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# *Sweet potato chlorotic stunt virus*: Studies on Viral Synergism and Suppression of RNA Silencing

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## ACADEMIC DISSERTATION

## in Plant Virology

Helsinki, 2008

*To be presented with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in auditorium B3 at the Metsätieteiden talo (Latokartanonkaari 7) on June 18th 2008, at 12 o´clock.*

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ISSN 1795-7079
ISBN 978-952-92-3969-6 (paperback)
ISBN 978-952-10-4733-6 (PDF)
Yliopistopaino
Helsinki, 2008

## ORIGINAL PUBLICATIONS

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

**I.** Kreuze, J.F., Savenkov, E.I., **Cuellar, W**., Li, X. & Valkonen, J.P.T. 2005. Viral class 1 RNase III involved in suppression of RNA silencing. *Journal of Virology* 79, 7227-7238.

**II.** **Cuellar, W.J.**, Tairo, F., Kreuze, J.F. & Valkonen, J.P.T. 2008. Analysis of gene content in sweet potato chlorotic stunt virus RNA1 reveals the presence of the p22 RNA silencing suppressor in only a few isolates: implications for viral evolution and synergism. *Journal of General Virology* 89, 573-582.

**III.** **Cuellar, W.J.**, Kreuze, J.F., Cruzado, K. R., Untiveros, M., Rajamäki, M-L. & Valkonen, J.P.T. 2008. Elimination of antiviral defence by a viral RNase III. *Submitted*.

**IV.** Kreuze, J.F., Samolski, I., Untiveros, M., **Cuellar, W.J.**, Lajo, G., Cipriani P.G., Ghislain, M. & Valkonen, J.P.T. 2008. RNA silencing mediated resistance to a crinivirus (*Closteroviridae*) in cultivated sweetpotato (*Ipomoea batatas* L.) and development of sweetpotato virus disease following co-infection with a potyvirus. *Molecular Plant Pathology* 9, doi:10.1111/j.1364-3703.2008.00480.x.

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# SUMMARY

## *Sweet potato chlorotic stunt virus*: Studies on Viral Synergism and Suppression of RNA Silencing

*Wilmer Cuellar 2008. Doctor's dissertation.*

The studies presented in this thesis aimed to a better understanding of the molecular biology of *Sweet potato chlorotic stunt virus* (SPCSV, *Crinivirus, Closteroviridae*) and its role in the development of synergistic viral diseases. The emphasis was on the severe sweet potato virus disease (SPVD) that results from a synergistic interaction of SPCSV and *Sweet potato feathery mottle virus* (SPFMV, *Potyvirus, Potyviridae*). SPVD is the most important disease affecting sweetpotato. It is manifested as a significant increase in symptom severity and SPFMV titres. This is accompanied by a dramatic sweetpotato yield reduction. SPCSV titres remain little affected in the diseased plants.

Viral synergistic interactions have been associated with the suppression of an adaptive general defence mechanism discovered in plants and known as RNA silencing. In the studies of this thesis two novel proteins (RNase3 and p22) identified in the genome of a Ugandan SPCSV isolate were shown to be involved in suppression of RNA silencing. RNase3 displayed a dsRNA-specific endonuclease activity that enhanced the RNA-silencing suppression activity of p22.

Comparative analyses of criniviral genomes revealed variability in the gene content at the 3´end of the genomic RNA1. Molecular analyses of different isolates of SPCSV indicated a marked intraspecific heterogeneity in this region where the *p22* and *RNase3* genes are located. Isolates of the East African strain of SPCSV from Tanzania and Peru and an isolate from Israel were missing a 767-nt fragment that included the *p22* gene. However, regardless of the absence of p22, all SPCSV isolates acted synergistically with SPFMV in co-infected sweetpotato, enhanced SPFMV titres and caused SPVD. These results showed that p22 is dispensable for development of SPVD.

The role of RNase3 in SPVD was then studied by generating transgenic plants expressing the RNase3 protein. These plants had increased titres of SPFMV (ca. 600-fold higher in comparison with non-transgenic plants) 2-3 weeks after graft inoculation and displayed the characteristic SPVD symptoms. RNA silencing suppression (RSS) activity of RNase3 was detected in agroinfiltrated leaves of *Nicotiana bethamiana*. *In vitro* studies showed that RNase3 was able to cleave small interferring RNAs (siRNA) to products of ~14-nt. The data thus identified RNase3 as a suppressor of RNA silencing able to cleave siRNAs. RNase3 expression alone was sufficient for breaking down resistance to SPFMV in sweetpotato and for the development of SPVD. Similar RNase III-like genes exist in animal viruses which points out a novel and possibly more general mechanism of RSS by viruses.

A reproducible method of sweetpotato transformation was used to target RNA silencing against the SPCSV polymerase region (RdRp) with an intron-spliced hairpin construct. Hence, engineered resistance to SPCSV was obtained. Ten out of 20 transgenic events challenged with SPCSV alone showed significantly reduced virus titres. This was however not sufficient to prevent SPVD upon co-infection with SPFMV. Immunity to SPCSV seems to be required to control SPVD and targeting of different SPCSV regions need to be assessed in further studies. Based on the identified key role of RNase3 in SPVD the possibility to design constructs that target this gene might prove more efficient in future studies.

*Keywords: sweetpotato, SPCSV, SPFMV, sweetpotato, viral synergism, RNase III, RNA silencing.*

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# ABBREVIATIONS

| | |
|---|---|
| aa | amino acid |
| AGO | Argonaute-like protein |
| BYV | *Beet yellows virus* |
| cDNA | complementary DNA |
| CMV | *Cucumber mosaic virus* |
| CP | coat protein |
| CPm | minor coat protein |
| CTV | *Citrus tristeza virus* |
| DCL | Dicer-like protein |
| dpi | days post inoculation |
| dRNA | defective RNA |
| dsRNA | double stranded RNA |
| GFP | jellyfish green fluorescent protein |
| GUS | β-glucuronidase |
| HC-Pro | potyviral helper component proteinase |
| Hel | helicase |
| hpRNA | hairpin (double stranded) RNA |
| Hsp70h | heat shock 70 family protein homologue |
| IB | inclusion bodies |
| L-Pro | papain-like leader proteinase |
| LIYV | *Lettuce infectious yellows virus* |
| Met | methyltransferase |
| MP | movement protein |
| nt | nucleotides |
| ORF | open reading frame |
| PDR | pathogen derived resistance |
| PVX | *Potato virus X* |
| PVY | *Potato virus Y* |
| RDR | host RNA-dependent RNA polymerase |
| RdRp | viral RNA-dependent RNA polymerase |
| RNase III | ribonuclease III |
| RSS | RNA silencing suppressor |
| sgRNA | subgenomic RNA |
| SPCSV | *Sweet potato chlorotic stunt virus* |
| SPFMV | *Sweet potato feathery mottle virus* |
| SPMMV | *Sweet potato mild mottle virus* |
| SPMSV | *Sweet potato mild speckling virus* |
| SPVD | sweet potato virus disease |
| ssRNA | single stranded RNA |

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**REPRINTS OF ORIGINAL PUBLICATIONS**

*“I´m goin´ta show you something mama, you never seen before*

*I got sweetpotatoes, ain´t goin´ta give you none,*

*If you want to get my sweetpotatoes you got to buy my stuff”*

Sweetpotato Blues

By Alonzo ‘Lonnie’ Johnson

(1927).

(http://www.redhotjazz.com/songs/ljohnson/sweetpotatoblues.ram)

# 1. INTRODUCTION

In the course of infection viruses must cope with host defence responses under which they must replicate and express their genes using the host metabolic machinery. The biological study of virus strategies to subvert the host metabolism has revealed important genetic and biochemical phenomena common to all living organisms (Hull, 2002). Viruses are found everywhere. They are associated with all major groups of organisms and are ecologically important components of the environment (Breitbart & Rohwer, 2005). Historically, viruses were studied and discovered owing to their association with the abnormal development observed in infected plants and animals and due to the devastating effects that viral infections can have on humans directly, or indirectly when they infect livestock and crops (Agrios, 1997; Hull, 2002).

Infection by a virus affects the physiology, metabolic activity or even structure of the plant and any of these induced changes may affect the ease with which the host will respond to a second virus challenge (Smith *et al*., 1931, 1960; Hull, 2002). Virus-infected plants can gain resistance to infection by close-related viruses (cross-protection) or they can gain susceptibility to unrelated viruses that otherwise would not cause disease in a single virus infection (Kassanis, 1963; Hull, 2002). In the latter case, a synergism is observed where the accumulation of one or both viruses, the severity of symptoms and the detrimental effects on the normal development of the plant are increased. The term synergism is used to indicate an effect greater than a simple addition of the effects produced by each virus alone (Kassanis *et al*., 1963; Latham & Wilson, 2008).

A number of disease syndromes are caused by synergistic mixed infections and the picture can get more complicated because triple or even quadruple infections are not uncommon in nature (Smith, 1960; Hull, 2002). Mix infections cause major economic losses in crop plants and it is important to understand these kinds of diseases. Knowledge of the virus-host interaction, genetic composition, variation, interaction and epidemiological information regarding virus spread, transmission efficiency by vectors, and the occurrence of alternative hosts for the viruses and/or the insect vectors, is essential. This is not a trivial task because viruses are continuously evolving, spreading and adapting to new hosts. In addition, mixed-infections may contribute to the evolution of new viruses by facilitating gene mutation, recombination and re-assortment (Roossinck, 1997, 2005; Worobey & Holmes, 1999), which result in the remarkable variation observed today (Simon & Bujarski, 1994; Hull, 2002).

This thesis is focused on the molecular biology of *Sweet potato chlorotic stunt virus* (SPCSV; *Closteroviridae*) in relation to its synergistic interactions with several heterologous viruses in

sweetpotato. Particular attention is given to its interaction with SPFMV, because the mixed infection of these two viruses causes sweet potato virus disease (SPVD), the economically most important disease of this crop.

## 1.1. SWEETPOTATO

Sweetpotato (*Ipomoea batatas* L.) belongs to the “morning glory” family (Convolvulaceae) of plants with funnel-shaped flowers. It is a dicotyledonous, perennial plant and the only *Ipomoea* species (out of 500) of economic importance as a food crop because it produces edible tuberous roots (Onwueme & Charles, 1994; Watson & Dallwitz, 2000). It probably has its origin somewhere between Central and northern South America (Huang & Sun, 2000) with a secondary centre of diversity in the South West Pacific (Zhang *et al.,* 2000, 2004). It may be among man´s earliest domesticates. By the time of European contact, sweetpotatoes were already cultivated throughout the American tropics and had spread to the Easter Islands, Hawaiian and other Polynesian islands, as well as New Zealand. Sweetpotato was introduced to Western Europe by Columbus after his first voyage in 1492. Soon thereafter, Portuguese explorers transported sweetpotato to Africa, India, South East Asia and the East Indies, whilst direct transfer of the plant was done by Spanish trading galleons from Mexico to the Philippines (Austin, 1987; Zhang *et al*., 2004; Srisuwan *et al*., 2006). Nowadays, thousands of sweetpotato cultivars are grown throughout the tropics, subtropics and warm temperate regions in several agro-ecological zones (He *et al*., 1995). Because of its high genetic diversity (with a ploidy level of 4x and 6x) the crop has a great potential for improvement to accommodate specific uses (Zhang *et al*., 2000).

Sweetpotato is commonly used in crop rotations, has a short growing period, stores well as a famine reserve crop, performs relatively well in marginal soils, and has a high yield per unit area per unit time (Woolfe, 1992). Genotypes can be selected to fit the needs of a particular consumer group. Storage roots are used as staple food, raw material for alcohol production and animal feed. High concentration of β-carotene in the orange-flesh cultivars can be used to alleviate vitamin A deficiency. Its anthocyanin content combined with the high stability of the pigments makes sweetpotato a healthier alternative to synthetic colouring agents in food systems and can create additional economic activities for farmers and rural households (Bovell-Benjamin, 2007).

Despite the many advantages offered by the cultivation of sweetpotato, production is mostly in developing countries, and by resource poor-farmers. Production is concentrated in East Asia, the Caribbean and tropical Africa, with the bulk of the crop (88%) being grown in China (Hijmans *et al*.,

2001). Uganda and Nigeria are the biggest producers of sweetpotato in Africa (Karyeija *et al*., 1998a; Hijmans *et al*., 2001; FAOSTAT 2006) where sweetpotato is the second most important tuber crop after cassava. The International Potato Center (CIP) has the international mandate for research on sweetpotatoes in developing countries with efforts focused in improving the agronomic qualities of sweetpotato. CIP has identified Africa as a continent where breeding sweetpotato for virus resistance should be priority particularly for the benefit of the most resource-poor sectors of the population in those countries (CIP, 2000). Despite remarkable success under constrained growing conditions, sweetpotato productivity is still limited by both abiotic and biotic constraints, which leads to poor yields at farm level. One of these major constraints is damage by weevils and viruses that are by far the most serious problem causing substantial losses worldwide (CIP, 2000).

Being a vegetatively propagated crop mixed virus infections are commonly found in sweetpotato. In the absence of a virus-indexing program, initial reports on several diseases of a suspected viral etiology relied on insect- or graft-transmission studies (or both). This was also the case with SPCSV. The word 'virus' was often appended to a disease name (Schaefer & Terry 1976; Winter *et al*., 1992) prior to the biological or biochemical characterization of the disease agent. The current combination of diagnostic host ranges, antisera and nucleotide sequence data for some of the more common viruses allows a rapid detection and characterization of the viral components that produce the main diseases in sweetpotato (Moyer, 1986; 1989; Clark & Moyer, 1988; Alicai *et al*., 1999; Colinet *et al*., 1996; Di Feo *et al*., 2000; Mukasa *et al*., 2003; 2006; Tairo *et al*., 2005; 2006; Untiveros *et al*., 2007, 2008).

## 1.2. SWEET POTATO VIRUS DISEASE (SPVD)

More than 20 viruses are known to infect sweetpotato worldwide (Loebenstein *et al*., 2003). Most of them cause only mild or no symptoms as single infections (Valverde *et al*., 2007). However plants infected with multiple viruses can develop severe symptoms and suffer from significant yield losses (Valverde *et al*., 2007). SPCSV has been identified as the critical component in these viral interactions resulting in severe diseases. Mixed infections with SPCSV and other unrelated viruses are characterized by an increased accumulation of the co-infecting virus or viruses, while SPCSV titres remain little affected (Gibson *et al*., 1998; Karyeija *et al*., 2000; Kokkinos & Clark, 2006; Mukasa *et al*., 2006; Untiveros *et al*., 2007). This is characteristic of viral synergistic interactions where infection by one virus affects the accumulation of a co-infecting virus. For example, SPCSV infection renders sweetpotato more susceptible to accumulation of SPFMV and *Sweet potato mild speckling potyvirus* (SPMSV). This virus complex causes a severe chlorotic dwarf disease reported in Argentina (Di Feo *et*

*al.,* 2000). SPCSV can also enhance accumulation of *Sweet potato mild mottle ipomovirus* (SPMMV) causing a severe mosaic disease reported in Uganda (Mukasa *et al*., 2006). A recent report has characterized the synergistic interactions of SPCSV also with carla- and cucumoviruses (Untiveros *et al*., 2007).

