AN ABSTRACT OF THE DISSERTATION OF

Ioannis E. Tzanetakis for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on March 19, 2004.

Title: Molecular Characterization of Criniviruses and Ilarviruses Infecting Strawberry

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Robert R. Martin

Pallidosis is a disease of strawberry identified in 1957 with an unknown etiology. Two previously uncharacterized viruses in strawberry, *Strawberry pallidosis associated virus* (SPaV) and *Beet pseudo-yellows virus* (BPYV), have been found associated with disease symptoms. The complete nucleotide sequence of both viruses was determined and molecular detection protocols developed. In addition, an immunological tissue blot assay was developed for SPaV. Phylogenetic analysis of SPaV placed it in the genus *Crinivirus*, family *Closteroviridae,* along with BPYV. Transmission studies identified *Trialeuroides vaporariorum*, the greenhouse whitefly, as a vector of SPaV, while thevirus was not transmitted by pollen or seed. The geographic distribution of both SPaV and BPYV in the United States was examined.

Two ilarviruses of strawberry were investigated. *Tobacco streak virus* (TSV) is the type member of the genus *Ilarvirus,* family *Bromoviridae,* and can cause severe yield

losses in small fruit crops. Sequence and phylogenetic analysis of 15 "TSV" isolates from *Fragaria* and *Rubus* revealed that they are homogeneous and represent a new virus species designated as *Strawberry necrotic shock virus* (SNSV). Nucleic acid-based protocols failed to identify any plants of the study infected with TSV an indication that the virus may not be a pathogen of *Fragaria* and *Rubus*.

Fragaria chiloensis latent virus (FClLV) is the second member of the genus *Ilarvirus* that infects strawberry. Previously, the virus had been found only in Chile and although many plants have been tested in North America using a serological test, none was found infected with the virus. A molecular test has been developed and used to confirm the presence of the virus in strawberries along the west coast of North America. Phylogenetic analysis of the coat protein gene of the virus place it in subgroup 4 of the genus along *Prune dwarf virus*, while it was previously thought to be related most closely to *Lilac ring mottle virus* and *Asparagus virus-2*.

The role of the newly identified viruses and other viruses infecting strawberry is discussed in association with the strawberry decline disease found to cause severe symptoms and yield losses in both California and British Columbia, Canada.

The significance of the work presented in this publication includes: the identification of two viruses associated with strawberry pallidosis disease; the complete nucleotide sequence of two criniviruses, an emerging group of plant viruses adds to the limited knowledge we have about this diverse virus genus; the identification of FClLV in North America; the identification of SNSV as a distinct virus species which demonstrates the need of further studies on TSV, a virus species that infect a wide range of plant species and may actually be a cluster of diverse species. The high incidence of the pallidosis associated viruses points to the significance of the disease and their role in strawberry decline. The development of fast, sensitive and reliable tests for four strawberry viruses will have an impact to the strawberry industry, since the need for high quality, virus-free plants is essential for a crop that is asexually propagated.

Molecular Characterization of Criniviruses and Ilarviruses Infecting Strawberry

by

Ioannis E. Tzanetakis

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APPROVED:

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Redacted for privacy

Major Professor, representing Molecular and Cellular Biology

Redacted for privacy

Director of the Molecular and Cellular Biology Program

Redacted for privacy

Dean of Graduate School

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Chapter 1

Introduction and literature review

Ioannis E. Tzanetakis

1.1 Strawberry

Strawberry (*Fragaria sp.* L.) belongs to the *Rosaceae* family (*Angiospermophyta*, Class *Dicotyledoneae*). The genus name *Fragaria* is a derivative of the latin word "fraga" (fragrant) and it was with this name that strawberry is mentioned in the *Natural History* of Pliny (Darrow, 1966). Although strawberries were harvested since at least the Roman times from the wild it was not until the 19th century that commercial production started. Strawberry may have acquired the name from the fact that merchants had fruit on straw while transporting it to the markets (Hancock, 1999). The predominant strawberry species grown today, *F.* x *ananassa*, is a hybrid of *F. virginiana* Duch. (Scarlet or Virginian strawberry) and *F. chiloensis* (L.) Duch. (Chilean strawberry) that were interplanted in European gardens during the 18th century (Maas, 1998). The species name *anannasa* was designated because of the strong, pineapple (*ananas*) fragrance of the fruit (Daubeny, 2003). Strawberry is planted in almost all countries around the world. The major strawberry producing countries are the United States, Mexico, Spain, Italy, Poland, Russia and Japan (Bowling, 2000). Annual fruit production exceeds 2.5 million metric tons (Hancock 1999).

