, F., and Jeger, M.J. 2006. Factors influencing Begomovirus evolution and their increasing global significance: Implications for sustainable control. Critical Reviews in Plant Sciences 25: 23-46.

Sim, J. 2001. Cytopathology, detection, and identification of viruses infecting sweetpotato. Doctoral Dissertation. Louisiana State University, Baton Rouge, USA.

Sim, J., Valverde, R.A., and Clark, C.A. 2000. Whitefly transmission of *Sweet potato chlorotic stunt virus*. Plant Disease 84: 1250.

Simon, A.E., Roossinck, M.J., and Havelda, Z. 2004. Plant virus satellite and defective interfering RNAs: New paradigms for a new century. Annual Review of Phytopathology 42: 415-438.

Smialowski, P., Martin-Galiano, A.J., Mikolajka, A., Girschick, T., Holak, T.A., and Frishman, D. 2007. Protein solubility: Sequence based prediction and experimental verification. Bioinformatics 23: 2536-2542.

Souto, E.R., Sim, J., Chen, J., Valverde, R.A., and Clark, C.A. 2003. Properties of strains of *Sweet potato feathery mottle virus* and two newly recognized potyviruses infecting sweetpotato in the United States. Plant Disease 87: 1226-1232

Sseruwagi, P., Rey, M.E.C., Brown, J.K., and Legg, J.P. 2004. The cassava mosaic geminiviruses occurring in Uganda following the 1990s epidemic of severe cassava mosaic disease. Annals of Applied Biology 145: 113-121.

Stanley, J. 2004. Subviral DNAs associated with geminivirus disease complexes. Veterinary Microbiology 98: 121-129.

Stanley, J., Bisaro, D.M., Briddon, R.W., Brown, J.K., Fauquet, C.M., Harrison, B.D., Rybicki, E.P., and Stenger, D.C. 2005. Family *Geminiviridae*. Pp. 301-326. In: Virus Taxonomy. Eighth Report of the International Committee on the Taxonomy of viruses. Eds: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., and Ball, L.A. Elsevier Academic Press. San Diego, California, USA.

Tairo, F., Jones, R.A.C., and Valkonen, J.P.T. 2006. Potyvirus complexes in sweetpotato: Occurrence in Australia, serological and molecular resolution, and analysis of the Sweet potato virus 2 (SPV2) component. Plant Disease 90: 1120-1128.

Untiveros, M., Fuentes, S., and Salazar, L.F. 2007. Synergistic interaction of *Sweet potato chlorotic stunt virus* (Crinivirus) with Carla-, Cucumo-, Ipomo-, and Potyviruses infecting sweetpotato. Plant Disease 91: 669-676.

USDA. 2008. Crop Production 2007 Summary. National Agricultural Statistics Service. United States Department of Agriculture.

Valverde, R.A., Clark, C.A., Kokkinos, C.D., and Gutierrez, D.L. 2008. Sweetpotato viral diseases. Pp. 251-272. In: Characterization, Diagnosis, and Management of Plant Viruses. Vol.1: Industrial Crops. Eds: Rao, G.P., Khurana, S.M.P., and Lenardon, S.L. Studium Press LLC. Houston, Texas, USA.

Valverde, R.A., Clark, C.A., and Valkonen, J.P.T. 2007. Viruses and Virus Disease Complexes of Sweetpotato. Plant Viruses 1: 116-126.

Valverde, R.A., Kokkinos, C.D., and Clark, C.A. 2004a. *Sweet potato leaf curl virus*: Detection by molecular hybridization. Phytopathology 94: S105.

Valverde, R.A., Sim, J., and Lotrakul, P. 2004b.Whitefly transmission of sweetpotato viruses. Virus Research 100: 123-128.

Varma, A. and Malathi, V.G. 2003. Emerging geminiviruses problems: A serious threat to crop production. Annals of Applied Biology 142: 145-164.

Wang, L.Y., Chen, K.C., Chen, T.C., and Yeh, S.D. 2007. Nucleotide sequence analysis of the coat protein genes of two Taiwan isolates of *Sweet potato feathery mottle virus* from central Taiwan. Plant Pathology Bulletin 16: 203-213.

Wartig, L., Kheyr-Pour, A., Noris, E., De Kouchkovsky, F., Jouanneau, F., Gronenborn, B., and Jupin, I. 1997. Genetic analysis of the monopartite tomato yellow leaf curl geminivirus: Roles of V1, V2, and C2 ORFs in viral pathogenesis. Virology 228 :132-140.

Winter, S., Purac, A., Leggett, F., Frison, E.A., Rossel, H.W., and Hamilton, R.I. 1992. Partial characterization and molecular cloning of a closterovirus from sweetpotato infected with the sweet potato virus disease complex from Nigeria. Phytopahology 82: 869-875.

Xie, Y. and Zhou, X.P. 2003. Molecular characterization of squash leaf curl Yunnan virus, a new begomovirus and evidence for recombination. Archives of Virology 148: 2047-2054.

Zhou, X., Liu, Y., Calvert, L., Munoz, D., Otim-Nape, G.W., Robinson, D.J., and Harrison, B.D. 1997. Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. Journal of General Virology 78: 2101-2111.

Zhou, X., Xie, Y., Tao, X., Zhang, Z., Li, Z., and Fauquet, C.M. 2003. Characterisation of DNA-β associated with begomoviruses in China and evidence for co-evolution with their cognate viral DNA-A. Journal of General Virology 84: 237-247.

Zvonic, S., Lefevre, M., Kilroy, G., Floyd, Z.E., DeLany, J.P., Kheterpal, I., Gravois, A., Dow, R., White, A., Wu, X., and Gimble, J.M. 2007. Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis. Molecular and Cellular Proteomics 6: 18-28.

APPENDIX A: SEROLOGICAL EXPERIMENTS

A.1 Introduction

Serological methods are based on the interaction between the viral coat protein and specific antibodies. Currently, serological methods, such as enzyme-linked immunosorbent assay (ELISA), to detect *Sweet potato leaf curl virus* (SPLCV) are not available due to the difficulties in obtaining purified virions that can be used as antigen for antiserum production. It is possible that either SPLCV virus particles occur in low concentration in plant tissues or viral particles are not stable after standard purification procedures.

