Genetic Variability and Interactions of Three Sweetpotato Infecting Viruses

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2004

Acta Universitatis Agriculturae Sueciae
Agraria 477

ISSN 1401-6249
ISBN 91-576-6764-0

Tryck: SLU Service/Repro, Uppsala 2004

Abstract

Mukasa, S.B. 2004. Genetic variability and interactions of three sweetpotato infecting viruses. Doctor's dissertation.
ISSN 1401-6249, ISBN 91-576-6764-0

The studies presented in this thesis contribute to improved understanding of genetic variability and interactions of the most prevalent sweetpotato infecting viruses in Uganda. *Sweet potato feathery mottle virus* (SPFMV; genus *Potyvirus*), *Sweet potato mild mottle virus* (SPMMV; genus *Ipomovirus*), *Sweet potato chlorotic stunt virus* (SPCSV; genus *Crinivirus*) and *Sweet potato chlorotic fleck virus* (SPCFV; genus *Carlavirus*) were serologically detected, and the positive results were confirmed by RT-PCR and sequence analysis. Molecular phylogenetic studies revealed high genetic variability in SPMMV and SPFMV whereas SPCSV isolates from East Africa seem less variable. At a molecular level, strains are now more recognised in SPFMV and SPMMV.

Co-infections of SPCSV with SPFMV and/or SPMMV were associated with persistent and more severe symptoms than infections with each of the viruses alone, and were responsible for over 90% of the field diseased plants. Novel and severe symptoms including chlorosis, rugosity, leaf strapping and dark green islands caused by the dual infection of SPMMV and SPCSV were observed. These symptoms could be differentiated from those caused by dual infection of SPFMV and SPCSV that is associated specifically with the sweetpotato virus disease (SPVD), characterised by often severe stunting of the plant, distortion and either chlorotic mottle or vein clearing of the leaves. These observations show SPCSV, SPFMV and SPMMV to be the most important viruses of sweetpotato in Uganda. The name "sweetpotato severe mosaic disease" (SPSMD) is proposed to describe the disease caused by the dual infection of SPMMV and SPCSV.

A comparative analysis of the interaction between the phloem-limited SPCSV and either SPFMV or SPMMV indicated more severe disease symptoms and much higher tuber yield reduction as compared to singly infected plants. Immunohistochemical localisation of SPMMV in sweetpotato plants suggest that it could be exploiting niches (mesophyll, companion and epidermal cells) similar to those of SPFMV. SPMMV and SPFMV RNA accumulation greatly increased by over 32 and 64 fold, respectively, in mixed infection with SPCSV. However, accumulation of SPCSV in mixed infection with SPMMV or SPFMV was reduced by 2-4 fold, indicating an antagonistic interaction. Neutral (non-detectable) interactions were observed between SPFMV and SPMMV. The synergistic, antagonistic and neutral interactions were observed in plants of the three sweetpotato cultivars, suggesting that they may represent typical interactions of three viruses. These findings further show the importance of SPCSV in elevating virulence of SPFMV and SPMMV, by suppressing the otherwise high levels of host resistance to single infections in East African sweetpotato cultivars. Efforts to particularly control SPCSV (e.g. through RNA-mediated resistance, use of clean planting materials) and improved knowledge on various aspects of the ecology of sweetpotato viruses in Uganda seem to be the next necessary steps towards better management strategies for sweetpotato viral diseases.

Keywords: Genetic variation; Viral synergism; Cultivar reaction; *Ipomoea batatas*

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Eri mukyala wange, *Sylvia*, n’ezadde lya ffe; maama *T.K Kiwanuka* ne banyinazze, era n’eri omubuze taata *S.K Luyimbazi-Kiwanuka*

Settumba Blasio Billy MUKASA

Yeebazibwe oyo ankuumye, nampa omukisa, amagezi n’amaanyi. Ekitiibwa, n'ettendo, n'obuyinza, biberenga eri Mukama.

Everything should be as simple as it can be yet no simpler
[*Albert Einstein*]

Contents

To raise new questions, new possibilities, to regard old problems from a new angle
requires creative imagination and marks real advances in science.
[*Albert Einstein*]

Appendix

Papers I-IV

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Mukasa, S.B**., Rubaihayo, P.R. and Valkonen, J.P.T. 2003. Incidence of viruses and virus-like diseases of sweetpotato in Uganda. *Plant Disease* 87, 329-335.

II. **Mukasa, S.B**., Tairo, F., Kreuze, J.F., Kullaya, A., Rubaihayo, P.R. and Valkonen, J.P.T. 2003. Coat protein sequence analysis reveals occurrence of new strains of SPFMV in Uganda and Tanzania. *Virus Genes* 27, 49-56.

III. **Mukasa, S.B**., Rubaihayo, P.R. and Valkonen, J.P.T. 2003. Sequence variability within the 3'-proximal part of the Sweet potato mild mottle virus genome. *Archives of Virology* 148, 487-496.

IV. **Mukasa, S.B**., Rubaihayo, P.R. and Valkonen, J.P.T. 2004. Viral synergism: a crinivirus enhances virulence of a potyvirus and an ipomovirus in sweetpotato plants. *Submitted*.

Abbreviations

aa	Amino acid
cDNA	Complementary DNA
CI	Cylindrical inclusion protein
CIP	International potato center
CP	Coat protein
cv.	Cultivar
DAS	Double antibody sandwich
dsRNA	double stranded RNA
ELISA	Enzyme linked immunosorbent assay
HC-Pro	Helper component proteinase
Hel	Helicase
Hsp70h	Heat shock 70 family protein homologue
kb	Kilo bases (nucleotides)
kDa	Kilo dalton
MAb	Monoclonal antibody
mCP	Minor coat protein
NCM	Nitrocellulose membrane
NIb	Nuclear inclusion protein b
nt	Nucleotide
ORF	Open reading frame
PAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDR	Pathogen derived resistance
P-Pro	Papain like proteinase
RdRp	RNA-dependent RNA polymerase
RNaseIII	Ribonuclease III
RT-PCR	Reverse transcription polymerase chain reaction
SPCFV	*Sweet potato chlorotic fleck virus*
SPCSV	*Sweet potato chlorotic stunt virus*
SPFMV	*Sweet potato feathery mottle virus*
SPMMV	*Sweet potato mild mottle virus*
SPVD	Sweet potato virus disease
SPVG	*Sweet potato virus G*
TAS	Triple antibody sandwich
VPg	Viral protein genome linked
WAP	Weeks after planting

Introduction

Sweetpotato

Sweetpotato (*Ipomoea batatas* L.) is a dicotyledonous, perennial plant that produces edible tuberous roots. It belongs to the morning glory family *Convolvulaceae*, and genus *Ipomoea* that is thought to contain over 500 species with ploidy levels ranging from 2x to 6x (Ozias-Akins and Jarret, 1994). Sweetpotato is the only *Ipomoea* species of economic importance as a food crop (Woolfe, 1992). It has both 4x and 6x forms (2n = 4x = 60 or 2n= 6x = 90) and probably originates from a cross between the ancestors of *I. trifida* and another wild *Ipomoea* sp., in Central or Northern South America, at least 5000 years ago (Huang and Sun, 2000).

Thousands of sweetpotato cultivars are grown throughout the tropics and subtropics (He *et al*., 1995). Because of the enormous genetic diversity of sweetpotato, the crop has great potential for improvement to accommodate specific uses (Zhang *et al*., 2000). Among the major starch staple crops, it has the largest rates of production per unit area per unit time (Woolfe, 1992). With an annual production of more than 133 million tons globally, sweetpotato ranks as the seventh most important food crop on a fresh-weight basis in the world, and fifth in over 50 developing countries after rice, wheat, maize, and cassava (CIP, 1999a). Production is concentrated in East Asia, the Caribbean and tropical Africa, with the bulk of the crop (88%) being grown in China (Hijmans *et al*., 2001). In Africa sweetpotato is the second most important tuber crop after cassava and production is concentrated in the East African countries around Lake Victoria. At an annual production of 1.8 m tons, Uganda is the biggest producer of sweetpotato in Africa and third in the world (Hijmans *et al*., 2001).

Sweetpotato is grown in several agro-ecological zones and usually plays significant roles in the farming and food systems. Farmers may leave vines in the field to improve soil fertility and is commonly used in crop rotations. It has a short growing period, stores well in soil as a famine reserve crop and performs relatively well in marginal soils, which makes it an ideal crop for food security. The crop has been the focus of global effort, steered by the International Potato Center (CIP, Lima, Peru) to realize its full potential as a source of food, feed, processed products, and source of income for millions of resource-poor farmers in developing countries. The yellow and orange-fleshed sweetpotato varieties are particularly a good source of the essential vitamin A that is frequently lacking in developing countries (CIP, 1999b). Processed products made from sweetpotato include starch, noodles, snacks, liquor, candy, desserts and flour. In addition to being used for human consumption, sweetpotato is also widely used as an animal feed (CIP, 2000) in some form and amount. Despite the advantages that the cultivation of sweetpotato offers, production is mostly in developing countries, and by resource poor-farmers. Because of its significance, the crop is one of the high priority commodities in the research and development agenda of National Agricultural Research Programmes of the East African countries.

Sweetpotato productivity is limited by both abiotic and biotic constraints, including those of viral origin, leading to poor yields at farm level. The sweetpotato virus disease (SPVD), usually caused by the dual infection of *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV), is the most severe disease affecting the crop and can cause 56-98% yield loss (Gibson *et al*., 1998). SPVD epidemics have been, in many cases, associated with the disappearance of the once elite cultivars (Gibson *et al*., 1997). This undermines previous and ongoing efforts in genetic improvement for yield and quality e.g. cultivars with increased starch content and vitamin A levels. These problems are further compounded by the gross lack of virus-indexing protocols and clean seed systems (planting materials are the common source of virus inoculum), and knowledge of the nature of the virus disease complexes. Development of strategies to control viral diseases requires knowledge on the molecular characteristics of the viruses, their interactions with the host plants and the different mechanisms by which they evolve and spread.

Viruses: the intriguing molecular pathogens

A virus is a set of one or more nucleic acid template molecules, normally encased in a protective coat or coats of protein or lipoprotein that is able to organize its own replication only within suitable host cells (Matthews, 1991). Within such cells, virus replication is (i) dependent on the host's protein synthesizing machinery, (ii) organized from pools of the required materials rather than by binary fission, (iii) located at sites that are not separated from the host cell contents by a lipoprotein bilayer membrane. Such a definition clearly distinguishes a virus from other disease agents such as viroids, mycoplasma and the rickettsia group of bacteria, which cause virus-like symptoms in plants. The Nobel Prize winner for Medicine and Physiology in 1960, Peter Medawar, defined a virus as a piece of nucleic acid surrounded by bad news! Indeed, most viruses cause disease. Viruses are sub-microscopic obligate intracellular parasites that infect every class of living organisms. By utilizing cellular substances and disrupting cellular processes, viruses upset the host metabolism leading to development of abnormal compounds and conditions injurious to the functions and life of the infected organism. Several epidemics of dreadful diseases in humans such as influenza, yellow fever, polio, rabies, smallpox, ebola hemorrhagic fever, human immunodeficiency virus (HIV)-AIDS and the severe acute respiratory syndrome (SARS) are caused by viruses. Viruses also cause devastating diseases in livestock and crops, thus causing enormous economic losses and human suffering throughout the world (Bos, 1999).

A complicating factor in understanding epidemiology of viruses and co-evolution with their hosts is the fact that viruses apparently can make big jumps in hosts (Rybicki, 1994). However, this is more likely to happen between more closely related hosts. An extreme case is that of virus species that belong to the family *Bunyaviridae* that infect plants (Tospoviruses) and animals (Bunyaviruses) (Kikkert *et al*., 1999). There is also evidence that a plant virus switched hosts to infect a vertebrate and then recombined with a vertebrate-infecting virus (Gibbs & Weiller, 1999).

