# Molecular Studies on the Sweet Potato Virus Disease and its Two Causal Agents

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# Abstract

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The studies presented in this thesis contribute to an increased understanding of the molecular aspects, variability and interaction of the two most important viral pathogens of sweet potato (*Ipomoea batatas* L): *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV), which cause the severe sweet potato virus disease (SPVD) when co-infecting sweet potato plants. SPVD is the most important disease affecting sweet potato in Africa, and may be the most important virus disease of sweet potato globally.

The coat protein gene sequences of several African SPFMV isolates were determined and compared by phylogenetic analyses. Results showed that East African SPFMV isolates were genetically distinct. They could furthermore be divided into two serotypes which differed in their ability to systemically infect the sweet potato cultivar Tanzania.

The aetiology of SPVD was studied in sweet potato plants co-infected with SPFMV and SPCSV using nucleic acid hybridisation, bioassays, tissue printing and thin section immunohistochemistry. Resistance to SPFMV in East African sweet potato cultivars was found to be due to inhibition of virus replication rather than movement and resistance was suppressed by infection with SPCSV, resulting in a ca. 600-fold increase in titres of SPFMV. Furthermore, in SPVD affected plants SPFMV is detected outside of the phloem, whereas SPCSV is detected only inside the phloem, which suggests novel as yet unknown mechanisms how SPCSV synergises SPFMV.

The genomic sequence of SPCSV was determined. It was composed of two RNA molecules (9407 and 8223 nucleotides), representing the second largest (+)ssRNA genome of plant viruses. The genomic organization of SPCSV revealed novel features for the genus *Crinivirus*, such as *i*) the presence of a gene putatively encoding an ribonuclease III-like protein, *ii*) near-identical, 208 nucleotides long 3'-sequences on both viral RNAs, and *iii*) the placement of the *SHP* gene at a new position on the genome of SPCSV relative to other closteroviridae. Northern analyses showed the presence of several sub-genomic RNAs, of which the accumulation was temporally regulated in infected tissues. The 5'-ends of seven sub-genomic RNAs were determined using a PCR based method, which indicated that the sgRNAs were capped.

Keywords: Sweet potato feathery mottle virus, Sweet potato chlorotic stunt virus, Ipomoea batatas, genetic variation, Crinivirus, Potyvirus, viral synergism, genome structure, expression strategy, virus resistance.

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# Contents

# Appendix

## Papers I-IV

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

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

II. Karyeija, R.F., **Kreuze, J.F.**, Gibson, R.W. & Valkonen, J.P.T. 2000. Two serotypes of *Sweetpotato feathery mottle virus* in Uganda and their interaction with resistant sweetpotato cultivars. *Phytopathology 90*, 1250-1255

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

IV **Kreuze, J.F.**, Savenkov, E. I. & Valkonen, J.P.T. 2002. Complete genomic sequence and analyses of subgenomic RNAs of *Sweet potato chlorotic stunt virus* reveals several new features for the genus *Crinivirus*. Submitted

# Abbreviations

| | |
|---|---|
| aa | amino acid |
| *Avr*-gene | avirulence gene |
| BYV | *Beet yellows virus* |
| CI | cylindrical inclusion protein |
| CMV | *Cucumber mosaic virus* |
| CP | coat protein |
| CTV | *Citrus tristeza virus* |
| cv. | cultivar |
| GFP | jellyfish green fluorescent protein |
| GUS | β-gluceronidase |
| HC-Pro | helper component proteinase |
| Hel | helicase |
| HR | hypersensitive response |
| Hsp70h | heat shock 70 family protein homologue |
| LIYV | *Lettuce infectious yellows virus* |
| LZ | leucine zipper |
| MAb | monoclonal antibody |
| mCP | minor coat protein |
| Met | methyltransferase |
| MP | movement protein |
| NIb | nuclear inclusion protein b |
| NLS | nuclear localisation signal |
| nt | nucleotides |
| ORF | open reading frame |
| PAb | polyclonal antibody |
| PDR | pathogen derived resistance |
| P-Pro | papain like proteinase |
| RdRp | RNA-dependent RNA polymerase |
| *R*-gene | resistance gene |
| RNaseIII | ribonuclease III |
| sgRNA | subgenomic RNA |
| SHP | small hydrophobic protein |
| SPCSV | *Sweet potato chlorotic stunt virus* |
| SPFMV | *Sweet potato feathery mottle virus* |
| sp. | species (singular) |
| spp. | species (plural) |
| SPVD | sweet potato virus disease |
| TMV | *Tobacco mosaic virus* |
| VPg | viral protein genome linked |

# Introduction

Viruses are sub-microscopic, obligate intracellular parasites that infect every class of living organisms known to date. Viruses themselves are not living organisms and they occupy a unique position in biology. Since they are not functionally active outside of their host cells, they lead, at most, only a kind of borrowed life (van Regenmortel *et al.*, 2000). Hull (2002) defines a virus as follows: *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. It can usually be horizontally transmitted between hosts. Within such cells, virus replication is (1) dependant on the host's protein synthesizing machinery, (2) organized from pools of the required materials rather than by binary fission, (3) located at sites that are not separated from the host cell contents by a lipoprotein bilayer membrane, and (4) continually giving rise to variants through various kinds of changes in the viral nucleic acid.*

Peter Medawar, awarded the Nobel Prize for Medicine and Physiology in 1960 had another definition for viruses: *A piece of nucleic acid surrounded by bad news!* Indeed, most viruses cause disease. By utilizing cellular substances and disrupting cellular processes, viruses cause the host metabolism to get upset, leading to development of abnormal compounds and conditions injurious to the functions and the life of the infected organism. Viral diseases such as influenza, polio, rabies, smallpox and AIDS have, and will continue to kill a countless number of people throughout the world. But viruses do not only cause human suffering directly. By infecting our livestock and crops, they can cause enormous economic losses and even hunger and starvation. Some virus diseases have destroyed entire plantings of certain crops in some areas, for example, geminiviruses in tomato, plum pox, hoja blanca of rice, Cacao swollen shoot, rice tungro, papaya ringspot, sugar beet yellows and citrus tristeza (Agrios, 1997; Bos, 1999). Because developing countries lack resources to control or limit damage caused by viruses, they often suffer most. Maize streak viruses cause severe yield losses yearly in Africa and since 1988 an epidemic of the *African cassava mosaic virus* (ACMV) has caused the complete collapse of cassava production in several districts of Uganda and western Kenya, leading to food shortages and famine (Otim-Nape *et al.*, 2000). How many lives were lost due to the indirect effects of malnutrition or what kind of impact these viruses have had on a largely subsistent society with an already weak economy is unknown, but it is bound to be significant. The loss of the cassava crop due to ACMV has lead farmers to switch to other crops, such as sweet potato. Sweet potato, however, in its turn, is also affected by a severe viral disease.

Virus infected plants cannot be cured, and the only way to adequately protect the crops of subsistence farmers is by the use of resistant cultivars. We, as scientist, can contribute to reducing human hardship by developing and making available such resistant cultivars to those who are in need of them. This is not an easy task, viruses are continually changing, exploring new sequence space to adapt

to the alterations in their hosts, and resistance-breaking strains appear. The development of durable resistance will be more likely if it is based on a thorough understanding of the pathogens involved, and the mechanisms by which they cause disease. The studies presented in this thesis contribute to an increased understanding of the molecular aspects, variability and interaction of the two most important viral pathogens of sweet potato: *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV), which cause the severe sweet potato virus disease (SPVD) when co-infecting sweet potato plants.

## Sweet potato

Sweet potato (*Ipomoea batatas* L.) is a dicotyledonous, perennial plant, producing edible tuberous roots. It belongs to the family *Convolvulaceae*, the Morning Glory (Austin 1987). This family contains about 55 genera (Watson & Dallwitz, 2000). The genus *Ipomoea* is thought to contain over 500 species with ploidy levels ranging from 2x to 6x (Ozias-Akins & Jarret, 1994). Sweet potato is the only *Ipomoea* species of economic importance as a food crop (Onwueme & Charles, 1994), and has both 4x and 6x forms (2n = 4x = 60 or 2n= 6x = 90). *I. batatas* probably originates from a cross between the ancestors of *I. trifida* (Huang & Sun, 2000; Jarret & Austin, 1994) and another wild *Ipomoea* sp., in Central or northern South America, at least 5000 years ago, and may be among "man's" earliest domesticates. By the time of European contact, sweet potato was cultivated throughout the American tropics and had spread to the Easter Islands, Hawaiian and other Polynesian islands, as well as New Zealand. Sweet potato was introduced several different times into Europe, Africa and Asia during the late 15th and 16th centuries. In Africa, sweet potato was introduced to at least two places, West Africa and East Africa (Austin 1987).

Today, thousands of cultivars of sweet potato are grown throughout the tropics and subtropics (He, Prakash & Jarret, 1995). With an annual production of more than 133 million tons globally, sweet potato currently ranks as the seventh most important food crop on a fresh-weight basis in the world, and fifth in developing countries after rice, wheat, maize, and cassava (CIP,1999a). The production is concentrated in East Asia, the Caribbean, and tropical Africa, with the bulk of the crop (88%) being grown in China (Fig. 1; Hijmans, Huaccho & Zhang, 2001). In Africa the production is concentrated in the countries around the Lake Victoria. Uganda is the biggest producer of sweet potato in Africa, and the third in the world. Sweet potato is processed into snacks, starch, liquor, flour and a variety of other industrial products. In addition to being used for human consumption, sweet potato is also widely used as an animal feed (CIP, 2000b). Because of the enormous genetic diversity of sweet potato (Zhang *et al.*, 1998, 2000), and the accompanying diversity in phenotypic and morphological traits (Woolfe, 1992), the crop has great potential for further development to accommodate specific uses. Sweet potato performs well in relatively poor soils, with few inputs, and has a short growing period. Among the major starch staple crops, it has the largest rates of production per unit area per unit time (Woolfe, 1992): in some areas up to three harvests per year can be achieved (Karyeija, Gibson & Valkonen, 1998a). Sweet potato tubers are rich in vitamin C and essential mineral salts. Due to the high

beta-carotene content of yellow and orange-fleshed tubers, they are being promoted to alleviate vitamin A deficiency in East Africa (CIP, 1999b).

Despite the advantages that the cultivation of sweet potato offers, production tends to be concentrated in countries with low per capita incomes, and within those countries in regions where income levels are relatively low. Because of this, sweet potato has commonly been categorized as a "subsistence", "food security", or "famine relief" crop. Efforts to improve the agronomic qualities of sweet potato will therefore be of most benefit to developing countries, and particularly the poor sectors of the population within those countries. The International Potato Center (CIP), in Lima, Peru, has the international mandate for research on sweet potatoes in developing countries. Woolfe (1992) has reviewed the general agronomic principles of sweet potato production. The subsistence production of sweet potato in Africa has been reviewed by Karyeija, Gibson & Valkonen (1998a).

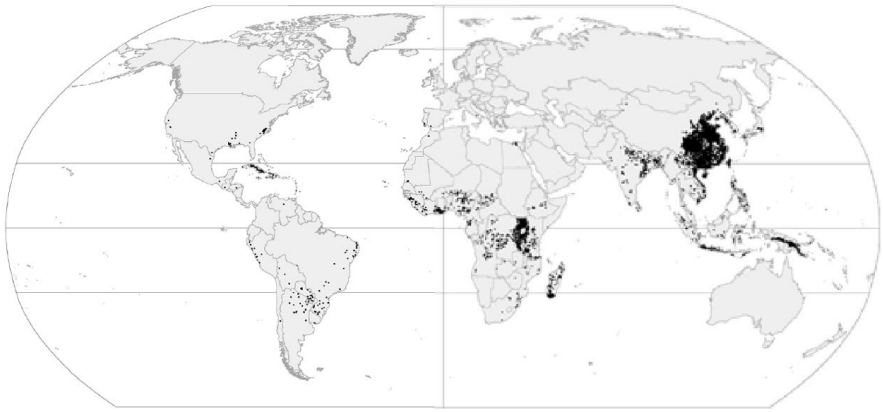

*Fig. 1.* Area cultivated with sweet potato over the period 1998-2000, each dot represents 1000 *ha.* (Hijmans, Huaccho & Zhang, 2001).

## Viruses of sweet potato

Although the sweet potato weevils (*Cylas brunneus* and *C. puncticolis*) are the most devastating pests of sweet potato worldwide (CIP, 2000a), diseases caused by viruses follow closely in importance wherever sweet potato is grown. Worldwide at least 19 different viruses have been described in sweet potato, but only 11 of these have currently been recognized by the International Committee of Taxonomy of Viruses (ICTV; Table 1). This number, however, will most likely increase by additional surveys. Vegetative propagation, usually by taking cuttings from a previous crop (Onwueme & Charles, 1994; Karyeija, Gibson & Valkonen, 1998a), increases the risk of a build-up of viruses. The importance of virus diseases and their build-up in farmers' planting material has been shown in China, where crops planted using pathogen tested sweet potato cultivars yielded 30-40% more, on average, than crops grown from farm-derived planting materials (Carey *et al.*, 1999; Fugli *et al.*, 1999).