The begomovirus coat protein (CP) is highly conserved and plays a key role in processes that are involved in virus infection, survival and spread (Harrison and Robinson, 1999; Harrison *et al*., 2002). Serological relationships have been reported among begomoviruses (Harrison and Robinson, 1999; Harrison *et al*., 2002; Stanley *et al*., 2005). The nature of these serological relationships has been explored in detail by assays with monoclonal antibodies (MAbs), especially the panels of MAbs to *African cassava mosaic virus* (ACMV), and *Indian cassava mosaic virus* (ICMV), and *Okra leaf curl virus* (Thomas *et al*., 1986; Swanson, 1992; Macintosh *et al*., 1992; Swanson *et al*., 1998). The numerous serological cross-reactions among begomoviruses in tests with polyclonal antisera and with a proportion of MAbs may be explained by the existence of many shared epitopes (antigenic determinants) present in the CP (Harrison *et al*., 2002). Serological relationships of SPLCV to *Bean golden mosaic virus* (BGMV) and *Mungbean yellow mosaic virus* (MYMV) by Western blotting have been reported by Onuki *et al*. (2000) using partially purified virions of SPLCV.

Antisera to *Tomato yellow leaf curl virus* (TYLCV) were tested for the ability to detect SPLCV from leaf extracts and partially purified preparations by ELISA and Western blot.

A.2 Materials and Methods

A.2.1 ELISA in Nitrocellulose Membranes (NCM-ELISA)

Foliar tissues of SPLCV infected plants (*I. nil*, *I. setosa*, and *I. aquatica*), as well as, tomato plants infected with TYLCV and *Dicliptera sexangularis* infected with *Dicliptera yellow mottle virus* (DiYMoV) (Begomovirus) were used for NCM-ELISA following the procedure described by Gutierrez *et al*. (2003). Two leaf disks (about 1 cm in diameter) for each sample, were ground in 2 ml of 1X TBS (0.02 M Tris, 0.5 M NaCl, pH 7.5) containing 0.2 % Na_2SO_3. Clarified sap extracts were blotted onto TBS buffer-saturated nitrocellulose membranes. Then membranes were blocked for 1 h with TBS containing 2 % nonfat dry milk and 2 % Triton X-100. Blocking solution was discarded, and membranes were rinsed with TBS. Membranes were probed using either TYLCV polyclonal antiserum #1214 (1:1,000 dilution) for 5 h, or TYLCV 4C13F7 monoclonal antiserum (1:500 dilution) overnight. Both antisera were kindly provided by Dr. J. Polston, University of Florida, Gainesville. The TYLCV antiserum #1214 was produced against the TYLCV coat protein expressed in *E. coli* (Abouzid *et al*., 2002). Membranes were washed 4 times, 3 min each with TBS containing 0.05% Tween 20, and then incubated for 1 h with alkaline phosphatase conjugates: goat anti-rabbit (GAR, 1:10,000 dilution) (Bio-Rad, Hercules, CA) and goat anti-mouse (GAM, 1: 3,333 dilution) (Agdia Inc., Elkhart, IN). Membranes were washed as before, and the presence of bound antibodies was visualized by the addition of BCIP/NBT color development solution (Bio-Rad). Positive reactions were determined by visual assessment where purple color reaction was recorded as positive.

The polyclonal antiserum was diluted and crossed-absorbed with healthy plant sap extracts. Tomato leaves were ground in 1/30 (w/v) TBS containing 0.2 % Na_2SO_3 and 2 % nonfat dry milk, and centrifuged at room temperature for 10 min at 10,000 RPM in a Beckman Coulter Avanti J-25 Centrifuge, JA 25.5 rotor. Supernatant was collected and the antiserum was

added at the appropriate dilution. The solution was incubated at 37 ˚C for 1 h, and applied to the blocked membrane. Monoclonal antiserum, GAR, and GAM were diluted in TBS containing 2% nonfat dry milk.

A.2.2 Partial Purifications

SPLCV was partially purified from *I. nil* and *I. aquatica*, following the minipurification method developed by Lane (http://lclane.net/text/minipurprotocol.html) with some modifications. TYLCV and DiYMoV were used as controls. Foliar tissues (1 g) were ground in cold buffer containing 22 ml of 0.2 M Sodium Citrate pH 6.5 plus 150 µl of 0.5 M Sodium diethyldithiocarbamate. Solutions were expressed through cheesecloth into polycarbonate centrifuge tubes; then, samples were centrifuged for 15 min at 30,000 RPM, 20 ˚C in a Beckman L8-70 Ultracentrifuge, 70 Ti rotor. Supernatants were transferred through cheesecloth into clean polycarbonate centrifuge tubes. Four drops of 10 % Triton X-100 were added to each sample. Solutions were mixed and centrifuged for 1h at 30,000 RPM, 15 ˚C in a Beckman L8-70 Ultracentrifuge, 70 Ti rotor. Supernatants were discarded immediately and tube walls were washed thoroughly with distilled water. Pellets were dried at room temperature for about 20-30 min, and resuspended in 200 or 250 µl (depending on the pellet size) of 50 mM $NaPO_4$, pH 7.0. Samples were mixed with an equal amount of 2X loading buffer (0.125 M Tris, pH 6.8, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.2 % bromophenol blue) and denatured in boiling water for 5 min. Proteins were separated by 10 % SDS-PAGE (stacking gel 4 % and resolving gel 10 %) as described by Laemmli (1970) and visualized with Coomassie Brilliant Blue staining solution (45 % methanol, 0.1 % Coomassie Brilliant Blue R-250, 10 % acetic acid).

A.2.3 Western Blot Analysis

Proteins electrophoresed on SDS-PAGE were transferred to nitrocellulose membranes. SDS-PAGEs were soaked in blotting buffer (0.025 M Tris, 0.192 M glycine, 20 % methanol)

before assembling the transfer cassette. Electroblotting was conducted at 4 ˚C, 35 V overnight in a TE 22 Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). All the following incubations and washings were performed at room temperature in a shaker with gentle agitation. Blotted membranes were blocked for 1 h 30 min with TBS (0.02 M Tris, 0.5 M NaCl, pH 7.5) containing 3 % nonfat dry milk and 1 % Triton X-100. Blocking solution was discarded, and membranes were rinsed with TBS. Membranes were probed using either TYLCV polyclonal antiserum #1214 (1:1,000 dilution) for 5 h, or TYLCV 4C13F7 monoclonal antiserum (1:500 dilution) overnight. Membranes were washed 4 times, 5 min each with TBS containing 0.05 % Tween 20, and then incubated for 1 h with alkaline phosphatase conjugates, GAR (1:10,000 dilution) (Bio-Rad) and GAM (1: 3,333 dilution) (Agdia Inc.), respectively. Antisera were diluted in TBS containing 2 % nonfat dry milk. Protein bands were visualized by the addition of BCIP/NBT color development solution (Bio-Rad).