Among the mixed virus infections, dual infection of SPCSV with SPFMV causes the most severe sweetpotato virus disease (SPVD) with yields losses of up to 98% in infected plants (Gibson *et al*., 1998). This disease is most severe in Africa (Clark & Moyer 1988; Lenné, 1991; Gibson *et al.,* 1998; Carey *et al.,* 1999; Njeru *et al*., 2004; Mukasa *et al*., 2006) and is also observed in other areas such as Peru (Guttierez *et al*., 2003) and Israel (Milgram *et al*., 1996). The study of SPVD is important considering that SPFMV is the virus most commonly found in sweetpotato crops worldwide and SPCSV is relatively widely distributed including the main production areas of Africa and South America (Gibson *et al*., 1998; Gutierrez *et al*., 2002; Tairo *et al*., 2005).

Karyeija *et al*. (2000) showed that in sweetpotato plants that show high levels of resistance to SPFMV, co-infection with SPCSV results in a 600-fold increase in SPFMV concentration and development of SPVD. Plants infected with SPFMV alone showed no symptoms. SPCSV-infected plants displayed only mild chlorosis and purpling of lower leaves. These data indicate that infection of sweetpotato by SPCSV causes a breakdown of resistance to SPFMV and to other viruses as diverse as those mentioned above. In addition, concentrations of SPCSV do not increase in SPVD-affected plants, as compared to plants infected with SPCSV alone. As observed in other reports on viral synergistic interactions, SPCSV most probably encodes proteins that can affect a common anti-viral defence pathway, thus facilitating accumulation of several heterologous viruses (Pruss *et al*., 1997).

The epidemics of SPVD have been in many cases associated with disappearance of elite cultivars (Gibson *et al*., 1997), which undermines previous and ongoing efforts for genetic improvement of sweetpotato. These problems have been compounded by the lack of virus-indexing protocols and clean seed systems (propagation by cuttings is the common source of virus inoculum), and scarce knowledge of the nature of the virus disease complexes (Valverde *et al*., 2007).

The search for resistance to SPVD in sweetpotato germplasm has not identified true resistance so far, but only a level of tolerance or resistance to vector-mediated transmission and infection in the field. Extreme resistance to SPFMV identified in the germplasm collections was lost after co-infection with SPCSV (Karyeija *et al*., 1998b). Although SPVD-resistant landraces characterized by fewer plants becoming infected in the field occur in East Africa, most give poor and late yield (Aritua *et al*., 1998a; 1998b). Furthermore, their resistance appears to be governed by multiple recessive genes (Mwanga

*et al*., 2002). Therefore alternative means of generating viral resistance must be pursued. In any case SPCSV appears to be a key factor for the development of severe viral diseases.

## 1.3. THE *CLOSTEROVIRIDAE*

The family *Closteroviridae* owns its name to the Greek word 'closter', which means "thread" referring to the very flexuous filamentous virions of viruses belonging to this family. They cause phloem-limited infections in their hosts. The positive single-stranded RNA (ssRNA) genomes of closteroviruses are the largest (15.5-19.5 kb) and the most complex of all plant viruses. They are comparable to the animal viruses in genera *Corona*- and *Torovirus*, which have the largest genomes among all positive stranded RNA viruses (Koonin & Dolja 1993; Agranovsky, 1996; Dolja *et al*., 2006). *Closteroviridae*´s RNA genomes contain many unique genes, show variability and posses distinct features as revealed by their molecular and functional comparison (Karasev *et al*., 1996; 2000; Kreuze *et al*., 2002; Aguilar *et al*., 2003; Dolja *et al*., 2006) (**FIGURE 1**).

### 1.3.1. Virion morphology and cytopathology

*Closteroviridae* particles range from 700-2000 nm in length. The flexuous virions show a uniform morphology with a short segmented tail (Bar-Joseph *et al*., 1979; Dolja *et al*., 1994). A virus particle contains one molecule of ssRNA genome encapsidated by two coat proteins (CP): the major CP and the minor CP (CPm). The majority of the RNA molecule is encapsidated by CP with only ~5% encapsidated by CPm. This results in a polar 'rattlesnake' appearance of the virions (Agranovsky, 1995; Tian, 1999). This unique morphology sets them apart from other elongated plant viruses (Napuli *et al*., 2000). It has been suggested that virus movement in the *Closteroviridae* requires the previous assembly of virions (Agranovsky, 1996; Alzhanova *et al*., 2001).

Closteroviral infections are associated with characteristic alterations of the phloem parenchyma and companion cells, including membrane proliferation and vesiculation of chloroplast and mitochondria (Pinto *et al*. 1988; Hoefert *et al*., 1988; Medina *et al*., 1998). Typically, these viruses induce the formation of inclusion bodies (IB) formed by aggregates of membranous vesicles. Sometimes these IB contain virions that form fibrous masses (Medina *et al*., 2003). Positive-strand RNA viruses may in general replicate their genomes in association with virus-induced intracellular membrane vesicles (Kopek *et al*., 2007). The closteroviral vesicular IB contain fibrils associated with dsRNA, suggesting that *Closteroviridae* virus replication may also happen in association with such vesicles. This

suggestion is supported by the observation that RNA1 of *Lettuce infectious yellows virus* (LIYV, *Closteroviridae*) (which contains genes for replication and lack genes for encapsidation or movement) is sufficient to induce the formation of such vesicles in infected cells (Medina *et al*., 1998).

### 1.3.2. Transmission by insect vectors

The majority of viruses in *Closteroviridae* depend on insect vectors for their transmission (few species can also be mechanically transmitted) (Karasev, 2000; Dolja, 2003). Transmission occurs semipersistently by specific homopteran vectors including species of families Aphididae (aphids), Aleyrodidae (whiteflies) and Pseudococcidae (mealybugs) (Nault, 1997; Karasev, 2000). The vectors require 30 to 60 min for acquisition of the virus and retain infectivity for days in the insect mouthparts depending on the virus and vector involved (Duffus, 1973; Wisler *et al.,* 1998; Ng & Falk, 2006). The virus-vector interactions might be generally mediated by receptor proteins, which probably contributes to the specificity observed in transmission (Uzest *et al*., 2007).

Whiteflies are particularly damaging. They can damage the plant by feeding, causing symptoms such as reduced vigor and growth, chlorosis and uneven ripening (ISSG Database, 2005). The whitefly species *Bemisia tabaci*, has over 900 host plant species and is a vector to more than 100 viruses including members of *Begomo-*, *Clostero-* and *Potyviridae* (Perring *et al*., 1993; Jones, 2003).

The type of vector needed for transmission is a key biological characteristic in *Closteroviridae.* Viruses in this family with similar genome organization are transmitted by a determined insect group, reflecting virus-vector co-adaptation (Karasev, 2000; Dolja *et al*., 2006).

### 1.3.3. Genome organization and taxonomic groups

The vector and the virus genome organization are the most important criteria to organize the members of *Closteroviridae* into three taxonomic groups: genus *Closterovirus* (monopartite, transmitted by aphids), genus *Ampelovirus* (monopartite, transmitted by mealybugs) and genus *Crinivirus* (bipartite, transmitted by whiteflies) (Karasev, 2000; Martelli *et al*., 2002).

- *Closterovirus:* members of this group such as *Beet yellows virus* (BYV) and *Citrus tristeza virus* (CTV) are among the best studied *Closteroviridae* and their properties were initially used to describe the whole family. They have a monopartite genome and a virion size over 1000 nm in length. In contrast with other *Closteroviridae*. Members of this group present the CP gene downstream of the CPm gene and are transmitted by aphids.

- *Ampelovirus* (from *ampelos*, greek for grapevine): *Grapevine leafroll associated virus* (GLRaV-3) is the type member (Ling *et al*., 2004). Similar to *Closterovirus*, they posses a monopartite genome, and a virion size over 1000 nm in length but their insect vectors are mealybugs. The order of CP and CPm is inverted with respect to *Closterovirus*.
- *Crinivirus: Lettuce infectious yellows virus* (LIYV) is the type member of this group. Criniviruses posses a bipartite genome (both RNAs encapsidated separately), a virion size smaller than 1000 nm in length, and are transmitted by whiteflies. These characteristics group them apart from the monopartite *Clostero*- and *Ampelovirus*. SPCSV, the virus under the main focus of this thesis, belongs to this genus and it will be described in more detail throughout this work.

The genomic organization includes two hallmark "gene blocks" that are conserved throughout the family *Closteroviridae*. The first gene block encodes proteins implicated in viral RNA replication: a papain-like leader proteinase (L-pro), methyltransferase (MET), RNA helicase (HEL) and RNA-dependent RNA polymerase (RdRp) (Peremyslov *et al*., 1998). The second hallmark is a quintuple gene block (QGB) (Dolja *et al*., 2006). It comprises a gene coding for a small hydrophobic protein, a homolog of hsp70 heat-shock proteins (hsp70h), a ~60-kDa protein, and the minor and a major coat proteins (CP and CPm, respectively). The QGB proteins are involved in cell-to-cell movement and virion formation (Peremyslov *et al*., 1999; Alzhanova *et al*., 2001) and can also be located along the short virion "rattlesnake" tail (Agranovsky *et al*., 1995; Peremyslov *et al*., 2004; Satyanarayana *et al*., 2004) (**FIGURE 1**).

### 1.3.4 Gene expression strategies

Proteins implicated in RNA replication are expressed directly from the genomic RNA in *Closteroviridae* (Karasev *et al*., 1989; Dolja *et al*., 2006). These include the products of 5'-end open reading frames (ORF) expressed as a polyprotein ("replication gene block", **FIGURE 1**) containing the L-Pro, HEL, MET and RdRp domains. The RdRp gene is fused to the other domains following a +1 ribosomal frameshift during translation. In criniviruses, RNA2 accumulation would require the previous synthesis of these RNA1 products (Yeh *et al*., 2000). L-pro, an enhancer of viral replication might cleave autocatalitically from the polyprotein (Agranovsky *et al*., 1994). As mentioned above, replication of these positive-strand RNA viruses may locate inside virus-induced intracellular membrane vesicles (Medina *et al*., 1998; Kopek *et al*., 2007).

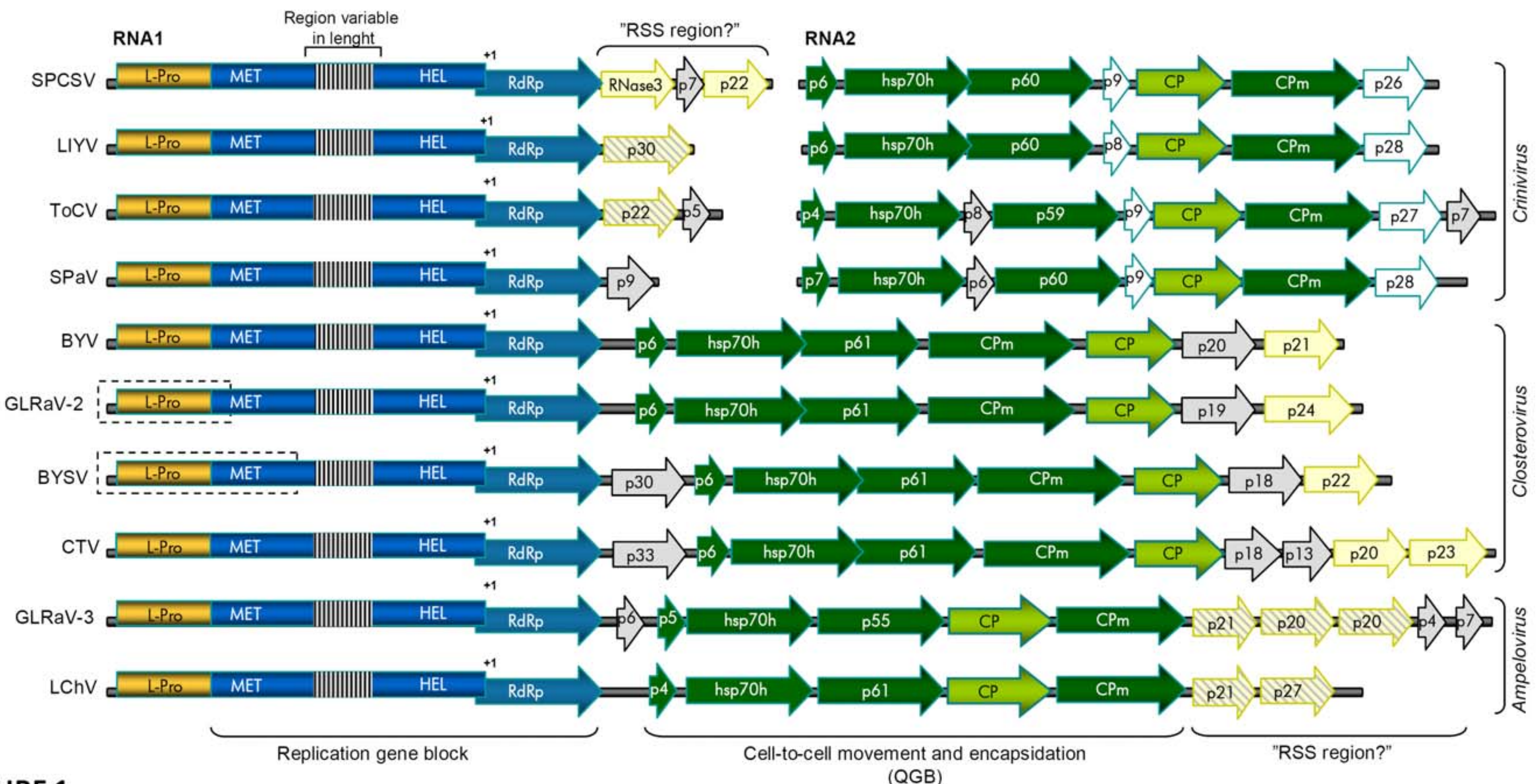
Translation of the viral genome is initiated at the 5'-end of the genomic RNA. This position corresponds to the tail of the virion and so proteins associated with it (*e.g.* CPm, hsp70h, p60) may be implicated in translation initiation (Zinovkin *et al*., 1999; Peremyslov *et al*., 2004; Satyanarayana *et al*., 2004). ORFs located downstream of the "replication gene block" are translated via a nested set of 3'-end co-terminal sub-genomic RNAs (sgRNA). sgRNAs are 5'-end capped and non-polyadenylated RNAs probably synthesized from a minus strand RNA that is also used as a template for replication of the genomic RNA (Dolja *et al*., 1994; 2006). Only the most 5'-proximal ORF in these sgRNAs is translated (Gowda *et al*., 2001). The possible role of sgRNAs in regulating closteroviral gene expression has received little attention. They accumulate at different rates (Bar-Joseph *et al*., 1997; Kreuze *et al*., 2002) suggesting that its timing and amount of synthesis are regulated. For example, sgRNAs encoding proteins with a role in enhancing RNA virus accumulation or counteraction host defence responses (not strictly required for virus replication) are produced early in infection by BYV and CTV (Hagiwara *et al*., 1999; Gowda *et al*., 2000). This is also observed with sgRNAs corresponding to the RNA1 of *Beet yellows stunt virus* (BYSV) and LIYV (Karasev *et al*., 1996; Yeh *et al*., 2000).

In addition, plants infected with *Closteroviridae* members often contain viral defective RNAs (dRNAs) (Rubio *et al*., 2000; Che *et al*., 2002; Kreuze *et al*., 2002; Eliasco *et al*., 2006). dRNAs appear as products of recombination between different viral genomic regions (Simon *et al*., 2004). They have been implicated in the evolution of the *Closteroviridae* (Che *et al*., 2003) including examples of genome segmentation as in *Crinivirus*, gene duplication as observed with the CP and CPm genes, and acquisition of novel genes such as *hsp70h*- and *RNase III*-like genes (Agranovsky *et al*., 1991; 1997; Klaasen *et al*., 1995; Kreuze *et al*., 2002; Napuli *et al*., 2003; Livieratos *et al*., 2004) (**FIGURE 1**).