*Table 1. Viruses that have been reported in sweet potato crops*

| Virus | genus | Reported distribution | Transmission | References |
|---|---|---|---|---|
| **Recognized by ICTV** | | | | |
| *Sweet potato feathery mottle virus* (SPFMV) | *Potyvirus* | Worldwide | aphids | 1, 2, 3 |
| *Sweet potato chlorotic stunt virus* (SPCSV) | *Crinivirus* | Worldwide | whiteflies | 1, 4, 5, 6, 7 |
| *Sweet potato mild mottle virus* (SPMMV) | *Ipomovirus* | Africa | whiteflies | 8, 9, 10 |
| *Sweet potato latent virus* (SwPLV) | *Potyvirus* | Africa, Taiwan, Peru, Indonesia | Unknown | 7, 11, 12 |
| *Sweet potato mild speckling virus* (SPMSV) | *Potyvirus* | Argentina, Peru, Indonesia | Aphids | 7, 13 |
| *Sweet potato leaf speckling virus* (SPLSV) | *Luteovirus* | Peru, Cuba | Aphids | 14, 15 |
| *Sweet potato yellow dwarf virus* (SPYDV) | *Ipomovirus* | Taiwan, Far East | Whiteflies | 11, 16 |
| *Sweet potato vein mosaic virus* (SPVMV) | *Potyvirus* | Argentina | Aphids | 17, 18 |
| *Cucumber mosaic virus* (CMV) | *Cucumovirus* | Israel, Egypt, Kenya | Aphids | 2, 19, 20 |
| *Tobacco streak virus* (TSV) | *Ilarvirus* | -* | Thirps, pollen | 2 |
| *Tobacco mosaic virus* (TMV) | *Tobamovirus* | - | Contact | 2 |
| **Not recognized by ICTV** | | | | |
| Sweet potato ringspot virus | *Nepovirus* | Papua New Guinea | Unknown | 21 |
| Sweet potato caulimolike virus | - | Puerto Rico | Unknown | 22 |
| Sweet potato leaf curl virus | *Geminivirus* | Taiwan, Japan, USA | Whiteflies | 23, 24, 25 |
| Sweet potato chlorotic fleck virus | | Africa | Unknown | 26 |
| Ipomoea crinkle leaf curl virus | *Geminivirus* | Israel | Whiteflies | 27 |
| C-6 virus | *Potyvirus* | South America | Aphids | 28 |
| C-8 virus | *Potyvirus* | South America | Aphids | 29 |
| Sweet potato virus G | *Potyvirus* | China | Unknown | 30 |

* not reported. References: 1) Sheffield, 1957; 2) Moyer & Salazar, 1989; 3) Sakai et al., 1997; 4) Winter et al., 1992; 5) Gibson et al., 1998; 6) Cohen et al., 1991; 7) Carey et al., 1999; 8) Hollings & Stone, 1976; 9) Colinet, Kummert & Lepoivre, 1996; 10) Colinet, Kummert & Lepoivre, 1998; 11) Liao et al., 1979; 12) Colinet, Kummert & Lepoivre, 1997; 13) Alvarez et al., 1997; 14) Nakano et al., 1992; 15) Fuentes et al., 1996; 16) Chung et al., 1986; 17) Nome 1973; 18) Nome, Shalla & Petersen, 1974; 19) Cohen & Loebenstein, 1991; 20) Ishak, 2002; 21) Brown, Brunt & Hugo, 1988; 22) Atkey & Brunt ,1987; 23) Chung et al., 1985; 24) Osaki & Inouye, 1991; 25) Lotrakul et al., 1998; 26) CIP 1993; 27) Cohen et al., 1997; 28) Fuentes, 1994; 29) Fuentes, Arellano & Meze, 1997; 30) Colinet, Kummert & Lepoivre, 1994

The most widespread virus infecting sweet potato, and the only one previously studied in detail is *Sweet potato feathery mottle virus* (SPFMV, genus *Potyvirus*, family *Potyviridae*) that occurs wherever sweet potato is grown (Brunt *et al.*, 1996; Moyer & Salazar, 1989). In many cases infection of sweet potato plants with SPFMV causes mild or no symptoms, although certain strains can cause qualitative damage due to internal cork or cracking of the tubers (;Mori *et al.*, 1995; Moyer, Kennedy & Abou-Ghadir, 1980; Ryu, Kim & Park, 1998). However, quantitative losses due to reduced plant vigour associated with chronic infection with SPFMV have been experienced (Esbenshade & Moyer, 1982; Moyer, 1987; Gibson *et al.*, 1997). Yet it is as a component of complex virus diseases that SPFMV probably causes the greatest damage.

Another widespread virus of sweet potato is the *Sweet potato chlorotic stunt virus* (SPCSV, genus *Crinivirus*, family *Closteroviridae*). The symptoms caused by this virus have often been confused with nutritional deficiencies, and it has therefore only recently been recognized as an important virus in sweet potato (Gibson *et al.*, 1998). As for SPFMV, the real importance of SPCSV probably lies in its role in several virus disease complexes of sweet potato.

### *Complex virus diseases of sweet potato*

Multiple virus infections are common in sweet potato (Carey *et al.*, 1999; Chung *et al.*, 1986; Clark *et al.*, 1998; Cohen, Milgram & Loebenstein, 1995; Di Feo *et al.*, 2000; Rossel & Thottappilly, 1987; Scheafers & Terry, 1976) and synergistic interactions are often involved. The most common of these disease complexes, known under the name sweet potato virus disease (SPVD), is caused by simultaneous infection with SPFMV and SPCSV (Scheafers & Terry, 1976; Ngeve & Bouwkamp, 1991; Winter *et al.*, 1992; Gibson *et al.*, 1998). It was probably first described around 1940 in Uganda, Burundi, Rwanda, and eastern Belgian Congo (Hansford, 1944). This disease is characterized by chlorosis, small, deformed leaves, and severe stunting, and can reduce yields of infected plants by up to 80% (Hahn, 1979; Mukiibi, 1977). Despite the apparent broad meaning of the name SPVD, the symptoms are so characteristic that the name has become restricted to the disease with these symptoms and caused by these viruses. SPVD is the most serious disease of sweet potato in Africa (Geddes, 1990), and may be the most important virus disease of sweet potato globally (Carey *et al.*, 1999).

Other viral disease complexes have also been described, which invariably seem to involve SPCSV. In Israel *Cucumber mosaic virus* (CMV, genus *Cucumovirus*, family *Bromoviridae*) was found infecting sweet potato together with SPCSV and usually also SPFMV, producing symptoms similar to SPVD and causing up to 80% reduction in yield (Cohen, Milgram & Loebenstein, 1995). It was shown that CMV could only infect sweet potato if the plants were first infected with SPCSV (Cohen & Loebenstein, 1991; Cohen, Milgram & Loebenstein, 1995). Interestingly, this seems not to be the case for CMV in Egypt, where it is found infecting sweet potato with or without SPCSV (Ishak, 2002). In Argentina, a disease locally known as chlorotic dwarf (CD) is caused by infection with SPCSV and SPFMV and/or *Sweet potato mild speckling virus* (SPMSV; genus *Potyvirus*, family *Potyviridae*), and is the most important disease of sweet potato in the

country (Di Feo *et al.*, 2000). Once again, the symptoms resemble those of SPVD and are most severe when all three viruses infect sweet potato simultaneously.

In all the mentioned disease complexes, infection with each virus separately causes only mild or no symptoms in sweet potato. They are thus caused by a synergistic interaction between the viruses. As both SPFMV and SPCSV are involved in all these diseases, the variation in the strains of these viruses should be important factors determining the pathology of disease.

## Sweet potato feathery mottle virus (SPFMV)

The genus *Potyvirus* and the family *Potyviridae*, of which SPFMV is a member, are the largest genus and family of plant viruses, respectively, to date. SPFMV has flexuous filamentous particles between 830-850 nm in length. They contain a single positive stranded RNA genome of about 10.6 kb (SPFMV-S; Sakai *et al.*, 1997), which is larger than the average (9.7 kb) of a potyvirus genome (Shukla, Ward & Brunt, 1994; van Regenmortel *et al.*, 2000). The coat protein (CP) of SPFMV 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, Conkling & Moyer, 1992). SPFMV is transmitted by several aphid species (*i.e. Aphis gossypii*, *A. craccivora*, *Lipaphis erysimi*, *Myzus persicae*) in a non-persistent manner. These aphids however do not colonize sweet potato and therefore itinerant alate aphids might be the means of transmission (Aritua *et al.*, 1998b; Kantack, Martin & Newsom, 1960). The host range of SPFMV is narrow and mostly limited to plants from the family *Convolvulaceae*, and especially to the genus *Ipomoea*, although some strains have been reported to infect *Nicotiana benthamiana* and *Chenopodium* spp. (Campbell, Hall & Mielinis, 1974; Moyer & Kennedy, 1978; Moyer, Kennedy & Abou-Ghadir, 1980; Nakashima, Salazar & Wood, 1993). Symptoms, host range, and serology have been used to group SPFMV isolates into two strains, the common strain (C) and the russet crack (RC) strain (Moyer, Kennedy & Abou-Ghadir, 1980; Moyer & Kennedy, 1978, Cali & Moyer 1981).

Traditionally potyviruses were recognized by their particle morphology and the typical 'pinwheel'-like cylindrical inclusions bodies, formed by viral proteins aggregating in the cytoplasm of infected cells. Nowadays, however, viral nucleic acid sequence data have replaced the cytopathological characteristics as the most important criterion for assigning viruses to the genus (Shukla, Ward & Brunt, 1994).

### *The infection cycle of a potyvirus*

Potyviruses normally enter their hosts via the stylet of an aphid. The acquisition of the virus by aphids may take seconds, and loss of virus transmissibility occurs after a short time (minutes). Virus acquisition by aphids is dependent on an N-terminal amino acid motif Asp-Ala-Gly in the CP (DAG; Shukla, Ward & Brunt, 1994), as well as the N-terminal motives Lys-Ile-Thr-Cys (KITC; Atreya *et al.*, 1992; Blanc *et al.*, 1998; Sasaya *et al.*, 2000) and Pro-Thr-Lys (PTK; Peng *et al.*,

1998) in the non-structural helper component protein (HC-Pro). Although evidence is mounting that HC-Pro forms a connection or 'bridge' between the virus particles and the inner surface of the aphid maxillary stylets (Blanc *et al.*, 1998), the possibility that HC-Pro enables a conformational change in the CP or an aphid factor in the stylet, thereby enabling virus binding to the stylet is not excluded (Salomon & Bernardi, 1995). Whatever the mechanism, upon feeding on the plant, the aphid regurgitates some saliva, and by this process inoculates the plant with the virus (Martin *et al.*, 1997).

As the virus enters the cell it starts to disassemble, and, being recognized by the host cell as an endogenous mRNA, is probably simultaneously translated in a process called "co-translational disassembly" (Shaw, Plaskitt & Wilson, 1986). By the time the virus has fully disassembled, the first viral proteins have already been produced and are ready to start replicating the viral RNA. Potyviruses contain a single large open reading frame (ORF) in their genome that is translated into a single polyprotein, which is then autocatalically digested into the several functional proteins (Fig. 2, page 16). Almost all of the potyvirus proteins have been shown to have multiple functions (Table 2, page 17). In addition, cleavage intermediates may have separate functions, and interactions with other viral and/or host proteins are probably required for certain functions, but this aspect has been little studied.

Upon infection potyviruses can cause the complete shut down, or up-regulation of several host genes (Aranda *et al.*, 1996; Escaler *et al.*, 2000; Wang & Maule, 1995). The conservation of virus-induced host gene shut down or induction between different viruses indicates some importance, but whether these changes in host gene expression are a prerequisite for viral replication or are an indirect consequence of viral protein functions is still unclear. Viral replication is restricted to a narrow zone of cells at the infection front (Aranda *et al.*, 1996; Wang & Maule, 1995). It occurs in tight association with membranous structures (Schaad, Jensen & Carrington, 1997), probably by a multimeric complex of viral and host proteins that specifically recognize and mediate replication of viral RNA, but not host RNA (reviewed by Lai, 1998). The role of other proteins than the NIb (RdRp), VPg, and CI (Hel) still remains unclear.

After infection of the inoculated cell, the virus moves to neighbouring cells (cell-to-cell, or short distance movement), and into the vascular tissue, where it spreads throughout the plant following the source-sink stream (systemic, or long distance movement). The mechanism of movement is not yet resolved, but several proteins are involved (Table 2, and references therein). The traditional view is that transport between cells requires active processes and receptor-like interactions between the virus and the host cell plasmodesmata. Both HC-Pro and CP have been shown to be able to increase the size exclusion limit of plasmodesmata in mesophyll cells (Rojas *et al.*, 1997). The CI is transiently located to the plasmodesmata at the infection front (Roberts *et al.*, 1998). However, many movement proteins (MPs) are also suppressors of host cell defence responses. There is also increasing evidence that a multitude of plant-encoded mRNAs travel through the phloem. Therefore it is possible that some viral movement proteins facilitate cell-to-cell or systemic movement of viruses by suppressing cell-to-cell

communication of plant defence responses, rather than by actively mediating transport through plasmodesmata (Carrington, 1999).

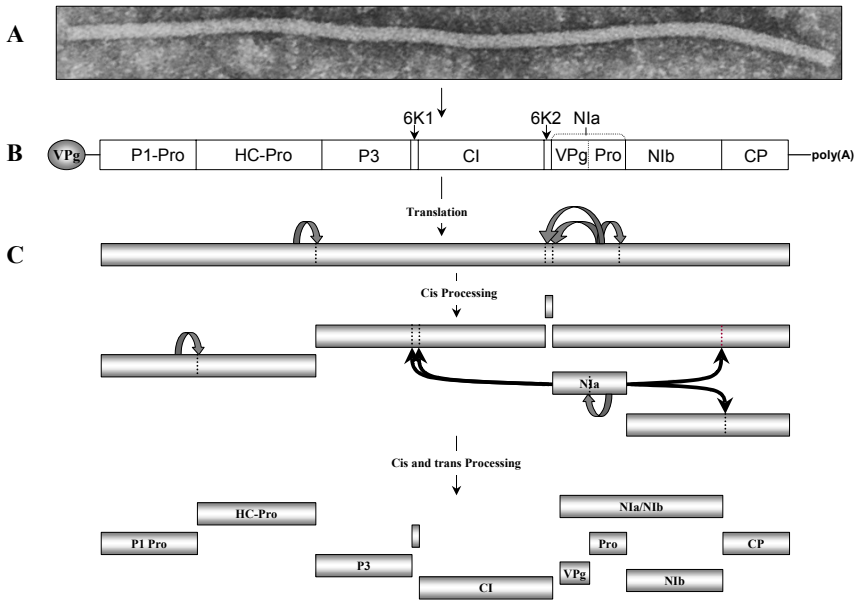


*Fig. 2*. Particle morphology (A), genome organization (B) and expression strategy (C) of the potyviruses (Shukla, Ward & Brunt, 1994). The potyvirus particle consists of flexuous filamentous rods (A) which are made up out of a single positive stranded RNA molecule (B) encapsidated by several thousand copies of a single coat protein. The genome contains one open reading frame, represented by the open box, in, or above, which the names of the final protein products are indicated, separated by lines that indicate the putative cleavage sites of the polyprotein. The 5' and 3' untranslated regions are represented by single lines. The genomic RNA is 3'-polyadenylated and has a viral protein (VPg) linked to it's 5'-end. The processing of the polyprotein by viral encoded proteinases is schematically depicted in C. The primary events are probably co-translational and autocatalytic, yielding precursors and mature products. There is no information about the sequential order of these events in plants, however in insect cells the 6K1/CI, 6K2/VPg, NIaPro/NIb and NIb/CP junctions are processed quickly, whereas the P3/6K1, CI/6K2 and the NIa-VPg/NIa-Pro junctions are processed at a slow rate (Merits *et al.*, 2002). The fully processed potyviral 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), 6kDa 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 coat protein (CP).