A.3 Results

Antisera to TYLCV reacted with the homologous antigen, but it did not react with SPLCV by NCM-ELISA or Western blot analysis. Attempts to electrophoretically detect SPLCV CP using partial purifications from *I. nil* and *I. aquatica* infected plants were not successful. The expected 29 kDa protein band (size of SPLCV CP) was not obtained when total protein extracts from infected plants were analyzed by SDS-PAGE (Figure E.1A). Moreover, Western blot analysis using TYLCV antisera did not result in cross reactivity with SPLCV (Figure E.1B).Weak reactions in NCM-ELISA and Western blot were observed between the TYLCV antisera (monoclonal and polyclonal) and DiYMoV.

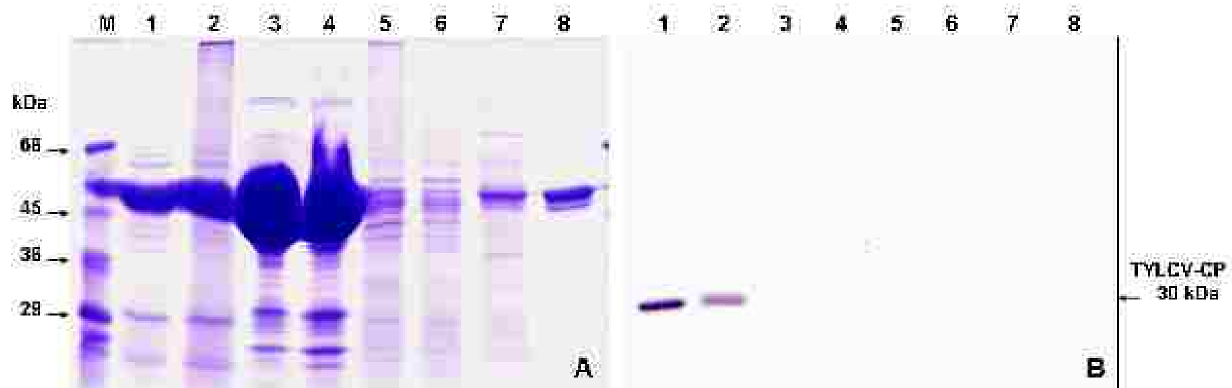


Figure E.1. Partial purification of *Sweet potato leaf curl virus* (SPLCV). (A) Protein analysis by 10 % SDS-PAGE stained with Coomassie Brilliant Blue. (B) Western blot analysis using *Tomato yellow leaf curl virus* (TYLCV) polyclonal antiserum #1214. Lanes 1-2, TYLCV (Tomato). Lane 3, healthy *Dicliptera sexangularis*. Lane 4, *Dicliptera yellow mottle virus* (*D. sexangularis*). Lane 5, healthy *I. nil*. Lane 6, SPLCV (*I. nil*). Lane 7, healthy *I. aquatica*. Lane 8, SPLCV (*I. aquatica*). Lane M, molecular weight marker.

A.4 Conclusions

The percent derived amino acid sequence identity and similarity between the CP of SPLCV and the CP of TYLCV is 49 and 65 %, respectively. This may explain the lack of reactivity with the antisera tested. Also, it is possible that epitopes recognized by these antisera are not present in the SPLCV CP, or perhaps, the epitopes on the SPLCV CP were not accessible to these antisera (Harrison *et al*., 2002).

The CP of SPLCV (about 29 kDa) was not observed when partial purifications of SPLCV from *I. nil* and *I. aquatica* were analyzed by SDS-PAGE. The presence of polysaccharides and other components in *Ipomoea* species interfered with pellet formation and recovery after centrifugation. Pellets obtained from *Ipomoea* species were smaller than those obtained from tomato or *D. sexangularis* plants. Consequently, the amount of proteins obtained from *Ipomoea* species was lower than those from other plant species. This is illustrated in Figure E.1. The use of a different plant host, such as *Nicotiana bethamiana*, may improve protein yields that could

facilitate the detection of SPLCV CP in polyacrylamide gels, as well as, its detection by Western blot.

The lack of cross reactivity of TYLCV antisera with SPLCV could also be due to the low concentration of the virus in plant tissues. Virus particles were not observed when minipurification preparations of SPLCV were subjected to electron microscope analysis.

It has been reported that *Sweet potato feathery mottle virus* (SPFMV) is distributed in the whole plant when detected by nucleic acid spot hybridization tests. However, the virus is serologically detected mainly in symptomatic leaves (Abad and Moyer, 1992; Cadena-Hinojosa and Campbell, 1981; Karyeija *et al*., 2000). It is possible that the virus moves as nucleic acid or as virions in very low concentration in symptomless leaves in the plant, but accumulation of detectable amounts of viral coat protein is directly associated with symptom expression (Abad and Moyer, 1992). The same phenomenon may happen with SPLCV, and for that reason it has been difficult to detect it by serological assays.

A.5 Literature Cited

Abad, J.A. and Moyer, J.W. 1992. Detection and distribution of *Sweet potato feathery mottle virus* in sweetpotato by in vitro-transcribed RNA probes (riboprobes), membrane immunobinding assay and direct blotting. Phytopathology 82: 300-305.

Abouzid, A.M., Freitas-Astua, J., Purcifull, D.E., Polston, J.E., Beckham, K.A., Crawford, W.E., Petersen, M.A., Peyser, B., Patte, C., and Hiebert, E. 2002. Serological studies using polyclonal antisera prepared against the viral coat protein of four begomoviruses expressed in *Escherichia coli*. Plant Disease 86: 1109-1114.

Cadena-Hinojosa, M.A. and Campbell, R.N. 1981. Characterization of isolates of four aphid-transmitted sweetpotato viruses. Phytopathology 71: 1086-1089.

Gutierrez, D.L., Fuentes, S., and Salazar, L.F. 2003. Sweet potato virus disease (SPVD): Distribution, incidence, and effect on sweetpotato yield in Peru. Plant Disease 87: 297-302.

Harrison, B.D. and Robinson, D.J. 1999. Natural genomic and antigenic variation in whitefly-transmitted Geminiviruses (Begomoviruses). Annual Review of Phytopathology 37: 369-398.

Harrison, B.D., Swanson, M.M., and Fargette, D. 2002. Begomoviruses coat protein: serology, variation and funtions. Physiological and Molecular Plant Pathology 60: 257-271.

Karyeija, R.F., Kreuze, J.F., Gibson, R.W., and Valkonen, J.P.T. 2000. Synergistic interactions of a potyvirus and a phloem-limited crinivirus in sweet potato plants. Virology 269: 26-36.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Macintosh, S., Robinson, D.J., and Harrison, B.D. 1992. Detection of three whitefly-transmitted geminiviruses occurring in Europe by tests with heterologous monoclonal antibodies. Annals of Applied Biology 121: 297-303.