## 1.4 *Sweet potato chlorotic stunt virus* (SPCSV; *Crinivirus*)

The symptoms described as a 'sweet potato chlorotic stunt' (SPCS) were originally associated with a disease agent transmitted by whiteflies in Nigeria (Schaefers & Terry, 1976). This whitefly-transmitted agent (WTA) was associated with veinclearing, leaf-strapping, puckering and stunting in sweetpotato plants, which had been reported also in other countries (Scheffield, 1957; Robertson, 1964). However, at that time no virus particles were detected in the diseased plants.

The SPCS 'agent' transmitted by whiteflies was associated with mixed virus infections and was given different names. Sheffield (1957) isolated "virus B" from mix infected plants. Clerk (1960) and Loebenstein & Harpaz (1960) described a "vein-clearing virus" isolated from mixed virus-infected plants in Ghana and Israel, respectively. Schaefers & Terry (1976) isolated the WTA from sweet




## FIGURE 1.

Genome organization in *Closteroviridae.* Open reading frames (ORFs) are shown as block arrows. Blue arrows indicate ORFs involved in replication. +1=ribosomal frameshit for RdRp synthesis. ORFs in green are part of the quintuple gene block (QGB). Note the inverted positions of CP and CPm in *Closterovirus.* White arrows represent ORFs characteristic among criniviruses with no assigned function. Yellow arrows represent ORFs for RNA silencing suppression (RSS) proteins located at the 3´-end of RNA1 in *Crinivirus* and at the 3´end of *Closterovirus.*"RSS regions" are expressed early in infection. Dashed yellow arrows indicate ORFs sharing marginal sequence similarity to other *Closteroviridae* RSS proteins. Grey arrows represent ORFs with no putative function assigned. Framed regions in GLRaV-2 and BYSV have not been characterized. *SPCSV=Sweet potato chlorotic stunt virus* (Kreuze *et al.*, 2002); LIYV=*Lettuce infectious yellows virus* (Klaassen *et al.*, 1995); ToCV=*Tomato chlorosis virus* (Wintermantel *et al.*, 2005); SPaV=*Strawberry pallidosis associated virus* (Tzanetakis *et al.*, 2005); BYV=*Beet yellows virus* (Agranovsky *et al.*, 1994); GLRaV-2=*Grapevine leafroll-associated virus 2* (Zhu *et al.*, 1998); BYSV=*Beet yellows stunt virus* (Karasev *et al.*, 1996); CTV=*Citrus tristeza virus* (Karasev *et al.*, 1995); GLRaV-3=*Grapevine leafroll-associated virus 3* (Ling *et al.*, 1998). LChV=*Little cherry virus* (Keim-Konrad & Jelkmann, 1996; Jelkmann *et al.*, 1997).

potatoes affected with a sweetpotato disease complex. Cohen & Loebenstein (1991) and Cohen *et al*. (1995) reported a WTA associated to CMV and SPFMV in Israel. Importantly, in some cases the co-infecting viruses were observed to be better acquired or transmitted by their vectors in the presence of the WTA, constituting probably the first indirect evidence for the synergistic characteristics of SPCSV.

Winter *et al*. (1992) reported the detection of the characteristic closteroviral membrane-enclosed vesicles and flexuous viral particles in *Ipomoea* tissues sampled from plants displaying SPCS symptoms. Transmission of the virus by whiteflies was not included in these experiments and the possibility remained that the WTA (Schaefer & Terry, 1976) and the observed closterovirus-like particles were not related (Winter *et al*., 1992). Later, particles were purified from SPCS-affected sweetpotato plants in Nigeria, Israel and Kenya and whitefly-transmission studies proved its identity as the causal agent of the SPCS disease (Winter *et al*., 1992; Cohen *et al*., 1992; Hoyer *et al*., 1996a,b). The newly identified virus had received several names (Cohen *et al*., 1992; Wisler *et al*., 1998) but because SPCS was the first symptom-descriptive name associated with it, the International Committee for Taxonomy of Viruses established the official name to be *Sweet potato chlorotic stunt virus*, a whitefly-transmitted member of the *Closteroviridae* (Fauquet *et al*., 2005).

The availability of purified particles facilitated molecular characterization of the conserved genomic regions of *Closteroviridae* (mainly CP and Hsp70 sequences) and their serological analysis using anti-CP antibodies (Hoyer *et al*., 1996b; Gibson *et al*., 1998; Alicai *et al*., 1999). The information was used to place SPCSV isolates to two phylogenetically distinct groups (Fenby *et al*., 2002; IsHak *et al*., 2003; Tairo *et al*., 2005). SPCSV isolates from Nigeria, Israel and USA were related and placed to the so-called WA (West African) strain (Hoyer *et al*., 1996b; Vetten *et al*., 1996; Pio-Ribeiro *et al*., 1996), whereas isolates from Kenya, Tanzania and Uganda were grouped into a EA (East African) strain (Kreuze *et al*., 2002; Tairo *et al*., 2005). Isolates of the EA strain have recently been found also in Peru (Gutierrez *et al*., 2003). SPCSV isolates act similarly in synergistic interactions with other viruses regardless of the strain to which they belong (Gibson *et al*., 1998; Alicai *et al*., 1999; Karyeija *et al*., 2000; Kokkinos & Clark, 2006; Mukasa *et al*., 2006; Untiveros *et al*., 2007).

The availability of a full genome sequence of SPCSV (Kreuze *et al*., 2002) allowed predicting its genome organization by comparison to other closteroviral genomes and assigning putative functions to different genomic regions. Intriguingly, the genomes of SPCSV and other plant viruses of the family *Closteroviridae* may have incorporated host genes, as suggested by the presence of *hsp70h*, a homolog of plant heat-shock proteins present in all *Closteroviridae* (Karasev, 2000) and an *RNase III*-like gene in SPCSV (Kreuze *et al*., 2002). The characterization of genes implied in SPCSV synergistic

viral interactions makes the function of SPCSV putative RNase III, a particularly interesting topic of study because this type of RNases have essential roles in RNA silencing, a fundamental antiviral defence mechanism in plants.

## 1.5. CROSS-PROTECTION AND RNA SILENCING

While viruses can be differentiated on the basis of symptoms induced, method of transmission, host range, and physicochemical properties, distinctions can also be made based on their co-interactions in plants (Kassanis, 1963; Hull, 2002). Close-related viruses seem to interact antagonistically whereas cases of synergism or lack of obvious interactions are more common among unrelated viruses (see below).

Antagonistic interactions in which a plant, after being invaded by one virus, is rendered immune or resistant to infection or invasion by a related virus have been described under different names including 'immunization', 'protection', 'interference', ´reciprocal protection', 'mutual antagonism', 'prophylactic inoculation', etc. (Wingard, 1928; McKinney, 1929; Salaman, 1933). Most commonly referred to as "cross-protection", these kind of interactions are virus specific (Price *et al*., 1936) and have been successfully implemented by inoculating plants with mild strains to protect them against infection by severe viral strains (Costa & Muller, 1980). There are, however, reservations about applying cross-protection on a practical scale (Fulton, 1986). Because it is virus-specific, cross-protection was initially useful to find relationships among virus groups. However, factors such as the order and site of the inoculation, virus dosage, environmental conditions and the host species used, can significantly influence the results (Bennett, 1951).

Hidden among the cross-protection results were hints of a sequence-specific RNA-degradation pathway that exists in plants (and other eukaryotes). However, it took many years and required development of new research tools for this mechanism to be uncovered. First of all, not even the nature of genetic material was known at the time of discovery of cross-protection (1920s-40s). *Tobacco mosaic virus* (TMV) was the best-studied plant virus and a subject of new emerging techniques (electron microscopy, X-ray diffraction, density gradient centrifugation, and crystallography). Stanley had identified TMV as a "purified protein" (Stanley, 1935) and although Bawden *et al*. (1936) soon reported the presence of RNA in TMV particles preparations, the role of viral nucleic acid as an infectious agent was recognized much later (Gierer & Schramm, 1956) in line with research developed in the 1940-50s uncovering the predominant role of nucleic acids as the genetic material (Avery *et al*., 1944; Hersey & Chase, 1952; Fraenkel-Conrat *et al*., 1957).

Development of novel molecular biology techniques (DNA cloning, cDNA synthesis, *in vitro* transcription, synthesis of virus infectious clones, etc.) allowed more detailed studies on cross-protection. The development of gene-transfer techniques in plants based on the Ti plasmid of *Agrobacterium tumefaciens* (Zambryski *et al*., 1983) offered the possibility to identify the virus factor(s) responsible for cross-protection, which was approached by introduction of viral genes into transgenic plants and sub-sequent challenge of the transgenic plant with the virus in question. Influenced by early works in bacteria and by the theory of pathogen derived resistance (PDR) (Sequeira, 1984; Sanford & Johnston, 1985), genes for viral replicases, coat proteins and movement proteins were transferred to transgenic plants so that their unregulated expression would interfere with the highly regulated infection cycle of the virus (Powell-Abel, *et al*., 1986; Golemboski *et al*., 1990; Carr & Zaitlin, 1991). Although cross-protection was achieved employing this concept, several observations pointed to a different picture than what the suggested PDR theory had predicted. Most importantly (review in Lindbo & Dougherty, 2005):

- Levels of transgene-derived protein accumulation and resistance were not correlated.
- Many transgenic lines accumulated CP but showed susceptibilities similar to untransformed plants, indicating that the phenotype observed was not explained by expression of the protein.
- Transgenes that did not encode a viral protein but were homologous to the virus provided resistance.
- In transgenic resistant plants the CP transgene was actively transcribed in the nucleus but very low levels if any of transgene mRNA was detected in cytoplasm. This indicated that resistance was associated with a post-trascriptional phenomenon.

The specificity of the transgenic resistance to viruses homologous to the transgene sequence resembled the situation in transgenic petunia plants engineered to express additional copies of the chalcone synthase gene (CHS) that is involved in flower pigment synthesis. Unexpectedly, the transgenic plants exhibited non-pigmented white flowers because in these plants both the transgene and the endogenous CHS mRNA levels were significantly reduced. The phenomenon was described as “co-suppression” (Napoli *et al*., 1990; van der Krol et al, 1990).

Co-suppression and PDR-related phenomena in transgenic virus resistance became two examples of post-transcriptional gene silencing (PTGS). The specificity of these phenomena reminded of the early cross-protection “immunization” work observed among related RNA virus strains (Price, 1936).

A working model for explaining the specificity of PTGS suggested a role for a host RNA-dependent RNA polymerase (RDR) which would make copies of the target RNA and thus synthesize dsRNA. dsRNA would subsequently be specifically detected and degraded together with homologous RNAs (Lindbo *et al*., 1993). The suggestion of dsRNA-synthesis in this process was not whimsical. Host RDR proteins were known to be induced upon virus infection (Dorssers *et al*., 1982; Fraenkel-Conrat, 1983; Khan *et al*., 1986). The hypothesis implicated the existence of an antisense RNA as a result of the RDR-mediated reverse-transcription of the inducer RNA in the PTGS-exhibiting plants. The antisense nature of this component would distinguish it from the degradation products of the target mRNA and would contribute to the sequence specificity of PTGS.

It was not until the late 1990s that specific antisense RNA was reported in plants exhibiting PTGS (Hamilton & Baulcombe, 1999). This RNA turned out to be of a small size (~25-nt) and not easily detected, which might explain why it was discovered a relatively long time after its existence had been postulated (Lindbo *et al*., 1993b). It was not yet known if the antisense small RNA was transcribed as a ~25-nt species or as a longer molecule that was later processed into 25-nt fragments. However, its detection validated the role of an RDR enzyme in PTGS. A role for host RDR enzymes (rev. in Ahlquist, 2002) in virus resistance mediated by PTGS was experimentally demonstrated in *Arabidopsis* and *Nicotiana* RDR-mutants in which increased accumulation of CMV and/or PVX was observed (Mourrain *et al*., 2000; Dalmay *et al*., 2000; Xie *et al*., 2001).

Almost in parallel, experiments in *Caenorhabditis elegans* discovered a highly sequence-specific RNA degradation mechanism induced by dsRNA and targeted against homologous cellular RNAs. This mechanism was less efficiently induced with sense or antisense ssRNA than dsRNA (Fire *et al*., 1998). Given the strong similarity with PTGS, the potential role of dsRNA as a trigger of RNA degradation was subsequently analysed in plants. Interestingly, transgenic plants deliberately expressing dsRNA showed the highest frequency of co-suppression as compared to different kinds of ssRNA-expressing plants (Smith *et al*., 2000). The identification of dsRNA as an efficient trigger of sequence-specific RNA degradation did not explain, however, the cases where PTGS was induced by ssRNA (´sense trangenes´). A branched initiation pathway was thus hypothesized, depending on the requirement of an RDR-activity in order to generate dsRNA (Voinnet, 2005).

It remains unclear how a host RDR would recognize viral or transgene ssRNA templates from other cellular sense transcripts. One hypothesis is that over-expressed transgene or viral RNA might lack some features of a “normal” mRNA (e.g. lack of a poly-A tail), which would “label” them for RDR recognition (Sijen *et al*., 1996). In the cases of cross-protection, replicative intermediates or

secondary structures with stretches of dsRNA would avoid the RDR step (Pantaleo *et al*., 2007). Once dsRNA is generated or detected the RNA-degradation pathway is triggered.

The sequence-complementary of the small RNAs detected in virus-infected plants indicated an antiviral role for PTGS (Hamilton & Baulcombe, 1999). In many transgenic plants expressing viral sequences, systemic infection with the virus initially occurred but plants later recover from infection (Germundsson & Valkonen, 2006). Recovery from virus disease (related to silencing) is not limited to transgenic plants. Also in non-transgenic plants young leaves may appear healthy and immune to secondary infection with the same virus while older inoculated leaves still display symptoms of infection (Wingard, 1928; Dougherty *et al*., 1994). The sequence-specificity was further demonstrated in novel experimental means. Non-viral genes were able to suppress virus infection if the virus was modified by insertion of the transgene sequence into the viral genome. Conversely, viruses could be used to silence host genes (Kumagai *et al*., 1995). Viruses were thus identified as inducers and targets of PTGS and therefore this post-transcriptional cross-protection phenomenon was considered to be an antiviral defence mechanism (Covey *et al*., 1997; Ratcliff *et al*., 1997).

Accumulation of small RNAs of ca. 20- to 25-nt corresponding in sequence to the targeted RNA are a diagnostic feature of RNA silencing. They are generally known as short interfering RNA (siRNA). Experiments with *Drosophila melanogaster* showed that siRNAs were 21- to 24-nt duplex RNA products of the digestion of long dsRNA (Tuschl *et al*., 1999; Zamore *et al*., 2000) and sufficient to initiate RNA silencing (Elbashir *et al*., 2000; 2001a). These siRNA shared characteristics with the cleavage products of RNase III-like enzymes including a 5'-terminal phosphate and 2-nt 3'-end overhangs (Robertson, 1982; Elbashir *et al*., 2001b). In addition, siRNAs are stabilized by methylation (performed by HEN1-like methylases) at the 2' position of their 3' terminal ribose (Li *et al*., 2005; Yang *et al*., 2006; Boutet *et al*., 2003).