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

| Protein | Functions | Reference |
|---|---|---|
| P1 | proteinase | 1, 2 |
| | modulator of gene silencing | 3, 4, 5 |
| | replication/virus propagation | 6, 7 |
| HC-Pro | proteinase | 1, 8 |
| | aphid transmission | 9, 10 |
| | seed transmission | 11 |
| | cell-to-cell and systemic movement | 12, 13 |
| | suppressor of gene silencing | 3, 4, 5 |
| | replication/virus propagation | 7 |
| P3 | replication/virus propagation | 7, 14 |
| 6K1 | replication/virus propagation | 7, 15 |
| CI | RNA helicase | 16 |
| | cell-to-cell movement | 17, 18 |
| | replication/virus propagation | 7 |
| 6K2 | Long distance movement | 19 |
| | replication/virus propagation | 7, 20 |
| NIa/VPg | binds to initiation factor eIF(iso)4E | 21, 22 |
| | cell-to-cell and systemic movement | 23 |
| | replication/virus propagation | 7, 24 |
| NIa/Pro | proteinase | 25 |
| | replication/virus propagation | 7, 26 |
| NIb | RNA-dependant RNA polymerase | 27 |
| | replication/virus propagation | 7 |
| CP | encapsidation of RNA | 28 |
| | cell-to-cell and systemic movement | 29 |
| | aphid transmission | 30 |
| | seed transmission | 11 |
| | replication/virus propagation | 31, 7 |

* Note that all coding regions and the 5'- and 3'-untranslated regions are essential for virus propagation (Kekarainen, Savilahti & Valkonen, 2002). Most proteins have also been identified as pathogenicity/avirulence determinants, or symptom modulators, in one or more viruses. References: 1) Carrington, Freed & Sanders, 1989; 2) Carrington, Freed & Oh, 1990; 3) Anandalakshmi *et al.*, 1998; 4) Brigneti *et al.*, 1998; 5) Kasschau & Carrington, 1998 ; 6) Verchot & Carrington, 1995; 7) Kekarainen, Savilahti & Valkonen, 2002; 8) Carrington *et al.*, 1989; 9) Atreya *et al.*, 1992; 10) Sasaya *et al.*, 2000; 11) Wang & Maule, 1994; 12) Klein *et al.*, 1994; 13) Kasschau, Cronin & Carrington, 1997; 14) Kasschau & Carrington 1995; 15) Riechmann, Laín & Garcia, 1992; 16) Laín, Riechmann & Garcia, 1990; 17) Carrington, Jensen & Schaad, 1998; 18) Roberts *et al.*, 1998; 19) Rajamäki & Valkonen, 1999; 20) Schaad, Jensen & Carrington, 1997; 21) Wittman *et al.*, 1997; 22) Schaad, Anderberg & Carrington, 2000; 23) Schaad, Lellis & Carrington, 1997; 24) Schaad *et al.*, 1996; 25) Dougherty *et al.*, 1989; 26) Daros & Carrington, 1997; 27) Hong & Hunt, 1996; 28) Jagadish, Huang & Ward, 1993; 29) Lopez-Moya & Pirone, 1998; 30) Atreya *et al.*, 1995; 31) Haldeman-Cahill, Daros & Carrington, 1998

One could imagine that the potyvirus translation strategy has a "downside", given that all proteins have to be produced in equimolar amounts, and as a result of that, their amounts are dictated by the protein that is required in the highest molarity (probably the CP). This must lead to the accumulation of huge amounts of "redundant" proteins, and is probably the cause of the typical inclusion bodies

found in potyvirus-infected cells. However, the success of this family of viruses (about 30% of all plant viruses are potyviruses) speaks for a successful strategy, despite of the apparent inefficient or extravagant genome expression strategy.

## The Closteroviridae

The family *Closteroviridae* contains the largest and most complex positive-stranded RNA viruses infecting plants (Koonin & Dolja, 1993). Currently the family *Closteroviridae* is divided into two taxa (van Regenmortel *et al.*, 2000). The monopartite viruses belong to the genus *Closterovirus*, whereas the bipartite viruses belong to the genus *Crinivirus*. Most of the closteroviruses and all criniviruses are phloem-limited. They are all transmitted semi-persistently by specific homopteran vectors: aphids, mealybugs or whiteflies. Their particles are extremely flexuous and filamentous, with lengths ranging from 650 to 2000 nm (van Regenmortel *et al.*, 2000). Particles of *Beet yellows virus* (BYV, genus: *Closterovirus*), *Citrus tristeza virus* (CTV, genus: *Closterovirus*), and *Lettuce infectious yellows virus* (LIYV, genus: *Crinivirus*) are coated at an extremity (probably the 5'-end of the genome; Zinovkin *et al.*, 1999) by a minor CP (mCP, referred to as CPd in the genus *Closterovirus*), giving rise to a distinct structure for which the name "rattlesnake" has been used (Agranovski *et al.*, 1995; Febres *et al.*, 1996; Tian *et al.*, 1999). Because mCP occurs in the genome of all members so far sequenced, this terminal structure is probably a general feature of the family. Clostero- and criniviruses cause the formation of conspicuous vesicles containing a fibrillar network, which is one of the hallmarks of this virus family (van Regenmortel *et al.*, 2000). The positive stranded RNA genome of closteroviruses has a 5'-cap structure ($m^7$GpppN) and contains no 3'-poly(A) tail or tRNA like structures (Karasev *et al.*, 1989; Agranovsky *et al.*, 1991).

The viruses of the family *Closteroviridae* exhibit an astonishing genetic diversity that suggests extensive, on-going evolution. Therefore, the current composition of taxa within the family is certain to change. By phylogenetic analyses of conserved clostero- and crinivirus proteins, Karasev (2000) could show that the members of the family clustered into three groups according to their insect vectors, irrespective of if they were mono- or bi-partite, and proposed a new classification into three genera accordingly.

The viruses of the family *Closteroviridae* have not been studied as much as those of, *e.g.*, the *Potyviridae*, and less is known about the function of their proteins. Also, the number of potential proteins encoded by the viruses can vary between species, ranging from eight in, *e.g.*, BYV to as many as 12 in, *e.g.*, *Grapevine leafroll associated virus 3* (GLRaV-3, genus: *Closterovirus*; Ling *et al.*, 1998). They do, however, have a similar layout of the genome (Fig. 3) and invariably contain a number of similar genes (Fig. 3, Table 3), which will be discussed in the following paragraph.

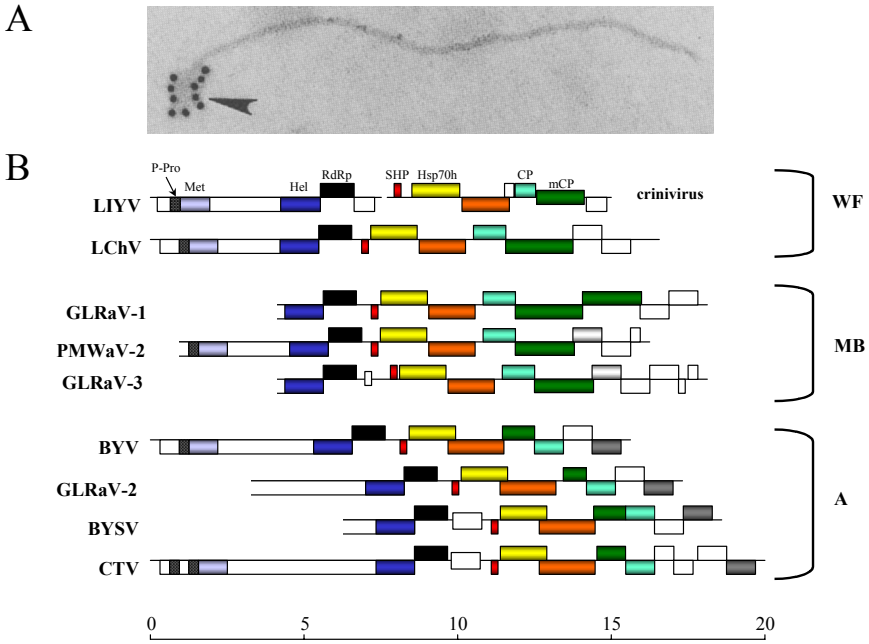


*Fig. 3.* (A) A particle of LIYV coated with gold-labelled antibodies against the minor CP located at one end of the particle, revealing the "rattlesnake" structure. (B) Genome structures of completely and partially sequenced closteroviruses and the only sequenced crinivirus LIYV. Rectangles correspond to ORFs, and a line indicates non-coding regions in the genomes. Conserved domains, or similar proteins in the different viruses, are indicated by the same colour or pattern. Open rectangles indicate no sequence data is available beyond what is shown. P-Pro: papain-like proteinase domain; Met, (putative) methyltransferase domain; Hel, (putative) helicase domain; RdRp, RNA-dependant RNA polymerase; SHP, small hydrophobic protein; Hsp70h, heat shock 70 family protein homologue; CP, coat protein; mCP, minor coat protein. The viruses are grouped according to the three lineages as determined by Karasev (2000); WF, whitefly-transmitted lineage; MB, mealybug-transmitted lineage; A, aphid-transmitted lineage. The vectors of LChV, GLRaV-1 and GLRaV-2 are unknown. BYV: *Beet yellows virus* (Agranovski *et al*., 1994), BYSV: *Beet yellow stunt virus* (Karasev *et al*., 1996), CTV: *Citrus tristeza virus* (Karasev *et al*., 1995), GLRaV-1: *Grapevine leafroll-associated virus-1* (Fazeli & Rezaian, 2000), GLRaV-2: *Grapevine leafroll-associated virus-2* (Zhu *et al*., 1998), GLRaV-3: *Grapevine leafroll-associated virus-3* (Ling *et al*., 1998), LChV: *Little cherry virus* (Jelkmann *et al*., 1997), LIYV: *Lettuce infectious yellows virus* (Klaassen *et al*., 1995), PMWaV-2: *Pineapple mealybug wilt-associated virus-2* (Melzer *et al.*, 2001).

*Table 3. Known functions/properties of the mature proteins, common to all closteroviruses and LIYV*

| Protein | Functions | Reference |
|---|---|---|
| L-Pro/P-Pro | proteinase | 1, 2 |
| | cell-to-cell movement | 2 |
| | replication | 3, 4 |
| Met-Hel-RdRp | RNA-dependent RNA polymerase | 5, 6 |
| | localized to membranes | 7, 8 |
| SHP | cell-to-cell movement | 9 |
| Hsp70h | cell-to-cell movement | 10 |
| | virion assembly | 11, 12 |
| | attached to virion | 13, 14 |
| P60 homologues | cell-to-cell movement | 11 |
| | virion assembly | 9 |
| CP | RNA encapsidation | 15 |
| | cell-to-cell movement | 9, 12 |
| mCP/CPd | RNA encapsidation | 13, 15 |
| | cell-to-cell movement | 9, 12 |
| | vector transmission | 13 |

References: 1) Agranovsky *et al*., 1994; 2) Peng *et al*., 2001; 3) Peremyslov *et al*., 1998; 4) Peng & Dolja, 2000; 5) Peremyslov, Hagiwara & Dolja, 1998; 6) Yeh *et al*., 2000; 7) Erokhina *et al*., 2000; 8) Erokhina *et al*., 2001; 9) Alzhanova *et al*., 2000; 10) Peremyslov, Hagiwara & Dolja, 1999; 11) Satyanarayana *et al*., 2000; 12) Alzhanova *et al*., 2001; 13) Tian *et al*., 1999; 14) Napuli, Falk & Dolja, 2000; 15) Agranovski *et al*., 1995

ORFs 1a and 1b encode a polyprotein (Fig. 4) and are sufficient to support replication of the viral RNA (Klaassen *et al.*, 1996; Peremyslov, Hagiwara & Dolja, 1998). A leader papain-like proteinase (P-Pro) encoded by the 5'-proximal part of ORF 1a autocatalytically cleaves itself from the rest of the protein. Downstream of the proteinase domain, ORF 1a encodes methyltransferase (Met) and helicase (Hel) domains. ORF 1b encodes an RNA-dependant RNA polymerase (RdRp). ORFs 1a and 1b are oriented in a 0/+1 configuration, and ORF1b (containing the RdRp) has been shown, or suggested, to be translated through a +1 ribosomal frameshifting mechanism (Agranovski *et al.*, 1994; Jelkmann *et al.*, 1997; Karasev *et al.*, 1995, 1996; Klaassen *et al.*, 1995; Ling *et al.*, 1998; Melzer *et al.*, 2001; Zhu *et al.*, 1998). Potentially, ORF1a and 1b could lead to the production of five different proteins (Fig. 4), or even nine in the case of CTV, where a duplication of the P-Pro has taken place (Fig. 3). ORFs 1a and 1b are translated from the genomic RNA, whereas all the other potential ORFs are translated from a set of 3' co-terminal subgenomic RNAs (sgRNA; Hilf *et al.*, 1995; Gowda *et al.*, 2001).

Besides ORFs 1a and 1b, closteroviruses and LIYV contain five additional common ORFs encoding proteins that are required for movement and have been referred to as the "quintuple gene block" (Alzhanova *et al.*, 2000). A small hydrophobic protein (SHP) is found in all viruses, but besides a predicted transmembrane helix there is little similarity between the SHPs of the different viruses. The heat shock protein 70 family homologue (Hsp70h) is highly conserved in closteroviruses and LIYV, and is unique for the family

*Closteroviridae*. Therefore, primers designed to the conserved phosphate domains encoded in the N-terminal part of the *Hsp70h* gene can be used to confirm closterovirus infections in plants (Saldarelli *et al.*, 1998; Tian *et al.*, 1996). Immediately downstream of *Hsp70h*, an ORF is found encoding a putative protein of variable size (48-63 kDa). In BYV and CTV, but none of the other viruses, the protein encoded by the ORF directly downstream of *Hsp70h* has similarities to heat shock protein 90 (Hsp90) family of proteins (Agranovski, 1996), and is known to have functions in both movement and virion assembly (Alzhanova *et al.*, 2000; Satyanarayana *et al.*, 2000). The last two genes common to all closteroviruses and LIYV encode the putative CP and the mCP. In the aphid-transmitted group of closteroviruses the *mCP* is located upstream of the CP and the two genes are of more-or-less equal size. In the other closteroviruses and LIYV the *mCP* is located downstream of the *CP*, and the *mCP* is considerably larger than the *CP* (Fig. 3). Besides a function in movement, there are indications that the mCP has a function in vector transmission, at least in the genus *Crinivirus* (Tian *et al.*, 1999).