The genomic chromosome number of the genus *Fragaria* is x=7. Strawberries are divided in four subgroups according to the polyploidy level - diploid, tetraploid, hexaploid or octaploid (Anon., 1982). The Scarlet and Chilean strawberries are octaploids and so is *F.* x ananassa. Strawberry is an herbaceous, perennial plant.The stem is shortened to ½ to 1" in length and is called a crown. Leaves develop spirally around the crown from nodes that are found on the apex. A leaf consists of three leaflets that attach to the petiole by a petiolule (short petiole) (Galletta and Bringhurst, 1990). Strawberries produce stolons (runners) that originate from axillary buds. Runners are the principal way of strawberry proliferation in nature since each mother plant may produce more than 15 runners per growing season. Roots form at the base of the runners when in contact with soil, generating daughter plants.

The strawberry flower is typical of the *Rosaceae* family with five sepals, five petals, 20-35 stamens and a receptacle with hundreds of pistils and ovaries embedded in

the receptacle. Strawberries can be either hermaphrodites or dioecious, containing either male or female reproductive organs, depending on the species or geographic location (Hancock, 1999). The inflorescences of the plant are modified stems and have primary flower secondary, tertiary etc. flowers. Strawberry fruit is not a true berry since the true fruit is an achene, which is referred to as 'seed'. The edible 'fruit' consists of the receptacle and the achenes.

Strawberry root system usually contains 20-30 primary roots and hundreds of higher order roots. The majority of the roots are found in the top 30-50 cm of soil (Shoemaker, 1978). The shallow root system in combination with the strawberry preference for sandy soils make the plants fairly sensitive to drought conditions that can greatly affect fruit production and growth.

1.2 Strawberry production systems in the United States

There are three major cultivar types of *F.* x *ananassa* (hereafter called strawberry) grouped by the climatic need for flower bud initiation (FBI) and optimal fruit production. Originally, strawberry was a short day plant that started FBI late in the season as day length decreased. The plants start FBI when day length is less than 14 h and or temperature is lower than 15^0C. This trait is found today in the June-bearing (JB) strawberries that give a single crop in late spring – early summer. The JB strawberries produce many runners and daughter plants. The ever bearing (EB) cultivars produce fruit when the day length exceeds 12 hours and give two main fruiting peaks in late spring and early fall although they bear some fruit between the two peaks. These cultivars do not produce many runners and their total yield is usually less than that of the JBs. The third type consists of the day-neutral (DN) cultivars. These plants are capable of fruiting at all times during the growing season with typically three peak time periods; the first in early summer and then 45 days thereafter until the end of summer – beginning of fall. DN plants stop FBI when temperature exceeds 24 ^{0}C. DN strawberries produce runners but to a lesser extent than the JB (Galetta and Bringhurst, 1990; Strik, 2003).

There are two main strategies in strawberry production applied today around the

world. The annual hill system that is replanted every year and harvested only for part of the year. Strawberries are planted usually in late August through late October (dates change depending on geographic region). The plants are planted through a plastic mulch, in two or four row raised beds. Drip irrigation under the mulch is used in the majority of cases, and fertilization and chemical pest management are applied through the irrigation. DN cultivars theoretically are ideal for the annual system since they do not produce many runners that cost the plant in energy resources and they yield heavily and for an extended period although JB can be used in mild climates were they can fruit heavily and for an extended period of time. Potential problems include poor fruit quality at high temperatures and problematic propagation via runners (Dale *et al.* 2002). The annual system is very popular in areas with mild winters where plants may produce most of the year providing growers with a high value crop that targets the fresh market. These areas include California, Florida and North Carolina in the United States.

The second system is that of the perennial matted row using JB cultivars. The plants are planted in rows in early spring. The first flush of flowers during the planting year are typically removed to enhance plant growth during establishment. The plants are allowed to runner freely and multiple daughter plants are established. Plants in this system remain in the field for three to five growing seasons depending on the vigor of the plantation. The input in resources in this system is not as great as in the annual hill system but the quality of the fruit in usually inferior because of the gradual decline of the plants caused by pests and pathogens (in many areas, viruses are a leading cause of this decline). The fruit in matted row production is used frequently for processing (Pacific Northwest) or for U-pick operations in the Northeast and Midwest. The Pacific Northwest and other strawberry producing areas in the northern United States use mainly this system.

Most of the strawberry plants grown in North America come from nurseries in northern California or in eastern Canada. There are high and low elevation nurseries, depending on the chilling demands for FBI and which production market is targeted. High and low elevation nurseries are isolated from major strawberry production areas of California which are found in the southern to central coastal parts of the state.

Strawberries that are grown for production in California and Mexico, are grown in the high elevation nurseries. The plants get the chilling requirements for FBI by late September to late October depending on elevation, are dug from the nurseries, processed (sorted, trimmed and packaged) and shipped to production fields for planting in a period of 48 hours. The low elevation nurseries produce plants for the eastern United States and the Pacific Northwest. Strawberries that are to be grown in the eastern United States (Florida and the Carolinas) are shipped from California in early summer to eastern Canada, where they are planted in nurseries for another cycle of plant increase and to obtain earlier chilling for FBI. The plants are then shipped as green plants (not trimmed) to production areas in September to November and planted in growing fields. The production in Florida targets the November-March market and are generally the first fruit appearing in the market.