Onuki, M., Honda, Y., and Hanada, K. 2000. Geminate particle morphology of *Sweet potato leaf curl virus* in partially purified preparation and its serological relationship to two begomoviruses by Western blotting. Journal of General Plant Pathology 66: 182-184.

Stanley, J., Bisaro, D.M., Briddon, R.W., Brown, J.K., Fauquet, C.M., Harrison, B.D., Rybicki, E.P., and Stenger, D.C. 2005. Family *Geminiviridae*. Pp. 301-326. In: Virus Taxonomy. Eighth Report of the International Committee on the Taxonomy of viruses. Eds: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., and Ball, L.A. Elsevier Academic Press. San Diego, California, USA.

Swanson, M.M. 1992. Antigenic variation and serological relationships of whitefly-transmitted geminiviruses. Doctoral thesis, University of Dundee, UK.

Swanson, M.M., Valand, G.B., Muniyappa, V., and Harrison, B.D. 1998. Serological detection and antigenic variation of two whitefly-transmitted geminiviruses: tobacco leaf curl and croton yellow vein mosaic viruses. Annals of Applied Biology 132: 427-435.

Thomas, J.E., Massalski, P.R., and Harrison, B.D. 1986. Production of monoclonal antibodies to African cassava mosaic virus and differences in their reactivities with other whitefly-transmitted geminiviruses. Journal of General Virology 67: 2739-2748.

APPENDIX B: ANALYSIS OF THE MBP*CP FUSION PROTEIN

ProtParam is a tool which allows the computation of various physical and chemical parameters that can be deduced for a protein sequence.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., and Bairoch, A. 2005. Protein identification and analysis tools on the ExPASy Server. Pp. 571-607. In: The Proteomics Protocols Handbook. Ed: Walker, J.M. Humana Press. Totowa, New Jersey, USA.

MBP*CP: Fusion protein comprising the maltose-binding protein and the coat protein of *Sweet potato leaf curl virus*

ProtParam

User-provided sequence: MBP*CP

```
        10         20         30         40         50         60
MKTEEGKLVI WINGDKGYNG LAEVGKKFEK DTGIKVTVEH PDKLEEKFPQ VAATGDGPDI

        70         80         90        100        110        120
IFWAHDRFGG YAQSGLLAEI TPDKAFQDKL YPFTWDAVRY NGKLIAYPIA VEALSLIYNK

       130        140        150        160        170        180
DLLPNPPKTW EEIPALDKEL KAKGKSALMF NLQEPYFTWP LIAADGGYAF KYENGKYDIK

       190        200        210        220        230        240
DVGVDNAGAK AGLTFLVDLI KNKHMNADTD YSIAEAAFNK GETAMTINGP WAWSNIDTSK

       250        260        270        280        290        300
VNYGVTVLPT FKGQPSKPFV GVLSAGINAA SPNKELAKEF LENYLLTDEG LEAVNKDKPL

       310        320        330        340        350        360
GAVALKSYEE ELAKDPRIAA TMENAQKGEI MPNIPQMSAF WYAVRTAVIN AASGRQTVDE

       370        380        390        400        410        420
ALKDAQTNSS SNNNNNNNNN NLGDDDDKVP MTGRMRVSPR FHPYGGRPVR RRLNFETAIV

       430        440        450        460        470        480
PYTGNAVPIA ARSYVPVSRG VRMKRRRGDR IPKGCVGPCK VQDYEFKMDV PHAGTFVCVS

       490        500        510        520        530        540
DFTRGTGLTH RLGKRVCVKS MGIDGKVWMD DNVAKRDHTN IITYWLIRDR RPNKDPLNFG

       550        560        570        580        590        600
QIFTMYDNEP TTAKIRMDLR DRMQVLKKFS VTVSGGPYSH KEQALIRKFF KGLYNHVTYN

       610        620        630        640
HKEEAKYENQ LENALMLYSA SNHASNPVYQ TLRCRAYFYD SHNN
```

Number of amino acids: 644
Molecular weight: 72214.0
Theoretical pI: 8.88

Amino acid composition:

Ala (A) 57	8.9%		Phe (F) 26	4.0%
Arg (R) 32	5.0%		Pro (P) 36	5.6%
Asn (N) 47	7.3%		Ser (S) 27	4.2%
Asp (D) 42	6.5%		Thr (T) 35	5.4%
Cys (C) 5	0.8%		Trp (W) 10	1.6%
Gln (Q) 15	2.3%		Tyr (Y) 29	4.5%
Glu (E) 35	5.4%		Val (V) 42	6.5%
Gly (G) 48	7.5%		Pyl (O) 0	0.0%
His (H) 12	1.9%		Sec (U) 0	0.0%
Ile (I) 31	4.8%			
Leu (L) 44	6.8%		(B) 0	0.0%
Lys (K) 54	8.4%		(Z) 0	0.0%
Met (M) 17	2.6%		(X) 0	0.0

Total number of negatively charged residues (Asp + Glu): 77
Total number of positively charged residues (Arg + Lys): 86

Atomic composition:

Carbon	C	3227
Hydrogen	H	5012
Nitrogen	N	890
Oxygen	O	952
Sulfur	S	22

Formula: $C_{3227}H_{5012}N_{890}O_{952}S_{22}$
Total number of atoms: 10103

Extinction coefficients:

Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.

Ext. coefficient 98460
Abs 0.1% (=1 g/l) 1.363, assuming ALL Cys residues appear as half cystines

Ext. coefficient 98210
Abs 0.1% (=1 g/l) 1.360, assuming NO Cys residues appear as half cystines

Estimated half-life:

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:

The instability index (II) is computed to be 26.69
This classifies the protein as stable.

Aliphatic index: 73.18
Grand average of hydropathicity (GRAVY): -0.537

APPENDIX C: ANALYSIS OF THE MBP*β-GAL PROTEIN

ProtParam is a tool which allows the computation of various physical and chemical parameters that can be deduced for a protein sequence.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., and Bairoch, A. 2005. Protein identification and analysis tools on the ExPASy Server. Pp. 571-607. In: The Proteomics Protocols Handbook. Ed: Walker, J.M. Humana Press. Totowa, New Jersey, USA.