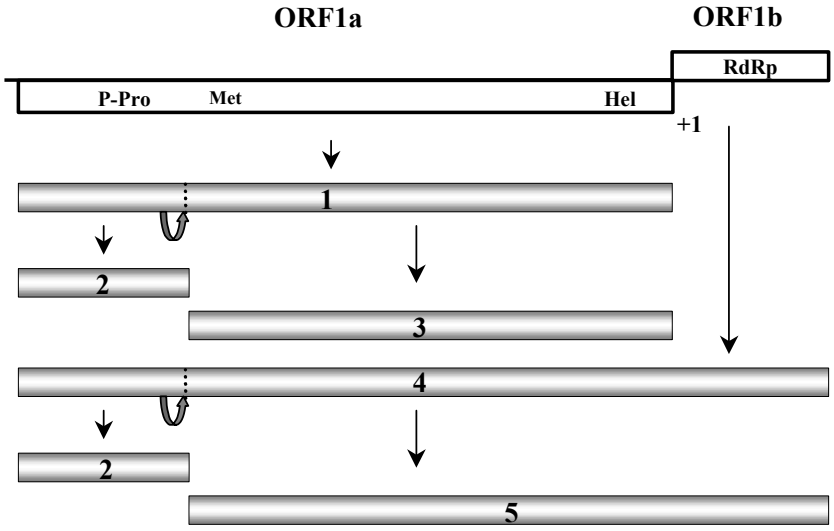


*Fig. 4.* Schematic representation of ORFs 1a and 1b, represented by boxes, and the five putative proteins that can be produced from it, indicated by the numbered and shaded boxes. Conserved domains are indicated. The site of putative +1 ribosomal frameshifting is also indicated. The curved arrow indicates the autoproteolytic cleavage site of the P-Pro.

In addition to the genes common in all closteroviruses and LIYV, the 3'-proximal gene of the aphid-transmitted closterovirus group (Karasev, 2000) is similar (Fig. 3). In BYV this gene is an enhancer of RNA replication (Peremyslov, Hagiwara & Dolja, 1998).

Because of its uniqueness, the Hsp70h of the *Closteroviridae* has been the subject of considerable attention. Cellular Hsp70 proteins in plants seem to have a special role during infection by viruses, as unrelated viruses induce expression of Hsp70 RNA at the infection front (Escaler *et al.*, 2000; Havelda & Maule, 2000).

The purpose of this induction of Hsp70s for the host and/or the virus is, however, unknown. Maybe the answer can be found by studies on clostero- and criniviruses. The viruses of the family *Closteroviridae* are unique among viruses, in that they have apparently recruited a cellular *Hsp70* gene into their own genome. Cellular Hsp70 proteins are ubiquitous molecular chaperones, involved in diverse tasks such as proper folding of proteins, guiding proteins across organellar membranes, disassembling oligomeric protein structures, facilitating proteolytic degradation of unstable proteins and in some cases controlling the biological activity of regulatory proteins, including transcription factors. All Hsp70s can be structurally divided into a N-terminal ATPase domain and a more variable C-terminal protein-binding domain (Bukau & Horwich, 1998). In vitro assays have demonstrated that Hsp70h of BYV has some similar characteristics as the cellular Hsp70s, including ATPase activity and association with microtubules. However Hsp70h does not bind protein chains known to stimulate cellular Hsp70s (Agranovski *et al.*, 1997; Karasev *et al.*, 1992). Hsp70h is physically associated with virions of LIYV and BYV (Napuli, Falk & Dolja, 2000; Tian *et al.*, 1999). It is localized to virion-containing vesicles and aggregates as well as plasmodesmata in BYV-infected plants (Medina *et al.*, 1999). Both BYV and CTV Hsp70h are necessary for proper virion assembly (Alzhanova *et al.*, 2001; Satyanarayana *et al.*, 2000). BYV Hsp70h is indispensable for movement, and assembly of the "rattlesnake" tail (Alzhanova *et al.*, 2001; Peremyslov, Hagiwara & Dolja, 1999). The current hypothesis predicts that mature, stable virions are guided toward plasmodesmata, presumably via an association of Hsp70h with the cytoskeleton, where it mediates translocation through the plasmodesmata in a similar manner as proposed for cellular Hsp70-mediated mitochondrial import (Alzhanova *et al.*, 2001).

The viruses of the genus *Closterovirus*, particularly CTV and BYV, have been extensively studied. In contrast, the only crinivirus sequenced to date is the type member LIYV. The number of reported diseases caused by the whitefly-transmitted criniviruses has increased over the past decades, which is probably due to the tremendous expansion of whitefly populations throughout the tropics and subtropics over the past 30 years (Wisler *et al.*, 1998). Increased knowledge on the genus *Crinivirus* will therefore be needed.

### *Sweet potato chlorotic stunt virus (SPCSV)*

SPCSV belongs to the genus *Crinivirus* within the family *Closteroviridae* (van Regenmortel *et al.*, 2000). The particles of SPCSV are 850 to 950 nm in length and 12 nm in diameter. The size of the major coat protein is 33 kDa, which is similar to other criniviruses (Cohen *et al.*, 1992; van Regenmortel *et al.*, 2000). SPCSV is transmitted by whiteflies (*e.g. Bemisia tabaci* and *Trialeurodes abutilonea*) in a semipersistent, non-circulative manner (Cohen *et al.*, 1992; Sim, Valverde & Clark, 2000). Similar to SPFMV, the host range of SPCSV is limited mainly to the family *Convolvulaceae* and 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 Lisianthus (*Eustoma grandiflorum*; Cohen *et al.*, 2001). SPCSV can be serologically divided into two major serotypes. One of the serotypes (designated serotype East Africa) occurs

only in East Africa, while the other serotype is found in all other parts of the world (Hoyer *et al.*, 1996; Vetten *et al.*, 1996). The genome structure and expression strategy of SPCSV are described in paper **IV** of this thesis.

## Genetic variability of RNA viruses

More than 90% of all plant viruses have an RNA genome (Hull, 2002). A hallmark of RNA genomes is the error-prone nature of their replication, which is thought to be due to the RdRps lacking 'proofreading' capabilities, typical to DNA-dependent DNA polymerases (Domingo & Holland, 1997). The error frequency of RdRps has been estimated to be $10^{-3}$ to $10^{-5}$ per nucleotide per round of copying (Domingo & Holland, 1997), which is about twice as high as measured for DNA replication (Roossinck, 1997). The high progeny yield, and short replication times, result in a heterogeneous population of viral RNAs that differ slightly from the population average within any given host and is termed a 'quasispecies' (Eigen, 1996; Smith *et al.*, 1997).

Evolution can be defined as the process by which the genetic structure of a replicating entity changes through time, and mutations create the variation upon which evolution can work. The viral quasispecies are a vast source of point mutations throughout the genome and provide RNA viruses with a potential for rapid evolution. However extreme bottlenecks in population size occur, *e.g.*, each time when a vector transmits a virus to a new host, and such bottlenecks often lead to a fitness loss, also called "Muller's ratchet" (Muller, 1964). Recombination is another source of variation and can occur between viruses or even between a virus and cellular RNA segments (reviewed in Simon & Bujarski, 1994), giving the viruses an opportunity to swap or recruit new genes or gene segments into their own genome. Furthermore, there is ample evidence that reassortment plays an active role in the adaptability of plant RNA viruses containing segmented genomes (Masuta *et al.*, 1998; Miranda, Azzam & Shirako, 2000; Qiu, Moyer & Qui, 1999).

Certain RNA virus proteins such as the RdRp or Hel are very conserved. Comparison of the amino acid sequences of the RdRp enable classifying all positive-stranded RNA viruses into three "supergroups" (Koonin & Dolja, 1993), which are further divided into families and genera. For the taxonomic assignment of viruses within families, genera, or even species, the more variable genes are usually used. Within the relatively homogenous family *Potyviridae*, CP sequence data has commonly been used to determine the phylogeny of different viruses and isolates (Shukla, Ward & Brunt, 1994). CP sequences have also been used for this purpose within the *Closteroviridae*, but, due to the enormous genetic variation within this family, it is more common to use the more conserved *Hsp70h* gene, which is readily amplified from any clostero- or crinivirus with degenerate primers.

## 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 & Inouye, 1986). The opposite, on the other hand, has been generally recognized to occur. One virus may assist a second, co-infecting virus, leading to increased titres and more severe symptoms and is referred to as viral synergism (Goodman & Ross, 1974; Pruss *et al.*, 1997; Savenkov & Valkonen 2001a; Vance *et al.*, 1995). In some cases, both of the co-infecting viruses may benefit from co-infection (Fondong *et al.*, 2000; Scheets, 1998). Synergism has also been known to occur between viruses and their satellite virus or RNA (Scholthof, 1999, Sanger *et al.*, 1994; Rodriquez-Alvarado, Kurath & Dodds, 1994), 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 (Hamilton & Nichols, 1977; Barker, 1989), thereby enabling it to invade tissues it otherwise could not. In other cases, viral replication and accumulation are enhanced. The best-studied viral synergisms are those where a potyvirus induces an increase in the titres of a second, unrelated virus (Goldberg & Brakke 1987; Poolpol & Inouye, 1986; Pruss *et al.*, 1997; Rochow & Ross, 1955; Ross, 1968; Savenkov & Valkonen, 2001a; Scheets, 1998; Vance, 1991; Vance *et al.*, 1995). In the potyvirus incited synergisms it has been found that the central region of the HC-Pro is the mediator of synergism, and the same region also suppresses the host RNA silencing mechanism, suggesting that these two phenomena are linked (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998; Savenkov & Valkonen, 2001a ; Shi *et al.*, 1997).

It is unusual to find the potyvirus component increased in synergistic diseases involving a potyvirus and another virus. However, Valkonen (1992) found an increase of about 1000 fold in the titres of *Potato virus Y* (PVY, genus: *Potyvirus*) in the wild potato species *Solanum brevidens* when co-infected with *Tobacco mosaic virus* (TMV, genus: *Tobamovirus*). The mechanism of this synergism has remained unresolved (Valkonen *et al.*, 1995).

## Natural virus resistance in plants

Resistance shown by an entire plant species to a specific parasite or pathogen is known as non-host resistance, and is expressed by every plant towards the majority of potentially pathogenic microbes (Heath, 2000). Non-host resistance is therefore the most common form of resistance exhibited by plants. As viruses are completely dependent on host factors, non host resistance to viruses can probably for a large part be attributed to incompatibility between viral and host proteins. However, there is increasing evidence that active defence responses, such as the hypersensitive resistance response (HR) constitute a major component in non-host resistance of plants against pathogens (Heath, 2000).

Individuals of a plant species that is usually a host to a certain pathogen can exhibit resistance. Such resistance can be due to environmental factors or plant age, but may also be genetically determined. Genetically determined resistance can be conferred by a single gene (monogenic resistance) or by a combination of several genes (oligogenic or multigenic resistance). Monogenic resistance genes usually confer strong resistance, but often only against a limited number of pathogen strains or races. Multigenic resistance is mediated through combined effects of many genes that each by them selves may confer only a rather insignificant resistance effect towards the pathogen in question. Multigenic resistance is generally not as strong as monogenic resistance, but it is typically equally effective against all strains or races of the pathogen (Agrios, 1997). Because of the complex inheritance of multigenic resistance it has long been under-utilized in breeding programs as compared to monogenic resistance and its mechanisms have been little studied.

### *Gene-for-gene resistance*

The gene-for-gene model, first proposed by the Dutch plant breeder Flor in the 1940s for flax and flax rust fungus (Flor, 1946), can explain many examples of resistance to fungi, bacteria, viruses, parasitic higher plants and insects in plants. In the gene-for-gene model, a plant containing a specific dominant resistance (*R*) gene is resistant to a pathogen-strain containing the corresponding dominant avirulence (*Avr*) gene. The plant and the corresponding pathogen are then called resistant and avirulent, respectively. Gene-for-gene plant disease resistance involves two basic processes: perception of a pathogen attack, followed by responses to limit disease.

Perception of the pathogen is mediated through the *R* genes, which can specifically recognize an elicitor that is directly or indirectly produced by a corresponding *Avr* gene in the pathogen. Several virus proteins including RNA-dependent RNA polymerase (Erickson *et al.*, 1999; Hamamoto *et al.*, 1997; Kim & Palukaitis, 1997; Padgett, Watanabe & Beachy,1997), movement protein (Meshi *et al.*, 1989; Weber, Schultze & Pfitzner, 1993; Weber, Schultze & Pfitzner, 1998), the coat protein (Bendahmane *et al.*, 1995; Berzal-Herranz *et al.*, 1995; de la Cruz *et al.*, 1997; Taraporewala & Culver, 1996), and a virus encoded suppressor of RNA silencing (Li *et al.*, 1999) have been identified as avirulence determinants. In a single case involving non-host resistance, viral RNA has been shown to act as the avirulence determinant (Szittya & Burgyán, 2000).

The responses mobilized after pathogen recognition are complex and described in a simplified manner in Figure 5. Although HR is common, the cell death response associated with HR is not an obligatory feature of resistance: some dominant *R* genes confer extreme resistance to viruses without the induction of cell death (Khan & Dijkstra, 2002), and virus resistance can be uncoupled from HR-associated cell death (Bendahmane *et al.*, 1999; Cole *et al.*, 2001).

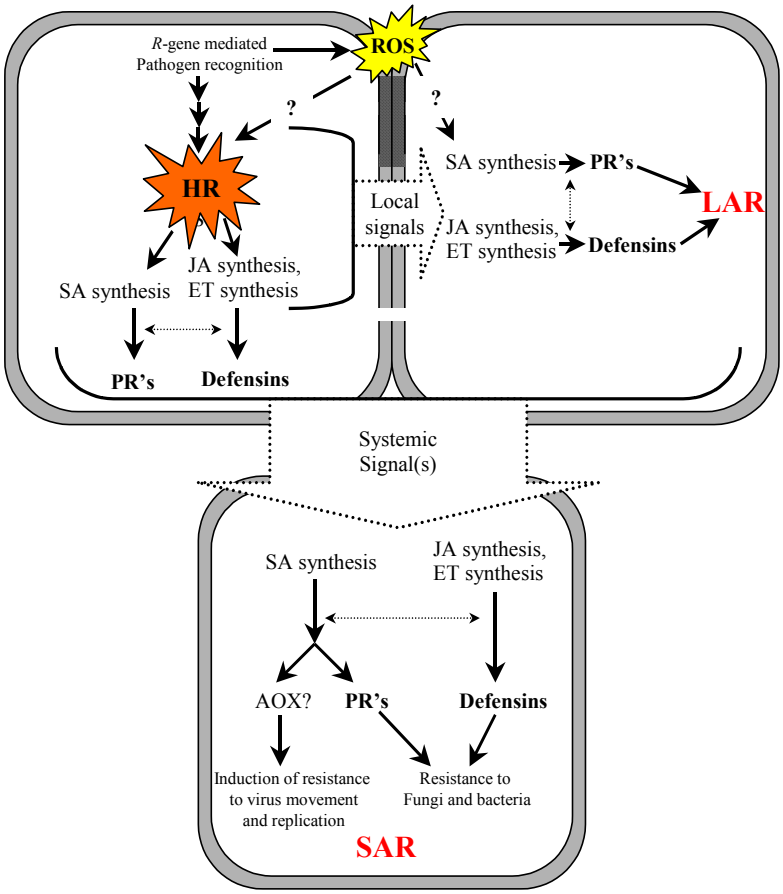


*Fig. 5*. Diagrammatic representation of the induction and expression of defence pathways in plants in response to recognition of avirulent pathogens (Costet *et al.*, 1999; Dangl & Jones, 2001; Feys & Parker, 2000; Graham & Graham, 1999; Hammond-Kosack & Jones, 1996; Lam, Kato & Lawton, 2001; Mauch-Mani & Métraux, 1998; Murphy *et al.*, 1999). Recognition of the pathogen mediated by a resistance gene (*R*-gene) is quickly followed by an oxidative burst characterized by the generation of extracellular reactive oxygen species (ROS) such as $H_2O_2$ and NO, which precedes the onset of the hypersensitive response (HR). The hypersensitive response is visually characterized by cell death at a later stage of the response. The effective defence responses include the production of salicylic acid (SA), jasmonic acid (JA) and ethylene, which in turn lead, through separate but cross-talking pathways to the production of anti-microbial compounds such as pathogen related (PR) proteins and defensins. The occurrence of HR evokes local signals, which lead to the induction of local acquired resistance (LAR) in adjacent or nearby cells, which is characterized by extreme resistance to superinfection by the same or different pathogens and coincides with the formation of PR proteins, defensins and cell wall modifications (textured area). A systemic signal is also produced in which SA may play a role. The systemic signal leads to systemic acquired resistance (SAR), which is characterized by increased resistance to infection by pathogens and a more rapid activation of defence responses to subsequent pathogen attacks. The model proposed by Murphy *et al.* (1999) to explain the induction of resistance to viruses in plants through a pathway where alternative oxidase (AOX) is involved is also indicated.