Virus families

The highest level of virus classification recognises six major groups (van Regenmortel *et al*., 2000), based on the nature of the genome: (i) Double-stranded DNA (dsDNA); which is defined to include viruses that replicate without an RNA intermediate. It includes viruses with the largest known genomes (up to about 400 kb) and there is only one genome component, which may be linear or circular. Well-known viruses in this group include the herpes and pox viruses. There are no plant viruses in this group, (ii) Single-stranded DNA (ssDNA); there are two families of plant viruses (*Geminiviridae* and *Nanoviridae*) in this group and both of these have small circular genome components, often with two or more segments, (iii) Reverse-transcribing viruses; these have dsDNA or ssRNA genomes and their replication is mediated the enzyme reverse transcriptase and many integrate into their host genomes. The group includes the retroviruses e.g. HIV. There is a single family of plant viruses (*Caulimoviridae*) in this group that is characterised by a single component of circular dsDNA, the replication of which is via an RNA intermediate, (iv) Double-stranded RNA (dsRNA); some plant viruses (e.g. *Partiviridae*, *Reoviridae*) and many of the mycoviruses are included in this group, (v) Negative sense single-stranded RNA (ssRNA-); in this group, some or all of the genes are translated into protein from an RNA strand complementary to that of the genome (as packaged in the virus particle). There are some plant viruses (e.g. *Rhabdoviridae*, *Bunyaviridae*) in this group and it also includes the viruses that cause measles, influenza and rabies, (vi) Positive sense single-stranded RNA (ssRNA+); this group includes many viruses that cause respiratory diseases (e.g. SARS *Coronavirus*), and the causal agents of polio and foot-and-mouth disease. The majority of plant virus families are included in this group e.g. *Bromoviridae*, *Closteriviridae*, *Flexiviridae*, *Comoviridae*, *Potyviridae*, *Sequiviridae* and *Tombusviridae*.

Within each of these virus groups, many different characteristics are used to classify the viruses into families, genera and species. Typically, a combination of characters is used and some of the most important are particle morphology, genome, serological, and biological properties (Matthews, 1991). Many viruses are good antigens (elicit strong antibody production when purified preparations are injected into a mammal) and this property has been widely exploited to produce specific antibodies that can be used for virus detection and for examining relationships between viruses. For example, the use of antibodies raised against the viral coat protein (CP) is widely applied for detection of plant viruses. It has also been used to identify new viruses and to estimate how closely related they are as compared to the previously described viruses. Although serological properties e.g. ELISA procedures are still important, their significance in taxonomy has declined to some extent now that nucleotide sequence data are available. Nucleotide sequence data, especially of more conserved genomic regions, is now widely used to group viruses in families, genera and species (van Regenmortel *et al*., 2000). The important viruses of sweetpotato belong to two families namely *Potyviridae* and *Closteroviridae*; there are also reported cases of *Geminiviridae* and *Flexiviridae* (*Carlavirus*) members infecting sweetpotato (see Table 1).

Genetic variability and population structure of plant viruses

Variation is an intrinsic property of living entities, a property long recognized in plant virology (McKinney, 1935; Kunkel, 1949). Indeed, a virus species constitutes a pool of variants termed "quasispecies" which are centred on a master sequence (Eigen, 1996; Roossink, 1997). The existence of genetic variants within a virus population increases the probability of survival and ability of viruses to adapt to different hosts. However, the high potential for genetic variation, through either mutation or genetic exchange by recombination or re-assortment of genomic segments, may not result in high diversity. Selection by factors such as interaction of the virus with host plants and vectors (Albiach-Marti *et al*., 1999) and random genetic drift (Fraile *et al*., 1997) may in fact reduce genetic diversity in virus populations. Plant virus populations often consist of a few genetic variants whose distribution may provide evidence of a population that is undifferentiated, differentiated by factors such as location, host plant or time (Garcia-Arenal *et al*., 2001).

The distribution of genetic variants in a population of an organism (i.e., population genetic structure) may change with time, a process called evolution. Evolution may lead to the rise of different taxonomic entities. The study of variability and changes in the population genetic structure of plant viruses is an important aspect of plant pathology. For instance, the effectiveness of control strategies can be compromised by evolution of the pathogens. In addition, plant viruses may be good models to address general questions of evolutionary biology (Garcia-Arenal *et al*., 2001).

Previous work show that virus mutants arise easily upon amplification of biologically or molecularly cloned inocula, giving raise to a heterogeneous population (Garcia-Arenal *et al*., 1984; Kurath and Dodds, 1995). Serial passaging under different hosts can generate variants and alter viral properties (Yarwood, 1979; Kearney *et al*., 1999). Even within the same host species and cropping system, viruses may change in incidence and virulence (Escriu *et al*., 2003). It is thought that the broad host range and worldwide distribution of *Cucumber mosaic virus* (CMV; genus *Cucumovirus*) is in part due to reassortment, contributing to its evolutionary success (Roossnick, 2002). Recombination events, which involve exchange of genetic material between two nearly identical or different RNAs, have been reported in various plant viruses (Bousalem *et al*., 2000; Moonan *et al*., 2000), and also demonstrated experimentally for *Potato virus A* (PVA; genus *Potyvirus*) isolates to express novel virulence phenotypes in plants (Paalme *et al*., 2004). Recombinations have also been reported for DNA viruses giving rise to more virulent strains (Chenault and Melcher, 1994; Fondong *et al*., 2000).

These observations suggest that the population of the "same" virus in different hosts or crop varieties could be different. This could have implications on the subsistence sweetpotato cultivation where farmers grow several varieties in the same field. Furthermore, the possible source of viruses from alternate hosts or wild plants is real given their abundance and proximity to sweetpotato fields. Therefore, the study of incidence, genetic variability and inferences to their evolutionary biology are important in development of strategies for control of viral diseases.

Sweetpotato-infecting viruses

Since early reports of suspected viral diseases of sweetpotato in USA and Eastern Africa in 1930s they are now reported worldwide wherever sweetpotato is grown (Moyer and Salazar, 1989). The first report of a suspected viral disease of sweetpotato in Eastern Africa was in 1939 from Ituri province of the Democratic Republic of Congo and then in 1944 from Uganda (Hansford, 1944). Later, sweetpotato viral diseases were reported in the present Kenya, Tanzania, Rwanda, Burundi, Malawi and Transvaal province of South Africa (Sheffield, 1957). Bioassays (host range, symptoms and vector transmission) were largely used to explain the aetiology of the various diseases. Since then many virus and virus-like diseases of sweetpotato have been described from several parts of the world (Moyer and Salazar, 1989). Advances in microscopy, serology and molecular biology techniques have resulted in better methods for virus detection, identification and characterisation. Worldwide, up to 20 different viruses have been described to infect sweetpotato (Loebenstein *et al*., 2003), but only 11 of them are currently recognized by the International Committee of Taxonomy for Viruses (see Table 1).

With the emerging sequence data for virus isolates from different parts of the world, it will be possible to more clearly distinguish the different viruses and virus strains that infect sweetpotato. Sequences for viruses isolated from sweetpotato are only available for 10 distinct viruses (Table 1). SPFMV and SPCSV probably occur wherever sweetpotato is grown (Moyer and Salazar, 1989; Table 1). In East Africa, SPFMV, SPCSV and SPMMV commonly occur in sweetpotato (Sheffield, 1957; Hollings, 1976; Aritua *et al*., 1998; Gibson *et al*., 1998; Kreuze *et al*., 2000). A few isolated cases of sero-occurrence of SPCaLV (Aritua *et al*., 1998; Gibson *et al*., 1998) and SPCFV (Gibson *et al*., 1998; Ateka *et al*., 2004; Tairo *et al*., 2004) have been reported in the region. Although reported elsewhere (Cohen *et al*., 1997; Lotrakul *et al*., 1998; Banks *et al*., 1999), begomoviruses (family *Geminiviridae*) have not been reported in East Africa.

Sweet potato feathery mottle virus (SPFMV)

SPFMV (genus *Potyvirus*, family *Potyviridae*) is the most common virus infecting sweetpotatoes worldwide (Moyer and Salazar, 1989). The family *Potyviridae* contains six genera: *Potyvirus*, *Macluravirus*, *Bymovirus*, *Rymovirus*, *Tritimovirus* and *Ipomovirus* that usually have monopartite genomes, with the exception of the bipartite bymoviruses (van Regenmortel *et al*., 2000). The different genera seem to be transmitted by different vectors (van Regenmortel *et al*., 2000). Potyviruses are transmitted by aphids, rymoviruses and tritimoviruses are transmitted by mites of the genus *Abacarus* or *Aceria*, respectively. Ipomoviruses are transmitted by whiteflies whereas bymoviruses are transmitted by plasmodiphorids (root-infecting parasites once considered to be fungi). Potyviruses constitute the largest and economically most important group of plant viruses (Shukla *et al*., 1994; Fauquet and Mayo, 1999). Currently, the genus *Potyvirus* contains 118 definite and 82 tentative species (van Regenmortel *et al*., 2000), which represent more than 30% of all known plant viruses. Traditionally potyviruses were recognized by their particle morphology (flexuous filaments of 11-15 nm diameter and 650-900

nm length) and the typical 'pinwheel'-like cylindrical inclusion bodies, formed by viral proteins aggregating in the cytoplasm of infected cells (Matthews, 1991).

Table 1. *Some viruses that have been reported from sweetpotato*

Virus species [a]	Virus genus	Virus distribution	Reference(s)
Sweet potato chlorotic stunt virus (SPCSV) [15]	*Crinivirus*	Worldwide	Cohen *et al*., 1992; Winter *et al*., 1992; Hoyer *et al*., 1996; Alicai *et al*., 1999.
Sweet potato mild mottle virus (SPMMV) [1]	*Ipomovirus*	East Africa	Hollings *et al*., 1976; Colinet *et al*., 1996.
Sweet potato feathery mottle virus (SPFMV) [24]	*Potyvirus*	Worldwide	Abad *et al*., 1992; Colinet & Kummert, 1993; Sakai *et al*., 1997; Kreuze *et al*., 2000.
Sweet potato latent virus (SPLV) [3]	*Potyvirus*	Taiwan, Peru, China, Japan	Liao *et al*., 1979; Colinet *et al*., 1997; Yun *et al*., 2002.
Sweet potato mild speckling virus (SPMSV) [1]	*Potyvirus*	Argentina, Peru, Brazil	Alvarez *et al*., 1997.
Sweet potato virus Y (SPVY) [3]	*Potyvirus*	Taiwan, USA	Ateka *et al*., 2004; Souto *et al*., 2004.
Sweet potato virus G (SPVG) [5]	*Potyvirus*	China, Egypt, USA	Colinet *et al*., 1994; IsHak *et al*., 2003; Souto *et al*., 2004.
Sweet potato leaf curl virus (SPLCV) [3]	*Begomovirus: Geminiviridae*	USA, Sicily	Lotrakul *et al*., 1998; Briddon R.W., Unpubl.
Sweet potato leaf curl Georgia virus (SPLCGV) [1]	*Begomovirus: Geminiviridae*	USA	Lotrakul P., Unpubl.
Ipomoea yellow vein virus (IYVV) [1]	*Begomovirus: Geminiviridae*	Spain	Banks *et al*., 1999.
Cucumber mosaic virus (CMV) [0]	*Cucumovirus*	Israel, Egypt, Kenya	Cohen & Loebenstein, 1991; Ishak *et al*., 2002.
Sweet potato chlorotic fleck virus (SPCFV) [0]	*Carlavirus*?	S. America, East Africa	CIP, 1993; Aritua *et al*., 2003; Ateka *et al*., 2004; Tairo *et al*., 2004.
Sweet potato leaf speckling virus (SPLSV) [0]	*Luteovirus*?	Peru, Cuba	Fuentes *et al*., 1996.
Sweet potato yellow dwarf virus (SPYDV) [0]	*Ipomovirus*?	Taiwan, Far East	Liao *et al*., 1979.
Sweet potato vein mosaic virus (SPVMV) [0]	*Potyvirus*?	Argentina	Nome *et al*., 1974.
Sweet potato ringspots virus (SPRSV) [0]	*Nepovirus*?	Papua New Guinea	Brown *et al*., 1988.
Sweet potato caulimolike virus (SPCaLV) [0]	*Caulimovirus*?	Puerto Rico	Atkey & Brunt, 1987.

[a] = Superscript values indicate number of isolates for which sequence data is reported.
? = Unassigned genus.