Strawberries grown for the Pacific Northwest are generally in production for three to four seasons and are mainly grown for processing. Planting timing in this case is not as critical as it is for the other two regions that use the annual hill system. Plants are grown in the low elevation nurseries, dug in mid-winter (December and January), placed in cold storage at -1^0C, and shipped to the fields where they are usually planted between late April and July. These plants grow vegetatively that first year without harvesting fruit.

The focal point of strawberry production in North America is California. Virtually all plants grown in commercial fields originate in nurseries of the state. The potential of infestation of the nursery material with viruses would signify widespread infestations in commercial fields. The trafficking of the plants enhances the potential of widespread dissemination of viruses. In addition to potential pathogen infection in California these plants grown in eastern Canada also are exposed to aphid and leafhopper vectors carried north and east on the Gulf Stream every spring and summer. Virus and phytoplasma vectors from the Mississippi River valley are transported on these air currents to the upper Mid-West and then east over the northeastern U.S. and eastern Canada (Miklasiewicz and Hammond, 2001). An effect of this movement may be the relatively high incidence of phytoplasma infection of strawberries (Jomantiene *et al.* 1998),

vectored by leafhoppers, in the southeast states in contrast to all other regions that strawberries are grown.

1.3 Strawberry viruses

To date 16 virus species that infect strawberry have been identified (Spiegel and Martin, 1998; this publication). Six of these viruses are aphid-borne, while five are vectored by nematodes. The rest are transmitted by whiteflies (this publication), thrips, pollen and an oomycete. All strawberry viruses cause very mild or no symptoms in most commercial strawberry cultivars grown today. Symptoms are usually in the form of gradual decline as multiple viruses accumulate in plants. Virus detection is based primarily on grafting of specimens onto *F. vesca* and *F. virginiana* indicators which develop mild to severe symptoms when infected with viruses. The grafting process has as follows: The petiolule of the middle leaflet of sample in trimmed to a 'V' shape while about ¾ of the leaf blade is removed. On the indicator plant the middle leaflet is removed and the main petiole is split in half to ~ ½" in depth with a razor blade. The leaflet from the sample is inserted in the petiole of the indicator plant and the graft is sealed with waterproof adhesive tape. Usually two grafts are performed per plant and all other leaves are removed (Fig. 1.1).

Fig. 1.1 Typical leaf graft of strawberry. Arrowhead points to the graft union.

The plants are placed in a moist chamber and after one week they are returned in the greenhouse. Symptoms usually develop within a month of grafting (Fig. 1.2).

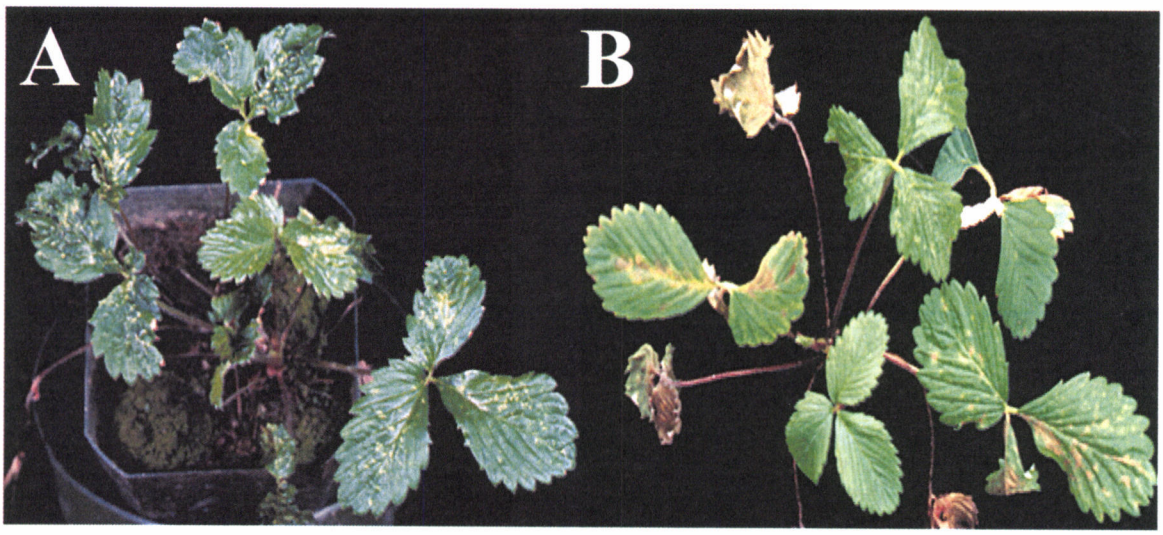

Fig. 1.2. Symptoms on strawberry indicator plants grafted with virus infected material. A. *Strawberry crinkle virus*; B. *Strawberry mild yellow edge virus.*

Over the last five years the partial or complete sequence of the genomes of most strawberry viruses have been determined. This allows for the development of sensitive and reliable molecular detection tests as an alternative to grafting. A brief review of the aphid-, nematode- and oomycete- transmitted viruses will be given here while the pollen/thrips – borne and whitefly transmitted viruses will be examined in more depth in individual chapters of this publication.