### *Recessive resistance*

Depending on the virus family, 20% to 40% of the known resistance genes to viruses are found to be recessive (Fraser, 1992). A large percentage of resistance genes to potyviruses are recessive and therefore many studies on recessive resistance concern resistance to this family of viruses. Recessive resistance to potyviruses can function by preventing cell-to-cell movement of the virus (PVY, Arroyo *et al.*, 1996; *Tobacco vein mottling virus* [TVMV], Nicolas *et al.*, 1997), systemic movement (*Pepper mottle virus* [PepMoV], Murphy *et al.*, 1998; *Tobacco etch virus* [TEV], Schaad, Lellis & Carrington, 1997; *Potato virus A* [PVA], Rajamäki & Valkonen 1999, 2002) or virus accumulation in the initially inoculated cells (*Pea seed-borne mosaic virus* [PSbMV], Keller *et al.*, 1998; PepMoV & TEV, Murphy *et al.*, 1998; PVA, Rajamäki & Valkonen 1999, 2002). In several cases, the VPg protein of potyviruses has been found to be the (a)virulence determinant in recessive virus resistance (TVMV, Nicolas *et al.*, 1997; TEV, Schaad, Lellis & Carrington, 1997; PSbMV, Keller *et al.*, 1998; PVA, Rajamäki & Valkonen 1999, 2002). Recessive resistance in pea (*Pisum sativum*), against PSbMV pathotypes corresponds to a gene-for-cistron interaction in which the *P3-6K1* cistron acts as the host specific pathogenicity determinant (Johansen *et al.*, 2001).

The cellular mechanisms of the recessive resistance genes have been little studied. One interpretation of resistance observed in plants carrying recessive resistance genes is that they do not provide a function essential for a particular step in virus infection (Revers *et al.*, 1999). However, the recessive gene *Mlo*, which confers resistance against powdery mildew in barley, is an example demonstrating that recessive resistance can also be caused by lack of a host factor that suppresses a resistance response (Büschges *et al.*, 1997).

### *RNA silencing*

Gene silencing is a basal and sophisticated biological network of interconnecting pathways involved in cellular defence against viruses and transposable elements and a mechanism to control development (reviewed by Fagard & Vaucheret, 2001; Matzke, Matzke & Kooter, 2001; Vaucheret & Fagard, 2001). Gene silencing in plants can be transcriptional, taking place in the nucleus, or post transcriptional (RNA silencing), taking place in the cytoplasm. Recent evidence, however, suggests that they are different phenomena of the same system (Bender, 2001; Finnegan, Wang & Waterhouse, 2001; Pal-Bhadra, Bhadra & Birchler, 2002; Waterhouse, Wang & Lough, 2001). The RNA silencing system recognizes and specifically degrades RNA it perceives as foreign or unusual/aberrant and sends a systemic signal, which induces RNA silencing to homologous RNA in distal parts of the plant. Although the exact mechanism involved in RNA silencing has yet to be determined, double stranded RNA (dsRNA) has an important role (Fire *et al.*, 1998). In addition, small interfering RNAs (siRNA) are invariably associated with RNA silencing (reviewed by Waterhouse, Wang & Finnegan). Crucial roles have also been shown for cellular RdRps, RNA helicases and ribonuclease III (RNaseIII)-like molecules (Bernstein

*et al.*, 2001; Dalmay *et al.*, 2001;Elbashir, Lendeckel & Tuschl, 2001; Xie *et al.*, 2001). Figure 6 presents a model of how RNA silencing may function.

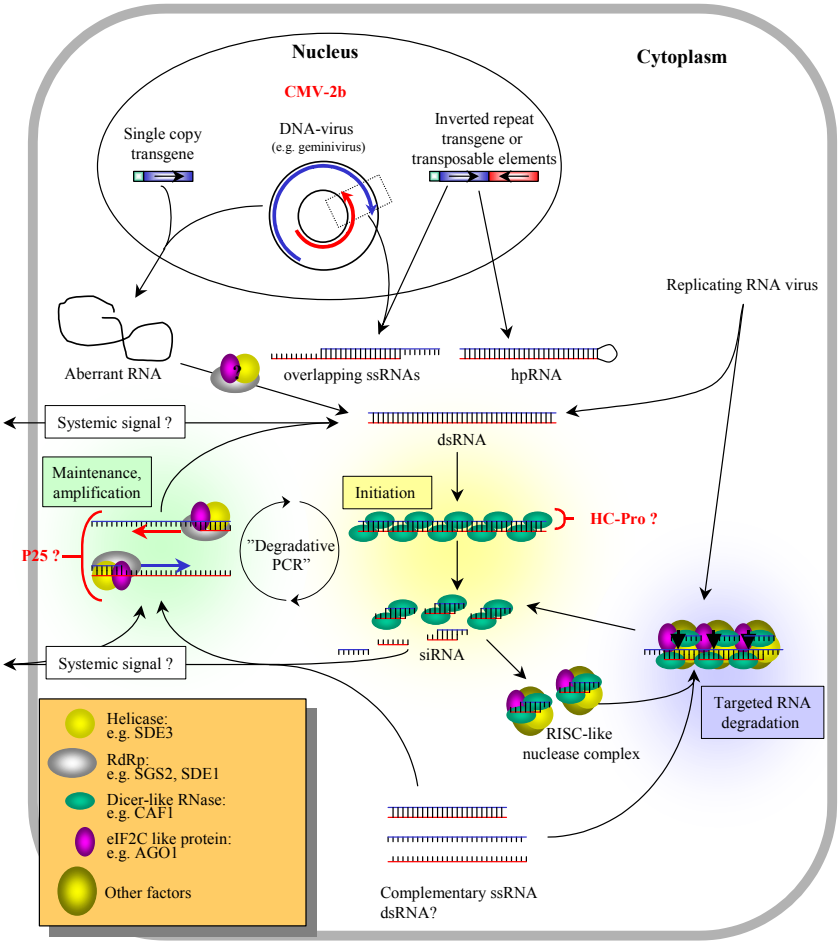


*Fig. 6.* A putative model for the mechanisms of RNA silencing in plants, based on knowledge from the related systems in plants, fungi and animals. The pathways leading to DNA methylation and transcriptional gene silencing are omitted for simplicity. RNA silencing is triggered by double stranded RNA (dsRNA). The dsRNA can originate from replicating RNA viruses, or from DNA viruses, *e.g.* through overlapping transcripts from the two DNA strands of geminiviruses, $tRNA_{met}$ priming from the 35S RNA (Voinnet, 2001) or the large stemloop structure in the pre-genomic RNA leader of caulimoviruses (Khane & Dijkstra, 2002). Also transgenes that have been designed to create inverted repeats, or, by chance, have integrated into the genome as inverted repeats can produce overlapping regions of dsRNA. In addition, single copy transgenes can be silenced, probably through the production of RNA that the cell somehow recognizes as being aberrant and replicates into a dsRNA form. In the initiation step, the dsRNA is recognized by a Dicer-like nuclease (Bernstein *et al.*, 2001; Knight & Bass, 2001) and is cleaved into 21-22 nt long (two helical turns), small interfering RNAs (siRNA), with 3' overhangs of 2-3 nucleotides (Elbashir,

Lendeckel & Tuschl, 2001). Following the initial cleavage into siRNA, first, the siRNA may serve as templates for a host RdRp complex which uses complementary single stranded RNA (ssRNA) and possibly dsRNA as a template to create more dsRNA which can be degraded into new siRNA in a cycle of 'degradative PCR' (Lipardi *et al.*, 2001; Nishikura, 2001; Sijen *et al.*, 2001). Alternatively, the siRNAs are mobilized into a multimeric RNase complex (RNA-Induced Silencing Complex, RISC; Hammond *et al.*, 2000), that is guided to the target RNA (ssRNA and possibly dsRNA) by base pairing of the siRNA. RISC cleaves the target RNA into siRNAs, at a position approximately in the middle of the guide sequence (black arrowheads; Elbashir, Lendeckel & Tuschl, 2001). The RISC complex is composed siRNA (Hammond *et al.*, 2000), a protein with similarity to initiation factor eIF2C (Hammond *et al.*, 2001) and additional factors, possibly including an RNA helicase (Nykänen, Haley & Zamore, 2001) and a Dicer-like nuclease (Hammond *et al.*, 2001). A systemic signal is also produced, which can confer the specific RNA silencing to distal parts of the plant. It has, however, still not been determined what composes the signal, but its specificity implicates some form of nucleic acid, probably RNA. While there is evidence that the siRNA may not be the silencing signal (Mallory *et al.*, 2001), the possibility is not excluded. Probably only very small amounts of the signal molecules are required to start the process of 'degradative PCR' and it may be below the current detection limits. Host proteins may be required in the transport of the signal (Waterhouse, Wang & Laugh, 2001). In the figure, the possible places where viral proteins may suppress RNA silencing at its different phases are indicated in red. HC-Pro of potyviruses eliminates the small RNAs but not the mobile signal (Mallory *et al.*, 2001). The 25 kDa movement protein (p25) of *Potato virus X* (PVX) suppresses the silencing signal and can suppress RNA silencing induced by a (sense) transgene but not RNA silencing induced by a virus (Voinnet, Lederer & Baulcombe, 2000). The 2b protein of cucumoviruses suppresses the initiation of silencing and cannot reverse silencing in already silenced tissue (Brigneti *et al.*, 1998). Nuclear localization is required for the activity of the CMV 2b protein (Lucy *et al.*, 2000).

Plant viruses are inducers and targets of RNA silencing, which poses a potent defence against them in all plants. 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. Several viral suppressors of RNA silencing have recently been identified among the virus-encoded proteins, namely the HC-Pro (potyviruses; Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998), 2b (cucumoviruses; Brigneti *et al.*, 1998), P1 (sobemoviruses; Voinnet, Pinto & Baulcombe, 1999), p19 (tombusviruses; Voinnet, Pinto & Baulcombe, 1999) and the AC2 (geminiviruses; Voinnet, Pinto & Baulcombe, 1999). The 25 kDa movement protein of *Potato virus X* (genus: *Potexvirus*) prevents the spread of the gene silencing signal (Voinnet, Lederer & Baulcombe, 2000). These proteins have all 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 different viral suppressors affect different phases of RNA silencing (Fig. 6); in fact the different phases have been determined based on how different viral RNA suppressors interfere with RNA silencing. The viral suppressors of RNA silencing can, in turn, be the targets of other host resistance mechanisms (Li *et al.*, 1999). For several synergistic viral diseases between a potyvirus and an unrelated virus, the silencing suppressing properties of HC-Pro

are enough to explain the enhanced accumulation of the non-potyviral component of the synergism (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998; Savenkov & Valkonen, 2001a; Shi *et al.*, 1997).

Recovery refers to the situation where a plant is initially susceptible to a virus and is systemically infected showing typical symptoms, but the new leaves, which develop later, are symptomless, with low virus titres or virus-free (reviewed by Pennazio, Roggero & Conti, 1999). Recovery can be the result of RNA silencing (Ratcliff, Harrison & Baulcombe, 1997) and the ability of plants to recover is then probably dictated by a balance between the RNA silencing inducing properties of a virus, and the ability of virus encoded factors to suppress RNA silencing.

As most viruses require insect vectors to spread to new hosts, resistance against the virus vector may also provide, indirectly, resistance to the virus. The emergence of new biotypes of whiteflies with wider host ranges and the subsequent problems with geminiviruses emerging in crops they did not previously infect, underline the importance of insect vectors in relation to virus host range (Morales & Anderson, 2001; Polston & Anderson, 1997).

## Genetically engineered virus resistance in plants

By inoculating a mild strain of a virus to a plant, it can be protected from infection with a more severe strain of the same virus, which is referred to as cross-protection. 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 cross-protection (Abel *et al.*, 1986). Since then, several different virus-derived genes and untranslatable sequences have been successfully used to obtain pathogen-derived resistance (PDR)(reviewed by Baulcombe, 1996; Beachy, 1997). The mode by which resistance is achieved can, however, be divided into two principally different mechanisms: protein-mediated and RNA-mediated.

### *Protein-mediated PDR*

In protein-mediated PDR, accumulation of the protein product of the transgene is required for the resistance phenotype. “Coat protein mediated protection” refers to the resistance caused by the expression of a viral CP in transgenic plants. For the CP mediated resistance against TMV, it was proposed that the large amounts of coat protein in the cells interfere with the uncoating of the virus upon initial infection of the inoculated cell and also may restrict virus long distance movement (reviewed by Bendahmane & Beachy, 1999). Another strategy uses the expression of mutated RdRps or MPs in transgenic plants to achieve resistance. In these cases it is thought that the mutated, dysfunctional proteins compete in binding to host factors needed for proper function of the corresponding virus protein (Baulcombe *et al.*, 1996; Beachy, 1997). This type of resistance has in some cases been shown to be active against a somewhat broader range of viruses than the CP-mediated resistance (Beck *et al.*, 1994; Cooper *et al.*, 1995).