The *Drosophila*'s RNase III producing these small RNAs (named Dicer) (Bernstein *et al*., 2001) and its homologous in plants have been identified as pivotal for the initiation of the RNA silencing response, cleaving silencing-inducing dsRNA molecules to 21- to 24-nt siRNA (Hammond *et al*., 2000; Berstein *et al*., 2001; Tijsterman *et al*., 2004).

An ancient origin of the mechanism guided by siRNA for RNA sequence-specific degradation is suggested due to its occurrence in animals (vertebrate and non-vertebrate), fungi and plants (Cogoni & Macino, 2000; Cerruti & Casas-Mollano, 2006). In adition to limit the accumulation of viral RNA, mechanisms mediated by small RNAs are also involved in protecting the host genome from transposons and in regulating gene expression (Grewal & Elgin 2007). Micro RNA (miRNAs) is another class of small regulatory RNAs that are endogenous to the host and regulate animal and plant

development (Carington *et al*., 2000; Lee *et al*., 2003). Both siRNA and miRNA can function as post-transcriptional negative regulators of target mRNAs by directing the sequence-specific degradation or translational repression (Brodersen *et al*., 2008) of specific mRNAs. They are also involved in DNA methylation and heterochromatic assembly, i.e. formation of "inactive" or silenced chromatin (Lippman & Martienssen, 2004; Bartel, 2004; Chen, 2005; Berstein & Allis, 2005). These diverse RNA-based mechanisms are generally referred to as RNA silencing pathways (Baulcombe, 2004).

The role of RNase III-like proteins in RNA silencing is nowadays well established (Tijsterman & Plasterk, 2004; Carmell & Hannon, 2004). A characteristic common to all of them is the presence of domains for specific binding and cleavage of dsRNA. The presence of additional domains allows their classification into 3 classes. RNase III proteins of *Thermotoga maritima*, *Aquifex aeolicus and E. coli* belong to class 1 containing one endonuclease (endoND) and one double-stranded RNA binding (dsRBD) domain. They have also been characterized from yeast and plants (Comella *et al*., 2008) but a role in RNA silencing for this class of RNases has not yet been addressed. RNase III enzymes of class 2 and 3 are distinguished by their N-terminal extensions and act as positive regulators of several RNA silencing pathways. Dicer and its homologues belongs to the RNase III class 3 and contains two endoND domains, one dsRBD, one N-terminal helicase and a PAZ domain also involved in binding of dsRNA (Tijsterman & Plasterk, 2004; Carmell & Hannon, 2004; MacRae & Doudna, 2007). Class 1 RNases generate small RNA cleavage products of less than 20-nt (Gan *et al*., 2006). In contrast, RNAses implicated in the positive regulation of RNA silencing pathways (class 2 and 3) generate cleavage products of 20-25-nt (**TABLE 1**).

Plants encode multiple, homologous Dicer-like RNase III enzymes (DCL). Four homologs are found in rice (a monocot) and *A. thaliana* (a dicot). *A. thaliana* DCLs are implicated in different RNA silencing pathways: DCL1 in miRNA, DCL2 in antiviral siRNA, DCL3 in endogenous siRNA biogenesis derive from heterochromatin, transposons and repeat elements and DCL4 in *trans*-acting siRNA (tasi-RNA), which are endogenous siRNAs whose synthesis requires the activity of RDR enzymes (Xie *et al*., 2004; 2005; Dunoyer *et al*., 2007). DCLs activities can have compensatory functions as exemplified in cases where the role of DCL4 in antiviral defence can be compensated by DCL2 (Deleris *et al*., 2006) or when the production of endogenous siRNA affected by mutations in DCL3 or DCL4, can be compensated by some of the other DCLs (Gasciolli *et al*., 2005). siRNA can in turn serve as "primers" for RDR activity, which would produce new target dsRNA for another cycle of dicing activity, thus amplifying the initial response (see below). Several reports indicate that RNA silencing induced by dsRNA occurs despite of mutated RDR genes, however, silencing induced by sense transgenes would require the host RDR (Boutet *et al*., 2003; Voinnet, 2005).

**TABLE 1.** Small regulatory RNAs identified in plants (Vaucheret *et al*., 2006; Ding & Voinnet, 2007)

| Class | Description | Size (nt) | Origin | Biological function |
|---|---|---|---|---|
| Primary siRNA (1st siRNA) | short interfering RNA | ~21, 22, 24 | Processing of dsRNA by DCL2-4 | Post-transcriptional regulation of transcripts; priming for RDR-dependent secondary siRNA synthesis. |
| Secondary siRNA (2nd siRNA) | short interfering RNA | ~21, 22, 24 | RDR-mediated synthesis and cleavage by DCL2-4 | Post-transcriptional regulation of transcripts; heterochromatin formation. |
| miRNA | micro RNA | ~21 | DCL1-dependent cleavage | Post-transcriptional regulation of transcripts |
| tasi-RNA | Trans-acting siRNA | ~21 | miRNA-mediated cleavage by DCL1, 4 | Post-transcriptional regulation of transcripts. |
| nat-siRNA | natural antisense transcript derived siRNA | ~21, 22 | RDR-depending cleavage by DCL1, 2 | Post-transcriptional regulation of defence and stress related transcripts. |

Genetic analyses and fractionation experiments indicated that degradation of homologous mRNAs could be separated from the Dicer activity. These studies identified 'siRNA-protein' complexes as the effectors of the RNA silencing response (Hammond *et al*., 2000; Baumberger & Baulcombe, 2005). To date two types of effector complexes have been described. One is a cytoplasmic complex called "RNA induced silencing complex" (RISC) and the other one is a nuclear complex known as "RNA-induced initiation of transcriptional gene silencing" (RITS) complex (Verdel *et al*., 2004). As a minimum these complexes contain an Argonaute-like protein and one strand of the siRNA (known as the guide strand) which is antisense to the target RNA. Argonautes (AGO) proteins contain a domain found in members of the RNase H family of proteins which degrade RNA in a DNA-RNA hybrid (Tolia & Joshua-Tor, 2007). Ten Argonautes (AGO1-10) have been identified so far in the genome of *Arabidopsis*, suggesting a diversified functions for these proteins (Vaucheret, 2006).

Consequently, experiments with AGO and DCL mutants (Morel *et al*., 2002; Deleris *et al*., 2006) in *Arabidopsis* show that dicing activity is not sufficient for limiting virus accumulation and that AGO1 is required for virus resistance. In *Drosophila*, Dicer activity was also found inssuficient for limiting virus accumulation in AGO-mutant embryos (Wang *et al*., 2006). A recently identified antiviral RISC in

plants infected with *Cymbidium ringspot virus* contains AGO1 and guide strands antisense to the virus (Pantaleo *et al*., 2007).

Dicers, RDRs and Argonautes are key players of the RNA silencing pathways. The Existance of four DCL, six RDR and ten AGO genes in *Arabidopsis* and the possibility of hierarchical and redundant roles among them suggests a significant diversification in RNA silencing pathways (Vaucheret, 2006).

### 1.5.1. Initiation, spread and maintenance of the silenced state

Once initiated by the processing of dsRNA into siRNA, one strand of the small RNA (known as the guide strand; antisense to the target RNA) is loaded into the effector complexes containing AGO (RISC) and is ready for the sequence specific recognition and degradation (slicing) or translational repression of target RNAs (Tolia & Joshua-Tor, 2007; Brodersen *et al*., 2008).

In plants, cell-to-cell movement of an RNA silencing signal from the cells in which RNA silencing-based RNA degradation is ongoing has been detected using transgenic, GFP-expressing *N. benthamiana* plants (e.g., line 16c) (Ruiz *et al*., 1998; Himber *et al*., 2003). This non-cell autonomous characteristic of RNA silencing can be induced by overexpressing *gfp*-homologous dsRNA in the leaves of the 16c line using agroinfiltration (infiltration of *A*. *tumefaciens* cells to the leaf tissue) (Kapila *et al*., 1997; Schöb *et al*., 1997). As a result, a silencing signal moving out through the plasmodesmata from the agroinfiltrated cells shuts down GFP expression in neighbouring tissue, which is observed as a narrow red border (of ~10-15 cells) at the edge of the infiltrated spot. Subsequently, silencing spreads systemically via phloem and shuts down GFP expression also in distant parts of the plant (Voinnet & Baulcombe, 1997; Palauqui *et al*., 1997). This pattern of silencing spreading resembles viral movement. In virus infections, the mobile and sequence-specific signal may immunize systemic tissue ahead of infection (Voinnet, 2005). The spread of silencing is associated with transitivity (the generation of siRNAs from sequences located outside the inducer sequence) and therefore dependent on RDR-mediated synthesis of secondary siRNAs (Himber *et al*., 2003, Sijen *et al*., 2001). Transitivity pathways are also involved in the reception of the silencing signal, where RDRs may readily synthesize new dsRNA substrates using the signal-sequence information and thus activate DCLs and AGOs to respond more efficiently to incoming viral RNA (Ding & Voinnet, 2007) (**FIGURE 2**).

Silencing at the nuclear level (Transcriptional Gene Silencing, TGS) is related to the maintenance of the silenced state via methylation of nuclear DNA corresponding to the transcribed region of the target endogenous RNA or silenced transgene (Jones *et al*., 1998; Bender, 2001).

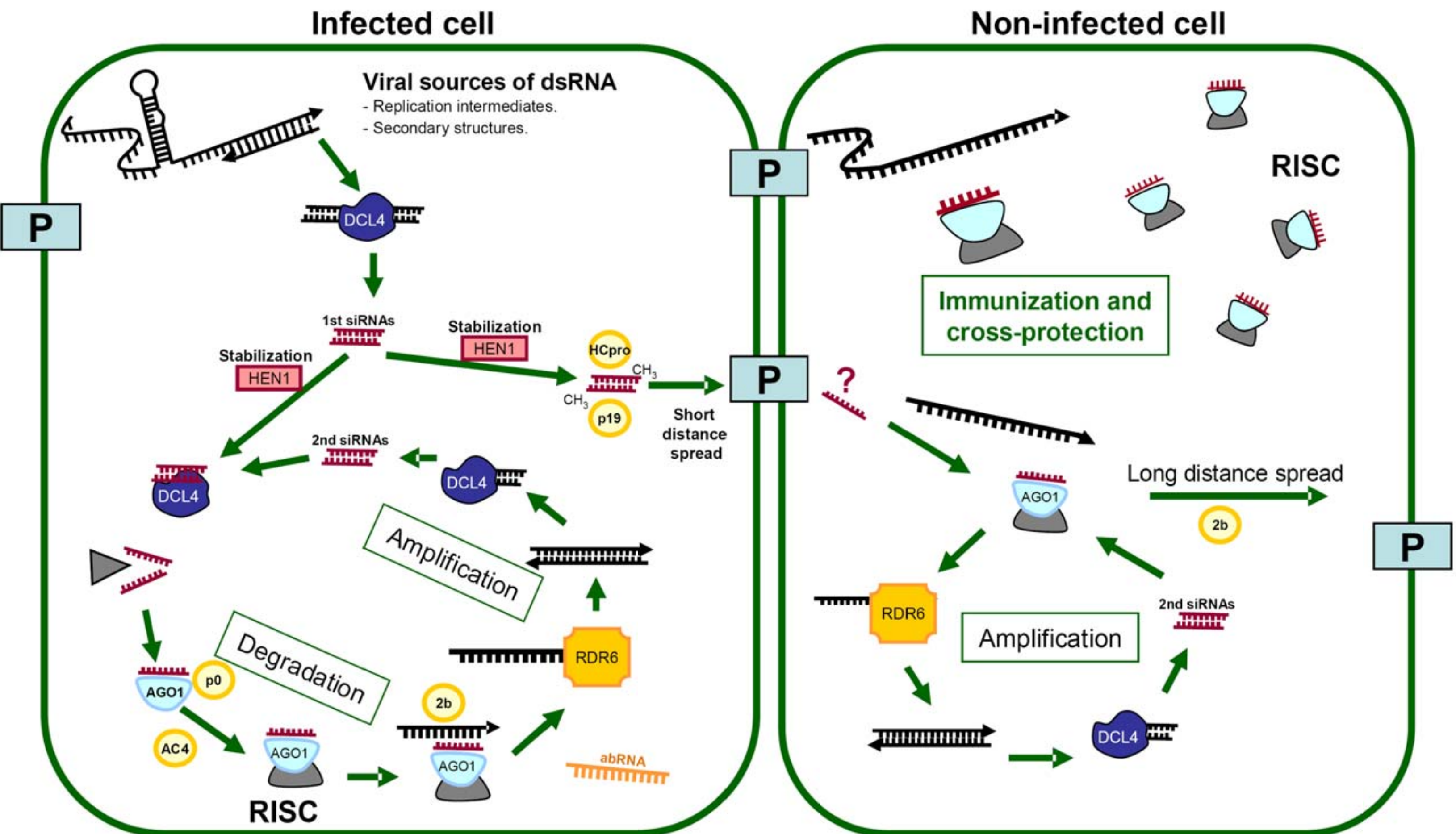



**FIGURE 2.**

A model of the RNA silencing pathway involved in antiviral defence in plants. Double-stranded RNA (dsRNA) from a replicating or secondary-structured virus initiates the pathway. DCL4 and DCL2 could detect this viral RNA. Alternatively single stranded "aberrant" RNA (abRNA) could initiate the pathway at the RDR6 level (*e.g.* from over-expressed transgenes or viral ssRNA). Several AGO proteins are likely to be involved in antiviral RNA silencing. Viral derived siRNAs are stabilized by 2´-O-methylation through the HEN1-dependent pathway. Silencing by primary (1st) siRNAs is amplified via synthesis of secondary siRNAs (2nd) via RDR6. Silencing spreads to neighbouring cells immunizing them against the same virus or related strains (cross-protection). The activity of different RSS proteins (Yellow balls; see Table 1) block the pathway. In these "susceptible" cells, viruses that otherwise would not be able to infect could accumulate to higher titres (synergism) (Adapted from Ding & Voinnet, 2007).

Components of the nuclear silencing pathways have also been associated to components of transitivity pathways (Himber *et al*., 2003; Dunoyer *et al*., 2007; Smith, 2007) and have been implicated in the reception of the silencing signal (Schwach *et al*., 2005; Brosnan *et al*., 2007). DNA methylation at defined genomic regions can be equally important in protecting the genome against damage caused by transposons and DNA viruses (Voinnet, 2005). The mainteneace of a silenced state via targeted methylation confers a 'memory' to RNA silencing (Lippman & Martienssen, 2004; Berstein & Allis, 2005).

In summary, RNA silencing pathways are specific, mobile and adaptive which are characteristic features of immune systems (Lecellier & Voinnet, 2004). This indicates that the suggested PDR theory actually acts by inducing an endogenous resistance mechanism and that cross-protection can be a result of the host using small pieces of viral genetic material to arm itself against sequence-related strains of the virus. Accordingly, for infection, viruses must cope with this resistance mechanism. They do so by acquiring proteins able to suppress RNA silencing at different points (Li & Ding, 2006). In suppressing specific steps of the RNA silencing pathways these viruses "immunocompromise" their hosts, making them vulnerable to opportunistic viral infections.

## 1.6. SUPPRESSORS OF RNA SILENCING: CAUSAL AGENTS OF VIRAL SYNERGISM

Despite of RNA silencing, RNA viruses are the predominant class of plant viruses and succeed to infect their hosts. This fact prompted researchers to look for the mechanisms by which viruses circumvent or suppress this host defence.