1.3.1 Aphid-borne viruses

1.3.1.1 *Strawberry mild yellow edge virus* (SMYEV)

The virus may be the first one identified to cause a disease in strawberry in the 1920s, and probably is found worldwide where strawberries are grown and aphid vectors are present (Converse *et al.* 1987). The symptoms of the virus on indicator plants range from leaf epinasty of leaves to vein necrosis and leaf scorching. Originally, the disease

was thought to be caused by a luteo-like virus due to vector transmission properties and isolation of isometric virions from infected plants. The disease has been shown to be caused by a potexvirus. Using a full-length infectious clone it has been shown that the potexvirus is able to cause disease but is not aphid-transmissable (Jelkmann *et al.*, 1992; Lamprecht and Jelkmann, 1997). The associated luteo-like virus probably is involved in the persistent transmission properties of SMYEV by *Chaetosiphon fragaefolii, C. jacobi, C. minor, Macrosiphum rosae* and the green peach aphid, *Muzus persicae.* The virus can cause yield losses up to 30% in the field (Spiegel and Martin, 1998) while the synergistic effect when found in complexes with other viruses increases impact.

1.3.1.2 *Strawberry vein-banding virus* (SVBV)

SVBV is a caulimovirus (Petrzik *et al* 1998) that was identified in 1955 (Frazier, 1955) and can be transmitted by *Cuscuta* sp. in addition to 10 aphid species in semi-persistant manner (Frazier and Morris, 1987). The typical symptoms of the virus on indicator plants include vein banding or clearing, leaf curling and necrosis. The virus has been proven recently to be the causal agent of the vein-banding disease (Mahmoudpour, 2003).

1.3.1.3 *Strawberry mottle virus* (SMoV)

SMoV belongs to the *Satsuma dwarf virus* (SDV) group of the family *Sequiviridae* (Thompson *et al.*, 2002), and is transmitted by more than 10 aphid species in semi-persistent manner (Mellor and Krczal, 1987). The virus is thought to be the most widespread of all strawberry viruses and is found everywhere that strawberries are grown (Spiegel and Martin 1998). It causes severe symptoms on indicator plants including mottling and stunting, unlike several other strawberry viruses that may be abundant but fail to cause symptoms on indicator plants after grafting assays (personal observation). The wide range of symptoms observed with SMoV may also be caused by multiple virus infections, thus grafting is not always a reliable method to identify the species of the virus

present. Reliable molecular detection methods with high sensitivity have been developed that can specifically detect the virus (Thompson *et al.* 2003; Thompson and Jelkmann 2003). SMoV strains show considerable variability depending on region of origin that may attribute to the different symptomatology on indicator plants (Thompson and Jelkmann 2003).

1.3.1.4 *Strawberry crinkle virus* (SCV)

SCV is one of the two rhabdoviruses identified today to infect strawberry. SCV is a cytorhabdovirus (Posthuma *et al.*, 2000) that is readily transmitted by aphids belong to the genus *Chaetosiphon* and the strawberry root aphid, *Cerosipha forbesi* (Babovic, 1976) and is found in strawberry everywhere that the vectors are found. The symptomology on indicators range from vein chlorosis and chlorotic spots on the leaves to crinkle and plant epinasty (personal observation). The virus is able to replicate in the aphid vectors (Sylvester *et al.*, 1974) that transmit the virus in a persistent manner. The latent period, time between acquisition and transmission of the virus, can exceed 30 days (Frazier, 1968). The latent period make the virus more abundant in climates where the vectors can survive for long periods of time. Under cooler conditions, the latent period exceeds the life span of the vectors, which die before they are able to transmit the virus. Therefore, SCV is rarely a problem in cool climates.

1.3.1.5 *Strawberry latent C virus* (SLCV)

SLCV is the second rhabdovirus identified to infect strawberry. Unlike SCV, SLCV is a nucleorhabdovirus. The virus seems to be isolated to eastern North America and Japan, regions where cultivar 'Premier' was distributed as it was infected universally with the virus. The properties of SCV and SLCV are very similar. Bacilloform virions are ~ 68 X 190-380 nm in size and the virus is transmitted persistently by aphids of the genus *Chaetosiphon* (Spiegel and Martin, 1998).