### *RNA-mediated PDR*

RNA-mediated PDR requires only the transcription of RNA and relies on activating the hosts RNA silencing machinery. In principle, any part of the viral genome could be used to induce RNA silencing. The minimum size of the RNA molecule required for RNA silencing induction seems to vary case by case, but 23 bases can be sufficient (Thomas *et al.*, 2001). Many cases of PDR have been shown to be RNA-mediated rather than protein-mediated (reviewed by Baulcombe, 1996; Beachy, 1997). Transgenic plants showing strong RNA silencing of desired genes at a high frequently (90-100% of transformants) can nowadays be obtained by the use of constructs containing self-complementary, intron-spliced, 'hairpins' (Smith *et al.*, 2000; Wesley *et al.*, 2001). The use of this particular technique to induce virus resistance in plants will probably increase in the coming years, due to the advantages it offers. For example, this technology is advantageous as compared to protein mediated PDR, because it minimizes some epidemiological risks that may be associated with the cultivation of transgenic plants expressing viral genes (reviewed by Hammond, Lecoq & Raccah, 1999). Little or no protein is produced in RNA-meditated PDR and RNA transcript levels are extremely low. As a consequence, the possibility of protein-mediated synergistic effects with other viruses is avoided and the chance of recombination of transgene transcripts with heterologous infecting viruses is greatly reduced.

Finally, some cases of PDR appear to be both protein- and RNA-mediated (Germundsson *et al.*, 2002) indicating that these mechanisms are not mutually exclusive.

### *Other approaches*

A general drawback of most PDR approaches is that they usually are effective only against a single virus or several closely related viruses. A number of alternative approaches to engineer resistance in plants have been tried. In some cases these have proven to be active against a wider range of viruses, *e.g.*, ribosome inactivating proteins (reviewed by Wang & Tumer, 2000), mammalian 2-5A system components (Ogawa, Hori & Ishida, 1996; Truve *et al.*, 1993), bacterial RNaseIII protein (Watanabe *et al.*, 1995), RNA capping enzymes (Masuta *et al.*, 1995), single chain antibodies expressed in plants (reviewed by Schillberg *et al.*, 2001) or cystein proteinase inhibitor (Gutierrez-Campos *et al.*, 1999). Syngenta-MOGEN, in collaboration with Pierre de Wit's group at Wageningen University have reportedly created broad-spectrum disease resistance, which is active against fungi as well as viruses, by transfer of a pathogen-derived elicitor under a tight control of a pathogen-inducible promoter, to plants containing the corresponding *R* gene (Stuiver & Custers, 2001).

One rationale coming from the study of *R* genes and their downstream signalling components, is that it might be possible to manipulate expression of these genes, or to transfer durable *R* genes from one species to another. Although this field is still very much in its infancy, there have been some promising reports (reviewed by Stuiver & Custer, 2001). The main problem with moving *R* genes among species is that they usually only function in closely related species. The

understanding of the recognition by the *R* genes and the downstream signalling pathways is still too limited to design regulation systems utilizing *R* gene-signalling systems for induction of general defence responses.

## Virus resistance in sweet potato

Little has been reported about virus resistance in sweet potato, and all that has been reported concerns SPFMV or SPVD (Aritua *et al.*, 1998a, 1998b; Gibson *et al.*, 1997; Hahn *et al.*, 1981; Mihovilovich, Mendoza & Salazar, 2000). Many sweet potato cultivars seem to be naturally quite resistant to most strains of SPFMV, showing only mild initial symptoms, from which they usually recover, and containing low virus titres (Abad & Moyer 1992; Cadena-Hinojosa & Campbell, 1981; Esbenshade & Moyer, 1982). East African sweet potato cultivars seem to be especially resistant to SPFMV (Gibson *et al.*, 1998). Also, sweet potato plants graft-inoculated with SPFMV and subsequently planted in a field in Uganda all became virus free by time, with exception of those that had become naturally infected with SPCSV (Aritua *et al.*, 1998b). This indicates that at least some East African sweet potato cultivars are able to eliminate SPFMV, but that infection with SPCSV somehow interferes with the recovery. Indeed, co-infection of SPFMV and SPCSV causes SPVD, even in the most resistant clones of sweet potato (Karyeija, Gibson & Valkonen, 1998a).

"Field resistance" to SPVD has been observed in East African cultivars, expressed as a lower number of plants affected by SPVD, although the plants that are affected are equally diseased as those of the more susceptible cultivars (Aritua *et al.*, 1998a, 1998b). The mechanism of this resistance is unknown, but cultivars with high levels of SPVD "field resistance" yield less than cultivars that are more susceptible, making the use of resistant cultivars attractive only in areas with exceptionally high disease pressure (Aritua, 1998a). Because resistance is correlated with low yield, it may be that resistance comes at a high energy cost to the plant. Alternatively, it may be that the genetic factors mediating "field resistance" are tightly linked to those conferring low yield. Karyeija, Gibson & Valkonen (1998b) identified several wild *Ipomoea* spp. that exhibited extreme resistance to SPFMV, SPCSV or both viruses. However, the incorporation of such resistance from the wild diploid species into polyploid sweet potato may not be an easy task.

### *Transgenic resistance*

As natural resistance to SPVD in sweet potato seems to be of limited use, it is legitimate to attempt alternative strategies for obtaining virus resistance through biotechnological means. At least four groups, namely the Kenya Agricultural Research Institute (KARI) in collaboration with Monsanto and the USAID-funded Agricultural Biotechnology Support Project at Michigan State University, the Center for Plant Biotechnology Research at Tuskegee University, the Japan International Research Center for Agriculture and CIP work on incorporating transgenic resistance to SPFMV into sweet potato. Most attempts involve the use of the viral *CP* gene to achieve resistance, while CIP is using both a cystein

proteinase inhibitor (Cipriani *et al.*, 2001), and more recently, another strategy, described in the results and discussion of this thesis. Several of these approaches have been successful (Cipriani *et al.*, 2001; Okada *et al.*, 2001). However, at least in East Africa, SPFMV itself is not the problem but the synergistic SPVD caused by co-infection with SPCSV. It is still unclear whether resistance to SPFMV only will hold in the field where infections with SPCSV will occur. On-farm field trials have been initiated in Kenya by the KARI/Monsanto group to address this question (Zeigler, 2001). The approach taken by CIP, using the cystein proteinase inhibitor, seems the most promising since both SPFMV and SPCSV rely on virus-encoded cystein-like proteinases for the production of functional viral proteins.

# Aims of the study

"Know thy enemy" is a classical saying, and it's meaning cannot be underestimated if one wishes to come out of battle victoriously. In this spirit, the strategy of combating virus diseases in sweet potato, or any other crop, requires a thorough knowledge of the pathogens involved. As SPFMV and SPCSV appear to be the most wide spread viruses of sweet potato, and the co-infection of the two consistently appears to cause the most severe virus disease (SPVD) in sweet potato, knowledge on their molecular variation, genetic composition, and interaction is essential. As the problems with SPVD are particularly severe in East Africa where the crop is of major importance for subsistence, the studies in this thesis have been focused on the viruses occurring there. The main aims of the work described in this thesis have been to:

1. determine the variation of SPFMV in East Africa as compared to the rest of the world and to relate the variability to possible differences in resistance expressed in the cultivars.

2. study the synergistic relationship between SPFMV and SPCSV and find out which virus is the main cause of the severe disease.

3. characterize SPCSV at a molecular level.

# Results and discussion

## Genetic and biological variability of SPFMV in East Africa (I, II)

Several different isolates of SPFMV from different parts of the world have been characterized (Abad & Moyer, 1992; Cadena-Hinojosa & Campbell, 1981; Cali & Moyer, 1981; Colinet & Kummert, 1993; Esbenshade & Moyer, 1982; Gibb & Padovan, 1993; Mori *et al.*, 1994; Moyer, 1986; Nakashima, Salazar & Wood, 1993). SPFMV strains have mostly been studied for symptoms in indicator hosts or local lesion hosts. The so-called russet crack (RC) strain of SPFMV causes internal corkiness in certain sweet potato cultivars, such as Jersey. Strains producing internal corkiness have been reported from Japan, Korea, China and USA (Cali & Moyer, 1981; Colinet & Kummert, 1993; Ryu, Kim & Park, 1998; Sakai *et al.*, 1997). East African strains of SPFMV have not been reported to cause russet crack symptoms in East African cultivars (Karyeija, Gibson & Valkonen, 1998a), and none of the ones tested in this study did so in the cultivar Jersey, suggesting that the East African isolates do not belong to the RC strain group (unpublished). None of the East African strains of SPFMV could produce any local lesions in *Chenopodium* spp., reported for the RC-strain (Cali & Moyer, 1981; Moyer *et al.*, 1980), nor could they infect *N. benthamiana*, reported as a host for West African SPFMV (Rossel & Thottappilly, 1987) and the C1 isolate from Peru (Nakashima, Salazar & Wood, 1993). Furthermore, a sweet potato cultivar that was resistant to Peruvian strains of SPFMV as well as SPFMV-C, was not resistant to East African SPFMV (**II**). It seems, therefore, that the East African SPFMV isolates are different from other characterized isolates and strains.

CP sequence data for SPFMV is available for strains from the USA, Japan, Korea, China and Argentina (**I**). The isolates of SPFMV from East Africa, where SPFMV causes major problems, had been little studied. Therefore, we decided to genetically characterize East African SPFMV strains by sequencing their *CP* genes and comparing them to the *CP* gene sequences from strains studied elsewhere in the world. Phylogenetic analyses revealed that East African isolates formed a distinct group, separate from SPFMV isolates from elsewhere in the world (**I**). Other isolates could be grouped into three additional groups of which the C group was the most distinct (75,8-78,3 nt identity to any other isolates; **I**). It could be classified as a virus subspecies according to the definition by Shukla, Ward & Brunt (1994). From the phylogenetic data it also was apparent that the symptoms of internal corkiness, produced by some strains is not a property limited to a single genetic strain group, as such strains are present in both the RC group (strains S, K1 and RC) and the O group (strains CH and K2). The monoclonal antibody (MAb) raised against a mixture of the Nigerian isolate SPV-1 and the American SPFMV-C (MAb 7H8; Hammond *et al.*, 1992) could not fully distinguish between the strain groups identified on a genetic basis, since strains reacting with this antibody (MAb+ strains) were present in the groups EA, RC and O. On the other hand, strains not reacting with MAb 7H8 (MAb- strains) were present in groups EA and C. The epitope for MAb 7H8 could not be determined

by comparison of the CP aa sequences of MAb+ and MAb- strains. There were, however, biological differences between MAb+ and MAb- strains from Uganda. The two serotypes differed in prevalence in crops in different districts of Uganda and in two common sweet potato cultivars. They could be simultaneously transmitted by a single aphid, but they differed in the rate to which they systemically co-infected sweet potato cv. Tanzania (**II**). Interestingly, out of 20 plants inoculated with single aphids (previously allowed access to MAb+, SPVD-affected plants), six were infected with only a MAb- strain and six with both the MAb+ and MAb- strains, but none were infected with only the MAb+ strain (see Fig. 2 in **II**). However, sequencing of eight individual *CP* gene fragments amplified from one of the MAb+ source plants (MBL), resulted only in the sequences of the MAb+ virus, implying that this virus represented the major component in the source plant (unpublished). This may suggest that the MAb+ strain is unable to infect cv. Tanzania by itself, even if the plant is already infected with SPCSV, *i.e.*, cv. Tanzania is completely resistant to MAb+ SPFMV strains. However, the infectivity of MAb+ strains in cv. Tanzania can be complemented by co-infection with a MAb- strain.

Two new SPFMV *CP* and *NIb* sequences have become available since the study (**I**) was completed. Therefore, a new phylogenetic analyses of the 5'-proximal 510 nt of the *CP* and a stretch 475 nt from the core-region of the *NIb* was carried out (Fig. 7). The results showed that a similar phylogenetic tree as determined in (**I**) was obtained for the CP sequence, but analysis of the *NIb* region placed the Ugandan isolate Nam1 together with isolate 956, belonging to the C strain group according to its *CP* sequence. This result may indicate that recombination has occurred between an EA strain and a C strain some time during virus evolution.

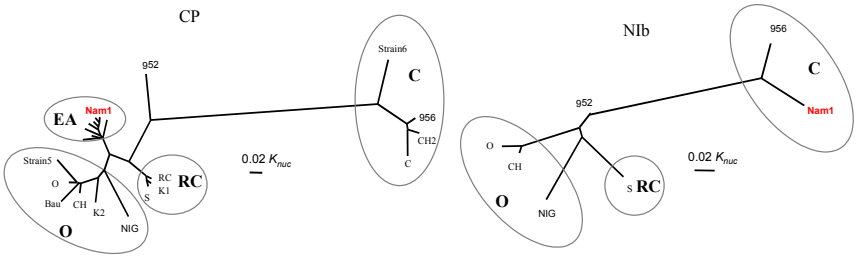


*Fig. 7.* Phylogenetic trees, generated by neighbour joining, based on the 5'-proximal 510 nt of the *CP*, and 475 nt from the core-region of the *NIb*. In the CP tree, only the Nam1 strain is indicated in the EA group for reasons of clarity. The strain-groups as determined in (**I**), are indicated by grey circles. Isolate Nam1, from East Africa is highlighted in red, and groups differently depending on if the *CP* or *NIb* sequence was used for phylogenetic analyses. The scale bar indicates 0.02 Kimura nucleotide units.

The high genetic diversity found for SPFMV in this study exemplifies the variable nature of RNA viruses. The overall CP amino acid sequence identities found between the C-strain group and the remaining strain groups of SPFMV are rather low and intermediate of what is found between individual virus species (< 71%) and between strains of a potyvirus (> 90%)(**I**; Shukla, Ward & Brunt, 1994; van Regenmortel *et al.*, 2000). In addition, recombination may have taken place

between the genetically distinct C and EA strain groups. Such differences are bound to express themselves in variable biological properties and has important implications for, *e.g*., resistance breeding in sweet potato and plant quarantine regulations. As shown in (**II**), breeding for resistance with local virus isolates may lead to the selection of plants that are not resistant to other isolates of the same virus. The determination of the genetic variability and geographic distribution of SPFMV isolates in this study have now made it possible for breeders to select for resistance to SPFMV in a more cognizant manner.

Containment of the different SPFMV isolates within their original geographic localities should be taken seriously, as the effects such strains may have on cultivars in other areas, where resistance to those strains may be lacking could be serious. This is particularly true for East Africa, where only one relatively homogenous group of isolates is found (**I**). The import of exotic isolates from elsewhere in the world could have a large impact on the East African crops that are quite resistant to the local isolates of SPFMV. As far as sweet potato is concerned, there are no borders in Africa and farmers exchange planting material freely across national borders. Considering this, it is important to determine where the geographic border between East Africa and West Africa goes as far as the SPFMV strain variability is concerned. Further sequencing of isolates collected throughout Africa will be necessary to resolve this question.