In contrast to cross-protection between closely related viruses, mixed infections with unrelated viruses sometimes cause an enhanced, more severe synergistic disease. Synergistic interactions can be manifested as an expansion of host range, an enhanced cell-to-cell and long distance movement, better transmissibility by insect vectors and elevated viral concentrations by one or several of the co-infecting viruses (Kassanis *et al*., 1963; Latham & Wilson, 2008). In fact, these manifestations can all be related to an increase in accumulation (or a decrease in viral degradation) of the synergizing virus. Since RNA silencing limits viral RNA accumulation, intereference with silencing pathways by viruses is a likely mechanism involved in viral synergism. Accordingly, one of the infecting viruses suppresses RNA silencing, which "helps" co-infecting heterologous viruses (that otherwise would not be able to cope with RNA silencing) to accumulate. The term synergism is used to indicate an effect greater than a simple addition of the effects produced by each virus alone (Kassanis *et al*., 1963).

Potyviruses typically “help” a broad range of heterologous viruses to accumulate to a higher concentration. *Potato virus Y* (PVY), *Tobacco etch virus* (TEV) and *Potato virus A* (PVA) interact synergistically with heterologous viruses such as PVX (*Potexvirus*), TMV (*Tobamovirus*) and CMV (*Cucumovirus*) (Smith, 1931; Rochow & Ross, 1954; 1955; Kassanis, 1963; Damirdagh & Ross, 1967; Vance, 1995; Savenkov & Valkonen, 2001). Co-infection with PVY and PVX in tobacco plants causes the classical example of viral synergism. The disease caused by this co-infection is characterized by severe symptoms and a dramatic increase in the accumulation of infectious PVX particles, with no significant alteration in the accumulation of PVY. The concentration of PVX genomic RNA, sgRNA for CP and the CP increase to the same extent (Goodman & Ross, 1974a; 1974b; Vance, 1991).

The PVX/PVY synergism or synergism of other potyviruses with PVX in tobacco plants does not require replication of the potyviral genome. Tobacco plants stably transformed with different parts of the potyviral genome revealed that expression of the potyviral 5'-proximal genomic region encoding the proteinase P1, helper component proteinase (HC-pro), and the third protein (P3) resulted in enhanced PVX symptoms resembling those observed in the synergistic mixed infections (Vance *et al*., 1995). Pruss *et al*. (1997) showed that P1 and P3 sequences are dispensable and identified the HC-pro protein as a sufficient mediator of the synergistic interactions of potyviruses with PVX, TMV and CMV.

HC-pro was subsequently shown to suppress RNA silencing in transgenic tobacco plants providing additional evidence of RNA silencing as a natural antiviral defense mechanism. In these plants HC-pro prevents recovery of viral infection and lowers the accumulation of siRNAs but does not seem to block spreading of silencing (Mallory *et al*., 2001; Yelina *et al*., 2002). HC-pro might also interfere with silencing by inhibiting RISC assembly (Silhavy *et al*., 2002; Kasschau *et al*., 2003). It also modulates 3´-modifications of small regulatory RNAs (Ebhardt *et al*., 2005). Interestingly, HC-pro interacts with a host protein able to downregulate RNA silencing (Anandalakshmi *et al*., 2000).

Since then many other RSS proteins have been identified in plant viruses. Several of them had been previously identified as viral pathogenicity determinants (Anandalakshmi *et al*., 1998; Brigneti *et al*., 1998; Kasschau & Carrington, 1998) suggesting that many such proteins could act by preventing RNA silencing. The role of RSS in PTGS was assayed using transgenic plants, chimeric viruses and temporal expression studies (Voinnet, 2005; Li & Ding, 2006). As predicted, many pathogenicity determinant proteins were found to suppress RNA silencing.

To date at least 40 RSS proteins from a number of different viruses from plants and animals have been identified (**TABLE 2**). The previously mentioned agroinfiltration assay is commonly used to identify RSS proteins. In the agroinfiltrated leaves, degradation of the GFP-specific transgene RNA

**TABLE 2.** Representative viral RSS proteins from different taxa of plant and animal viruses.

| Positive-strand RNA viruses in plants | | | |
|---|---|---|---|
| *Aureusvirus* | *Pothos latent virus* | P14 | Merai *et al.*, 2005 |
| *Carmovirus* | *Turnip crinkle virus* | CP | Qu *et al.*, 2003 |
| *Closterovirus* | *Beet yellows virus* | P21 | Reed *et al.*, 2003 |
| | *Citrus tristeza virus* | P20, P23, CP | Lu *et al.*, 2004 |
| | *Grapevine leafroll-associated virus-2* | P24 | Chiba *et al.*, 2006 |
| | *Beet yellow stunt virus* | P22 | Reed *et al.*, 2003 |
| *Comovirus* | *Cowpea mosaic virus* | Small CP | Liu *et al.*, 2004 |
| *Cucumovirus* | *Cucumber mosaic virus* | 2b | Brigneti *et al.*, 1998 |
| *Furovirus* | *Soil-borne wheat mosaic virus* | 19K | Te *et al.*, 2005 |
| *Hordeivirus* | *Barley stripe mosaic virus* | γb | Yelina *et al.*, 2002 |
| *Pecluvirus* | *Peanut clump virus* | P15 | Dunoyer *et al.*, 2002 |
| *Polerovirus* | *Beet western yellows virus* | P0 | Pfeffer *et al.*, 2002 |
| *Potexvirus* | *Potato virus X* | P25 | Voinnet *et al.*, 2000 |
| *Potyvirus* | *Tobacco etch virus* | Hc-Pro | Anandalakshmi *et al.*, 1998 |
| *Sobemovirus* | *Rice yellow mottle virus* | P1 | Voinnet *et al.*, 1999 |
| *Tobamovirus* | *Tobacco mosaic virus* | P130 | Kubota *et al.*, 2003 |
| *Tobravirus* | *Tobacco rattle virus* | 16K | Liu *et al.*, 2002 |
| *Tombusvirus* | *Cymbidium ringspot spot virus* | P19 | Silhavy *et al.*, 2002 |
| *Tymovirus* | *Turnip yellow mosaic virus* | P69 | Chen *et al.*, 2004 |
| *Vitiviruses* | *Grapevine virus A* | P10 | Chiba *et al.*, 2006 |
| **Negative-strand RNA viruses in plants** | | | |
| *Tenuivirus* | *Rice hoja blanca virus* | NS3 | Bucher *et al.*, 2003 |
| *Tospovirus* | *Tomato spotted wilt virus* | NSs | Takeda *et al.*, 2002 |
| **Double-stranded RNA viruses in plants** | | | |
| *Phytoreovirus* | *Rice dwarf virus* | Pns10 | Cao *et al.*, 2005 |
| **DNA viruses in plants** | | | |
| *Begomovirus* | *Tomato leaf curl virus* | C2 | van WR *et al.*, 2002 |
| | *African cassava mosaic virus* (KE)/(CM) | AC4/AC2 | Vanitharani *et al.*, 2003 |
| *Curtovirus* | *Beet curly top virus* | L2 | Wang *et al.*, 2005 |
| **Positive-strand RNA viruses in animals** | | | |
| *Nodavirus* | *Flock house virus* | B2 | Li *et al.*, 2002 |
| **Negative-strand RNA viruses in animals** | | | |
| *Orthomyxovirus* | *Influenza virus A* | NS1 | Li *et al.*, 2004 |
| *Orthobunyavirus* | *La Crosse virus* | NSs | Soldan *et al.*, 2005 |
| **Double-stranded RNA viruses in animals** | | | |
| *Orthoreovrivus* | (no specified) | σ3 | Lichner *et al.*, 2003 |
| **Retroviruses in animals** | | | |
| *Lentivirus* | HIV-1 | Tat | Bennasser *et al.*, 2005 |
| *Spumavirus* | PFV-1 | Tas | Lecellier *et al.*, 2005 |
| **DNA viruses in animals** | | | |
| *Poxvirus* | *Vaccinia virus* | E3L | Li *et al.*, 2004 |

and suppression of GFP fluorescence can be prevented by co-expression of RSS proteins (Johansen & Carrington, 2001). Because viruses encode one or more RSS proteins (Moissiard & Voinnet, 2004; Lu *et al*., 2004) acting at different levels and on different RNA silencing pathways, alternative assays in addition to agroinfiltration are necessary to identify them (Lu *et al*., 2004).

As seen with the HC-pro of potyviruses, viral RSS proteins act at various points throughout the RNA silencing pathways including initiation, spread and the maintenance of the silenced state (Li & Ding, 2006). For example, the 2b protein of CMV prevents the initiation of PTGS by interfering with the spreading of the signal to new tissues (Brigneti *et al*., 1998) and p25 of PVX interferes with the accumulation of a systemic silencing signal (Voinnet *et al*., 2000). Lately, many RSS proteins have been implicated in binding siRNA, which may be a common mechanism for interference with RNA silencing (Silhavy *et al*., 2002; Lakatos *et al*., 2006; Mérai *et al*., 2006). Accordingly, plant mutants defective in certain components of the RNA silencing pathway show hypersusceptibility to specific virus infections (Mourrain *et al*., 2000; Dalmay *et al*., 2001; Morel *et al*., 2002; Yu *et al*., 2003; Qu *et al*., 2005; Schwach *et al*., 2005). These results point to RSS as a general mechanism for viral synergism in plants.

The high diversity of RSS proteins and their ways of action (Diaz-Pendon & Ding, 2008) seems like a necessary adaptation against the equivalent high diversity in host RNA silencing pathways (Ding & Voinnet, 2007). In addition, the accumulation of functionally distinct RSS proteins in mixed virus infections could have a cooperative activity in suppressing different RNA silencing pathways resulting in higher accumulation of more than one virus (Vanitharani *et al*., 2004) (**FIGURE 2**).

### 1.6.1 RSS proteins identified in *Closteroviridae*

The 3´ most proximal gene of BYV encodes a 21 kDa protein (p21) first dentified as an enhancer of viral RNA accumulation which was not essential for viral replication (Peremyslov *et al*., 1998). p21 is early expressed during infection (Hagiwara *et al*., 1999) and its RSS activity was demonstrated using the agroinfiltration assay in leaves of *N. benthamiana* (Reed *et al*., 2003). In these assays p21 expression allowed the accumulation of GFP mRNA but did not reduce siRNA accumulation. It has recently been shown that p21 can bind siRNA and miRNA, probably sequestering them and interfering with their incorporation in RISC (Chapman *et al*., 2004). Distantly related functional analogous of p21 have been identified in other members of *Closteroviridae* such as p20 of CTV and p22 of BYSV (Reed *et al*., 2003).

The sgRNA that encodes p20 of CTV is among the highest expressed and its encoded protein also accumulates at high levels in infected plant tissues (Gowda *et al*., 2000). As BYV's p21, the p20 of CTV is not required for CTV replication (Satyanarayana *et al*., 1999) and interferes with the local (Reed *et al*., 2003) and systemic spread (Lu *et al*., 2004) of RNA silencing. Two additional RSS proteins (p23 and CP) are encoded and expressed by CTV. A series of tests using agroinfiltration assays and transgenic plants expressing p23 and CP showed that they are functionally non-reduntant to p20 RSS activity. The p23 protein acts locally but, unlike p20, it does not prevent spreading of silencing. Compared to p20 and p23, CTV's CP consistently interferes with spreading of silencing but is unable to suppress RNA silencing locally (Lu *et al*., 2004).

The p24 gene of GLRaV-2 (*Closterovirus*; Zhu *et al*., 1998) shows sequence similarity to BYV's p21 and CTV's p20 (Reed *et al*., 2003). Its RSS activity has been identified on RNA silencing induced by a mini-viral replicon (which had a low infectivity due to the absence of RSS proteins) or agroinfiltrated dsRNA (Chiba *et al*., 2006). Karasev *et al*. (1996) identified the p22 ORF at the 3'-terminal part of BYSV and indicated its marginal similarity with BYV p21 and CTV p20 (**FIGURE 1**). BYSV p22 was studied in parallel with other closteroviral proteins and its RSS activity was found to be functionally similar to BYV p21 in agroinfiltration assays (Reed *et al*., 2003).

To our knowledge, there are no RSS proteins reported from genus *Ampelovirus* and, previous to our studies with SPCSV, RSS proteins from *Crinivirus* have not been characterized. However, the presence of analogous ORFs at the 3'-end of their genome sharing characteristics with the identified RSS proteins of other members of *Closteroviridae* (Kreuze *et al*., 2002; Ling *et al*., 2004; Rott & Jelkmann, 2005) suggests that they might also encode RSS proteins (**FIGURE 1; TABLE 2**).

# 2. AIM OF THE STUDY

The aim of the study was to identify SPCSV proteins associated with suppression of RNA silencing and evaluate the role of such proteins in the development of the synergistic sweet potato virus disease (SPVD). The specific aims were:

1. Identification of SPCSV proteins involved in RNA silencing suppression (RSS)
2. To study the genetic variability of SPCSV isolates of geographically different origins to detect possible differences in the genomic region encoding RSS proteins
3. Test whether SPCSV RSS proteins play a role in synergism with SPFMV.
4. Engineer transgenic resistance to SPCSV in sweetpotato.

# 3. METHODS

Protocols for the methods used along this work, listed below, are described in more detail in the indicated publications.

| Method | Publication |
|---|---|
| Agroinfiltration | **I, III** |
| Cross-protection studies | **I** |
| DNA cloning and sequence analyses | **I, II, III, IV** |
| Northern blotting | **I, II, III, IV** |
| Plant transformation | **III, IV** |
| Real-time PCR | **II** |
| Recombinant protein expression and purification | **I, III** |
| RNA cleavage assay | **I, III** |
| Serological detection of viruses | **I, II, III, IV** |
| SDS-PAGE | **I, III, IV** |
| Southern blotting | **IV** |
| Western blotting | **I, III, IV** |

# 4. RESULTS

## 4.1. Identification of SPCSV proteins involved in RSS (I)

Plant viruses need to be able to suppress or escape RNA silencing in order to increase their probabilities for successful invasion of the host. The role of RSS proteins in virus-host interactions, establishment of infection, pathogenesis and virus synergism prompted us to screen for SPCSV genes encoding RSS proteins. Given the high-titre accumulation of co-infecting heterologous viruses in the presence of SPCSV (Gibson *et al*., 1998; Karyeija *et al*., 2000), it was hypothesized that the conspicuous synergistic effects of this crinivirus might be related to its interference with RNA silencing (Pruss *et al*., 1997; Anandalakshmi *et al*., 1998; Vanitharani *et al*., 2004).

The full genome sequence analysis of a Ugandan isolate of SPCSV (SPCSV-Ug) predicted that it encodes two novel proteins expressed from sgRNAs early in infection (Kreuze *et al*., 2002). These genes were located at the 3' end of RNA1, a region showing a high interspecific variability in gene content among criniviruses. Sequence analyses predicted that these sgRNAs encode: (1) A putative RNase III-like protein (RNase3; 26 kDa) belonging to a family of endoribonucleases that specifically recognize and cleave dsRNAs (Conrad & Rauhut, 2002) and of which some play a role in RNA silencing; and (2) a putative 22-kDa protein (p22) which showed no homology to other sequences in databases. The previously described RSS proteins in *Closteroviridae* are similarly encoded by ORFs with no obvious sequence homology to other proteins. These ORFs are located at the 3'-terminal region of the genome and share a similar size range as SPCSV p22 (e.g. p21 of BYV, and p20 and p23 of CTV) (Reed *et al*., 2003; Lu *et al*., 2004).