1.3.1.6 *Strawberry pseudo mild yellow edge virus* (SPMYEV)

SPMYEV belongs to the genus *Carlavirus,* of the family *Flexiviridae.* The virus was identified in Japan (Yoshikawa and Inouye, 1989) and there is a single report of the virus in the United States. SPMYEV causes symptoms on *F. vesca* indicator plants, while *F. virginiana* plants remain asymptomatic. Symptoms include yellow to red discoloration at the leaflet edge. SPMYEV is readily transmitted by *Aphis gossyppi* and *Chaetosiphon* sp. in a semi-persistent manner (Spiegel and Martin, 1998).

1.3.2 Nematode-borne viruses

1.3.2.1 *Tomato ringspot virus* (TomRSV)

TomRSV is the most widespread of the nematode vectored viruses in strawberry in the United States (Converse 1981; Frazier et al. 1961). Nepoviruses have spherical (icosahedral) particles of ~ 30 nm in diameter. The genome is divided in two genomic RNA molecules that encode single polyproteins that are proteolytically processed. The 5'ends have a genome-linked virus protein (VPg) while the 3' end terminates with a poly adenosine tail. The virus belongs to subgroup B of the *Nepovirus* genus, has a wide host range and is transmitted by *Xiphinema* sp. as well as through seed at rates that can approach 100%. Symptoms on *F.vesca* can range from mottling and stunting to leaf necrosis while *F. virginiana* develop large red lesions on the leaves.

1.3.2.2 *Strawberry latent ringspot virus* (SLRSV)

SLRSV is transmitted by nematodes of the genus *Xiphinema* (Allen *et al.* 1970). Phylogenetic analysis based on the nucleotide sequence available, places the virus in the *Satsuma dwarf virus* (SDV) group of the *Sequiviridae* instead the *Comoviridae* (Everett, 1994). The virus has wide host range that exceeds 125 plant species in 27 families and is efficiently transmitted by seed, in some cases over 70% (Murant, 1974). The symptoms

on indicators include chlorosis and stunting. There are two reports of the virus in North America (Allen *et al.*, 1970; Hanson and Campbell, 1979) but in both cases no movement of the virus was reported. The virus has been found in strawberry plants in California and British Columbia (Martin *et al.*, 2004) and it seems to be associated with the symptomatology in *Mentha x gentilis* L. 'Variegata' as all samples tested were infected with the virus, although two additional previously unknown viruses were present also (Postman *et al.*, 2004). The extent of SLRSV in other hosts and areas, the wide distribution of *Mentha x gentilis* L. 'Variegata' and the host range of the virus make it a potential threat for the strawberry and other agricultural industries in the future.

1.3.2.3 *Arabis mosaic, Raspberry ringspot* and *Tomato black ring* viruses

All three viruses are not known to occur in the United States. *Arabis mosaic* (ArMV) and *Raspberry ringspot* (RRSV) viruses belong to subgroup A of the *Nepovirus* genus while *Tomato black ring* (TBRV) to subgroup B as TomRSV. ArMV is transmitted with *Xiphinema* sp. while RRSV and TBRV are transmitted with *Longidorus* sp. All viruses have wide host range and they are transmitted efficiently by seed, approaching 100% in some hosts (Murant, 1970 a; b; 1978). The symptoms on indicators may vary from mottling and chlorosis of the leaves to plant stunting.

1.3.3 Oomycete- borne virus - *Tobacco necrosis virus* (TNV)

TNV belongs to the genus *Necrovirus* of the family *Tombusviridae,* and is transmitted by the oomycetes *Olpidium brassicae* (Hull, 2002). The virus has many strains that where determined utilizing serology while sequence analysis reveal that they may be considered individual viruses. The icosahedral virions are 28 nm in diameter and encapsidate the genomic RNA of about 3.7 kilobases. A satellite RNA is usually associated with the virus that is capable to alter symptomatology drastically. The virus is very persistent in soil and has a very wide host range. The symptoms usually observed on strawberry indicator plants are similar to those caused by SMYE and SPMYE viruses. Information on the incidence of the virus in commercial field is limited (Spiegel and

Martin 1998) since detection is problematic as the virus is found in higher titre in plant roots (Martin R.R., personal communication).

1.4 Overview of the dissertation research

The original planning of my research project included determining the complete nucleotide sequence, development of detection methods and epidemiological characterization of the agent that is associated with the pallidosis disease of strawberry. The name of the disease is derived from the symptoms of marginal chlorosis and epinasty that it causes on indicators (Frazier and Stubbs, 1969). By definition, pallidosis is a disease caused by a graft-transmittable agent(s) and causes symptoms on *F. virginiana* indicators 'UC-10' and 'UC-11' and is asymptomatic on *F. vesca* plants.