SPFMV contains a single positive stranded RNA genome of about 10.8 kb (SPFMV-S; Sakai *et al*., 1997), which is larger than the average (9.7 kb) of a potyvirus genome (Shukla *et al*., 1994). SPFMV CP is also exceptionally large (38 kDa) as compared to other potyviruses, which is largely due to the insertion of a contiguous sequence at the 5′-end of the CP cistron (Abad *et al*., 1992). Like other potyviruses the genome contains a single ORF, flanked by a UTR at both 5′- and 3′-ends (Fig. 1) and it encodes a large polyprotein (3493 aa) that is processed to mature proteins by virus-encoded proteases: P1, HC-Pro and NIa-Pro (Riechman *et al*., 1992). The P1 and HC-Pro mediate their own cleavage from the polyprotein (Carrington *et al*., 1989) whereas the NIa-Pro is responsible for the cleavage of the C-terminal two-thirds of the polyprotein (Dougherty and Carrington, 1988). The primary events are probably co-translational and autocatalytic, yielding precursors and mature products. The fully processed proteins are: P1 proteinase (P1-Pro), helper component proteinase (HC-Pro), the third protein (P3), 6 kDa protein 1 (6K1), cylindrical inclusion protein that is an RNA helicase (CI), 6 kDa protein 2 (6K2), nuclear inclusion protein a (NIa), which can be further processed into the viral protein genome linked (VPg) and the NIa proteinase (Pro). The last two proteins are the nuclear inclusion protein b (NIb) and the CP. The function of each mature protein has been described (Table 2).

The co-infection of SPFMV and *Sweet potato chlorotic stunt virus* (SPCSV; genus *Crinivirus*), is associated with SPVD, the most important disease of sweetpotato crops in Africa (Gibson *et al*., 1998; Karyeija *et al*., 1998a). SPFMV is transmitted by several aphid species (e.g. *Aphis gossypii*, *A. craccivora*, *Myzus persicae*) in a non-persistent manner. The host range of SPFMV is narrow and mostly limited to plants of the family *Convolvulaceae*, and especially to the genus *Ipomoea*. Some strains have been reported to infect *Nicotiana benthamiana* and *Chenopodium* spp. (Campbell *et al*., 1974; Moyer *et al*., 1980).

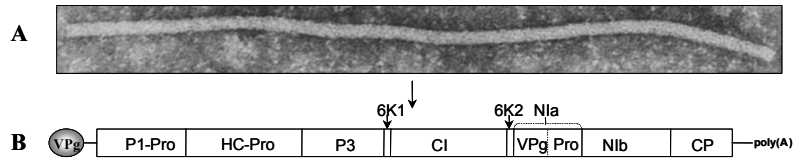


Fig. 1. Particle morphology and genome organization of potyviruses (Shukla *et al*., 1994), which is similar to that of ipomoviruses (Colinet *et al*., 1998). The particle consists of flexuous filamentous rods (**A**) that encapsidate a single positive stranded RNA molecule (**B**). The genome contains one open reading frame and names of the final protein products are indicated as boxes, separated by lines that indicate the putative cleavage sites of the polyprotein. The 5′- and 3′-untranslated regions are represented by single lines. The RNA genome is 3′-polyadenylated and has a viral protein (VPg) linked to the 5′-end.

Discrimination between potyvirus species and isolates was predominantly based on serology and biological properties such as host range, cross protection and symptomatology (Shukla *et al*., 1994). However, when dealing with potyviruses, use of serological methods has sometimes been hampered by serological relationships between virus species (cross-reactions) or lack of detection of all strains of a given virus (Spetz *et al*., 2003). Cross-reactions are probably due to

the recognition by antibodies of highly conserved core of the CP. Lack of detection of some strains is probably due to the variability of the N-terminus of the CP, which also may be lost due to proteolysis during virus extraction from plant tissues (Shukla *et al*., 1994). Current approaches have adopted the use of the genomic sequences, mainly the CP and the 3´-UTR sequences as a criterion for virus classification (Shukla *et al*., 1994; Badge *et al*., 1997; Bousalem *et al*., 2000; van Regenmortel *et al*., 2000;).

Table 2. *Known functions of the mature potyviral proteins*

Protein	Functions	Reference
P1	Protease	Carrington *et al*., 1990.
	Modulator of gene silencing	Anandalakshmi *et al*., 1998.
	Virus replication	Verchot and Carrington, 1995.
HC-Pro	Protease	Carrington *et al*. 1989.
	Aphid transmission	Atreya *et al*., 1992; Sasaya *et al*., 2000
	Seed transmission	Wang and Maule, 1994.
	Suppressor of gene silencing	Kasschau and Carrington, 1998.
	Cell-to-cell and systemic movement	Klein *et al*., 1994; Kasschau *et al*., 1997; Rojas *et al*., 1997.
	Virus replication	Kasschau and Carrington, 1995.
P3	Genome amplification	Merits *et al*., 1999.
	Movement	Suehiro *et al*., 2004.
6K1	Virus replication	Riechmann *et al*., 1992.
CI	RNA helicase	Lain *et al*., 1990.
	Cell- to-cell movement	Carrington *et al*., 1998; Roberts *et al*., 1998.
6K2	Symptoms	Spetz and Valkonen, 2004.
	Long distance movement	Rajamäki and Valkonen, 1999.
	Virus replication	Restrepo-Hartwig & Carrington, 1994.
NIa/VPg	Binds to initiation factor eIF(iso)4E	Wittman *et al*., 1997.
	Cell-to-cell and systemic movement	Schaad *et al*., 1997
	Virus replication	Schaad *et al*., 1996.
NIa/Pro	Protease	Dougherty *et al*., 1989.
	Virus replication	Daros and Carrington, 1997.
NIb	RNA-dependent RNA polymerase	Hong and Hunt, 1996.
CP	Encapsidation of RNA	Jagadish *et al*., 1993.
	Cell-to-cell and systemic movement	Dolja *et al*., 1994; 1995.
	Aphid transmission	Atreya *et al*., 1995.
	Seed transmission	Wang and Maule, 1994.
	Virus replication	Haldeman-Cahill *et al*, 1998.

Phylogenetic analysis of the CP sequences have shown that SPFMV isolates can be placed to four strain groups (Kreuze *et al*., 2000). Isolates of the russet crack strain group (RC) may cause internal corkiness in the tuberous roots of certain sweetpotato cultivars and have been found in China (Chen *et al*., 2001), Japan (Sakai *et al*., 1997), Egypt (IsHak *et al*., 2003), Korea (Ryu *et al*., 1998) and USA (Cali and Moyer, 1981; Abad *et al*., 1992). Isolates of strains group O have been described from Niger, Nigeria, Japan, Korea, China and Argentina (see Kreuze *et al*., 2000). Strain group C contains the most different SPFMV isolates, showing only 75.8 – 78.3 % nt sequence identity to other strains (Kreuze *et al*., 2000), and has been reported only in Argentina, China and USA (Abad *et al*., 1992; Colinet *et al*., 1998). SPFMV isolates from East Africa were exclusively placed to the fourth

strain group (EA). No SPFMV isolate belonging to the EA strain group has been found outside East Africa, and no strain of the other strain groups has been reported in East Africa, which makes East Africa unique as far as the occurrence of SPFMV strains is concerned (Kreuze *et al*., 2000). However, these results are exclusively based on analysis of ten SPFMV isolates from Uganda and one isolate from Madagascar.

Sweet potato mild mottle virus (SPMMV)

SPMMV (genus *Ipomovirus*, family *Potyviridae*) was first isolated in East Africa from sweetpotato plants exhibiting leaf mottling, vein chlorosis, dwarfing and poor growth (Hollings *et al*., 1976). SPMMV has flexuous filamentous particles between 830-850 nm in length. It contains a single positive stranded RNA genome of about 10.8 kb (Colinet *et al*., 1998), which is comparable to that of SPFMV (Sakai *et al*., 1997). SPMMV is the type member of the genus *Ipomovirus* (van Regenmortel *et al*, 2000). Other Ipomoviruses include *Cassava brown streak virus* (CBSV) (Monger *et al*., 2001) and *Cucumber vein yellowing virus* (CVYV) (Lecoq *et al*., 2000). SPMMV was reported to be transmitted by whiteflies (*Bemisia tabaci*) in a persistent manner (Hollings *et al*., 1976).

The morphology and size of SPMMV virions (Hollings *et al*., 1976), the cytoplasmic inclusions SPMMV forms in infected cells (Moyer and Salazar, 1989) and the viral genome organisation (Colinet *et al*., 1998) are similar to those of potyviruses. However, sequences of the CP core region show only limited similarity with other members of the *Potyviridae* (Colinet *et al*., 1996, 1998). Another notably divergent feature of SPMMV as compared to most other viruses infecting sweetpotato is the wide host range that includes species in 14 plant families (Hollings *et al*., 1976). This is striking when compared to SPFMV and SPCSV whose host range is limited to *Ipomoea* species. Unlike for SPCSV (Alicai *et al*., 1999) and SPFMV (Kreuze *et al*., 2000), and using isolates from East Africa, genetic variability studies of SPMMV have not been carried out. Available molecular data on this virus is based on a single isolate from Kenya (Colinet *et al*., 1996) and little is known about SPMMV incidence and prevalence, and genetic variability of SPMMV in Uganda and in the East African region in general.

Sweet potato chlorotic stunt virus (SPCSV)

SPCSV (genus *Crinivirus*) is the only member of the family *Closteroviridae* that is reported to infect sweetpotato (see refs. in Table 1). The family *Closteroviridae* is characterised by its very flexuous and long particles ranging from 950 to up to 2000 nm and 10-13 nm in diameter (Koonin and Dolja, 1993). The family is currently divided into three taxa: *Ampelovirus* (monopartite genome; mealybug-transmitted), *Closterovirus* (monopartite genome; mostly aphid-transmitted) and *Crinivirus* (bipartite genome; whitefly-transmitted). The genomes of different genera (mono- or bi-partite) are linear positive sense ssRNA with up to about 12 ORFs, including two coat proteins towards (but not at) the 3´-terminus. The 3´-terminus has no poly(A) tract and the 5´-terminus probably has a methylated nucleotide cap(m7GpppN) (Karasev *et al*., 1989; Agranovsky *et al*., 1991). The ORFs are translated from a set of overlapping, 3´-coterminal subgenomic RNAs

(Kreuze *et al*., 2002; Hilf *et al*., 1995). Compared to *Potyviridae* less is known about the functions of different proteins encoded by the *Closteroviridae* genome. Some of the known functions of the proteins encoded by the *Closteroviridae* genome are indicated (Table 3). The number of proteins encoded by the viruses can vary between species, ranging from eight in, e.g., *Beet yellows virus* (BYV; genus *Closterovirus*) to as many as 12 in, e.g., *Grapevine leafroll associated virus 3* (GLRaV-3, genus: *Ampelovirus*). They do, however, have a similar genome layout (Fig. 2) and invariably contain a number of similar genes (Kreuze *et al*., 2002). However, SPCSV (genus *Crinivirus*) genome contains some unique genetic features (Fig. 2; Kreuze *et al*., 2002), and also described later.

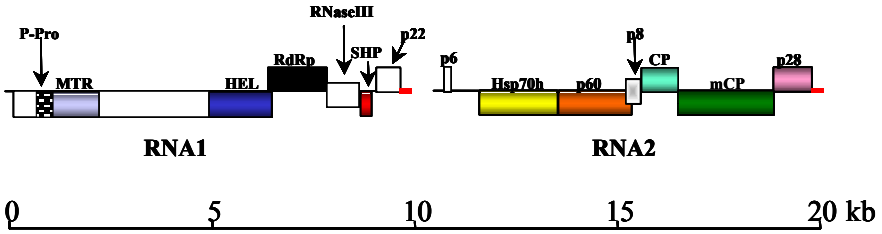


Fig. 2. Genome structure of the crinivirus SPCSV (RNA1 and RNA2). The functional domains in each ORF are indicated above the boxes; if no function is known the approximate molecular weight of the putative protein is indicated. Boxes correspond to ORFs: P-Pro; papain-like protease domain; MTR, (putative) methyltransferase domain; HEL, (putative) helicase domain; RdRp, RNA-dependent RNA polymerase; SHP, small hydrophobic protein; Hsp70h, heat shock 70 family protein homologue; CP, coat protein; mCP, minor coat protein; and a line indicates non-coding regions. RNaseIII denotes the ribonuclease III domain. The red line at the 3´-ends of SPCSV RNA1 and RNA2 indicates the near-identical 3'-sequences.