SPCSV RNase3 and a mutated form, RNase3-Ala$^{37,44}$, containing substitutions E37A and D44A to abolish endonuclease activity were tested for cleavage of nucleic acid substrates. These substrates included ssRNA, dsRNA, ssDNA, dsDNA, and an RNA-DNA hybrid. RNase3 endonuclease activity was specific for dsRNA and required the presence of divalent cations ($Mg^{2+}$ and $Mn^{2+}$). These result were consistent with earlier reports on the activity of analogous RNase III enzymes (Robertson *et al*., 1968; Nicholson, 1999). As with *E. coli* RNase III (Calin-Jageman *et al*., 2001), low concentrations of EtBr (125 µM) inhibited RNase3 cleavage of the dsRNA substrate. In contrast, none of the tested nucleic acid substrates were cleaved by RNase3-Ala$^{37,44}$, but this mutant was fully able to bind dsRNA, indicating that no other property of RNase III was affected by the introduced mutations (dimerization, for instance).

The substitutions E37A and D44A that abolished the endonuclease activity of the protein were introduced based on the crystal structure and functional model of class 1 RNase III of *Aquifex aeolicus* and *E. coli* (Blaszczyk *et al*., 2001). In particular, D44 is a very well conserved residue in the "RNase III signature motif" of this class of RNases (see Suppl. Fig 2 in **III**). We concluded that RNase3 exhibits the characteristic endonuclease activities of the RNase III family of proteins (**I**). The RNase III family of proteins has a pivotal role in different RNA silencing pathways (Bernstein *et al*., 2001; Hammond *et al*., 2001). They are divided into three classes according to their structure (Carmell & Hannon, 2004) where SPCSV-Ug RNase III (RNase3) together with the RNase III proteins of *Thermotoga maritima*, *Aquifex aeolicus and E. coli* belong to the simplest class (class 1) containing one endonuclease (endoND) and one double-stranded RNA binding (dsRBD) domain.

A possible role of p22 and RNAse3 in RNA silencing was tested using different approaches. The genes for p22 and RNase3 were expressed from an infectious PVX cDNA by mechanical inoculation of the plasmids (PVX-p22, PVX-RNase3, respectively) to *N. benthamiana* plants. Following inoculation with PVX-RNase3, the plants developed similar symptoms and high PVX titres as observed with the control PVX-GF (containing a fraction of the GFP gene) in upper non-inoculated leaves. In contrast, PVX-p22 and the PVX vector carrying the HC-Pro gene of PVA (genus *Potyvirus*, family *Potyviridae*) caused necrotic symptoms in systemically infected young leaves. Due to quick necrotic reactions caused by p22 starting at ~7 d.p.i samples from all treatments were taken around this time. Frameshift mutants of the p22 gene in PVX-p22 accumulated in titres similar to those of the control PVX-GF (determined by DAS-ELISA), indicating that the necrotizing ability was most probably due to the p22 protein. RT-PCR cloning and sequencing of the p22 insert from the viruses in systemically infected leaves confirmed that lack of necrosis was not associated with loss or partial deletion of the inserts during virus replication (*not shown*).

Subsequently, a cross-protection assay was used, in which *N. benthamiana* leaves are inoculated with PVX-GF and, 3 days later, with TMV encoding the *gfp* gene (TMV-GFP; Yelina *et al*., 2002). In co-inoculated leaves, replication of the two viruses with partially homologous viral genomes (the GFP sequence) in the same tissue triggers RNA silencing and prevents long-distance transport of the latter inoculated virus, in this case TMV-GFP (Ratcliff *et al*., 1999). Derivatives of PVX-GF were designed to express p22 or RNase3 (PVX-p22-GF and PVX-RNase3-GF respectively) and they were co-inoculated with TMV-GFP into *N. benthamiana* plants, as described above. The p22 mutants described earlier were also used. PVX-GF and a PVX chimera (PVX-HCpro-GF) expressing the HC-Pro of PVA were used as controls. In plants inoculated with PVX-RNase3-GF, the PVX-p22-GF frameshift-mutant constructs, or PVX-GF and subsequently with TMV-GFP, green fluorescence was confined to the inoculated

leaves. In contrast, following inoculation with PVX-p22-GF or PVX-HCpro-GF and subsequently TMV-GFP, green fluorescence spread to the stem and the upper non-inoculated leaves by 6 to 8 d.p.i. in most of the inoculated plants. The plants soon developed apical necrosis, as in previous assays. p22 therefore interfered with cross-protection, which suggested that it suppresses RNA silencing. Such an activity for SPCSV RNase3 was not evident in this assay (**I**).

The *Agrobacterium tumefaciens* infiltration assay was used to test whether p22 and/or RNase3 could suppress short- or long distant spread of RNA silencing triggered by a hairpin construct (i.e., dsRNA). Co-infiltration of GFP, hpGFP, and p22 or HC-Pro resulted in strong fluorescence in the infiltrated leaf areas, starting 2 d.p.i. and increasing until 5 d.p.i. The fluorescence observed with HC-pro was stronger than with p22 and continued up to 10 days without any noticeable effect on the leaf. However p22 induced chlorosis by 4-5 d.p.i. and necrosis by 5-6 d.p.i. (*not shown*). Other constructs did not induce chlorosis, and did not suppress RNA silencing; neither did the p22 mutants described earlier (*data not shown*). These data indicated that a full-length p22 protein suppresses RNA silencing triggered by dsRNA. Experiments with the transgenic *N. benthamiana* line 16c revealed short distance movement of the silencing signal following infiltration with hpGFP and GUS or RNase3 but not HC-Pro or p22 (see Fig 4 in **I**). These data indicated that p22 as well as HC-pro interfere with short-distance intercellular spread of silencing. Assays designed to test RSS on the systemic spread of GFP silencing in 16c plants by these proteins indicated that expression of p22 interfered with long distance spread of silencing.

Because the sgRNAs for both RNase3 and p22 accumulate early during SPCSV infection (Kreuze *et al*., 2002) it was tested whether RNase3 influences the p22-mediated RSS and whether it also affects the RSS functions of an RSS protein encoded by a heterologous virus (PVA HC-Pro) in co-infiltrated leaves. The constructs expressing RNase3 were co-infiltrated into leaves of *N. benthamiana* with constructs expressing p22, HC-Pro or GUS along with those for GFP and hpGFP. At 5 dpi, northern blot analyses readily detected GFP mRNA in leaves co-infiltrated with GFP, hpGFP, and p22 or HC-Pro but not RNase3 or GUS (see Fig. 5 in **I**). Importantly, leaves co-infiltrated with p22 plus RNase3 showed at least a 2-fold-higher *gfp* mRNA levels than the leaves co-infiltrated with p22 and an approximately 5-fold decrease in the levels of 21-nt *gfp* siRNA, compared with leaves infiltrated with p22 alone. Thus, even though RNase3 itself exhibited no detectable RSS activity in these assays, it was able to enhance the RSS activity of p22 (**I**). Co-expression of p22 with individual RNase3 domains or with an endonuclease mutant (endoND, dsRBD, or RNase3-Ala[37,44]) resulted in no apparent change in siRNA accumulation compared to leaves infiltrated with p22 alone (Fig. 6C in **I**). These results resembled those of a previously reported work with *E. coli* RNAse III where a mutant that binds

dsRNA but lacks the RNA endonuclease activity exhibited a lower RSS activity as compared to the wild-type enzyme in a comparable agroinfiltration assay (Lichner *et al*., 2003). The enhancing effect of RNase3 on RSS was specific to p22, as it was not observed upon co-infiltration with HC-Pro (Fig. 6C in I, compare lanes 3 and 10), despite the detectable amounts of RNase3 and RNase-Ala[37,44] proteins expressed in all infiltrated leaves (Fig. 5B in **I**).

These results indicated that the dsRNA-binding activity of SPCSV RNase3 was not sufficient to enhance p22 RSS activity. The endonuclease activity of RNase3 was however required for its ability to enhance RSS by p22. The unpublished data on co-immunoprecipitation and yeast-two hybrid interactions (M. Ala-Poikela *unpublished results*) suggest that p22 and RNAse3 do not interact directly. Therefore the RNase3 endonuclease activity probably affects another step than p22 in the RNA silencing pathway.

To investigate whether p22 or RNase3 affects transitivity (the generation of secondary siRNAs from sequences located outside the inducer sequence) (Himber *et al*., 2003, Sijen *et al*., 2001), the membranes previously tested with the entire GFP probe were stripped and hybridized with two non-overlapping probes (I, Fig. 6B). The 3'-GFP probe was expected to reveal the secondary siRNA resulting from transitivity. Northern blot hybridization and subsequent quantification showed similar relative siRNA accumulation in different samples, regardless of the probe used for siRNA detection. Thus, the expression of p22 had no detectable influence on transitivity, implying that RNA-dependent RNA polymerase-mediated amplification of the silencing signal was unlikely affected by this RSS protein (Dalmay *et al*., 2000; Himber *et al*., 2003; Mourrain *et al*., 2000).

## 4.2. Characterizing the genetic variability of SPCSV (II)

Viruses of the family *Closteroviridae* show a high level of variability in gene content at the 3'-end of their genomic RNA (closteroviruses and ampeloviruses) or RNA1 (criniviruses) (Fig. 2), . Because the RSS protein p22 and its enhancer, RNase3, of SPCSV-Ug were found to locate within this variable genomic region, it became relevant to study whether these genes and proteins showed any level of variability between SPCSV isolates.

It was known that isolates of SPCSV from different geographical locations differed in symptomatology and the severity of synergistic interactions with other viruses (Milgram *et al*., 1996; Alicai *et al*., 1999; Gutierrez *et al*., 2003). In addition, data reported in a conference abstract (Hoyer et al., 1996a) and a review on molecular organization of *Closteroviridae* (Agranovsky, 1996)

suggested that a Kenyan SPCSV isolate might differ in gene content from the Ugandan isolate characterized by Kreuze *et al*. (2002). The Kenyan isolate was indicated to contain a similar ORF organization as LIYV and harbour only one ORF downstream the RdRp gene (3'end of SPCSV RNA1) in contrast to the Ugandan isolate in which two ORFs (*RNase3* and *p22*) were found (Kreuze *et al*., 2002).

Given the potential genetic variability in a region implicated in RSS and probably also in SPCSV virulence we set to analyze different SPCSV isolates for the gene content and variability of *p22* and/or *RNase3* sequences at the 3´-end of RNA1. The EA strain isolates Tug2 from Uganda, Unj2 and Mis1 from Tanzania (Tairo *et al*, 2005) and m2-47 from Peru (Gutierrez *et al*., 2003), and a WA strain isolate (Is) from Israel (previously called sweet potato sunken vein virus, SPSVV; Milgram *et al*., 1996) were included. Isolate Tug2 contained an RNA1 3'-end region (nt 7197–9277) that was nearly identical in sequence and identical in gene content as compared to Ugandan isolate SPCSV-Ug (Kreuze *et al*., 2002). In contrast, all other isolates were lacking a 767-nt region containing the gene for the p22 RSS protein and their genetic structure conformed to the notes on the Kenyan isolate (Hoyer *et al*., 1996a). They harboured only one ORF after the *RdRp* gene which corresponded to *RNase3*.

Varibility in the number and size of ORFs is very well reported among members of *Closteroviridae* (Tzanetakis & Martin, 2004; Dolja *et al*., 2006). In contrast, the functional characterization or these putative encoded proteins has received much less attention. P22 and RNase3 sgRNAs have been detected in SPCSV infected plants (Kreuze *et al*., 2002) and their activities as proteins implicated in RSS was uncovered using different functional mutants in agroinfiltration and cross-protection assays (**I**). The lack of p22 in some SPCSV isolates was rather surprising. The enhancing activity of RNase3 augmenting RSS by p22 suggested that the missing gene would be the one for *RNase3* rather than *p22*. These data constitute the first report in *Closteroviridae* on the intraspecific variability in the number of genes implicated in suppressing the host antiviral defence.

All isolates analyzed contained *RNase3* and expressed its corresponding sgRNA early in infection (see Fig. 1 in **II**). Hybridization with a probe for *p22* showed signals only for isolate Tug2 (and Ug, as expected). These analyses also revealed that the p22-encoding isolate SPCSV-Ug accumulated in much higher titres than isolates lacking the *p22* gene in young leaves of *I. setosa*, as could be expected given the RSS activity of the p22 protein (see Fig. 2 in **I**).

Most importantly, all SPCSV isolates synergized with SPFMV in sweetpotato plants (cv. Tanzania) regardless of the presence or absence of p22. With all isolates the severe symptoms and high SPFMV

titres characteristic of SPVD were observed (**II**, Fig. 4 and Fig. 5). In the indicator plant *I. setosa*, SPVD symptoms were more severe with the p22-encoding isolates and eventually lethal to plants at 20 d.p.i. Necrosis caused by isolates Ug and Tug2 in co-infection with SPFMV may be due to a higher SPCSV or SPFMV accumulation in infected tissues and/or other p22 related activities. This reminded of the necrosis induced in leaf-spots of *N. benthamiana* agroinfiltrated with p22 expressing constructs and necrosis caused by infection with the PVX vector expressing p22 (**I**). Real-time PCR and DAS-ELISA data indicated that viral titres for SPCSV and SPFMV in SPVD-affected sweetpotato (in the presence of *p22*) were significantly lower than with isolates lacking the p22 gene, probably reflecting again the detrimental effects of p22 activity (**II**, Table 2).

Deletion of functionally redundant sequences has been observed in viruses of genera *Pomovirus* and *Tobravirus* (Torrance *et al*., 1999; Sandgren *et al*., 2001; Hernandez *et al*., 1996) and in chimeric viral vectors (Chung *et al*., 2007) and could in principle explain the results with p22, i.e., most SPCSV isolates would have lost a segment of RNA1. On the other hand, gene gain via virus-host RNA recombination has been suggested as a frequent phenomenon in the evolution of the *Closteroviridae* (Che *et al*., 2003; Dolja *et al*., 2006). Given that genetically distinct strains that are geographically widely distributed in East Africa, Israel and Peru all lack exactly the same RNA1 segment containing the p22 RNA silencing suppressor gene, it is hypothesized that *p22* might rather be recently acquired by some EA isolates of SPCSV. Sequence comparisons with other criniviruses identified another putative RNase III of class 1 that may be encoded by the ORF2 of LIYV (downstream the *RdRp* gene) originally reported as a putative 30-kDa protein (Hoyer *et al*., 1996a). Interestingly, this LIYV gene has been identified as an enhancer of viral RNA accumulation (Yeh *et al*., 2000). Since LIYV and most of the SPCSV isolates showed an analogous gene content and identity in RNA1, the results suggest close relatedness of these criniviruses and further support the idea that *p22* is a recent acquisition in SPCSV. This suggestion is in line with accumulating evidence on viral RSS proteins where the lack of a significant sequence-based homology among them is also taken as an indication of their relatively recent and independent acquisitions by viruses to counteract the evolving host defence responses that limit viral RNA accumulation (Li & Ding, 2006). Many RSS proteins are dispensable for basic viral functions such as replication, encapsidation or systemic movement, but their activity allows more efficient viral RNA accumulation, which may be more important in some hosts than others (Peremyslov *et al*., 1998; Qu & Morris, 2002; Silhavy *et al*., 2002; Stenger *et al*., 2005; Scholthof, 2006).