MBP*β-gal: Fusion protein comprising the maltose-binding protein and the β-galactosidase α fragment protein

ProtParam

User-provided sequence: MBP*β-gal

```
        10         20         30         40         50         60
MKTEEGKLVI WINGDKGYNG LAEVGKKFEK DTGIKVTVEH PDKLEEKFPQ VAATGDGPDI

        70         80         90        100        110        120
IFWAHDRFGG YAQSGLLAEI TPDKAFQDKL YPFTWDAVRY NGKLIAYPIA VEALSLIYNK

       130        140        150        160        170        180
DLLPNPPKTW EEIPALDKEL KAKGKSALMF NLQEPYFTWP LIAADGGYAF KYENGKYDIK

       190        200        210        220        230        240
DVGVDNAGAK AGLTFLVDLI KNKHMNADTD YSIAEAAFNK GETAMTINGP WAWSNIDTSK

       250        260        270        280        290        300
VNYGVTVLPT FKGQPSKPFV GVLSAGINAA SPNKELAKEF LENYLLTDEG LEAVNKDKPL

       310        320        330        340        350        360
GAVALKSYEE ELAKDPRIAA TMENAQKGEI MPNIPQMSAF WYAVRTAVIN AASGRQTVDE

       370        380        390        400        410        420
ALKDAQTNSS SNNNNNNNNN NLGDDDDKVP EFGSSRVDLQ ASLALAVVLQ RRDWENPGVT

       430        440        450        460
QLNRLAAHPP FASWRNSEEA RTDRPSQQLR SLNGEWQLGC FGG
```

Number of amino acids: 463
Molecular weight: 50960.2
Theoretical pI: 5.04

Amino acid composition:

Ala (A)	51	11.0%		Phe (F)	18	3.9%
Arg (R)	13	2.8%		Pro (P)	26	5.6%
Asn (N)	36	7.8%		Ser (S)	22	4.8%
Asp (D)	31	6.7%		Thr (T)	22	4.8%
Cys (C)	1	0.2%		Trp (W)	11	2.4%
Gln (Q)	15	3.2%		Tyr (Y)	15	3.2%
Glu (E)	32	6.9%		Val (V)	25	5.4%
Gly (G)	36	7.8%		Pyl (O)	0	0.0%
His (H)	4	0.9%		Sec (U)	0	0.0%
Ile (I)	21	4.5%				
Leu (L)	40	8.6%		(B)	0	0.0%
Lys (K)	37	8.0%		(Z)	0	0.0%
Met (M)	7	1.5%		(X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 63
Total number of positively charged residues (Arg + Lys): 50

Atomic composition:

Carbon	C	2283
Hydrogen	H	3525
Nitrogen	N	609
Oxygen	O	700
Sulfur	S	8

Formula: $C_{2283}H_{3525}N_{609}O_{700}S_{8}$
Total number of atoms: 7125

Extinction coefficients:

Extinction coefficients are in units of M^{-1} cm^{-1}, at 280 nm measured in water.

Ext. coefficient 82850
Abs 0.1% (=1 g/l) 1.626, assuming ALL Cys residues appear as half cystines

Ext. coefficient 82850
Abs 0.1% (=1 g/l) 1.626, assuming NO Cys residues appear as half cystines

Estimated half-life:

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:

The instability index (II) is computed to be 25.04
This classifies the protein as stable.

Aliphatic index: 78.06
Grand average of hydropathicity (GRAVY): -0.483

APPENDIX D: TRYPSIN DIGESTION OF *SWEET POTATO LEAF CURL VIRUS* COAT PROTEIN

PeptideCutter predicts potential cleavage sites cleaved by proteases or chemicals in a given protein sequence.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., and Bairoch, A. 2005. Protein identification and analysis tools on the ExPASy Server. Pp. 571-607. In: The Proteomics Protocols Handbook. Ed: Walker, J.M. Humana Press. Totowa, New Jersey, USA.

PeptideCutter

The sequence to investigate: *Sweet potato leaf curl virus* coat protein

```
        10         20         30         40         50         60
VPMTGRMRVS PRFHPYGGRP VRRRLNFETA IVPYTGNAVP IAARSYVPVS RGVRMKRRRG

        70         80         90        100        110        120
DRIPKGCVGP CKVQDYEFKM DVPHAGTFVC VSDFTRGTGL THRLGKRVCV KSMGIDGKVW

       130        140        150        160        170        180
MDDNVAKRDH TNIITYWLIR DRRPNKDPLN FGQIFTMYDN EPTTAKIRMD LRDRMQVLKK

       190        200        210        220        230        240
FSVTVSGGPY SHKEQALIRK FFKGLYNHVT YNHKEEAKYE NQLENALMLY SASNHASNPV

       250
YQTLRCRAYF YDSHNN
```

The sequence is 256 amino acids long.

The enzyme(s) that you have chosen: Trypsin

Trypsin cleaves C-terminal to Arginine (R) and Lysine (K) residues.

This enzyme cleaves the sequence:

No. of cleavages	Positions of cleavage sites
40	6 8 12 22 24 44 51 54 56 57 59 62 65 72 79 96 103 106 107 111 118 127 128 140 142 146 166 168 172 174 179 180 193 199 200 203 214 218 245 247

Map of Trypsin cleavage sites:

Cleavage occurs at the right side (C-terminal direction) of the marked amino acid.
Highlighted fragments were identified by mass spectrometry.

```
                                                              Tryps
                                                            Tryps |
               Tryps                                       Tryps| |
           Tryps   |       Tryps                         Tryps || |
         Tryps |   |     Tryps |               Tryps  Tryps  | || |

             | |   |         | |                   |      |  | || |
        VPMTGRMRVSPRFHPYGGRPVRRRLNFETAIVPYTGNAVPIAARSYVPVSRGVRMKRRRG
   1    ---------+---------+---------+---------+---------+---------+   60


                                                      Tryps
                                                  Tryps   |
        Tryps                                    Tryps|   |
     Tryps  |  Tryps  Tryps            Tryps  Tryps  ||   |  Tryps

         |  |      |      |                |      |  ||   |      |
        DRIPKGCVGPCKVQDYEFKMDVPHAGTFVCVSDFTRGTGLTHRLGKRVCVKSMGIDGKVW
  61    ---------+---------+---------+---------+---------+---------+   120


                                                               Tryps
                                                              Tryps|
                                                         Tryps    ||
                             Tryps                     Tryps |    ||
           Tryps         Tryps   |                 Tryps   | |    ||
          Tryps|       Tryps |   |               Tryps |   | |    ||

              ||           | |   |                   | |   | |    ||
        MDDNVAKRDHTNIITYWLIRDRRPNKDPLNFGQIFTMYDNEPTTAKIRMDLRDRMQVLKK
 121    ---------+---------+---------+---------+---------+---------+   180


                          Tryps
                       Tryps  |          Tryps
                Tryps Tryps|  |      Tryps   |

                    |     ||  |          |   |
        FSVTVSGGPYSHKEQALIRKFFKGLYNHVTYNHKEEAKYENQLENALMLYSASNHASNPV
 181    ---------+---------+---------+---------+---------+---------+   240


          Tryps
        Tryps |

            | |
        YQTLRCRAYFYDSHNN
 241    ---------+------   256
```

APPENDIX E: MASS SPECTROMETRY ANALYSIS REPORT

Proteomics Core Facility at Pennington Biomedical Research Center, Louisiana State University, Baton Rouge.