SPCSV has flexuous particles of 850 to 950 nm in length and 12 nm in diameter and its genome consists of two RNA molecules. With a total length of 17630 nt, SPCSV is the second largest positive sense ssRNA virus after *Citrus tristeza virus* (CTV; genus *Closterovirus*) (Kreuze *et al*., 2002). RNA1 (9407 nt) contains five putative ORFs and RNA2 (8223 nt) contains seven putative ORFs (see Fig. 2). The size of the major coat protein is 33 kDa, which is similar to other criniviruses (Cohen *et al*., 1992). Analysis of the genomic sequence of SPCSV show some unique features as compared to other *Closteroviridae*. The most striking may be the apparent recruitment of a novel gene encoding a putative ribonuclease III protein (Kreuze *et al*., 2002). Such a putative protein is known to be present only in one other virus, *Paramecium bursaria chlorella virus 1* (PBCV-1), a 330 kb dsDNA virus that infects green algae (Kreuze *et al*., 2002). As cellular RNase III is involved in the maturation of eukaryotic and prokaryotic RNA (Conrad and Rauhut, 2002) and in RNA silencing (Bernstein *et al*., 2001), the function of SPCSV RNaseIII-like protein could range from modification of its own RNAs to interference with host RNAs, including those involved in defence responses (Kreuze *et al*., 2002). The genome structure and expression strategy of SPCSV have been described (Kreuze *et al*., 2002).

SPCSV is transmitted by whiteflies (e.g. *Bemisia tabaci* and *Trialeurodes abutilonea*) in a semi persistent, non-circulative manner (Sim *et al*., 2000). The host range of SPCSV is limited mainly to the genus *Ipomoea*, although *Nicotiana* spp. and *Amaranthus palmeri* are reportedly susceptible (Cohen *et al*., 1992). SPCSV has also been detected in the wild species of Lisianthus (*Eustoma grandiflorum*; Cohen *et al*., 2001). There are no reports of strains based on plant infectivity assays. However, using polyclonal and monoclonal antibodies East African SPCSV isolates were distinguished from isolates from West Africa, America and Asia (Hoyer *et al*., 1996).

The heat shock protein 70 family homologue (Hsp70h) is highly conserved and is unique to viruses of the family *Closteroviridae* (Agranovsky *et al*., 1997). Therefore, primers have been designed to the conserved phosphate domains encoded in the N-terminal part of the Hsp70h gene and used to confirm closterovirus infections in plants (Tian *et al*., 1996; Fenby *et al*., 2002). Previous phylogenetic analyses based on partial Hsp70h nt or aa sequences of SPCSV isolates from different parts of the world further demonstrate a two-group division (Kreuze, 2002; Fenby *et al*., 2002). These groups are in agreement with the previously determined serological division (Hoyer *et al*., 1996; Alicai *et al*., 1999). All East African isolates and no isolate from elsewhere, clustered in the East African serotype (SEA). Isolates from elsewhere in the world cluster together in a distant group (NEA) (IsHak *et al*., 2003). As more sequences become available e.g. CP sequences from other parts of the world, there is a need to re-analyse genetic variability in this very important virus probably by targeting a larger and different genome segment than the 446 nt Hsp70h segment that was previously used (Alicai *et al*., 1999; IsHak *et al*., 2003).

Table 3. *Known functions of the mature proteins common to closteroviruses*

Protein	Functions	Reference
L-Pro/P-Pro	Proteinase	Agranovsky *et al*., 1994; Peng *et al*., 2001
	Cell-to-cell movement	Peng *et al*., 2001.
	Replication	Peremyslov *et al*., 1998; Peng & Dolja, 2000.
Met-Hel-RdRp	RNA dependent RNA Polymerase	Peremyslov *et al*., 1998; Yeh *et al*., 2000.
	Localized to membranes	Erokhina *et al*., 2001.
SHP	Cell-to-cell movement	Alzhanova *et al*., 2000.
Hsp70h	Cell-to-cell movement	Peremyslov *et al*., 1999.
	Virion assembly	Alzhanova *et al*., 2001.
	Attached to virion	Napuli *et al*., 2000.
P60	Cell-to-cell movement	Satyanarayana *et al*., 2000.
	Virion assembly	Alzhanova *et al*., 2000.
CP	RNA encapsidation	Agranovsky *et al*., 1995.
	Cell-to-cell movement	Alzhanova *et al*., 2000, 2001.
mCP/CPd	RNA encapsidation	Agranovsky *et al*., 1995; Tian *et al*., 1999.
	Cell-to-cell movement	Alzhanova *et al*., 2000, 2001.
	vector transmission	Tian *et al*., 1999.

Sweetpotato virus complexes and diseases

Sweetpotato virus disease (SPVD) is the name used to describe a range of severe symptoms in sweetpotato comprising overall plant stunting, leaf narrowing and distortion, chlorosis, mosaic or vein-clearing (Schaefers and Terry, 1976; Aritua *et al*., 1998; Gibson *et al*., 1998). Mixed infections of SPFMV and SPCSV are the usual cause of SPVD (Gibson *et al*., 1998; Karyeija *et al*., 2000a). In fact, single virus infections commonly cause mild or no symptoms in many sweetpotato cultivars (Milgram *et al*., 1996; Karyeija *et al*., 2000a). In Uganda, SPFMV and SPCSV have been detected in the major sweetpotato growing districts (Gibson *et al*., 1998). Virus-like diseases are known in sweetpotato crops also in other parts of East Africa (Hansford, 1944; Sheffield, 1957; Karyeija *et al*., 1998a; Ateka *et al*., 2004; Tairo *et al*., 2004). In Israel, co-infection of sweetpotatoes with *Cucumber mosaic virus* (CMV; genus *Cucumovirus*, family *Bromoviridae*) and SPCSV is reported to cause SPVD (Cohen and Loebenstein, 1991). Other potyviruses, such as *Sweet potato mild speckling virus* (Di Feo *et al*., 2000) and *Sweet potato virus G* (IsHak *et al*., 2003) have been detected in severely diseased sweetpotato plants in Argentina and Egypt, respectively. However, co-infection with an isolate of *Sweet potato virus Y* (Ateka *et al*., 2004) or SPFMV strain C (Souto *et al*., 2004) and SPCSV caused no detectable disease symptoms in sweetpotato. These observations suggest that co-infection of sweetpotatoes with SPCSV and a potyvirus (or another unrelated virus) may result in viral synergism leading to development of severe symptoms and significant yield losses, but the actual occurrence of viral synergism should be verified in each case.

Viral synergism

When two or more viruses co-infect a plant they may influence each other in several ways. They compete for host resources but, however, there are few reports indicating that unrelated viruses suffer a disadvantage during mixed infection (Poolpol and Inouye, 1986). Often, one virus may assist a second, co-infecting virus, leading to increased titres and more severe symptoms and this phenomenon is referred to as viral synergism (Goodman and Ross, 1974; Vance *et al*., 1995; Pruss *et al*., 1997). In some cases, the two viruses may benefit from the co-infection (Scheets 1998; Fondong *et al*., 2000). Synergism has also been known to occur between viruses and their satellite virus or RNA (Rodriguez-Alvarado *et al*., 1994; Sanger *et al*., 1994; Scholthof, 1999), or even between viruses and viroids (Valkonen, 1992). The mechanisms behind synergism may vary. In some cases the helper virus may aid another virus in movement (Barker, 1989), thereby enabling it to invade tissues it otherwise could not. In other cases, viral replication and accumulation are enhanced (Savenkov and Valkonen, 2001a).

The best-studied viral synergism cases are those where a potyvirus induces an increase in the titres of a second, unrelated virus (Rochow & Ross, 1955; Poopol & Inouye, 1986; Goldberg & Brakke, 1987; Scheets, 1998). In many known examples of viral synergism, one of the component viruses is a potyvirus, the titres of which are unaffected or decline (Poopol & Inouye, 1986) while titres of the co-infecting virus increase (Pruss *et al*., 1997; Vance *et al*., 1995). Only in three reported cases has the co-infection with another virus (Valkonen, 1992; Scheets,

1998; Karyeija *et al*., 2000a), led to enhancement of the potyvirus titres. One of the cases involve a dual infection of SPFMV and SPCSV, the usual cause of the severe sweetpotato virus disease (SPVD) in which potyvirus titres increase by 600 fold whereas no increase is observed in titres of SPCSV (Karyeija *et al*., 2000a). It is not known if SPCSV interacts in the same way with other sweetpotato infecting viruses. The mechanism of this synergism is yet to be solved (Kreuze, 2002).

Virus resistance in sweetpotato

Plant viruses are inducers and targets of RNA silencing (post transcriptional gene silencing), which poses a potent defence against them in plants (Matzke *et al*., 2001; Vaucheret and Fagard, 2001). The RNA silencing system recognizes and specifically degrades RNA it perceives as foreign, unusual or aberrant and sends a systemic signal, which induces degradation of homologous RNA in distal parts of the plant (Waterhouse *et al*., 2001b). However, many viruses still manage to infect their host plants quite successfully. The explanation may be that plant viruses have developed mechanisms to counter the effects of RNA silencing, e.g., by encoding suppressors of RNA silencing (Voinnet *et al*., 1999). Several viral suppressors of RNA silencing have been identified among the virus-encoded proteins, namely the HC-Pro (potyviruses; Kasschau and Carrington, 1998), 2b (cucumoviruses; Brigneti *et al*., 1998), P1 (sobemoviruses; Voinnet *et al*., 1999), p19 (tombusviruses; Voinnet *et al*., 1999), the 25 kDa movement protein (Potexviruses; Voinnet *et al*., 2000) and the AC2 (geminiviruses; Voinnet *et al*., 1999). These proteins have previously been identified as viral pathogenicity determinants. They also have, with the exception of AC2, important roles in viral long distant movement, suggesting a link between long distance movement and RNA silencing. The viral suppressors of RNA silencing can, in turn, be the targets of other host resistance mechanisms (Li *et al*., 1999) raising possibilities of diseased plants to recover and thereby resulting in plants that are symptomless, with low virus titres or virus-free (Lindbo *et al*., 1993).

Although the RNA silencing mechanism has yet to be determined, dsRNA has an important role (Fire *et al*., 1998). In addition, small interfering RNAs are invariably associated with RNA silencing (Waterhouse *et al*., 2001a). Crucial roles have also been shown for cellular RdRps (Xie *et al*., 2001), RNA helicases (Dalmay *et al*., 2001) and ribonuclease III-like molecules (Bernstein *et al*., 2001). Different mechanisms of pathogen-derived resistance (PDR) have been reviewed (Baulcombe, 1996) and they are related to gene silencing, which requires only the transcription of RNA and relies on activating the host RNA silencing machinery. In principle, any part of the viral genome could be used to induce RNA silencing (Thomas *et al*., 2001). Transgenic plants showing strong RNA silencing can be obtained by use of constructs containing self-complementary 'hairpins' (Smith *et al*., 2000; Wesley *et al*., 2001). The first example of transgenic resistance to a virus was based on the transformation of plants with a virus-derived gene for the CP in an attempt to mimic CP-mediated cross-protection (Abel *et al*., 1986). Since then, several different virus-derived genes and untranslatable sequences have been successfully used to obtain PDR (Lindbo & Dougherty, 1992; Beachy, 1997; Germundsson *et al*., 2002).

Many sweetpotato cultivars seem to be naturally quite resistant to SPFMV strains, showing only mild initial symptoms, from which they usually recover and may contain low virus titres (Cadena-Hinojosa and Campbell, 1981; Esbenshade and Moyer, 1982; Gibson *et al*., 1998). Similarly, though East African cultivars differ significantly in their reaction to SPMMV infections, they usually recover from symptoms (Hollings *et al*., 1976). However, co-infection of SPFMV with SPCSV could somehow interfere with the recovery and cause SPVD even in the most resistant clones of sweetpotato (Karyeija *et al*., 2000b; Mwanga *et al*., 2002). The genetic basis of resistance to SPVD was investigated and it was hypothesized that resistance to SPCSV and SPFMV is controlled by two separate recessive genes inherited in a hexasomic or tetradisomic manner (Mwanga *et al*., 2002). Together with the complex reproductive biology of sweetpotato (e.g. being a hexaploid crop and several cultivars poorly set seed), improvement of the crop may not be easy. Karyeija *et al*. (1998b) identified wild *Ipomoea* species that exhibited extreme resistance to SPFMV, SPCSV or both viruses. However, according to Diaz *et al*. (1996) incorporation of such resistance from wild diploid species into sweetpotato may not be an easy task (partly as explained above) and probably several accessions of the same species were not studied to verify whether this was not an aspect of non-host resistance as described (Heath, 2000).