Since the first identification of the disease in 1957, there were several attempts to characterize the causal agent(s) of the disease but no definitive agent was identified. There has been identification of inclusion bodies associated with the disease, similar to those formed in plants infected with *Beet yellows virus*, the type member of the family *Closteroviridae* (Henriques and Schlegel 1975) and dsRNA has been isolated from pallidosis positive plants (Yoshikawa and Converse, 1990) but there was not a consistent band pattern of dsRNAs visualized on gels.

At the beginning of my studies, dsRNA from infected plants was obtained and cloned. Several of the clones obtained had segments from a virus belonging to the family *Closteroviridae,* genus *Crinivirus* and was designated as *Strawberry pallidosis associated virus* (SPaV). A molecular test was developed based on the sequence data and used to verify the presence of the virus in 37 out of the 38 plants that were pallidosis positive based on symptoms in grafting assays. dsRNA was extracted from the single plant that tested negative and showed a pattern similar to but distinct from that of SPaV. Oligonucleotide primers designed from the sequence of four criniviruses were utilized to test the plant for the presence of a second crinivirus. An amplicon was obtained after reverse transcriptase-polymerase chain reaction (RT-PCR) utilizing primers based on the sequence of *Beet pseudo-yellows virus* (BPYV). BPYV has a wide host range including

important agricultural crops in the *Cucurbitaceae, Solanaceae, Rosaceae* and *Chenopodiaceae.* Since there was limited sequence data of BPYV in the database (less than 1200 base pairs at the beginning of the project), it was decided that I would try to acquire the complete nucleotide sequence of the BPYV isolate from strawberry.

In the efford to determine BPYV genome, several clones were attained with sequence that showed similarity at the nucleotide level with *Tobacco streak virus*, an *Ilarvirus* that infects small fruits, but high conservation in the amino acid level. Utilizing immunological methods we determined that this plant was coinfected with TSV. The complete nucleotide sequence of RNA 3 of the TSV isolate from strawberry was obtained and showed considerable variation from the published sequences of TSV, including different gene sizes and low nucleotide identities. A total of 14 'TSV' isolates from *Fragaria* and *Rubus* were obtained from the National Clonal Germaplasm Repository, in Corvallis, Oregon and the coat protein gene of each was sequenced. The coat proteins of these 15 small fruit isolates of 'TSV' were very similar to each other but quite distinct from TSV. This unique virus was designated as *Strawberry necrotic shock virus* (SNSV), the name used when the virus first was isolated from strawberry (Frazier *et al.*, 1962).

Fragaria chiloensis latent virus (FClLV), is another *Ilarvirus* that was identified in *F. chiloensis* that originated from Chile (Spiegel *et al.*, 1993). Since *F. chiloensis* is used in breeding programs and no testing is performed for the virus, I felt that it would be appropriate to acquire sequence data that would help in the better detection of the virus after development of molecular tests. The sequence data obtained revealed that the virus is related more closely to *Prunus dwarf virus* in contrast to what was reported previously. Several strawberry plants from California, Oregon, Washington and British Columbia were sampled and tested positive for FClLV by RT-PCR but negative in ELISA. Therefore, the virus is more widespread in North America than previously thought.

The epidemiological studies of SPaV included seed and pollen transmission as well as transmission studies with whiteflies that are known vectors of criniviruses. Whitefly transmission studies were done in collaboration with Dr. W.M. Wintermantel, at USDA-ARS, Salinas, California, and *Trialeuoides vapororium* was identified as a vector.

In addition, several plants species other than *Fragaria* spp. were identified as hosts for the virus and may act as a reservoir for the virus.

The dissertation is divided in seven chapters. Two chapters are devoted to the identification, detection, molecular characterization and epidemiology of SPaV. One chapter is devoted to the determination of the complete nucleotide sequence of BPYV. The properties of SNSV and its' molecular detection is discussed in an additional chapter as is the molecular detection, geographic distribution and phylogenetic relationships of FCILV within the genus. The final chapter contains information on the importance of these viruses in strawberry decline a problem that is becoming more common in Western United States.

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Chapter 2

Identification and Detection of a Virus Associated with Strawberry Pallidosis Disease

Ioannis E. Tzanetakis and Robert R. Martin

2.1 Abstract

The etiology of pallidosis, a disease of strawberry identified more than 45 years ago, remains unknown. We report a putative agent of the disease, a virus belonging to the *Crinivirus* genus of the *Closteroviridae* family. A sensitive RT-PCR test has been developed. Polyclonal antibodies that can be used to detect the virus in petiole tissue blots were developed using a recombinant virus coat protein. The nucleotide sequences of regions of the viral genome that encode the heat shock protein 70 homolog and the major coat protein were obtained. Alignments of the major coat protein show that the virus isolated from strawberry plants positive for pallidosis is most closely related to *Cucumber yellows virus* (syn. *Beet pseudo-yellows virus*) and *Cucurbit yellow stunt disorder virus,* members of the *Crinivirus* genus.