Mass Spectrometry Analysis

Accession: gi|29294540
Description: Coat protein AV1 [*Sweet potato leaf curl virus*]
MW: 29,436
Peptides: 26 (Table E.1)
Coverage: 34%

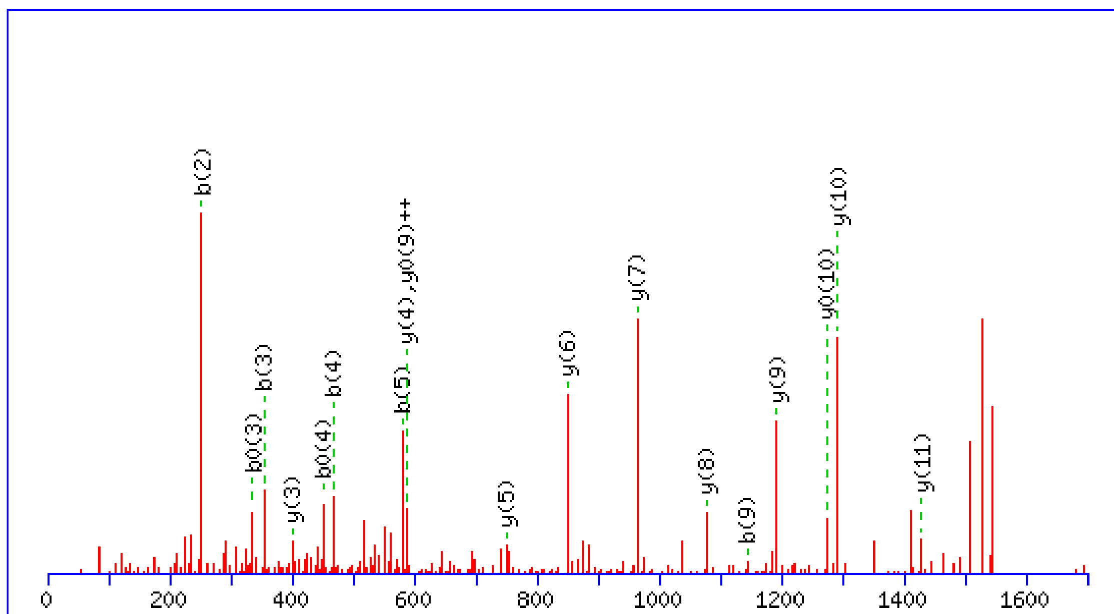
Table E.1. Peptide sequences of *Sweet potato leaf curl virus* coat protein identified by mass spectrometry analysis

Fragment	Peptide Sequence	m/z	Z	Peptide MW
13-20	PYGGRPVR*	451.223	2	900.43
22-42	RLNFETAIVPYTGNAVPIAAR	758.347	3	2272.02
23-42	LNFETAIVPYTGNAVPIAAR	1058.959	2	2115.90
23-42	LNFETAIVPYTGNAVPIAAR	1059.020	2	2116.03
28-42	AIVPYTGNAVPIAAR	756.882	2	1511.75
30-42	VPYTGNAVPIAAR	664.818	2	1327.62
30-42	VPYTGNAVPIAAR	664.819	2	1327.62
31-42	PYTGNAVPIAAR	615.275	2	1228.54
31-42	PYTGNAVPIAAR	615.265	2	1228.52
86-94	FV(CamC)VSDFTR	565.725	2	1129.44
87-94	V(CamC)VSDFTR	492.192	2	982.37
88-94	(CamC)VSDFTR	442.668	2	883.32
117-125	VW(OxM)DDNVAK	547.224	2	1092.43
117-125	VWMDDNVAK	539.226	2	1076.44
118-125	W(OxM)DDNVAK	497.680	2	993.35
119-126	(OxM)DDNVAKR	482.701	2	963.39
127-138	DHTNIITYWLIR	772.854	2	1543.69
127-138	DHTNIITY(OxW)LIR	780.873	2	1559.73
179-191	FSVTVSGGPYSHK	683.304	2	1364.59
180-191	SVTVSGGPYSHK	609.779	2	1217.54
181-191	VTVSGGPYSHK	566.249	2	1130.48
204-216	YNHVTYNHKEEAK	544.894	3	1631.66
204-212	YNHVTYNHK	588.275	2	1174.54
205-212	NHVTYNHK	506.736	2	1011.46
246-254	AYFYDSHNN	565.712	2	1129.41
248-254	FYDSHNN	448.658	2	895.30

MS/MS Fragmentation of DHTNIITYWLIR peptide of *Sweet potato leaf curl virus* coat protein

Mascot MS/MS Matrix Science (http://www.matrixscience.com/)
Mascot is a powerful search engine that uses mass spectrometry data to identify proteins from primary sequence databases.

Accession: gi|29294540
Description: Coat protein AV1 [*Sweet potato leaf curl virus*]



Monoisotopic mass of neutral peptide Mr(calc): 1543.8147
Fixed modifications: Carbamidomethyl (C)
Ions Score: 63 **Expect:** 0.0046
Matches (Bold Red): 18/116 fragment ions using 32 most intense peaks

#	b	b^{++}	b^{*}	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y^{*}	y^{*++}	y^{0}	y^{0++}	#
1	116.0342	58.5207			98.0237	49.5155	D							12
2	**253.0931**	127.0502			235.0826	118.0449	H	**1429.7950**	715.4012	1412.7685	706.8879	1411.7845	706.3959	11
3	**354.1408**	177.5740			**336.1302**	168.5688	T	**1292.7361**	646.8717	1275.7096	638.3584	**1274.7256**	637.8664	10
4	**468.1837**	234.5955	451.1572	226.0822	**450.1732**	225.5902	N	**1191.6885**	596.3479	1174.6619	587.8346	1173.6779	**587.3426**	9
5	**581.2678**	291.1375	564.2413	282.6243	563.2572	282.1323	I	**1077.6455**	539.3264	1060.6190	530.8131	1059.6350	530.3211	8
6	694.3519	347.6796	677.3253	339.1663	676.3413	338.6743	I	**964.5615**	482.7844	947.5349	474.2711	946.5509	473.7791	7
7	795.3995	398.2034	778.3730	389.6901	777.3890	389.1981	T	**851.4774**	426.2423	834.4509	417.7291	833.4668	417.2371	6
8	958.4629	479.7351	941.4363	471.2218	940.4523	470.7298	Y	**750.4297**	375.7185	733.4032	367.2052			5
9	**1144.5422**	572.7747	1127.5156	564.2615	1126.5316	563.7694	W	**587.3664**	294.1868	570.3398	285.6736			4
10	1257.6263	629.3168	1240.5997	620.8035	1239.6157	620.3115	L	**401.2871**	201.1472	384.2605	192.6339			3
11	1370.7103	685.8588	1353.6838	677.3455	1352.6997	676.8535	I	288.2030	144.6051	271.1765	136.0919			2
12							R	175.1190	88.0631	158.0924	79.5498			1

Note: There are three different types of bonds that can fragment along the amino acid backbone: the NH-CH, CH-CO, and **CO-**NH bonds. Each bond breakage gives rise to two species, one neutral and the other one charged, and only the charged species is monitored by the mass spectrometer. The most common cleavage sites are at the CO-NH bonds which give rise to the b ions (charge retained on the N-terminal fragment) and/or the y ions (charge retained on the C-terminal fragment). The mass difference between two adjacent b ions, or y ions, is indicative of a particular amino acid residue.