Because of the difficulty in identifying and incorporating of natural resistance in elite cultivars, use of the viral CP gene to achieve resistance has been sought (Cipriani *et al*., 2001; Okada *et al*., 2001; Zeigler *et al*., 2001). Reports of the field evaluations are yet to be published. However, it is not known whether transgenic resistance to SPFMV will be sufficient to prevent the development of SPVD, i.e., whether the resistance to SPFMV will break down following co-infection of the plants with SPCSV, which is experienced with natural resistance to SPFMV (Gibson *et al*., 1998). On the other hand, since SPCSV seems to be the mediator of synergism and SPVD (Karyeija *et al*., 2000a), resistance to at least SPCSV may be appropriate to control SPVD. The exact mechanism of synergism is not yet understood, whereas it is known that potyviruses can suppress RNA silencing (Kasschau and Carrington, 1998). Therefore, infection with SPFMV might suppress RNA silencing-mediated transgenic resistance against SPCSV similar to what has been reported for transgenic resistance to *Potato virus A* (PVA; genus *Potyvirus*), following infection with *Potato virus Y* (PVY; genus *Potyvirus*) (Savenkov and Valkonen, 2001b) or transgenic resistance to PVY following infection with CMV (Mitter *et al*., 2001).

Whatever the mechanism for synergism or whether it will be feasible to deploy transgenic sweetpotato lines, we need to address the fundamental population biology problem of the viruses that can cause disease in sweetpotato. Currently, there is no convincing data that we have good knowledge of the genetic variability of at least the major sweetpotato viruses i.e. SPCSV, SPFMV and SPMMV. Since SPCSV is the mediator of SPVD, in mixed infection with SPFMV, we also need to have an understanding of the interaction of SPCSV with other sweetpotato infecting viruses e.g. SPMMV. The possible consequences of these interactions with respect to epidemiology and sweetpotato productivity also need to be understood at the onset.

Aims of the study

This study is part of the "Molecular Markers for Identification of Viruses and Virus Resistance in Sweetpotato" project. The project is under the auspices of the East African Regional Network for Biotechnology, Biosafety and Biotechnology Policy Development programme (BIO-EARN) that aims to build capacity in biotechnology in Ethiopia, Kenya, Tanzania and Uganda and promote appropriate research and related policies for sustainable development. Sweetpotato being one of the priority crops in the research and development agenda of the Uganda National Agricultural Research Programmes (and also other BIOEARN countries), control of the devastating viral diseases was considered an appropriate intervention for increased productivity. The primary requirements in control of any disease include a good understanding of the pathogens. Knowledge on their molecular variation, population structure and interaction among themselves and with the host will be essential. Thus, this thesis focuses on understanding molecular aspects, genetic variability and virus-host interactions of the major viruses of sweetpotato in East Africa. The epidemiological implications of these studies are also discussed.

The specific aims of this study were:

1. To provide a quantitative assessment of the incidence of sweetpotato viruses and virus-diseased plants, and associations of co-occurrence between different viruses in the major agro ecological zones of Uganda
2. To determine the genetic variability and population structure of SPMMV and SPFMV and SPCSV in Uganda
3. To determine the sweetpotato virus-virus and virus-host interactions and their implications on sweetpotato productivity

Results and discussion

Incidence of Sweetpotato-infecting viruses in Uganda

Sweetpotato plants were surveyed for viruses and virus-like diseases in the four major agro-ecological zones of Uganda as described (**I**). The highest average disease incidence of 20% was observed in Mukono district that lies in the tall grass-forest mosaic zone (**I**). The lowest disease incidence level of 2.7% was recorded in Soroti district that lies in the short grassland zone. However, for some districts that had high disease incidences, it was possible to find sweetpotato fields that were devoid of symptomatic plants. Types of virus-like diseases and the virus-like symptoms observed in the plants in the field are presented (**I**).

Sero-prevalence and incidence of sweetpotato viruses was carried on vines sampled during the survey using antisera kit provided by CIP. The kit contained polyclonal antibodies to 8 viruses, but only four viruses namely; *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato mild mottle virus* (SPMMV) and *Sweet potato chlorotic fleck virus* (SPCFV) were reliably detected.

Besides using virus-specific antisera in ELISA, SPCSV was detected in a few plants by RT-PCR using the SPCSV-specific primers CP1 and CP2 that amplify a 1.15 kb fragment of the SPCSV genome (Alicai *et al*., 1999). Also, several plants that tested positive for SPMMV and SPFMV in NCM-ELISA were tested using RT-PCR with potyvirus-specific degenerate primers (Gibbs and Mackenzie, 1997), resulting in the expected 1.8 kb product. However, the potyvirus-specific degenerate primers did not amplify any PCR products from plants that were only positive for SPCFV in ELISA, which is not consistent with the previous suggestion that it could be a potyvirus (Fuentes and Salazar, 1992). More recent information, after publication of paper **I**, suggested that SPCFV could be a *Carlavirus* (sequence information for one SPCFV isolate kindly was provided by Dr H.J. Vetten, BBA, Braunschweig, Germany). Thence, carlavirus specific degenerate primers were designed (reverse primer, CF00R 5´-GCT CAA AAG TAC TTT AAA AC-3´ and forward primer, CF1580 5´-GTC TTT AGR TTK TRA GAY TTA-3´) and used to amplify the 3´-end region of SPCFV genome. Cloning and sequencing of the PCR products and comparison to sequences available in databanks further confirmed virus identification.

Sequence analysis of 1.6 kb from SPCFV revealed an ORF for the CP (370 aa) and a nucleic acid-binding protein (133 aa) and a short 3´-end UTR of 52 nt. The CP aa sequence is 62% identical to the *Melon yellow associated virus* (MYaV; genus *Carlavirus*, family *Flexiviridae*). SPCFV isolate Mas sequence described here is available in the EMBL databank (accession no. AJ781295). More detailed sequence analysis of the three important viruses of sweetpotato in the region is provided in the following sections. No amplification products were obtained using the primers designed for detection of geminiviruses, and negative control samples (virus-free leaves) gave no PCR products. Thus this study has provided robust procedures for detecting, identifying and discriminating sweetpotato viruses and virus strains (see methods in **I**, **II**, **III** and **IV**).

SPCSV (67.5%) followed by SPFMV (63.8%) recorded very high incidence in diseased plants and were detected in all the surveyed sweetpotato fields, suggesting their occurrence in Uganda wherever sweetpotatoes are grown. SPMMV was the third most prevalent virus. It was detected in 24.6% of the diseased plants and in 13 out of the 14 districts, and was more frequently detected than earlier reported (Gibson *et al*., 1997). Incidence of SPMMV was relatively high in Mukono, Rukungiri and Tororo districts. SPCFV was detected in 5.2% of the diseased plants and was the least frequent of the four detected viruses. It was prevalent in 8 out of 14 districts, especially in the districts of Hoima, Masindi and Lira. From screenhouse observations, all viruses reported in this thesis can induce symptoms that may be appreciated when the infected plant is compared with a similar healthy one during the 3-5 weeks after replanting the vine. Some diseased plants (11.1%) did not react with any available virus antisera yet they displayed virus-like symptoms (Bos, 1999). The leaf-clearing syndrome (Fig. 3, and also described in **I**) that was prevalent in Soroti district is now associated with some physiological disorder. We could not detect any pathogen using molecular techniques (total nucleic acid display and RT-PCR) and plant infectivity assays (graft inoculation to other sweetpotato cultivars and indicator plant, *Ipomoea setosa*) and total protein profiling (2D PAGE analysis). Furthermore, it was mostly restricted to one local sweetpotato cultivar "Araka", that is popular in Eastern Uganda. However, for one of such plants, when scions were graft-inoculated to *I. setosa*, vein chlorosis was induced and the subsequent RT-PCR with potyvirus specific primers indicated presence of a potyvirus (later identified as SPFMV strain C isolate, see **II**) that was not serologically related to other SPFMV isolates or SPMMV.

Fig. 3. A healthy plant (right) and a plant showing the leaf distortion and clearing syndrome (left) at 5 weeks after planting. Both plants belong to the same cultivar.

Almost 90% of the symptomatic plants tested positive with at least one of the four virus-specific antisera used, which suggests that the four viruses studied are largely responsible for the virus diseases of sweetpotato in Uganda. Emerging reports also show occurrence of these four viruses in the neighbouring countries of Kenya (Ateka *et al*., 2004) and Tanzania (Tairo *et al*., 2004) at more or less the same incidence levels. Thirty five percent of the diseased plants tested positive for only one of the four viruses studied, whereas 54% were naturally infected with two or more viruses. The most common of the multiple infections was double infection with SPFMV and SPCSV (32.4%) followed by the triple infection of SPFMV, SPMMV and SPCSV (12.6%). SPCSV was the most frequently detected virus, both in single and mixed infections, and it was associated with severe and persistent symptoms. These observations indicate SPCSV to be the most important virus that infects sweetpotato in Uganda, followed by SPFMV and SPMMV.

SPCSV (Wisler *et al*., 1998) and possibly SPMMV (Hollings *et al*., 1976) are transmitted by whiteflies (*Bemisia tabaci*) that are more abundant in sweetpotato fields than aphids, which do not colonize sweetpotato (Byamukama *et al*., 2004). SPFMV (Schaefers & Terry, 1976) and SPCFV possibly (Fuentes & Salazar, 1992) are transmitted by aphids. It was hypothesized that viruses (e.g. SPCSV and SPMMV) with a common vector could exhibit a high frequency of co-occurrence. However, this was not supported by our data on co-occurrence of SPCSV and SPMMV (**I**). The incidence data and co-occurrence analysis suggest lack of association between SPCSV and SPMMV in sweetpotato plants in the field. For instance, the odds (number of events divided by the number of non-events) for detecting double infections of SPCSV and SPFMV were significant (odds ratio = 2.90, P = 0.000). Furthermore, the odds for co-occurrence of SPFMV and SPMMV were significant (odds ratio = 1.74, P = 0.008). Our data may suggest that SPCSV and SPMMV are transmitted by at least different biotypes of *B. tabaci*, or may reflect differences in vector transmission efficiency, which requires further study. Preliminary attempts to transmit SPMMV using whiteflies or aphids were not successful. Unexpectedly, there was a significant association with the aphid- and whitefly-transmitted viruses (SPFMV and SPCSV, and SPFMV and SPMMV) (**I**). The explanation for the observed common co-occurrence of SPFMV and SPCSV may in part be that infection with SPCSV suppresses the natural resistance to SPFMV in sweetpotato cultivars, which results in elevated titres of SPFMV and makes it more readily detectable (Karyeija *et al*., 2000a). On the other hand, SPCSV synergizes also with SPMMV (also discussed later, **IV**), which, however, was not reflected as a commonly found co-occurrence of SPCSV and SPMMV in sweetpotato plants in the field.

Genetic variability of the major sweetpotato infecting viruses

Variability in SPFMV

Additional sampling of some SPFMV isolates was done in Uganda to improve our understanding on genetic variability in SPFMV, following data reported in **II**. RT-PCR amplification and cloning of the 3´-proximal part (1.8 kb) of the SPFMV genome from additional samples were carried out as described (**II**). Primers were also designed to specifically amplify SPFMV strain C isolates (reverse primer, SPVC-CPRE 5´-GGT GAA ACA GAG CGC AT-3´ and forward primer, FM10820 5´-GGC TCG ATC ACG AAC CAA AA-3´). The clones were 1814 nt, excluding the poly(A) tail except isolate MPg1 (and Sor), whose amplified products were 6 nt shorter than other isolates. Translation of nt sequences of the SPFMV cloned fragments (Table 4) revealed an open reading frame ending up to a stop codon (UAG) at position 1585 in strain C isolates and position 1591 in all strain EA isolates. The stop codon was followed by a non-coding region of 221 nt in all sequences. Molecular characteristics are correspondingly described in **II**.