## Studies on the synergistic interaction between SPFMV and SPCSV (III)

Increased titres of SPFMV in SPVD-affected plants have been reported based on ELISA measurements, whereas the titres of SPCSV do not seem to be much changed (Gibson *et al.*, 1998). The mechanism lying behind these observations is not known. In this study (**III**), we quantitatively determined the changes in viral RNA concentrations, showing that SPCSV caused an enormous increase in the titres of SPFMV even in tissues where SPCSV itself was not detected, *e.g.*, in the youngest leaves. Titres of SPCSV did not significantly change and SPCSV remained limited to the phloem, as based on nucleic acid hybridisation and *in situ* immunohistochemical microscopy, respectively. In contrast, the high SPFMV titres were found in tissues outside of the phloem. The rate of movement of SPFMV in sweet potato plants was not affected by co-infection with SPCSV, indicating that other mechanisms must cause the synergistic effect.

One explanation of the results may be that SPCSV-encoded proteins exit from the phloem and assist SPFMV replication outside of the phloem, leading to the observed higher titres. For example, the P-Pro of clostero- and criniviruses seems to be functionally analogous to the potyviral HC-Pro as they both mediate proteolytic cleavage and genome amplification (Kasschau, Cronin & Carrington, 1997; Peng *et al.*, 2001). Also, the crinivirus-encoded Hsp70h may aid SPFMV: translation of host Hsp70s is induced in plants while the expression of many other host genes is shut off at the initial stages of potyvirus infection (Aranda *et al.*, 1996), suggesting some role for Hsp70s in potyvirus infections.

Alternatively, SPCSV-encoded factors may interfere with host defence responses that actively inhibit SPFMV replication. Resistance to CMV in sweet potato in Israel is broken by co-infection with SPCSV (Cohen & Loebenstein, 1991; Cohen, Milgram & Loebenstein, 1995). SPFMV and CMV are unrelated viruses, which suggests that SPCSV may affect an fundamental mechanism in sweet potato that is normally capable of suppressing infection of different kinds of viruses. One obvious candidate for such a mechanism is RNA silencing. Both cucumoviruses and potyviruses encode suppressors of RNA silencing (2b and HC-Pro respectively; Brigneti *et al.*, 1998), but the suppressors may differ in their activity in different host plants, or even be inactive in non-hosts plants (Voinnet, 2001). The SPCSV-encoded P-Pro may complement inefficient function of SPFMV HC-Pro, or even possess RNA silencing suppressing activities itself, similar to HC-Pro. RNA silencing involves signals that follow the same route through the phloem as used by viruses (Santa Cruz, 1999). Therefore, it may also be possible that SPCSV interferes with the systemic signalling required for efficient RNA silencing.

The determinations of viral RNA amounts in (**III**) were done using RNA probes spanning the *CP* gene, or antibodies detecting the CP. We now know that the sgRNAs corresponding to the genes encoded by SPCSV RNA2, including the *CP* gene, accumulate later in infection than the sgRNAs of SPCSV RNA1 in *Ipomoea setosa* (**IV**). In fact, RNA1 sgRNAs are present even in the youngest leaves of *I. setosa*, and it is possible that the same applies for sweet potato. This has yet to be tested, but if it is so, it means that, at least SPCSV RNA1 is present in all leaves where high SPFMV titres are observed, and may indicate that the SPCSV-encoded factors mediating the synergistic effect have to be sought on RNA1.

## Characterization of SPCSV (IV)

The viruses belonging to the genus *Crinivirus* have not been studied in much detail and their economic importance has only recently been recognized (Wisler *et al.*, 1998). The only crinivirus studied in detail is LIYV. It is therefore unknown if this virus is a 'characteristic' representative of the genus.

Any measures, aimed at controlling the synergistic viral diseases of sweet potato, in which SPCSV has emerged as a key player, will benefit from better understanding of the molecular mechanism of the SPCSV-induced synergism. Therefore, SPCSV was characterized at a molecular level (**IV**). The complete genomic sequence was determined and several sgRNAs were detected.

The genome of SPCSV consists of two RNA molecules. With a total length of 17630 nt, SPCSV is the second largest positive stranded RNA virus infecting plants, after CTV, sequenced to date. RNA1 (9407 nt) contains five putative ORFs and RNA2 (8223 nt) contains seven putative ORFs. Analysis of the genomic sequence of SPCSV exposed a number of new features as compared to LIYV (Fig. 8). The most striking may be the apparent recruitment of a novel gene encoding a putative RNaseIII-like protein in the genome of SPCSV. Such a putative protein is known to be present only in one other virus, *Paramecium bursaria Chlorella virus*

*1* (**IV**) containing a 330 kb large dsDNA genome. As cellular RNase III is involved in the maturation of almost any class of eukaryotic and prokaryotic RNA (Conrad & Rauhut, 2002) and also has an essential role in the process of RNA silencing (Bernstein *et al.*, 2001), the function of the putative SPCSV RNaseIII-like protein could range from modification or regulation of expression of its own RNAs to interference with host RNAs, including those involved in defence responses.

Another remarkable feature of SPCSV is the presence of near-identical, 208 nt long 3'-sequence on SPCSV RNA1 and RNA2, which have predicted stable RNA secondary structures. It is possible that the 3'-sequences have a regulatory role in replication, gene expression or particle assembly (Dreher, 1999). The fact that LIYV does not contain near-identical 3'-regions, or similar predicted RNA secondary structures in the 3'-region, suggests regulatory differences between the two viruses.

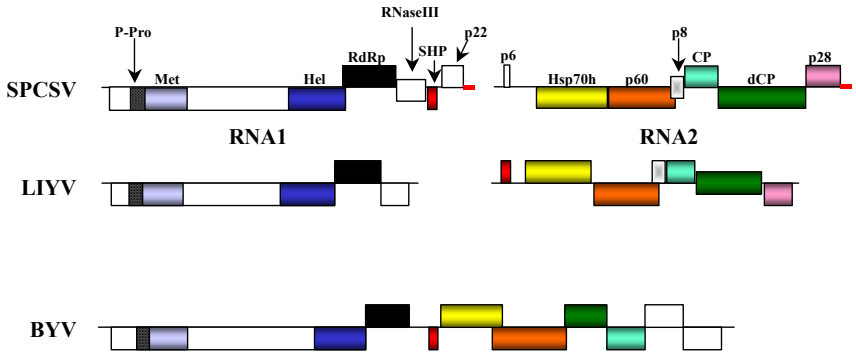


*Fig. 8*. The genome structures of the two criniviruses SPCSV (**IV**) and LIYV (Klaassen *et al.*, 1995) as compared to the closterovirus type member BYV (Agranovski *et al.*, 1994). Rectangles correspond to ORFs, and a line indicates non-coding regions in the genomes. The functional domains, predicted in the deduced amino acid sequence of each ORF, are indicated above the boxes in SPCSV. Alternatively, if no function could be predicted, the approximate molecular weight of the putative protein is indicated. RNaseIII denotes the ribonuclease III domain, whereas the other domains are as in Fig. 3. Conserved domains, or similar proteins in the different viruses, are indicated by the same colour or pattern. The red line at the 3'-ends of SPCSV RNA1 and RNA2 indicates the near-identical 3'-sequences.

The *SHP* gene, found immediately upstream of the *Hsp70h* gene in all closteroviruses and LIYV (Fig. 3), was found at a new relative position on the genome of SPCSV (Fig. 8). The only function known for the SHP is that it is required for viral movement (Table 3). Therefore it would have been logical to find *SHP* together with the other genes encoding movement proteins (Hsp70h, p60, CP and mCP; Table 3) on RNA2, as is the case for LIYV. Remarkably, however, *SHP* is found on RNA1 in SPCSV (Fig. 8).

The putative Hsp70h protein found encoded on SPCSV RNA2 has novel features as compared to its counterparts present in clostero- and criniviruses. Such features include a bipartite nuclear localization signal (NLS) and a C-proximal

leucine zipper (LZ) motif. LZs are thought to be DNA-binding domains and/or involved in protein dimerisation. They are found at the C-proximal part of many transcription factors (Busch & Sasson-Corsi, 1990; Ellenberger *et al.*, 1992). The presence of an NLS and a putative DNA binding domain (LZ) may suggest that SPCSV Hsp70h has some functions in the nucleus.

The 5'-ends of seven sgRNAs were determined, using a method based on the amplification of only 5' ($m^7$GpppN)-capped RNAs. Thus, the experiments indicated that the sgRNAs are 5' ($m^7$GpppN)-capped (**IV**), similar to the genomic RNA of BYV (Karasev *et al.*, 1989). Northern analysis of the sgRNAs at different stages of infection revealed that their production is temporally regulated: RNA1 sgRNAs accumulate earlier in infection than RNA2 sgRNAs. This suggests that genes encoded on RNA1 may have functions required early in the infection. Besides the requirement of the "replication module" encoded by ORFs 1a and 1b, it is not obvious what the role of the other putative proteins encoded on RNA1 (RNaseIII, SHP and p22) may be. The most 3'-proximal gene of LIYV RNA1 is an enhancer of RNA2 accumulation (Yeh *et al.*, 2000). Although the putative gene found at the same position of SPCSV (*p22*) is similar in size to the LIYV gene, there is little similarity in the deduced amino acid sequences between the two genes (**IV**).

Two putative proteins (p8 or p9 and p28 or p26 in SPCSV or LIYV respectively) are conserved in LIYV and SPCSV (Fig. 8) that are not found in any closteroviruses, and therefore appear to be specific to the genus *Crinivirus*.

The data presented in this study constitute a comprehensive basis for the detailed analysis of genome functions in the genus *Crinivirus* and, specifically, those of SPCSV. Some future lines of studies may be proposed. For example, the role of the RNaseIII-like protein putatively encoded by SPCSV could be examined by testing whether this molecule indeed has any RNaseIII activity, and if so, what the specificities for its substrate may be. Answers to these questions can give clues to the role of this protein in the infection cycle of SPCSV. Another interesting aspect to test is whether the SPCSV Hsp70h is localized to the nucleus, as the presence of NLS and a LZ motif may suggest. Finally, the creation of an infectious clone of SPCSV would open possibilities for many interesting studies to be conducted, shedding light on the life cycle of criniviruses.

### *Genetic variability of SPCSV*

Using monoclonal and polyclonal antibodies, East African SPCSV isolates can be serologically distinguished from isolates from West Africa, America and Asia (Hoyer *et al.*, 1996; Vetten *et al.*, 1996). East African isolates can be further distinguished into two groups, designated serotype East Africa 1 ($S_{EA1}$) and East Africa 2 ($S_{EA2}$), by a panel of MAbs (Alicai *et al.*, 1999). Although the complete *CP* and partial *Hsp70h* gene sequences of several East African isolates have been published in another study (Alicai *et al.*, 1999), and several additional partial *Hsp70h* sequences from other parts of the world are available from the genebank, there are no published comparisons, or phylogenetic analyses based on nucleotide or amino acid sequences of SPCSV strains from different parts of the world.

The *Hsp70h* sequence obtained in this study (**IV**) was compared to other sequences available from the genebank and a phylogenetic analysis was carried out (Fig. 9). The resulting phylogenetic tree shows that SPCSV can be divided into two genetically distinct groups (76.0%-78.3% nt and 91.1%-92.5% aa sequence identity between the two groups) based on partial *Hsp70h* nt sequences (Fig. 9). This grouping is constistent with the previously determined serological division (Hoyer *et al.*, 1996; Vetten *et al.*, 1996; Alicai *et al.*, 1999).

Similar to SPFMV, SPCSV strains from East Africa seem to be genetically unique and the implications of this finding is similar to what has already been discussed for SPFMV.

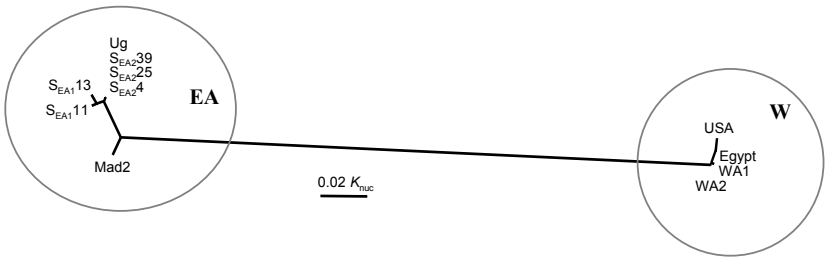


*Fig. 9.* Phylogenetic tree generated using a 446 nucleotide sequence stretch from the 5'-proximal ATPase domain of SPCSV *Hsp70h* genes. The bar represents 0.02 Kimura nucleotide units. All East African isolates and no isolate from elsewhere are clustered in group EA and belong to the Serotype $S_{EA}$. Isolates from elsewhere in the world cluster together in a distant group, designated W and are of the non East African serotype. Origin of isolates: all $S_{EA1}$ and $S_{EA2}$ are from Uganda (Alicai *et al.*, 1999), Mad2 is from Madagascar, WA1 and WA2 are from Nigeria, Ug is from Uganda (**III**, **IV**).

## Current and future studies

### *Attempts to identify the SPCSV genes involved in synergism*

The availability of the complete genomic sequence of SPCSV enabled us to select for candidate genes to be tested for induction of the synergistic effects with SPFMV. Four SPCSV genes were selected and were cloned into a plant expression vector (Fig. 10). The P-Pro of clostero- and criniviruses seems to be functionally analogous to the potyviral HC-Pro as they both mediate proteolytic cleavage and genome amplification (Kasschau *et al.*, 1997; Peng *et al.*, 2001). It was hypothesized that the SPCSV P-Pro may complement possible inefficient functions of SPFMV HC-Pro, or even possess RNA silencing suppressing activities, similar to HC-pro. Therefore *P-Pro* was included in our trials. The *RNaseIII-like* gene was selected because of the role assigned to the host RNaseIII-like proteins in the process of RNA silencing. Accordingly, the SPCSV putative RNaseIII-like protein may interfere in some way with the host RNA silencing mechanism, or alternatively SPCSV RNaseIII may interfere with host processing of mRNAs involved defence responses. Transcription of host *Hsp70s* is induced in plants while the expression of many other host genes is shut off at the initial stages of potyvirus infection (Aranda *et al.*, 1996), suggesting some positive role for

Hsp70s in potyvirus infections. Hence, the *Hsp70h* gene was selected to be tested. Other molecular chaperones have also been shown to be involved in virus infections (reviewed by Sullivan & Pipas, 2001), *e.g*., Hsp90 is required for *Hepatitis B virus* replication (Hu & Seeger, 1996, 1997). The homologues of the putative SPCSV p60 protein (one of the closteroviral hallmark genes) in BYV and CTV were found to have some similarity with the Hsp90 family of chaperones (Agranovski, 1996), indicating that these proteins and their homologues in other clostero- and criniviruses may be a type of molecular chaperones. SPCSV *p60* was therefore also included in our trials.