APPENDIX F: WHITEFLY TRANSMISSION EXPERIMENTS

F.1 Introduction

Sweet potato leaf curl virus (SPLCV), a begomovirus that has been reported infecting sweetpotato breeding lines in the United States, is transmitted by the sweetpotato whitefly *Bemisia tabaci* at very low levels under greenhouse conditions (Lotrakul *et al*., 1998; Valverde *et al*., 2004). Most transmission experiments of SPLCV reported in the literature used indicator plants (*Ipomoea setosa*, *I. nil*, and *I. aquatica*) as acquisition and transmission hosts. However, in order to better understand epidemiological aspects of SPLCV spread in natural conditions, sweetpotato cultivars need to be used as acquisition and recipient hosts in whitefly transmission experiments.

F.2 Materials and Methods

F.2.1 Whitefly Transmissions

Transmission experiments were conducted using sweetpotato whitefly *B. tabaci* colonies that were reared on *I. nil* and tomato plants kept in plexiglass cages under greenhouse conditions (From June to September, Baton Rouge, LA). Whiteflies were allowed to feed for 72 or 96 h on SPLCV infected *I. batatas* cv. Beauregard and 48 h on SPLCV infected *I. nil*. Groups of five and 10 whiteflies were then transferred to single *I. nil* seedlings (6-day-old) (Figure G.1). After 48 h, whiteflies were removed and plants were maintained in an insect-free cage in the greenhouse. Between two and four weeks after inoculation, plants were evaluated for SPLCV infection by symptom development and polymerase chain reaction.

F.2.2 Polymerase Chain Reaction (PCR)

Total DNA was extracted from foliar tissues of *I. nil* using DNeasy Plant Mini Kit (Qiagen, Valencia, CA), following the procedure provided by the manufacturers.

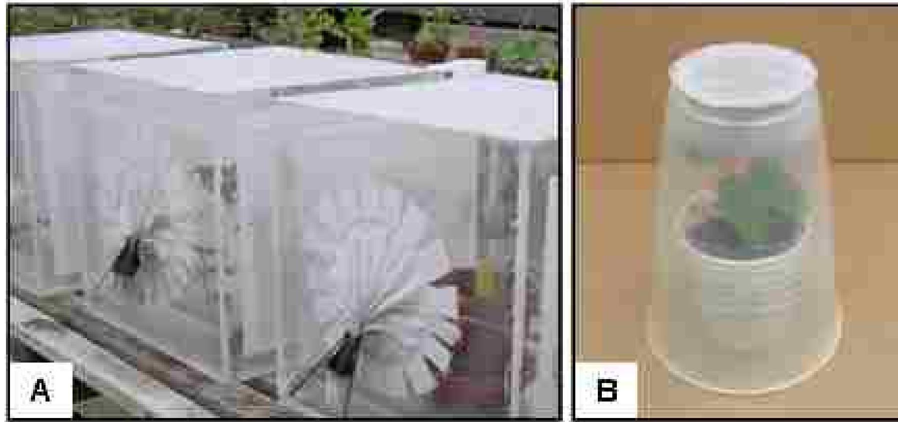


Figure G.1. Whitefly transmission of *Sweet potato leaf curl virus*. (A) Whitefly colonies reared on *Ipomoea nil* plants. (B) Transmission of individual *I. nil* plant.

Degenerate primers SPG1 and SPG2 described by Li *et al*. (2004) were used to detect SPLCV. PCR reaction mixtures were conducted as described by Lotrakul *et al*. (1998) in 50 µl volume containing: 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.2 µM of each primer, 2.5 U of Taq DNA polymerase (Promega, Madison, WI) and 1 µl of DNA sample. PCR was performed in a Genius Thermocycler (Techne, Cambridge, UK) with an initial cycle of 94 ˚C for 1.5 min followed by 35 cycles of 94 ˚C for 40 s, 56 ˚C for 40 s, and 72 ˚C for 1.5 min, and a final extension cycle of 72 ˚C for 10 min. PCR products were assessed by electrophoresis in 1.2 % agarose gels in TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light.

F.3 Results

Two and 4 weeks after whitefly inoculations, *I. nil* began showing typical leaf curl symptoms (Figure G.2). Asymptomatic and symptomatic *I. nil* plants were tested by PCR. Degenerate primers SPG1/SPG2 amplified a 912 bp DNA fragment only from symptomatic plants (Figure G.3).

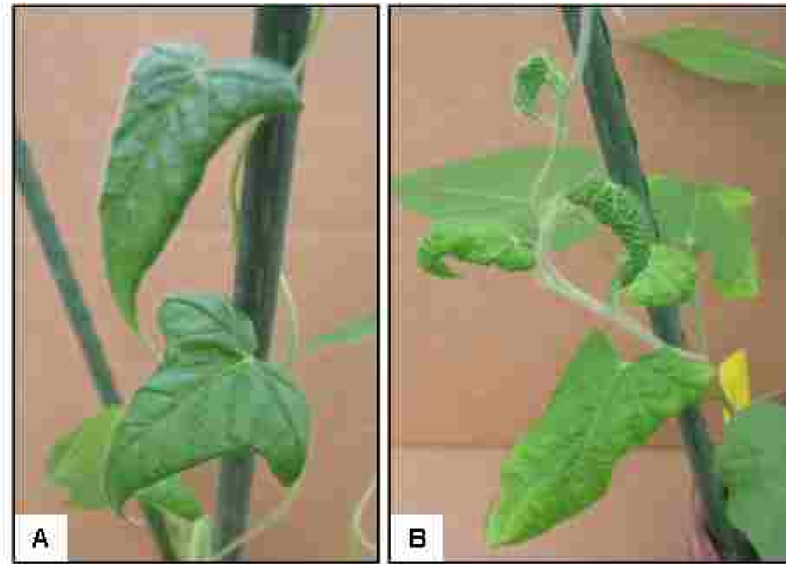


Figure G.2. Symptoms caused by *Sweet potato leaf curl virus* on *Ipomoea nil* after whitefly transmission. Leaf curl symptoms (A) and chlorosis with severe leaf curl symptoms (B).