Unlike SPFMV strain EA isolates, strain C isolates were not detected with the polyclonal antisera to SPFMV. They showed moderately high CP nt (93.3 – 96.7%) and aa (93.6 – 96.8%) sequence identity to isolates of SPFMV strain group C available in databank (Table 4; Table 1 in **II**). In contrast, nt (78.1 – 80.1%) and aa (79.9 – 83.1%) identities to isolates of SPFMV strain groups EA, RC, and O were low. The CP aa sequences of the SPFMV isolates of this study were clearly distinct from other potyviruses known to infect sweetpotatoes such as SPVG (<72%), SPVY (<71%), SPMSV (<65%) and SPLV (<60%).

Phylogenetic analyses were carried using the complete CP-encoding nt sequences of SPFMV isolates described in this study and those available in the databanks (Table 4; Table 1 in **II**). Four main groups, supported by high bootstrap values (>98%), were observed and as shown (Fig. 3 in **II**), corresponding to the previously described strain groups C, EA, O and RC (Kreuze *et al*., 2000). Phylogenetic analysis using 3´-UTR gave trees with topologies similar to those of CP sequences but with lower bootstrap values (<60), except the 3´-UTR of one strain C isolate (SPFMV-C, Abad *et al*., 1992) is clearly that of EA strain group (e.g. 98% identical to SPFMV isolate Bny). This probably indicates that SPFMV-C isolate may be a recombinant between EA (or RC) and strain C isolates. Other strain C isolates are very distinct from SPFMV strain groups EA, RC and O, and other sweetpotato infecting potyviruses for the 3´-UTR. Since plant population structure is usually built of a major genotype plus a set of minor variants generated by mutation, or kept at low frequencies by selection (Rodriguez-Cerezo and Garcia-Arenal, 1989), the SPFMV-C isolate may represent a variant case.

The EA strain group was previously reported to be unique to the East African region. However, recently an isolate of EA strain group has been detected in Spain (Valverde *et al*., 2004). On the other hand, it became more evident that SPFMV isolates of other strain groups (C, RC and O) do not show consistent differences according to geographic regions but nearly identical isolates are distributed worldwide. For example, isolates of RC and C strains were detected in other parts

of the world (refs. in Table 1 in **II**), while they were originally described in the USA (Abad *et al*., 1992). Molecular data on RC strain isolates are available also from Asia, North Africa and East Africa, whereas C strain isolates have been detected in Asia and South America (refs. in Table 1 in **II**) and now in Uganda.

Table 4. Coat protein-encoding sequences of *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato mild mottle virus* (SPMMV) and *Sweet potato chlorotic stunt virus* (SPCSV) isolates used in this study

Virus sp./ isolate	Strain group	Geographic origin (country, region)	Accession no.	Reference
SPFMV*:				
Mpg1	C	Uganda, Mpigi	AJ 781793	This study
Apa	EA	Uganda, Apach	AJ 781787	This study
Kby1 [a]	EA	Uganda, Mpigi	AJ 781791	This study
Kby2 [a]	EA	Uganda, Mpigi	AJ 781792	This study
Mbl2	EA	Uganda, Mbale	AJ 781788	This study
Mpg2	EA	Uganda, Mpigi	AJ 781789	This study
Nak	EA	Uganda, Nakasongola	AJ 781790	This study
SPMMV:				
EAK	-	Kenya	Z 73124	Colinet *et al*., 1996
Bny	-	Uganda, Bushenyi	AJ 459314	**III**
Bus	-	Uganda, Busia	AJ 459319	**III**
Ish	-	Uganda, Ishaka	AJ 459318	**III**
Kam	-	Uganda, Kamuli	AJ 459317	**III**
Kum	-	Uganda, Kumi	AJ 459313	**III**
Ruk	-	Uganda, Rukungiri	AJ 459315	**III**
Ruk2	-	Uganda, Rukungiri	AJ 459316	**III**
Tor	-	Uganda, Tororo	AJ 459312	**III**
Kam2	-	Uganda, Kamuli,	AJ 717732	This study
Kum2	-	Uganda, Kumi	AJ 717734	This study
Tor2	-	Uganda, Tororo	AJ 717733	This study
SPCSV:				
SPSVV**	EA	Kenya	X80995	Hoyer *et al*., 1996
S2EA-41	EA	Uganda, Kabalore	AJ010768	Alicai *et al*., 1999
S2EA-39	EA	Uganda, Kabalore	AJ010767	Alicai *et al*., 1999
S2EA-4	EA	Uganda, Rakai	AJ010762	Alicai *et al*., 1999
S2EA-25	EA	Uganda, Rakai	AJ010764	Alicai *et al*., 1999
S1EA-9	EA	Uganda, Rakai	AJ010760	Alicai *et al*., 1999
S1EA-2	EA	Uganda, Rakai	AJ010759	Alicai *et al*., 1999
S1EA-1	EA	Uganda, Rakai	AJ010754	Alicai *et al*., 1999
S1EA-3	EA	Uganda, Tororo	AJ010757	Alicai *et al*., 1999
EA2	EA	Uganda, Mpigi	AJ428555	Kreuze *et al*., 2002
BUS	EA	Uganda, Busia	AJ811970	This study
C14	EA	Uganda, Mpigi	AJ811971	This study
KIB	EA	Uganda, Kiboga	AJ811972	This study

[a] = The isolates Kby1 and Kby2 were obtained from *I. setosa* plants planted outdoors at Makerere University Agricultural Research, Kabanyolo and which became infected by natural vector transmission of SPFMV

* = Only additional samples (isolates) are listed, other SPFMV isolates used in phylogenetic analysis are shown in **II**

** = Previously described as Sweet potato sunken vein virus (Hoyer *et al*., 1996)

Strain group C grossly deviates from the other SPFMV strain groups at sequence level, which is emphasized by the 2-aa shorter CP sequence. Only the originally described isolate SPFMV-C from the USA (Abad *et al*., 1992) seems to be an exception in this respect. The unique feature of the C strain CP-encoding sequence provides possibilities for specific PCR-based detection, which is important since the polyclonal and monoclonal antibodies nearly globally used for detection of SPFMV do not recognize these C strain isolates. Thus using SPFMV strain C specific primers, we were able to discriminately detect the strain C isolates both in single and mixed infection with isolates of EA or RC strain groups. Pathogenicity of one strain C isolate is described in **IV**. In general, due to the distant relationship to other strain groups of SPFMV, strain group C may eventually need to be classified as a different virus species or subspecies.

Variability in SPMMV

Since completion of the study described in **III** additional sampling was carried out in Uganda for 3 SPMMV isolates (Table 4). The accumulated sequence information was used to update our knowledge on molecular characteristics and genetic variability of SPMMV. As described in **III**, the 3´-terminal 1813-1820 nucleotides (excluding the poly(A) tail) of 12 SPMMV isolates (Table 4) were aligned. Translation of the SPMMV nucleotide sequences revealed an ORF ending up to a stop codon (UAA in all isolates) at position 1504 of the cloned fragment, followed by a non-coding region. The 3´-UTR was variable in length among the isolates (305-314 nucleotides) due to deletions within the first 42 positions (*data not shown*). Nonetheless, the SPMMV 3´-UTR is much longer than that of reported sweetpotato infecting potyvirids like SPFMV (221 nt, all strains), SPLV (194 nt), SPVG (218 nt), SPVY (213 nt) and SPMSV (245 nt).

In general, molecular characteristics of the additional samples for the 3´-terminal genomic region are as in **III**. However, we previously proposed another putative NIb/CP cleavage site (VYVE/P) for SPMMV 29 amino acids upstream from the VVQ/RE motif proposed by Colinet *et al*. (1998). In one of the additional sequences, arginine (R) was substituted with lysine (K) in the VVQR/E motif. Kang *et al*., (2001) determined the substrate specificity of *Turnip mosaic virus* NIa protease and suggested the VXHQ/S as the most favourable cleavage sequence of the possible cleavable sequence in which histidine (H) was always conserved. Considering this new information and with availability of new sequence data, a third cleavage site is possible at VEPHA/S (Fig. 2 in **III**). The proposed motifs are unique to SPMMV and have not been observed in other *Potyviridae* genera. Further studies are underway to determine the NIb/CP cleavage site and the N-terminus of CP in SPMMV by direct amino acid sequencing. Knowledge of the NIb/CP is important in production of bacterially expressed CPs that can be used in production of antibodies for routine sero-diagnosis.

The CP-encoding region of 12 isolates (Table 4) showed nt and aa sequence identities of 88.2-100% and 93.0-100%, respectively. Isolates Bus and Kum were identical. However, all isolates were different for the 3´-UTR sequences that showed identities of 91.6-98.1%. This level of intraspecific genetic variability is high and comparable to many potyviruses (Shukla *et al*., 1994; Rajamäki *et al*.,

1998; Bousalem *et al*., 2000). According to the sequence identity levels corresponding to different genera (<31%), species (55-75%), subsets of species (74-88%) and strains of the same virus (90-99%) (Schubert *et al*., 1999), the studied isolates of SPMMV can all be considered as strains of SPMMV. The low CP aa sequence similarity of SPMMV isolates characterised so far with other characterised viruses as described in **III**, and the unusual putative proteolytic cleavage site at the NIb/CP junction further demonstrate SPMMV as a very distinct virus in the family *Potyviridae*.

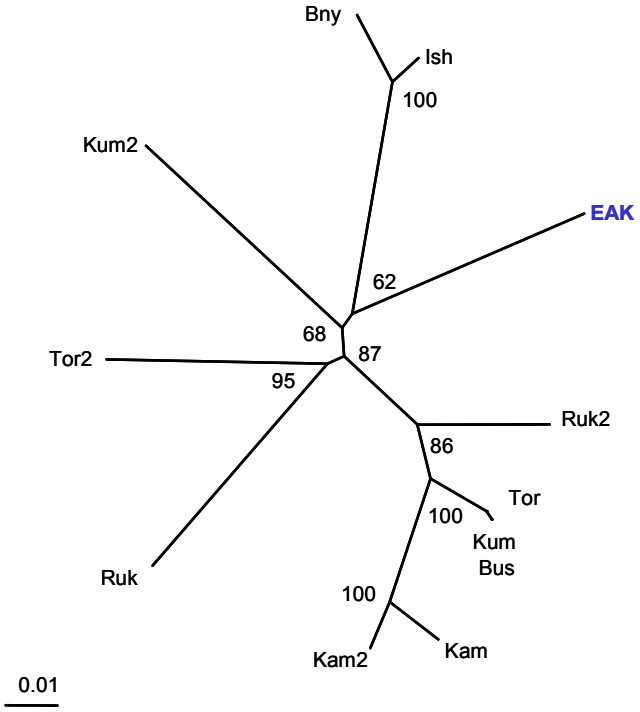


Fig. 4. Phylogenetic analysis of nt sequences of the CP-encoding region of 12 SPMMV isolates using neighbour-joining method. The trees are inferred from Kimura two-parameter estimates of nt substitutions per site (as in **II**). Branch lengths are proportional to genetic distance. Numbers on branches represent bootstrap values out of 100 replicates.