Taken together, the results showed that p22 is dispensable for development of SPVD. In fact, Untiveros *et al*. (2007) used isolate m2-47 of SPCSV to co-inoculate sweetpotato plants with poty-,

cucumo-, ipomo- or carlaviruses and detected synergism in all cases. The Israelian isolate SPCSV-Is has been reported to synergize with CMV and SPFMV (Cohen *et al*., 1995; Milgram *et al*., 1996). In addition, the Kenyan isolate, for which the whole genome has been analyzed (but not published) and that also seems to lack p22, was isolated from an SPVD-affected plant (Hoyer *et al*., 1996 a,b). Hence, p22 is not required for synergistic interactions with heterologous viruses. However, it would be important to test whether any difference in the range of synergizing co-infecting viruses exists between SPCSV isolates that differ in their gene content.

## 4.3. The role of RNase3 in SPVD and RNA silencing suppression (III)

Since p22 was found to be dispensable for development of SPVD (**II**), the focus was returned to RNase3 as the putative mediator of synergism with SPFMV. Transgenic sweetpotato plants expressing the RNase3 protein of SPCSV isolate Ug were generated in collaboration with the International Potato Center (CIP). The transgenic sweetpotato lines were phenotypically undistinguishable from the wild-type plants and no major developmental abnormalities where observed (Fig. 1 in **III**). In contrast, transgenic plants expressing *E. coli* RNase III class 1 enzyme suffer reduced growth and are stunted (Lagenberg *et al*., 1997; Zhang *et al*., 2001).

SPVD symptoms were observed in the RNase3-transgenic sweetpotatoes 2-3 weeks after inoculation with SPFMV. DAS-ELISA and real-time PCR data indicated high concentrations of SPFMV in the symptomatic leaves, whereas little if any SPFMV could be detected in the leaves of non-transgenic plants of the highly SPFMV-resistant variety 'Huachano' used for these experiments. These results indicated that the RNase3 protein alone was sufficient to mediate development of SPVD in plants infected with SPFMV (**III**). The symptoms were characteristic of SPVD except that the plants were less chlorotic than following co-infection with SPCSV and SPFMV. The typical chlorosis in SPCSV-infected plants (Gibson *et al.*, 1998) might therefore be a symptom caused by additional SPCSV factors.

RNase3 was also tested for silencing suppression activity using different silencing inducers. Our experiments in *N. benthmiana* 16c line using a sense GFP transcript as the inducer of silencing (co-suppression) showed a clear RSS activity for RNase3 (Fig. 2 in **III**) contrasting with previous results where dsRNA (hairpin RNA) was used to induce silencing (see Fig. 3 and 5 in **I**). The RSS activity was consistently observed in several experiments and started 3-4 days post infiltration (d.p.i.) in young leaves. However by 7 d.p.i. RNase III RSS activity in terms of enhanced GFP fluorescence became more difficult to observe in the 16c background. Similar results were obtained for RNase3 of SPCSV

isolates m2-47 and Is which are somewhat different, sequence-wise, from RNase3 of SPCSV-Ug (see Fig. 1 in **II**). RNase3 from SPCSV-Is seemed to show a more apparent RSS effect than the other two RNases and was the first one detected for RSS activity in different assays (*not shown*). In repeated experiments, the RNase3-Ala$^{37,44}$ mutant failed to display RSS activity (**III**) in accordance with previous results where the endonuclease mutant RNase3 was shown unable to enhance the RSS attributed to p22 (see Fig. 6 in **I**). These results are supported by those obtained with *E. coli* RNase III in a previous study (Lichner *et al*., 2003). The work on *E. coli* RNase III was focused on the RSS activity of proteins that bind dsRNA, but the results also provide evidence that the wild-type RNase III protein (encoded by *rnc*+) is more efficient in RSS than the endonuclease-impeded mutant (rnc70) when silencing is induced with ssRNA. In addition, weak or no RSS was observed with rnc+ when silencing was induced with a hairpin RNA. The results of *E. coli* RNase III support the results of this thesis suggesting that regardless of the dsRNA-binding function, the loss of endonuclease activity significantly affects RSS caused by RNase III enzymes of class 1.

Several viral silencing suppressors have been previously shown to target siRNAs by binding and sequestering them from their effector complexes and thus inhibiting their functions (Mérai *et al*., 2006; Lakatos *et al*., 2006). Cleavage of siRNA by RNase3 was considered as the putative mechanism of RSS, given that the endonuclease activity of RNase3 was needed for the enhancement of p22-mediated RSS activity (**I**). Furthermore, accumulation of siRNAs was significantly reduced in the presence of RNase3 (see Fig. 5 and 6 in **I**). In a series of different assays described in (**I**), RNase3 and its endonuclease-mutant could bind siRNAs, but the RNase3 showed no detectable cleavage activity on most of the siRNAs. Out of several small RNAs tested (analogous to siRNAs) results indicated endonuclease activity of RNase3 only on the synthetic 25-nt siRNA. Because siRNA cleavage might require different reaction conditions than used in these experiments optimized for cleavage of long dsRNA substrates (**I**), it seemed worthwhile to re-test whether RNase3 could target the siRNAs by cleaving them. New cleavage assays were carried out using a purified RNase3 and its mutant RNase3-Ala$^{37,44}$ proteins. In these experiments, as before (**I**), the mutant RNase3-Ala$^{37,44}$ was unable to cleave high molecular weight dsRNA (600 bp) or any of the tested siRNAs (*not shown*). However, cleavage studies on small dsRNAs showed that 21-, 22-, and 24-bp siRNA were readily cleaved to products of ~14-bp which is in the size-range of products expected for this class of enzymes (RNase III) (Zhang *et al*., 2003; Gan *et al*., 2006).

RNAse III class 1 genes have been identified in several organisms (Suppl. Fig. 2 in **III**) where their products play different roles in the metabolism of cellular RNA (Giorgi *et al*., 2001; Spasov *et al*., 2002; Zhang *et al*., 2003; Comella *et al*., 2008). In bacteria, RNase III processes precursor ribosomal

RNA (rRNA) and although its dsRNA-specific activity first suggested an antiviral role against bacterial viruses (phages), it turn out that phages indeed can use RNase III to properly regulate their gene expression (Robertson, 1982). These results provide evidence that viruses can exploit host RNase III activity.

Putative functions for class 1 RNase III enzymes in relation to viral infection in eukaryotes are still unknown. Recently, an RNase III enzyme encoded by *Paramecium bursaria Chlorella virus 1* has been characterized (Zhang *et al*., 2003). In addition, dsDNA-containing viruses infecting fish, insects, or algae also encode putative RNase III proteins (Tidona & Darai, 1997; Van Etten & Meints, 1999; Stasiak *et al*., 2000; Jakob *et al*., 2001). The possibility that these class 1 RNase III endonucleases target siRNAs, points to a novel viral mechanism for interfering with the antiviral RNA silencing response.

## 4.4. Engineering transgenic resistance to SPCSV in sweetpotato (IV)

Although some resistance to SPVD has been observed in certain sweetpotato varieties in the field, true or durable sources of natural resistance to SPCSV are rare (Aritua *et al*., 1998a,b; Mwanga *et al*., 2002). In addition, the basis of this resistance is largely unknown, and most probably multigene-based (Mwanga *et al*., 2002). Incorporation of resistance detected in diploid wild *Ipomoea* species (Karyeija *et al*., 1998b) to polyploid sweetpotato may not prove an easy task. These characteristics limit utilization of natural resistance in sweetpotato.

The discovery of RNA silencing as a natural defence mechanism against viruses in plants (reviewed in Wang & Metzlaff, 2005; Lindbo & Dougherty, 2005) and the subsequent development of plant transformation constructs which are highly efficient in activating RNA silencing, has made it possible to induce resistance in transgenic plants against targeted viruses with a high rate of success (Smith *et al*., 2000; Bucher *et al*., 2006). This technology relies on activation of the natural anti-viral defence response (Fusaro *et al*., 2006) and does not require foreign protein or high levels of mRNA to be expressed. This is of particular importance due to the phenomenon of trans-complementation between viruses (Latham & Wilson, 2008) –most notably synergism that can be caused by single viral proteins as shown in this study (**III**).

Genetic transformation of sweetpotato using *A. tumefaciens* is not easy but the methods have been improved (Cipriani *et al*, 2001; Luo *et al*., 2006; Xing *et al*., 2008). A new protocol for transformation of sweetpotato cultivar 'Huachano' was used in this study (**IV**) to express an intron-spliced hairpin

(dsRNA) targeting conserved RdRp-encoding sequences of SPCSV and SPFMV. The chosen cultivar 'Huachano' shows a high level of natural resistance to SPFMV but no resistance to SPCSV. Therefore, main target in these experiments was to test resistance to SPCSV in the transgenic plants.

The modified transformation protocol of Cipriani *et al*. (2001) allowed obtaining a transformation efficiency of 4.7% and transgenic sweetpotato regenerants within 6 months of work. Transgenic status and independent transformation events were verified by PCR and Southern blot analysis. Up to 7 transgene loci were present in some plants. Although the transformation efficiency was rather low, it was not surprising as low transformation efficiencies have been reported for sweetpotato in all previous studies (Cipriani *et al*, 2001; Luo *et al*., 2006; Xing *et al*., 2008). However, the reproducibility of the protocol used in this work on 'Huachano' is a promising improvement as compared to previous studies carried out at CIP.

Transgenic plants were challenged with SPCSV using inoculation with whiteflies. Significant reduction in virus titres was detected in 10 transgenic events as compared to non-transgenic plants (see Table 1 and Fig. 3 in **IV**). These results indicated that resistance to SPCSV can be obtained by engineering plants to express dsRNA, which offers an alternative to generate sources of resistance to SPCSV. Additionally, the results are significant in the light of few reports on successful engineered resistance to *Closteroviridae* in cultivated plants (**IV**).

Expression of the transgene and RNA silencing-mediated degradation of the transcript were confirmed in these transgenic plants by detecting variable amounts of siRNAs corresponding to the hairpin construct (made of sequences of both SPCSV and SPFMV) (see Fig. 2 in **IV**). Hence, plants were 'primed' for antiviral defence by silencing. siRNAs were also detected in SPVD affected non-transgenic control plants, but plants infected only with SPCSV or SPFMV did not show detectable levels of siRNA (*not shown*).

The observed accumulation of transgene-derived siRNA and SPCSV showed no apparent correlation in the transgenic plants. It seems that other factors besides RNA silencing induced by the transgene may affect the observed resistance. The hairpin construct sequences were from SPCSV (and SPFMV) isolates of the EA strain whereas the SPCSV isolate used in the experiments was SPCSV-m2-47 that is not 100 % identical to the transgene sequence (see Table 1 in **II**). It is probable that the degree of protection through this strategy depends on the degree of homology between the sequences. It is also possible that the early expression of the sgRNA that encodes the RNase3 RSS protein (Kreuze *et al*., 2002; **III**) may protect the SPCSV genome from being efficiently targeted by silencing in the transgenic plants.

Despite of the level of resistance to SPCSV in these plants, infection with SPFMV resulted in SPVD in all transgenic events 3 weeks after inoculation, even in the transgenic resistant plants accumulating the lowest SPCSV titres. Given that expression of an RNase3 endonuclease renders sweet potato plants susceptible to SPFMV infection (**III**), it is possible that the low levels of SPCSV accumulation still produce catalytic RNase3 activity that is sufficient for RSS in these plants. Hence, even low levels of accumulation of SPCSV are sufficient to breakdown high levels of resistance to SPFMV, such as those of 'Huachano'.

The exploitation of RNA silencing to induce virus resistance is becoming a versatile strategy in order to combat viral pathogens. The combination of transgenic technologies with traditional breeding approaches can provide a synergistic benefit in thwarting viral diseases. Currently, efforts towards these goals are carried out by several laboratories including the Kenya Agricultural Research Institute (KARI), the Japan International Research Center for Agriculture, and the International Potato Center (CIP), among others. Furthermore, accessibility of technologies that take environmental and consumer concerns into consideration (Miki & McHugh, 2003; Cuellar *et al*., 2006) and development of highly reproducible and more efficient transformation protocols for additional sweetpotato cultivars will allow exploring new strategies to engineer virus resistance in this crop. Constructs targeting RNase3 in a specific manner should be evaluated in continuation of this line of work.

# 5. DISCUSSION

RNase III endoribonucleases are necessary for RNA silencing and have thereby a fundamental role in gene regulation in eukaryotic organisms (Carrington & Ambros, 2003). However, the data of this study show that their functions are more diversified than previously thought.

An RNase activity specific for dsRNA (RNase III) was first isolated and characterized from *E. coli* (Robertson *et al*., 1968). Its substrate specificity readily distinguished it from other RNA degrading enzymes like RNase I endonuclease and RNase II exonuclease, both specific for ssRNA. All members of the RNase III family have, as a minimum, a characteristic endonuclease domain (endoND) containing a conserved core of 9-aa residues known as the RNase III signature motif (Supplementary Fig. 2 in **III**) and at least one dsRNA binding domain (dsRBD) (Gan *et al*., 2006). Members of this family of proteins can be placed to three classes depending on the presence of additional domains. Class 1 represents the simplest and the smallest of these proteins, containing a single ribonuclease domain and a single dsRBD domain. Bacterial and viral RNAse III belong to this class. Class 2 proteins contain a long terminal domain, one dsRBD and two endoND domains. Class 3, also referred to as Dicer, contains one N-terminal helicase, one PAZ domain, one dsRBD and two ribonuclease domains, (MacRae & Doudna, 2007). Comella *et al*. (2008) have suggested a further division of members of the class 1 based on the presence of a highly variable N-terminal domain extension in yeast Rnt1 and PacI RNases (Lino *et al*., 1991; Rotondo *et al*., 1997; Giorgi *et al*., 2001; Spasov *et al*., 2002) and in the recently characterized *Arabidopsis* RNase III (Comella *et al*. 2008).

RNase III need the coordinated action of two endonucleases domains for the production of their distinct products. In case of class 1 RNase III, this means that a homodimer is needed for dsRNA cleavage. Class 2 and 3 RNases act as monomers but their duplicated endonuclease domains form an internal 'dimer' structure for cleavage of dsRNA (Zhang et al., 2004). Bacterial and yeast RNases of the class 1 characterized so far produce cleavage products of ~12- to 15-bp (Rotondo *et al*., 1997; Blaszczyk *et al*., 2001; Giorgi *et al*., 2001; Spasov *et al*., 2002). The minimum dsRNA substrate length for *E. coli* RNase III is ~20-nt, which is approximately equivalent to two turns of A-form dsRNA (Dunn, 1982) and close to the size of the small regulatory RNAs generated by Dicer RNases (**TABLE 1**). Biochemical and structural studies have shown that the catalytic properties of RNase III enzymes are conserved in all three classes. The longer cleavage products of class 2 and 3 are produced in part due to mutation of the RNase III signature motif in one of their duplicated endonuclease domains (Zhang *et al*., 2004). The cleavage products of RNase III enzymes (class 1-3) contain a 3'-OH 2-nt overhang and a 5' terminal phosphate (Robertson, 1982; Rotondo *et al*., 1997). The structural similarity of

siRNAs with the cleavage products of RNase III enzymes first suggested that siRNAs are produced by an RNAse III-like activity (Elbashir *et al*., 2001b).