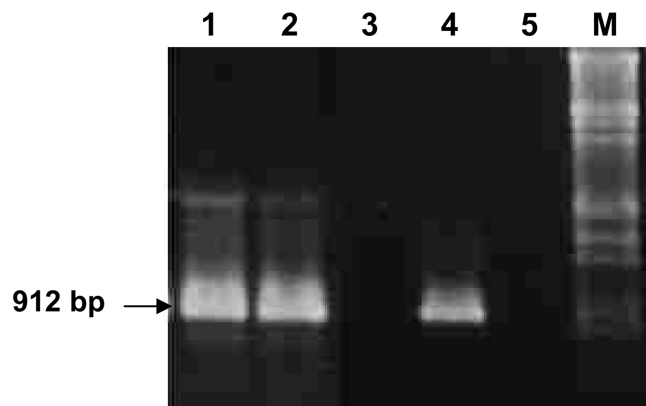


Figure G.3. PCR products resulting from amplification with primers SPG1/SPG2. Lanes 1-2, *Ipomoea nil* infected with *Sweet potato leaf curl virus* (SPLCV) by whitefly transmission. Lane 3, healthy *I. nil.*Lines 4, SPLCV positive control (*I. setosa*). Lane 5, water control. Lane M, DNA ladder.

Low whitefly transmission rates were obtained when *I. nil* plants infected with SPLCV were used as the acquisition hosts. However, transmission was not obtained when *I. batatas* cv. Beauregard was used as acquisition host (Table G.1).

Table G.1. Transmission of *Sweet potato leaf curl virus* (SPLCV) by *Bemisia tabaci*

Experiment	SPLCV source[a]	Number of whiteflies[b]	Acquisition access period	SPLCV transmission[c]
1	*Ipomoea batatas* cv. Beauregard	5	72 h	0/6
2	*I. batatas* cv. Beauregard	5	96 h	0/2
1	*I. nil*	10	48 h	5/9
2	*I. nil*	10	48 h	3/7
3	*I. nil*	10	48 h	2/8
4	*I. nil*	10	48 h	10/20

[a] Transmissions were made from SPLCV infected plants to *Ipomoea nil*. Acquisition access periods of 48, 72 and 96 h, and an inoculation access period of 48 h were tested.
[b] Individual *I. nil* plants were exposed to groups of five and ten whiteflies. [c] Infected plants/plant tested.

F.4 Conclusions

Several attempts to transmit SPLCV from *I. nil* infected plants to *I. nil* seedlings using single whiteflies were not successful. However, when the number of whiteflies was increased (groups of 10), SPLCV was transmitted at very low rates. Similar results were reported by Lotrakul *et al*. (1998) and Valverde *et al*. (2004). Whitefly transmissions were not successful when *I. batatas* cv. Beauregard was used as acquisition host.

Environmental conditions play an important role in symptom expression of SPLCV on *I. nil* and other indicator plants. Typical leaf curl symptoms were consistent on *I. nil* from June to September under greenhouse conditions in Baton Rouge, LA. For this reason, whitefly transmissions were conducted during this period.

The results of these preliminary experiments suggest that whiteflies were more efficient in transmitting SPLCV from *I. nil* to *I. nil*, when they were reared on *I. nil* than on tomato plants. Hosts can play an important role in whitefly transmission of begomoviruses. Antony *et al*. (2006) have reported that *Indian cassava mosaic virus* was successfully transmitted from cassava to cassava by whiteflies reared on cassava, but not whiteflies reared on sweetpotato. They found that the activities of the cyanide detoxifying enzymes rhodanese and β-cyanoalanine synthase in *B. tabaci* were significantly higher in the cassava-reared whitefly population compared with the sweetpotato-reared population suggesting a possible reason for higher mortality of the sweetpotato-reared whiteflies feeding on cassava.

Experiments dealing with SPLCV whitefly trasmision efficiency such as temperature, biotypes and age of whiteflies, and hosts, need to be conducted.

F.5 Literature Cited

Antony, B., Lisha, V.S., Palaniswami, M.S., Sugunan, V.S., Makeshkumar, T., and Henneberry, T.J. 2006. *Bemisia tabaci* (Homoptera: Aleyrodidae) and *Indian cassava mosaic virus* transmission. International Journal of Tropical Insect Science 26: 176-182.

Li, R., Salih, S., and Hurtt, S. 2004. Detection of geminiviruses in sweetpotato by polymerase chain reaction. Plant Disease 88: 1347-1351.

Lotrakul, P., Valverde, R.A., Clark, C.A., Sim, J., and De La Torre, R. 1998. Detection of a geminivirus infecting sweet potato in the United States. Plant Disease 82: 1253-1257.

Valverde, R.A., Sim, J., and Lotrakul, P. 2004. Whitefly transmission of sweetpotato viruses. Virus Research 100: 123-128.

VITA

Dina Lida Gutiérrez Reynoso, daughter of David F. Gutiérrez Michuy and Dina C. Reynoso de Gutiérrez, was born in September 1975, in Lima, Peru. She graduated from La Universidad Nacional Agraria, La Molina, in 1998 with a Bachelor of Science degree in biology, concentration biotechnology. Her entry into the world of plant viruses began with her training in plant virus detection at the International Potato Center in Lima, under the guidance of M.S. Segundo Fuentes and Dr. Luis F. Salazar. Due to her outstanding performance, the International Potato Center awarded her a scholarship to conduct research on *Sweet potato chlorotic stunt virus* and sweet potato virus disease in Peru. As a result of her research, she received the title of Biologist in 2001 from La Universidad Nacional Agraria, La Molina. In 2003, she joined the Department of Plant Pathology and Crop Physiology at Louisiana State University to pursue her doctoral degree under the direction of Dr. Rodrigo A. Valverde. Throughout her time at LSU, she has had the opportunity to learn new techniques for cloning and sequencing viral genes, as well as, expressing viral genes in *E. coli*. She joined the American Phytopathological Society and presented her findings in various conferences. Also, she served as teaching assistant for a general plant pathology course and as secretary of the Plant Pathology and Crop Physiology Graduate Student Association. Ms. Gutiérrez's research efforts were recognized by the Department of Plant Pathology and Crop Physiology by making her the recipient of the 2008 C.W. Edgerton Award. She will receive the degree of Doctor of Philosophy at the Fall 2008 Commencement.