Phylogenetic analysis using the CP-encoding nt sequences did not reveal distinguishable groups within SPMMV isolates described in this study (Fig. 4). Rather, analysis indicated a high genetic variability of isolates. A largely similar organization of the phylogenetic tree was obtained using the 3´-UTR sequences (*data not shown*) but low bootstrap value (<60) for most branches. Variation in length of 3´-UTR and non-statistically significant clustering of SPMMV isolates using the 3´-UTR may suggest that the region is less appropriate for SPMMV phylogenetic studies as compared to the CP sequences. Furthermore, clustering of the isolates was not correlated with the geographical origin. For instance, isolate EAK from Kenya (East of Uganda) is more distinct, but phylogenetically more close to isolates BNY and ISH from western Uganda (Fig. 4). These results

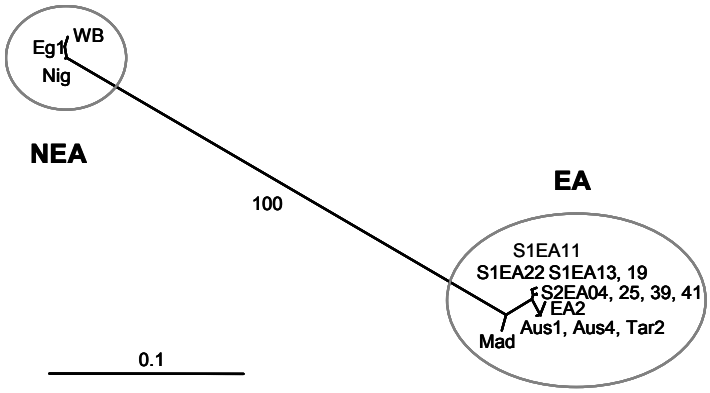
indicate existence of distinct genetic strains of SPMMV in Uganda. The characterized isolates may represent a population that is either undifferentiated, differentiated according to some factor such as host plant, or fluctuate randomly in composition. Data may also be indicative of the role of epidemiological factors, such as mode of transmission and the perennial nature of the host plant. These factors would determine the extent of isolation among subpopulations (Albiach-Marti *et al*., 1999) that seem to indicate low rates of spread. In short, no clear conclusions can be drawn from the present data. Epidemiological studies are needed to address these points.

Variability in SPCSV

We sampled 1.18 kb of the SPCSV genome, including the CP encoding region, from 3 isolates. One isolate was sampled from central (Mpigi district), western (Kiboga district) and eastern (Busia district) Uganda (Table 4) and used in RT-PCR, cloning and sequencing. CP sequences obtained in this study were compared with 10 CP sequences available from the genebank (Table 4). Unfortunately, no CP sequence was available from other parts of the world, particularly for non-East African (NEA) isolates for comparative analysis. Phylogenetic analysis showed very low variability even within the CP (identity range for aa 98.5%-100%; for nt 96.9-100%). However, clustering of isolates did not match the previous grouping based on serotype (SEA1 or SEA2) (Alicai *et al*., 1999; Fenby *et al*., 2002) (Fig. 5). Together with sequence identity data using the Hsp70h (96-100%), these observations may suggest stability of the SPCSV genome of the EA group. This is not surprising since similar studies using the CP gene have shown low genetic diversity in the geographically distant isolates of *Cucurbit yellow stunting disorder virus* (CYSDV; genus *Crinivirus*) (Rubio *et al*., 2001). Sequences of *Citrus tristeza virus* (CTV; genus *Closterovirus*) separated in time and space were also essentially identical (Albiach-Marti *et al*., 2000).

The non-East African (NEA) and East African serotype (SEA) groups were genetically distinguished (76.0%-78.3% nt and 91.1%-92.5% aa sequence identity between the two groups) based on the Hsp70h sequence analysis (IsHak *et al*., 2003) and also shown here for comparison (Fig. 5A). Using the Hsp70h, previous studies emphasized the uniqueness of the East African SPCSV (strain group EA) as compared to the SPCSV isolates (strain group NEA) described from other parts of Africa or the rest of the world (Alicai *et al*., 1999; IsHak *et al*., 2003). However, the SEA isolates are no more geographically restricted to East Africa since some SPCSV isolates in Australia were found to belong to this strain group (F. Tairo, S.B. Mukasa, R.A.C Jones, A. Kullaya, P.R. Rubaihayo & J.P.T. Valkonen, *unpublished data*). Furthermore, serological detection of SPCSV in Peru (Gutierrez *et al*., 2003) suggests that the EA serotype of SPCSV may have a more global distribution.

A)



B)

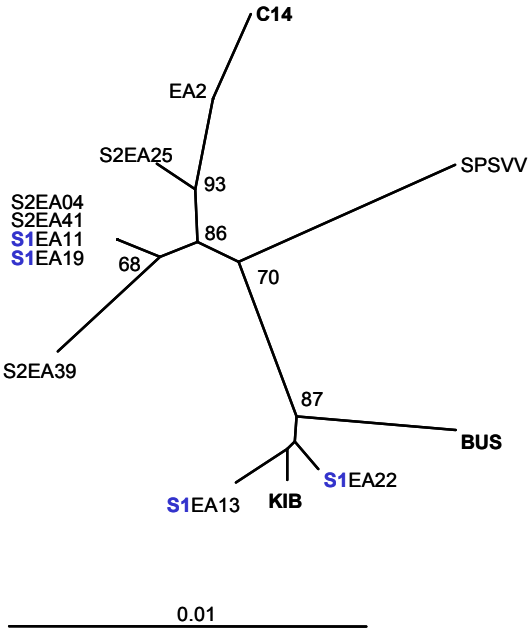


Fig. 5. Phylogenetic analysis of SPCSV EA and NEA isolates using the partial Hsp70h encoding region (**A**). Isolates Aus1 and Aus5 are from Australia and, Tar2 is from Tanzania. NEA isolates Eg1, Nig and WB are from Egypt, Nigeria and USA, respectively (see IsHak *et al*., 2003) and are compared with SPCSV EA isolates (described in Table 4) using CP-encoding region nt sequences (**B**). The trees are inferred from Kimura two-parameter estimates of nt substitutions per site (as in **II**). Branch lengths are proportional to genetic distance. Numbers on branches represent bootstrap values out of 100 replicates.

Although CP sequences of 10 SPCSV East African isolates have been published in previous studies (Alicai *et al*., 1999; Kreuze *et al*., 2002; Hoyer *et al*., 1996), there were no published phylogenetic analyses based on CP sequences of SPCSV. For studies on genetic diversity and molecular diagnostics, it is important that sequence characterizations are consistently carried out on the same genomic regions (e.g CP gene). Like with Hps70h sequences, the CP-encoding sequences did not reveal wide genetic differences within the EA group (short genetic distances and high identity levels of 97-100%). Among the SEA isolates, isolate Mad (from Madagascar) was the most genetically distinct (Fig. 5A). Like with other viruses, the phylogenetically distinguishable lineages of SPCSV (Fig. 5B) may be associated with differences in host adaptation and phenotypic differences in virus-host interactions, which is important to know in order to choose the appropriate sources for resistance and to use the appropriate virus isolates for challenge inoculation in the resistance breeding programs. For studies aiming to develop RNA silencing based transgenic resistance to SPCSV this sequence information will assure that resistance covers the locally prevailing viruses and virus strains.

Virus-virus interactions and cultivar reaction to virus infection

Three sweetpotato cultivars, Dimbuka, New Kawogo and Tanzania, were selected to study interactions between the three important but taxonomically different viruses of sweetpotato. Ugandan SPCSV, SPFMV and SPMMV isolates were used as described (**IV**). Sweetpotato cultivars were selected based on their grossly different phenotypes reflecting genetic differences and their importance in sweetpotato production in Uganda. SPMMV mostly induced mild vein chlorosis and/or diffuse mottling in cv. Tanzania and mottling in cv. Dimbuka. SPFMV also induced mottling in cvs. Tanzania and Dimbuka, or no symptoms were observed. Symptoms induced by these viruses were transient, lasting for 2-4 weeks in the young leaves of the re-planted vines, and then faded. Recovery from disease occurred faster with SPFMV than SPMMV. The new symptomless leaves sometimes tested virus-negative in NCM-ELISA. However, recovery from disease did not result in complete elimination of the virus since when cuttings were taken from the symptomless upper parts of the vines and replanted, the symptoms re-appeared in the young shoots.

On the contrary, no symptoms were observed and no virus was detected by NCM-ELISA (or TAS-ELISA) in cv. New Kawogo following graft-inoculation with SPFMV or SPMMV. However, vein chlorosis and mottling developed and the viruses were readily detected in leaves of *I. setosa* scions top-grafted on the graft-inoculated cv. New Kawogo plants. These results indicate that cv. New Kawogo is more resistant to SPMMV or SPFMV than cvs. Tanzania and Dimbuka, which is manifested as lower virus titres in infected plants, as previously shown with other Ugandan isolates of SPFMV (Karyeija *et al*., 2000b). SPCSV caused diffuse chlorotic patches or general mild chlorosis that gave a pale appearance of the leaves in the three cultivars at 5-7 weeks after planting (WAP). The symptomatic leaves readily tested positive for SPCSV in TAS-ELISA. At later stages of growth, purpling of the older leaves was observed. Symptoms due

to single or multiple virus infections are described (**IV**). Symptom recovery has also been observed in *I. setosa* for SPFMV and SPMMV, whereas the SPMMV susceptible hosts (e.g. *N. banthamiana* and *N. tabacum* cv sumsun) did not recover.

Resistance to SPMMV or SPFMV was broken down following co-infection with SPCSV, resulting in severe symptoms regardless of the different resistance levels expressed by cvs. Dimbuka, Tanzania or New Kawogo to infection with SPMMV and/or SPFMV. Novel disease symptoms including chlorosis, rugosity, leaf strapping and dark green islands were induced by the dual infection of SPMMV and SPCSV. These symptoms could be differentiated from those caused by dual infection of SPFMV and SPCSV that is associated specifically with the sweetpotato virus disease (SPVD), characterised by often severe stunting of the plant, distortion and either chlorotic mottle or vein clearing of the leaves (Gibson *et al*., 1998). The name "sweetpotato severe mosaic disease" (SPSMD) is proposed to describe the disease caused by the dual infection of SPMMV and SPCSV.

These observations indicate synergistic interactions between SPCSV and SPFMV or SPMMV, whereas neutral (non-detectable) interactions were observed between SPFMV and SPMMV in plants of the three cultivars. Like in western blot analysis (Fig. 4 in **IV**), we did not detect signals of the SPMMV CP in immunohistochemical localisation experiment (Fig. 6) in plants infected with SPMMV alone. SPMMV was readily detected in mixed infection with the phloem limited SPCSV. Like for SPFMV (Karyeija *et al*., 2000), we detected SPMMV in mesophyll, companion and epidermal cells, including the guard cell but not in the phloem tissue. SPCSV was detected exclusively in the cells, in the major and minor veins. This is interesting since a crinivirus increases titres of the non-phloem tissue and ipomovirus as observed in this study, and a potyvirus (Karyeija *et al*., 2000). Immunohistochemical localisation of SPMMV in sweetpotato plants (Fig. 6) suggests that it could be exploiting or infecting cells similar to SPFMV.

Use of the nucleic acid spot hybridisation (NASH) method allowed more accurate comparison of the relative virus amounts in cv. Tanzania plants as described (**IV**). SPMMV and SPFMV RNA accumulation greatly increased by over 32 and 64 fold, respectively, in mixed infection with SPCSV. However, accumulation of SPCSV in mixed infection with SPMMV or SPFMV was reduced by 2-4 fold, indicating an antagonistic interaction. Furthermore, our data indicated that SPMMV and SPFMV had an additive effect on synergism with SPCSV, since symptom severity was further increased in triple-infection of the viruses, while SPMMV and SPFMV showed no detectable mutual interaction or synergism when they co-infected sweetpotato cultivars in the absence of SPCSV. These data indicated that SPMMV and SPFMV, both of which are members of the family *Potyviridae*, are beneficiaries of the synergistic interaction with SPCSV. Most of these virus-virus interactions have not been previously studied, except the synergistic interaction between SPFMV and SPCSV (Karyeija *et al*., 2000a; Gutierrez *et al*., 2003).

Although the severe symptoms may be merely due to the potyvirus or ipomovirus component that exhibits greatly increased titres in SPVD or SPSMD,

the actual cause of the disease problem seems to be SPCSV. Many East African sweetpotato cultivars, such as New Kawogo, Tanzania and Dimbuka, show high or moderate levels of resistance to SPFMV (Mwanga *et al*., 2002) and, as shown here, also to SPMMV. However, no sweetpotato cultivar highly resistant to SPCSV has been found (Mwanga *et al*., 2002). It is intriguing that infection with SPCSV (family *Closteroviridae*) suppresses resistance to the unrelated viruses of family the *Potyviridae* in many different sweetpotato genotypes. The underlying mechanism is unknown and will be the key issue for understanding the synergistic effects (Karyeija *et al*., 2000a). Working with SPFMV-Sor isolate (strain group C) we did not observe foliar symptoms on cv. Tanzania but co-infection with SPCSV induced severe disease symptoms. These results suggest that, in general, co-infection of sweetpotatoes with a potyvirid and SPCSV imposes a high risk for development of severe symptoms and significant yield losses, but the actual occurrence of synergism must be verified in each case.