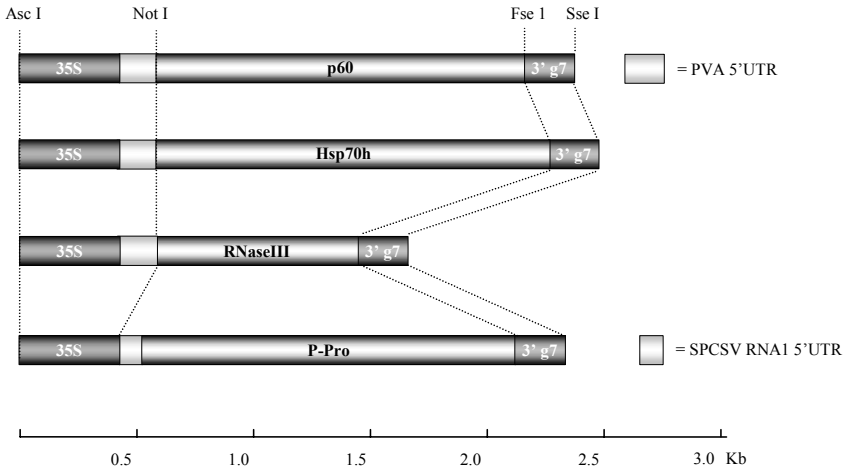


*Fig. 10.* Constructs made to test the effects of SPCSV-encoded proteins on the titres of SPFMV in sweet potato leaves. The SPCSV genes were amplified by RT-PCR from purified viral RNA with primers including *NotI* or *FseI* restriction sites. The amplified sequences were checked by sequencing and cloned under control of the CaMV 35S promoter fused with a translational enhancer (PVA 5'UTR), and upstream of the 3'g7 polyadenylation site, except *P-Pro* with which no enhancer was used. The constructs were transferred to the binary vector pKOH200 and transformed into *Agrobacterium tumefaciens* strain C58C1/pGV3850.

The selected genes were to be expressed in SPFMV-infected sweet potato plants by the *Agrobacterium tumefaciens*-mediated transient expression system (Agro-infiltration) as described by Kapila *et al.* (1996). If any of the SPCSV proteins, or a combination of proteins, would cause a rise in the titres of SPFMV, such a result might implicate that the SPCSV protein(s) concerned were causing the synergism with SPFMV.

A method for infiltrating sweet potato leaves with *A. tumefaciens* was developed, but we were unable to get any significant expression of the marker gene *GUS* (Fig. 11). Apparently, the *A. tumefaciens* strain used showed a low virulence and inefficient transfer of the T-DNA into sweet potato cells. In contrast, *GUS* expression was high in the infiltrated leaves of *N. benthamiana*, and the indicator plant *I. setosa* (Fig. 11). The experiments on sweet potato need to be continued. Possibly the *A. tumefaciens* strain used at CIP to transform sweet potato

(Otani *et al.*, 1998) may be more virulent on sweet potato and could be used for Agro-infiltration experiments in sweet potato leaves.

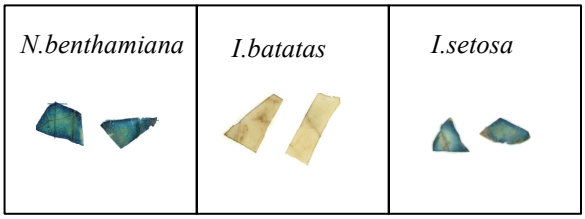


*Fig. 11.* Pictures of X-gluc stained leaves from *N. benthamiana*, sweet potato (*I. batatas*), and *I. setosa*, three days after infiltration with an *A. tumefaciens* strain containing the p35SGUSINT (Vancanneyt *et al.*, 1990) binary plasmid. Both *N. benthamiana* and *I. setosa* are stained blue indicating that the leaves were transformed with the *GUS* gene by *A. tumefaciens*. *I. batatas* was however not transformed, as indicated by the lack of staining.

*I. setosa* is initially highly susceptible to SPFMV and virus titres are equally high as in plants co-infected with SPFMV and SPCSV. However, the plants recover after prolonged infection, leading to lower SPFMV virus titres and loss of symptoms in the new leaves. Therefore, the selected SPCSV genes were expressed in the recovered leaves of *I. setosa* by Agro-infiltration. Table 4 summarizes the results from two experiments, showing that there was an increase in titres of SPFMV in almost all infiltrated leaves, apparently as a result of the infiltration procedure itself. Therefore, the *I. setosa* plants did not prove to be a helpful system for identification of SPCSV proteins causing the synergistic effect with SPFMV.

*Table 4. Absorbances ($A_{450nm}$) generated in TAS-ELISA, specific for SPFMV, of I. setosa leaves which had recovered from SPFMV infection and subsequently been infiltrated with A. tumefaciens containing plant expression constructs with the GUS gene (Fig. 11), or 4 different SPCSV genes (Fig. 10). Mix indicates that A. tumefaciens strains with all the different constructs were infiltrated simultaneously. Numbers between brackets indicate the percentage of the measured absorbances as compared to the SPVD affected plants. SD: standard deviation; dpi: days post infiltration (maximum GUS staining is observed 3dpi)*

| Agro construct | Experiment 1 (3dpi) | | Experiment 2 (5dpi) | |
|---|---|---|---|---|
| | Average | SD | Average | SD |
| GUS | 0.134 (33%) | 0.091 | 0.390 (41%) | 0.043 |
| P-pro | 0.222 (54%) | 0.037 | 0.361 (38%) | 0.048 |
| RnaseIII | 0.182 (44%) | 0.107 | 0.435 (45%) | 0.044 |
| Hsp70h | 0.236 (58%) | 0.118 | 0.395 (41%) | 0.025 |
| P60 | 0.328 (80%) | 0.170 | 0.060 (6%) | 0.050 |
| Mix | 0.061 (15%) | 0.079 | 0.249 (26%) | 0.022 |
| Recovered *I. setosa* | | | 0.013 (1%) | 0.013 |
| healthy | 0.003 | 0.02 | 0.004 | 0.010 |
| SPVD affected | 0.409 (100%) | 0.016 | 0.961 (100%) | 0.003 |

However, using "silencing on the spot" technology (Johansen & Carrington, 2001) based on Agro-infiltration, silencing of a marker gene, *e.g.*, *GFP* by a co-delivered gene directing production of a double-stranded *GFP* (dsGFP) transcript, could be induced in *I. setosa*. The selected genes of SPCSV (Fig. 10) can then be

tested for their ability to suppress the silencing induced by the dsGFP by introducing them simultaneously using Agro-infiltration. In addition, induction of silencing by a dsGFP transcript in one leaf should lead to a systemic signal. A *GFP* gene subsequently introduced by Agro-infiltration to an upper leaf should therefore be silenced. If one of the SPCSV genes co-delivered in the initially infiltrated leaf produces proteins that interfere with the systemic signalling of RNA silencing, the *GFP* introduced to the systemic leaf may not be silenced.

### *Towards transgenic resistance to SPVD*

As mentioned previously, transgenic resistance to SPFMV has been reported. However, it is unknown 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 (**II**). On the other hand, since SPCSV seems to be the mediator of synergism and SPVD, one could argue that it is enough to create resistance to just SPCSV. However, the exact mechanism of synergism is not yet understood, whereas it is known that potyviruses can suppress RNA silencing (Anandalakshmi *et al.*, 1998; Kasschau & 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 PVA, following infection with PVY (Savenkov & Valkonen, 2001b) or transgenic resistance to PVY following infection with CMV (Mitter *et al.*, 2001). The safest way to proceed seems, therefore, to develop resistance to both viruses simultaneously. We have started a project in collaboration with CIP, with the aim to create RNA silencing-based resistance against SPFMV and SPCSV simultaneously in sweet potato.

A fusion of a fragment from the SPFMV *NIb* (=*RdRp*) and SPCSV *RdRp* gene sequences, and an inverted repeat of this construct, separated by an intron (CSFMhr; Fig. 12) was made and is now being transformed to sweet potato cultivars at CIP. Transcription of the construct should lead to the formation of a perfect dsRNA specific to both viruses. This strategy has recently been shown to consistently lead to a very high frequency (98-100%) of transformants showing silencing of the targeted sequences (Smith *et al.*, 2000; Wesley *et al.*, 2001).

The *RdRp* gene sequences of RNA viruses are more conserved than the commonly used *CP* gene sequences, which should provide resistance to a larger range of virus strains. An additional advantage of using the SPCSV *RdRp* is that it resides on RNA1, which accumulates earlier in the infection than RNA2 (**IV**), and is essential for RNA2 replication.

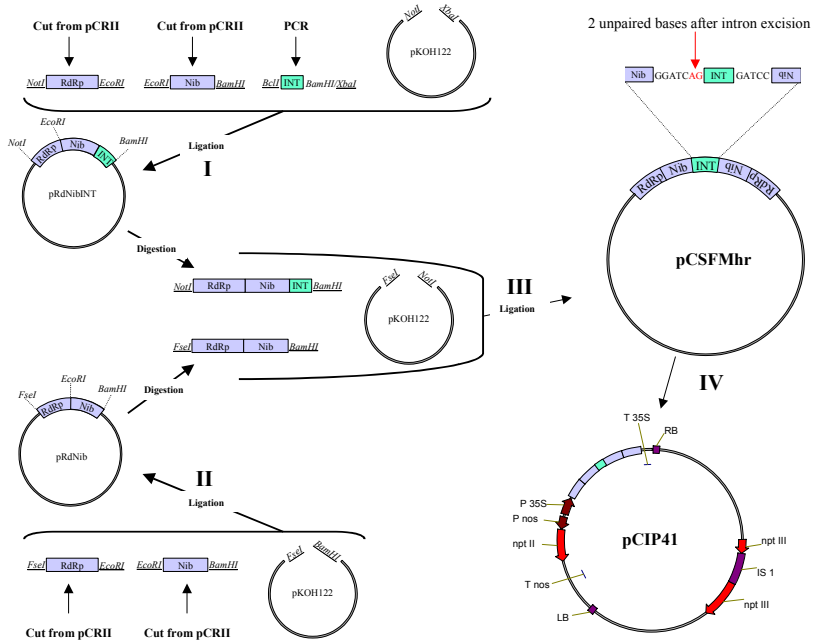


*Fig. 12.* The cloning strategy of pCSFMhr and the final vector, pCIP41. The 479 nt RdRp fragment of SPCSV was amplified by RT-PCR from purified viral RNA from the strain SPCSV-Ug (**IV**), using degenerate primers elongated with either *NotI* and *EcoRI* sites or *FseI* and *EcoRI* sites. The 532 nt SPFMV NIb fragment was amplified in the same way from the strain SPFMV-Nam1 (**I**,**III**), with degenerate primers containing *EcoRI* or *BamHI* sites. The 201 nt intron IV2, from the *ST-LS1* gene, with border sequences optimised in respect of the consensus sequence for plant introns, was amplified from the vector p35SGUSINT (Vancanneyt *et al.*, 1990), with primers containing *BclI* or *BamHI* + *XbaI* sites. All PCR products except IV2 (intron) were first cloned into the TA-cloning vector pCRII where they were checked by sequencing. The IV2 sequence was checked by sequencing from the plasmid pRdNibInt. Subsequently, the RdRp and Nib fragments were cut from pCRII and cloned into the vector pKOH122 in three steps: I) *NotI*-RdRp-*EcoRI* + *EcoRI*-NIb-*BamHI* + *BclI*-IV2-*BamHI*-*XbaI* were ligated into pKOH122 between *NotI* and *XbaI* sites. The resulting plasmid was designated as pRdNIbInt. II) *FseI*-RdRp-*EcoRI* + *EcoRI*-NIb-*BamHI* were ligated into pKOH122 between *FseI* and *BamHI* sites. The resulting plasmid was designated as pRdNIb. III) The RdRp-NIb-IV2 fragment was cut out of pRdNIbInt by *NotI* and *BamHI*, and the RdRp-NIb fragment was cut out of pRdNIb by *FseI* and *BamHI*. *NotI*-RdRp-NIb-IV2-*BamHI* + *FseI*-RdRp-NIb-*BamHI* were then ligated into pKOH122 between the *NotI* and *FseI* sites. The resulting plasmid was designated as pCSFMhr. To enable directional ligation of the IV2 intron the construct was designed so it would produce 2 unpaired bases after intron excision. In the final step the CSFMhr fragment was transferred to the binary plasmid pMOG800 under the control of the CaMV 35S promoter and CaMV 35S terminator, the resulting plasmid was designated pCIP41.

Based on the theoretical minimum requirement of a 23 nt stretch of perfect sequence conservation between the silencing inducer- and target-sequences (Thomas *et al.*, 2001), we can predict that the construct based on the SPFMV isolate-Nam1 *NIb* sequence used in our project will probably not mediate resistance against strains from the O or the RC strain groups of SPFMV (Fig. 7; **I**). However, the project is indeed directed to create resistance to SPVD in East-Africa, which was the reason to select sequences from the East African strains of SPFMV and SPCSV.

# Conclusions

The main conclusions that can be drawn from the studies in this thesis are:

1. SPFMV isolates from East Africa form a genetically distinct group compared to isolates present elsewhere in the world.

2. East African SPFMV isolates can be divided into two serotypes by MAb 7H8, and these two serotypes differ in their ability to systemically infect the sweet potato cultivar Tanzania.

5. The resistance to SPFMV found in East African sweet potato cultivars is due to inhibition of virus replication rather than virus movement, and this resistance can be suppressed by co-infection with SPCSV. The synergistic virus disease caused by dual infection with SPFMV and SPCSV is attributable to a ca. 600 fold increased titres of SPFMV, while the titres of SPCSV remain unchanged as compared to single infection with either virus.

3. The genomic organization of SPCSV shows similarities to the crinivirus LIYV: RNA1 (9407 nt) contains two overlapping ORFs encoding the typical closteroviral "replication module", whereas RNA2 (8223 nt) contains the *Closteroviridae* hallmark gene array (*Hsp70h*, *p60*, *CP* and *mCP*). However, the complete nucleotide sequence of SPCSV has also revealed novel and unique features for the genus *Crinivirus*, such as the apparent recruitment of a novel gene, putatively encoding an RNaseIII-like protein, on SPCSV RNA1, the presence of near-identical, 208 nt long 3'-sequences on both SPCSV genomic RNAs, and the placement of the *SHP* at a new relative position on the genome of SPCSV. In addition, we were able to identify two genes that were conserved within the genus *Crinivirus*, but absent from the genus *Closterovirus*.

4. SPCSV isolates from East Africa form a genetically distinct group compared to isolates present elsewhere in the world.

6. In sweet potato plants co-infected with SPFMV and SPCSV the viruses are found in different tissues: high titres of SPFMV are detected outside of the phloem, whereas SPCSV is detected only inside the phloem.

7. Durable resistance to SPVD requires that the role of SPCSV in the disease be taken into account. Efforts should be concentrated on resistance to both SPFMV and SPCSV.

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# Acknowledgements

I have been told that, next to the abstract, this is the most important part of the thesis, as that is all that most people can bear to read. So, I will do my best and hope to mention everyone who deserves it. If you do not find your name here, you are welcome to complain to me.

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