2.2 Introduction

Pallidosis is a disease of strawberry first reported in California and Australia in 1957 (Frazier and Stubbs, 1969). Since then it has been reported in eastern Canada (Craig, 1981), Arkansas (Fulton and Moore, 1982) and Maryland (Hokanson *et al.*, 2000). Pallidosis disease is an unrecognized or under-recognized problem in the strawberry industry but there are recent studies showing that the disease is very widespread in the Mid-Atlantic states (Hokanson *et al.*, 2000). In greenhouse grown plants of 'Northwest' strawberry the disease reduces runner production and root growth by 15-20% (Converse and Volk, 1990). However, its major impact in yield loss is believed to result when it occurs in mixed infections with other strawberry viruses (Mullin *et al.*, 1975).

The pallidosis agent is graft-transmitted and high molecular weight dsRNA species have been purified previously from infected plants (Yoshikawa and Converse, 1990). Inclusion bodies similar to those of *Beet yellows virus* (BYV), the type member of the *Closteroviridae* family of plant viruses, have been observed in plants that indexed positive for pallidosis (Henriques and Schlegel, 1975). Several lines of evidence suggest that the pallidosis disease is of viral etiology. Pallidosis is defined as a disease caused by

a graft transmissible agent(s) that causes distortion, chlorosis of leaves and dwarfing of grafted *Fragaria virginiana*, 'UC-10' or 'UC-11' plants, while *F. vesca* indicator plants and commercial strawberry cultivars remain symptomless. It is important to note that because of the latent infection in most cultivars the detection of the disease has only been possible by grafting onto both *F. virginiana* and *F. vesca* indicator plants (Fulton, 1987). We report a putative causal agent of pallidosis disease, a virus in the *Crinivirus* genus of the *Closteroviridae* family, and tentatively designate it as Strawberry pallidosis associated virus (SPaV). In addition, the complete nucleotide sequences of the heat shock protein 70 homolog (HSP70h) and major coat protein (CP) genes of the virus, are presented and molecular and immunological assays developed for detection of SPaV are described.

2.3 Materials and Methods

2.3.1 Plant material

Strawberry plants (*Fragaria* x *ananassa)* from commercial fields in Maryland were tested at the USDA-ARS Fruit Laboratory in Beltsville, MD for virus infection by grafting onto indicator plants *F. vesca* ('UC-4' or 'UC-5') and *F. virginiana* ('UC-10' or 'UC-11') as described previously (Frazier, 1974). Source plants that resulted in symptoms when grafted onto *F. virginiana* plants (yellowing, distortion of leaves, cachexia) but failed to cause any visible symptoms on *F. vesca* plants were considered to have pallidosis. Twenty-nine individual strawberry plants from commercial fields in Maryland (M1-M29), two from California (C1 and C2) that indexed positive for pallidosis by grafting at USDA-ARS, Corvallis, OR, seven pallidosis isolates from the National Clonal Germplasm Repository (NCGR) in Corvallis, OR (CFRA # 9006, 9037, 9038, 9064, 9065, 9067, 9087), and 12 certified virus-free plants from USDA-ARS, Corvallis, OR were used.

2.3.2 Mechanical transmission

Plants belonging to 24 species and eight families (Table 2.1) were inoculated mechanically with a mix of leaf tissue of the seven NCGR pallidosis isolates. Four plants of each species were inoculated twice. The wt/vol ratio was 1:10 to 1:20 in phosphate buffered saline (PBS) pH 7.4, with the addition of 2% nicotine. Carborundum (600 mesh) was added on the leaf surface to facilitate delivery of the pallidosis agent(s) into the indicator plants.

2.3.3 Purification of virus and dsRNA

Virus was purified as described previously (Klaassen *et al.*, 1995) with the initial wt/vol ratio changed to 1:6 or 1:10 due to the viscosity of the strawberry tissue. Double-stranded RNA (dsRNA) was purified as described previously (Yoshikawa and Converse, 1990) from 22 of the available pallidosis isolates in addition to four certified virus-free strawberry cultivar plants. At least 100 dsRNA extractions were carried out in this study and subjected to gel electrophoresis. After extraction the dsRNA was aliquoted and precipitated by centrifugation at 16,000 x *g* for 30 min at room temperature in 70% ethanol and 0.1 M sodium acetate. The dsRNA was resuspended in 10 µl of tris borate ethylenediaminetetraacetic acid buffer, pH 8.0, (TBE) and was resolved by electrophoresis through a 1% agarose gel containing 100 ng/ml of ethidium bromide.