Infection with SPFMV or co-infection of SPMMV and SPFMV resulted in ca. 25% lower tuber yields than obtained from non-infected plants. Infection with SPCSV caused a more significant ($p<0.05$) yield reduction (54%). Severe disease symptoms developed following co-infection of SPMMV or SPFMV with SPCSV and resulted in poor yields that were 79-86% lower than tuber yields obtained from healthy plants, and 62-72% lower than yields of plants infected with SPCSV alone. Triple-infection nearly prevented tuber formation, and only very small and poorly developed tubers were produced. Thus co-infection of SPMMV and SPCSV can induce severe disease symptoms and cause up to 80% reduction in tuber yield as compared to the healthy plants. This is remarkable since while yield reduction caused by SPCSV alone reached 50%, infection with SPMMV alone caused no significant yield reduction. Thus, when SPMMV co-infects with SPCSV, it seems to pose a significant risk to sweetpotato productivity, as reported for the co-infection of SPFMV and SPCSV (Ngeve, 1991; Milgram *et al*., 1996; Gibson *et al*., 1998; Guiterrez *et al*., 2003). Effects of viruses (in single or mixed infection) on plant growth attributes were i.e. plant height, number of internodes, fresh weight of foliage and number of branches, recorded at 20 WAP are described (**IV**). Since our experiments were done in an insect-proof screenhouse and in a geographic area typical for sweetpotato production, the results of this study may provide a fair estimate of the potential negative impact that SPMMV, SPFMV, SPCSV and their mixed infections can cause on sweetpotato yields, especially when virus-infected vines are used as planting material.

The genome of SPCSV contains an open reading frame for a putative RNaseIII enzyme that could interfere with the host responses (Kreuze *et al*., 2002). The gene functions of SPCSV in relation to the suppression of host resistance to viruses are currently under study. One of the important questions is whether SPCSV directly suppresses host resistance, thereby increasing susceptibility of sweetpotato plants to virus infection, or whether it stimulates the heterologous virulence factors of other viruses to more efficiently interfere with virus-specific host resistance. On the other hand, the helper component proteinase (HC-Pro) of potyviruses is a multifunctional protein required for synergism with other viruses (Pruss *et al*., 1997; Shi *et al*., 1997; Vance *et al*., 1995). It is known to interfere with the basal anti-viral host defence mechanism (RNA silencing) that is induced

by dsRNA, including replicating viruses, and subsequently targets homologous RNA in cytoplasm to degradation (Brigneti *et al*., 1998; Kasschau and Carrington, 1998). DGI are an outcome of locally induced RNA silencing that targets the viral RNA to degradation (Ratclif *et al*., 1997), and it can be inhibited in virus-susceptible hosts by expression of HC-Pro (Yelina *et al*., 2002). The top leaves that developed DGI in this study contained low titres of SPCSV, and in absence of SPCSV the plants recovered from all SPMMV-induced symptoms, which is also attributable to locally induced RNA silencing. Thus, SPCSV may be responsible for elevating virulence of SPFMV and SPMMV, by suppressing the otherwise high levels of host resistance to single infections in the local East African sweetpotato cultivars.

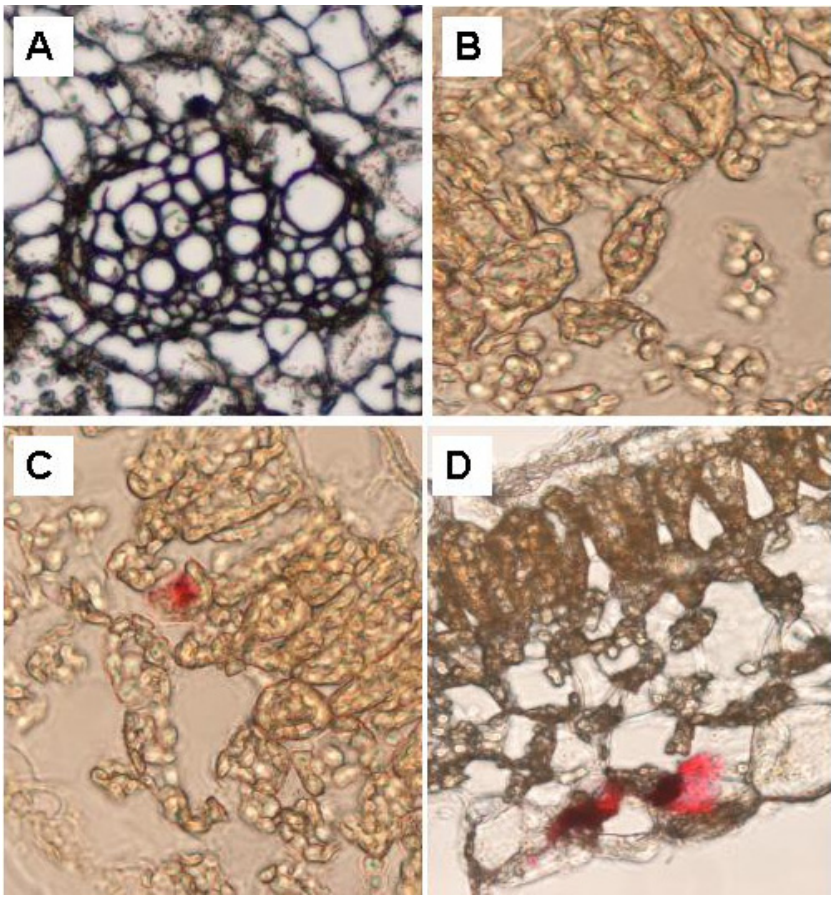


Fig. 6. Immunohistochemical localisation, as described in Karyeija *et al*. (2000a), of SPMMV using thin sections (10 μm) prepared from the fifth leaf below the uppermost fully opened leaf of sweetpotato cv. Tanzania plants infected with SPMMV+SPCSV. Leaf sections were probed with AP-conjugated antibodies to SPMMV CP and stained with Sigma *FAST* Red (Sigma), visible as red colouration in **C** and **D**. SPMMV CP was detected in mesophyll cells (**C**), companion cells and epidermal cells, including the guard cells (**D**) but not in phloem cells, minor or major veins (**A**). Healthy plants did not show any staining (**B**). Magnification of section A is x400, and B-D is x200

Conclusions and future perspectives

The survey for sweetpotato viruses provides a quantitative assessment of the incidence and distribution of viruses and viral diseases in sweetpotato plants in Uganda, taking into account the major agro-ecological zones and cropping systems. The description of symptoms associated with sweetpotato viruses may be a useful guide to diagnosis of sweetpotato diseases. The molecular studies improved our knowledge on possible use of molecular techniques in diagnostic methodologies that could be used by downstream partners in crop protection (e.g. plant quarantine, virus indexing for virus elimination and clean-seed production programs). For instance, it is now possible to detect SPFMV strain C isolates that had hitherto not been reported in the East African region due to the current problems with serological detection.

The extensive sequence analyses of sweetpotato virus isolates from Uganda and in relation to isolates from other parts of the world have provided improved resolution of the genetic variability of the three most important viruses of sweetpotato. For instance, SPMMV showed high genetic diversity with no clear genetic structure using the available sequence data. SPCSV isolates from East Africa have displayed low sequence diversity (identity range 98-100%) but very distinct (76-78% and 91-92% nt and aa sequence identity, respectively) from some isolates originating from other parts of the world. Conversely, SPFMV (all strain groups) showed high genetic variability but with evidence of a population differentiated into at least two strain groups (subspecies) of C and RC regardless of geographical location. The RC group can further be phylogenetically split into 3 subgroups (EA, O and RC) that seem to be genetically well differentiated, except for members of the subgroup O. SPFMV variants may probably be differentiated according to some yet clear factors. More sequence information is required to resolve the taxonomic position of the C and RC strain groups, and to understand the possible recombination between the different strains.

Although the severe symptoms may be merely due to the potyvirus or ipomovirus component that exhibits greatly increased titres during synergistic interactions, the actual cause of the disease problem seems to be SPCSV. Many East African sweetpotato cultivars show high or moderate levels of resistance to SPFMV and/or SPMMV. Co-infection of SPCSV with SPFMV or SPMMV poses a high risk for development of severe symptoms, spread of the potyvirid components and significant yield losses, but the actual occurrence of synergism must be verified in each case. The underlying mechanism is unknown and will be the key issue for understanding the synergistic effects.

Synergism has implications on the spread and survival of the component viruses in the field. Use of apparently diseased sweetpotato vines as planting material is often avoided by farmers. Therefore, synergistic disease expression due to mixed virus infection, as described in this study, may decrease the opportunities for viruses to be transmitted to new crops through cuttings. On the other hand, increase in virus titres following the synergistic interactions may enhance virus transmission by vectors. Where the effects of synergism via disease severity are

greater than those manifested on virus transmission, the viruses in multiple infections may gradually exclude each other, especially when the transmission rate of the component viruses is low. The final outcome may be determined by the vector abundance, efficacy of vector transmission, and the initial disease (inoculum) state. Thus, in developing epidemiological models, several factors must be considered. Therefore, future studies need to establish the implications of synergism between SPCSV and other viruses (e.g. SPFMV and SPMMV) on virus spread and its subsequent effects on sweetpotato productivity.

Rather than just summarize the findings of this thesis and what is known of the genetic variability, population structure and interactions of the three most important sweetpotato viruses in East Africa, I have also put emphasis on the aspects where current knowledge is weaker. The hope is that this thesis shows the reader that molecular and evolutionary biology of plant virus populations are exciting areas of research, both intellectually challenging and relevant to everyday life.

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Acknowledgement

I would like to express my gratitude to all people and institutions that helped, encouraged or facilitated my PhD study. The list is long but I will mention some.

First, I am very much indebted to my supervisors Professor *Jari Valkonen* (Department of Plant Biology and Forest Genetics, SLU, and Department of Applied Biology, University of Helsinki, Finland) and Professor *Patrick Rubaihayo* (Department of Crop Science, Makerere University, Kampala, Uganda) for their guidance throughout my study.

My sincere appreciation goes to Dr *Ivar Virgin* and Ms *Benita Forsman*, Stockholm Environment Institute, for coordinating the logistical support during the study period while in Uganda and in Sweden. I also appreciate the roles of all BIO-EARN partners, who have steered the BIO-EARN programme.

My thanks to all colleagues at the Genetics Centre, Uppsala, SLU, and particularly the present and past members of the virology group (*Aldo*, *Anna G*, *Anders*, *Andrey*, *Elin*, *Eugene*, *Fred*, *Gassan*, *Ingela*, *Jon*, *M.L Mohan*, *Nina, Viiu,* and *Jan*, *Carl*, *Hannele*, *Minna*, *Mkumbo*, *Robert*). Thanks to all BIO-EARN colleagues, during all the meetings, at Alnarp, KTH-Stockholm, Lund and Uppsala (*Elisabeth B.K*, *Joel Mutisya, Patrick Okori* and *Yona Baguma*). I thank the SLU academic staff, particularly *Christer J*, *Christina D*, *Marie N*, *Per B* and *Urban G* for all BIO-EARN related activities. I thank the administrative and technical staff (Proteomics, Histology, and Sequencing Laboratories; *Håkan L*, *Ingrid E* and *Ingrid S*) of the Department of Plant Biology and Forest Genetics, SLU. Björn N, Lena J and Mona M for your active roles. Tack så mycket. My thanks to Dr *S Kyamanywa* and all staff of the Department of Crop Science and colleagues in the Faculty of Agriculture, Makerere University.

I gratefully acknowledge the Swedish International Development Cooperation (Sida) for the financial support to the BIO-EARN programme through its Department for Research Cooperation (SAREC) in collaboration with the Stockholm Environment Institute (SEI). I am grateful to Makerere University, my employer, for giving me the opportunity and the 5-year study leave.

Finally, I honour my family that persevered during my study. I appreciate the responsibilities of my wife, *Sylvia*, for running our home and for taking care of our daughter, *Susan Benny N*. This achievement is a blessing to our family and God will bless us with more children. To my family, to my mother *T.K Kiwanuka* and to my late father *S.L Kiwanuka*, I dedicate this effort.

Kind words do not cost much. Yet they accomplish much!
[*Blaise Pascal*]