RNase III products smaller than 20-nt are inefficient in triggering gene silencing regardless of whether they contain the hallmark 3'-OH 2-nt overhangs or not (Elbashir *et al*., 2001; Paddison *et al*., 2002; Yang *et al*., 2002). For example, although partial or limited cleavage of long dsRNA with *E. coli* RNase III (resulting in 18-25-nt siRNAs) successfully induce RNA interference (RNAi) in mammalian systems, the products of complete digestion of dsRNA and siRNA by this enzyme (~15-nt) are unable to trigger RNAi. (Paddison *et al*., 2002; Yang *et al*., 2002). In addition, stabilization of siRNAs by HEN1-mediated methylation of their 3´-overhangs is more efficient on siRNAs longer than 20-nt *in vitro* (Li *et al*., 2005; Yang *et al*., 2006; Boutet *et al*., 2003). These data indicate that size of small RNAs is another critical factor in their ability to trigger RNA silencing.

It is proposed that RSS proteins could interfere with RNA silencing at least by the following means: (i) inhibiting viral siRNA generation; (ii) preventing the incorporation of siRNAs into effector complexes; or (iii) interfering with silencing effector complexes (Silhavy & Burgyán, 2004) (**FIGURE 2**). For example, tombusvirus p19 targets siRNA incorporation, and potyviral HC-pro might inhibit RISC assembly (Silhavy *et al*., 2002; Kasschau *et al*., 2003; Chapman *et al*., 2004). Small regulatory RNAs (**TABLE 1**) are central players in the initiation, spreading and maintenance of RNA silencing (Vaucheret, 2006; Ding & Voinnet, 2007). Consequently several RSS proteins interfere with silencing by binding and sequestering siRNA, a suggested general strategy for RSS (Lakatos *et al*., 2006; Mérai *et al*., 2006). Cleavage of siRNAs may constitute an additional mechanism adopted by SPCSV RNase3 for suppression of RNA silencing (**III**).

Promiscuous degradation of important regulatory small RNAs by RNAse III enzymes might be expected to cause detrimental effects on the normal development of plants as may be the case with bacterial RNase III when expressed in transgenic plants (Lagenberg *et al*., 1997; Zhang *et al*., 2001) or upon agroinfiltration (Lichner *et al*., 2003). However, RNAse III of yeast (*Saccharomyces*) (Watanabe *et al*., 1995; Sano *et al*., 1997) and SPCSV (**III**) expressed in transgenic plants do not seem to cause such detrimental effects. These observation could indicate that the yeast and SPCSV RNase III proteins may be more target-specific. RNAse3 may cleave siRNAs bound by a specific RISC. In addition, their functions are probably regulated by interactions with cellular proteins as shown with yeast RNase III (Goirgi *et al*., 2001; Spasov *et al*., 2002).

RNA silencing induced by ssRNA relies on RDR-mediated pathways in order to generate dsRNA for DCLs and AGOs activities (Voinnet, 2005). RDR proteins are necessary for transitivity-dependent

amplification and systemic spread of RNA silencing, two pathways deeply overlapping with antiviral defence (Ding & Voinnet, 2007). In addition, HEN1 activity, which targets small RNAs longer than 20-nt (Yang *et al*., 2006) has been implicated in sense-transgene silencing and dispensable for dsRNA-induced silencing (Boutet *et al*., 2003). These observations, together with the fact that SPCSV RNase3 silencing suppression activity is more efficient in ssRNA- rather than in hairpin (dsRNA)-induced RNA silencing (**I**; **III**) would suggest a further mechanism for SPCSV RNase3 RSS activity. In this model, RNase3 would target siRNA originating from RDR-dependent pathways, the generated smaller cleavage products are no substrates for HEN1-mediated methylation, unstable and rapidly removed and/or unable to prime further RDR-mediated RNA silencing, which results in RSS. Given the phloem-limited nature of SPCSV (Karyeija *et al*., 2000), siRNAs generated or transported through vascular tissue (Yoo *et al*., 2004; Buhtz *et al*., 2008) might be particularly prone to be targeted by RNase3.

The viral RSS proteins known to date (**TABLE 2**), including p22 identified here, show no significant similarity to cellular proteins, in contrast to SPCSV RNase3 which is similar to the recently characterized RNase III class 1 protein of *Arabidopsis thaliana* (Comella *et al*., 2008) and a putative homologue found in rice (*Oryza sativa*; Genebank ID: BAD36550). The role of host RNases in RNA silencing has not yet been addressed. A role as negative regulators of RNA silencing could be hypothesized involving mechanisms for the turnover of small RNAs, an area that has received relatively little attention. For example, RNase3 activity could act similarly to *C. elegans* ERI-1, an exonuclease able to degrade siRNA *in vitro*. Worms containing an *eri-1* mutation show an enhanced RNA silencing response (Kennedy *et al*., 2004; Grishok, 2005). Whether or not the SPCSV *RNase3* gene is of host origin its activity could exemplify an additional strategy by which viruses incorporate, subvert or mimic host defence mechanisms in order to enhance virulence (Peremyslov *et al*., 1999; Anandalakshmi *et al*., 2000; Bilgin *et al*., 2003).

Among viruses, the closest relative of SPCSV in genome organization, gene content and sequence is LIYV. Interestingly, LIYV also encode a putative RNase III-like class 1 protein from ORF2 at the 3'-end of RNA1 (Cuellar WJ, *unpublished observations*). Although the function of this LIYV protein as a suppressor of RNA silencing remains to be assayed, previous experiments identified LIYV ORF2 associated with the differential accumulation of viral RNA1 and RNA2 (Yeh *et al*., 2000). A similar activity was previously found for the RSS protein p23 of CTV (Lu *et al*., 2004) which has a role in the asymmetrical accumulation of CTV sgRNAs (Satyanarayana *et al*., 2002).

RNase III class 1 proteins have also been reported in other viruses such as PBCV-1 (Zhang *et al*., 2003) and in animal viruses members of family *Iridoviridae* (e.g. *Frog Virus 3*) (Supplementary Fig. 2 in **III**). Interestingly, we have found that an iridoviral RNase III-like gene product show a similar

endonuclease-dependent RSS activity as SPCSV RNase3 in agroinfiltrated leaves of *N. benthamiana* (Cuellar *et al., In preparation*). Therefore, cleavage of siRNA may constitute a more general RSS mechanism in viral pathogenesis (**III**).

Closteroviruses, such as CTV, can encode more than one RSS protein which have non-redundant functions in suppressing the RNA silencing pathway (Lu *et al*., 2004) (**FIGURE 1**). This seems to be also the case for certain isolates of SPCSV that express two RSS proteins, RNase3 and p22 (**I**), and which accumulate at higher concentrations than isolates lacking p22 (**II**) in *Ipomoea* plants. p22 but not RNase3 can suppress RNA silencing induced by dsRNA suggesting that p22 probably interferes with RNA silencing downstream of RDR activity (Boutet *et al*., 2003; Ebhardt *et al*., 2005; Ding & Voinnet, 2007). As with analogous RSS proteins in *Closteroviridae*, p22 might also be able to bind siRNAs (Reed *et al*., 2003) but this hypothesis remains to be tested. In addition, the effect of RNase3 can be additive with RSS mediated by p22 (**I**; **II**), which would further suggest that RNase3 and p22 are not mutually redundant in RSS, as is the case for CTV RSS proteins (Lu *et al*., 2004).

The variability in number of encoded RSS proteins found among isolates of SPCSV could be related to previously observed differences in incidence and severity of geographically distinct SPCSV synergistic interactions. Sweetpotato cultivars with low incidence of SPVD in West Africa develop higher incidence of SPVD when cultivated in East Africa (Carey *et al*., 1999). In addition, local SPCSV can cause more severe symptoms in East Africa than in West Africa in a determined cultivar (Gibson *et al*., 1998). In Uganda, distinct SPCSV isolates can be distinguished based on the severity of symptoms induced during SPVD in sweetpotato plants (Alicai *et al*., 1999). These differences might reflect the occurrence of SPCSV isolates expressing additional RSS proteins which may be associated with a distinction in symptom severity and virus accumulation (**II**). SPCSV isolates containing RNase3 and p22 have been identified so far only in the Mpigi region in Uganda (**II**).

By suppressing degradation of viral RNA (and thus enhancing virus accumulation), RSS proteins might have a direct effect on the rate of vector transmission, virulence and in the host-range of viruses. For example, tombusviral P19 RSS protein is involved in the systemic invasion of alternate hosts, such as pepper (*Capsicum annuum*) and spinach (*Spinacia oleracea*) (Scholthof, 2006). However, studies have largely focused on viruses of cultivated crops; consequently viruses present in alternate hosts, including wild plants, have gained much less attention (Hull, 2002; Roossinck, 2005).

Wild plant species are potential perennial reservoirs for viruses that can also infect sweetpotato (Tugume *et al*., 2008). By infecting wild and cultivated sweetpotato these viruses might have evolved different strategies in order to adapt to a changing environment including co-infections and the acquiring of alternate RSS proteins with non-redundant functions (Vanitharani *et al*., 2004; Lu *et al*.,

2004; **II**). these adaptations would allow viruses to cope with the multilayered and hierarchical antiviral RNA silencing defence (Deleris *et al*., 2006; Blevins *et al*., 2006) and its variations, present in different hosts. Studies on the occurrence, variability and interactions of viruses such as SPFMV and SPCSV in wild *Ipomoea* species are therefore of special interest for future studies.

Chronic latent virus infections are commonest in vegetatively propagated plants such as sweetpotato where single infections with viruses usually cause mild diseases (Valverde *et al*., 2007). The continuos propagation of a virus in the same host and the common practice of roguing field plants showing conspicuous viral symptoms would leave only mild strains of the virus. The result is positive, because in these plants the more severe virus strains are eliminated or "diluted" due to cross-protection (Smith *et al*., 1960; Hull, 2002). However, the presence of a chronic mild-virus infection might render the plant susceptible to heterologous viruses, as evidenced in viral synergism and trans-complementation studies (*reviewed in* Latham & Wilson, 2008). Results of this study show that expression of a single SPCSV protein (RNase3) is sufficient to enhace SPFMV accumulation characteristic of the synergistic SPVD (**III**).

The suppression of host defence by SPCSV RNase3 is consistent with no SPCSV-resistant sweetpotato genotype identified so far (**IV**). Disturbance of host physiology and the development of severe symptoms in sweetpotato may be caused by the increased accumulation of the synergizing co-infecting viruses (Untiveros *et al*., 2007; **II**; **IV**). In SPVD the accumulation of SPFMV HC-pro can interfere with several small RNA-mediated regulatory pathways (Chapman *et al*., 2004). Identification of the RSS activity of RNase3 and its role in viral synergism during SPVD, together with the lack of sources of natural resistance in sweetpotato, stress the importance of targeting SPCSV and particularly its RNase3 gene product in order to generate SPVD resistance in sweetpotato plants.

Taken together, our results underline the role of SPCSV in viral synergism and in suppression of RNA silencing. The data presented show that RNase3 is sufficient for the development of SPVD in SPFMV-infected sweetpotato plants. A valuable working hypothesis is provided on how this type of viral proteins may severely interfere with the plant immune defence rendering the host vulnerable to co-infecting viruses. The novel role for RNase III class 1 enzymes in RSS is an area in which they were previously not known to be involved.

# 6. CONCLUDING REMARKS

1. A Ugandan isolate of SPCSV (Ug) encodes two novel proteins involved in RNA silencing suppression (RSS). These genes are located at the 3' end of RNA1. They showed different RSS characteristics: p22 showed a consistent RSS activity induced by dsRNA, interfered with viral cross-protection in *N. benthamiana* plants and its overexpression caused necrosis in leaves. RNase3 enhanced the RSS activity of p22 but could not suppress silencing induced by dsRNA.

2. The number of RSS proteins in SPCSV varies. Isolates that lack the p22 gene were found in Tanzania, Peru, and Israel. They were able to synergize with SPFMV and induce SPVD but accumulated less than isolates containing p22. Isolates lacking p22 had a gene arrangement identical to LIYV (*Crinivirus*) in RNA1. Data suggested that LIYV also may encode for a class I RNase III. It seems that the p22 RSS protein has been a relatively recent incorporation into the SPCSV genome.

3. Expression of the RNase3 protein is sufficient to induce SPVD symptoms as shown in transgenic sweetpotato plants infected with SPFMV. The RSS activity of RNase3 was dependent on its endonuclease activity which in turn is required for its detected ability to cleave siRNAs. We suggest that the cleavage of siRNA could be related to RSS and viral synergism. The presence of genes for functional analogous RNase III proteins in animal viruses was revealed. The data constitute the first report on siRNA cleavage related to RSS as a additional strategy in viral pathogenesis.

4. Transgenic resistance to SPCSV was developed in sweetpotato plants. Production of transgenic dsRNA specific for SPCSV caused a significant reduction in SPCSV accumulation. However, a synergistic interaction with SPFMV was still observed in the transgenic plants, even in the plants where the SPCSV accumulation was the lowest.

5. Identification of SPCSV RNase3 as a sufficient factor for SPVD induction and the improved sweetpotato transformation protocols developed in this work will allow developing additional strategies for resistance to SPVD in sweetpotato.

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# 8. ACKNOWLEDGMENTS

I would like to thank Jari Valkonen for the opportunity, patience and guidance. Mart Saarma and Jaakko Kangasjärvi for the following up of this work and to Eeva Sievi from the Viikki Graduate School in Molecular Biosciences (VGSB) for the help with academic and extra-academic activities. Thanks to Erkki Truve for reviewing this work and to Jan Kreuze for the friendship and good collaboration. To Ian Barker, for the kind invitation and the facilities to start the writing of this thesis at CIP.

Kiitos to my friends, colleagues and co-authors (kaikille) from the KPAT and SLU Labs. To “Roope” Hurme for his help with radioactive work and Minna Rajamäki for our “Lab-duets“. To my office mates and friends Katrin “Weigmannen”, Arthur “Tug2” Tugume and Elisa Goytia. To Qiao-Chung Wang for the collaboration, the good laugh and the fishing stories. Thanks to Arturol Salinas, Anselmo V, Carolo Spetz, Cesar Zevallos, Oscar Santaolalla, Gisella Canessa, Jani K, Ricardo “Apetito”, Sanna Markkula, Zuzichka, Yang Peng Ng, Marjo A-P, Sascha “el curtidor”, Adrien, Olga, Konstantin, Rasa, Anatoly, Deus, Tuuli, Luis Santos, Miguel “el cordobés”, Ana G, “su excelentisima” Victor Casillas, *et al.*, for the friendship, parties, dinners and other very needed distractions and for making my stay in Finland even more enjoyable.

Gracias a los amigos y colegas del CIP que han colaborado de una u otra manera para que este trabajo se realice, Ilanit Samolski, Milton Untiveros, Karin Cruzado, Luis Ñopo, Dennis “The Bear”, Gaby Burgos, Jose Rodriguez, Max Fernandez, Luciano, Marleni y a mi ‘antiguo’ supervisor Marc Ghislain. A Jorge “Lox” Nuñez y Armando “Demolition Man” Casas, por el Lab-training y la amistad.

A los muchachos del barrio Chachu, Paul, Alejo y Jeca por la música, los “viajes” y la buena comida. A la memoria de Vice y Ruben, a quienes todos extrañamos.

Ačiū Alma, tu geriausia (you know). Thanks to Albertas ir Jurate from Gelvonu, for being always welcoming and to the Vilnius-crew for the fun cultural-crash, the ham and beer. Į sveikatą!

Finalmente, agradezco el cariño incondicional de toda mi familia, en especial el apoyo de mis viejos, Mamaberna y Chalo, mis hermanos y sobrinas, con los que comparto tantos buenos momentos.