2.3.4 Cloning and sequence analysis

An amount of dsRNA equivalent to that extracted from four grams of fresh strawberry leaf tissue of Maryland field isolate M1 was incubated with 20 mM methyl mercuric hydroxide and random nucleotide hexamer primers (0.5 µg) (Invitrogen, Carlsbad, CA) for 20 min at room temperature in water. The cDNA synthesis was performed according to the manufacturer's instructions using Thermoscript reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) in a final volume of 50 µl. The cDNA was

ethanol precipitated, air-dried and second-strand synthesis carried out as described previously (Jelkmann *et al.*, 1989). The products were then adenylated at the 3' ends using *Taq* polymerase (Invitrogen, Carlsbad, CA) (1unit) added to the second-strand synthesis reaction and incubated for 15 min at 72^{0}C. The cDNA products then were purified utilizing the rapid polymerase chain reaction (PCR) purification system (Marligen Biosciences, Ijamsville, MD) prior to ligation. The products were concentrated to a volume of 4 µl and cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The recombinant plasmids were purified, digested with EcoRI (New England Biolabs, Beverly, MA) and analyzed using agarose gel electrophoresis. Several clones with inserts ranging from 600 to 2,500 nucleotides were sequenced in the Central Services Laboratory at Oregon State University using an ABI 377 DNA sequencer. The BLAST databases (blastn and blastp) at the National Center for Biotechnology Information (Altschul *et al.,* 1997) were used to compare the unknown sequences to the Genbank's nucleotide and protein databases, respectively. Three clones (SP 21, SP 37 and SP 44) corresponding to the HSP70h gene and two clones (SP 60 and SP 63) corresponding to the CP gene of the virus were identified after using BLAST by comparison to related sequences in the database. Oligonucleotide primers HSP 5', HSP 3', CP 5'and CP 3' (Table 2.2) were developed, which allowed the amplification of the complete HSP70h (HSP 5'/HSP 3') and CP (CP 5'/CP 3') genes by RT-PCR. The first-strand cDNA was prepared using reverse transcriptase as described above from dsRNA, and 5 µl of the RT reaction was used as template in a 50 µl PCR reaction that consisted of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM dNTPs, 400 nM each of 5' and 3' primers, and 1 unit *Taq* Polymerase (Invitrogen, Carlsbad, CA). The PCR program we used consisted of a 5 min denaturation step at 94^{0}C followed by 40 cycles of 30 sec denaturation at 94^{0}C, 30 sec annealing at 50^{0}C and 2.5 min extension at 72^{0}C, followed by a final extension time of 15 min at 72^{0}C. For DNA sequencing purposes we used primers SP 37, SP 44F and CP R (Table 2.2). We used isolates M1 and C1, a Maryland and a California field isolate, respectively, and CFRA 9064 from the NCGR, one of the N.W. Frazier clones in which the virus was first identified. The consensus of the sequences of both genes was

constructed after sequencing two individual PCR reactions at least twice in both directions. However, for the HSP70h gene of isolate CFRA 9064, a PCR product was cloned into the pCR2.1 TOPO vector (Invitrogen Carlsbad, CA), and three individual clones were sequenced twice using the primers mentioned above as well as the M13 forward and reverse primers. All consensus sequences were assembled using the ClustalW program (European Bioinformatics Institute, http://www.ebi.ac.uk/ clustalw).

2.3.5. Detection by RT-PCR

RNA was extracted from 100 mg of strawberry leaf tissue as described previously (Hughes and Galau, 1988) and was resuspended in 40 µl of RNAse-free water. Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen, Carlsbad CA) according to the manufacturer's instructions using RNA as 1/10 of the volume of the reaction and using 0.3 µg of random nucleotide hexamers (Invitrogen, Carlsbad CA). The reaction was terminated by heating at 75^0C for 20 min followed by incubation with one unit of RNAse H (Invitrogen, Carlsbad CA) for 30 min at 37^0C. For PCR we used primers SP 44F and SP 44 R that amplify a 517 base pair (bp) fragment of the HSP70h gene and CP 5' and CP n731R (Table 2.2) that amplify a 752 bp fragment containing the largest part of the CP gene. We developed primers SPL F and SPL R (Table 2.2) that amplify a 301 bp fragment of the strawberry pectate lyase B gene which we used as an internal control in our PCR reactions. The program consisted of initial denaturation for 5 min at 94^0C followed by 40 cycles with denaturation for 30 sec at 94^0C, annealing for 30 sec at 50^0C for the CP primers or 55^0C for the HSP70h primers and extension for 1 min at 72^0C. The PCR reaction was essentially the same as described above with the addition of acetamide to a final concentration of 5% (Chakrabarti and Schutt, 2001). To verify the amplification of the viral genes several of the PCR products were sequenced and the sequences showed greater than 99% sequence identity to the M1 isolate.

2.3.6 CP expression

Primers were developed for the cloning of the CP gene into the pET 21b expression vector (Novagen, Madison WI). Primer CP exp. F introduces an NdeI restriction site at the 5' end of the CP ORF while primer CP exp. R (Table 2.2) introduces an XhoI site at the 3' end of gene just prior to the stop codon. Single-stranded RNA extracted as described above from leaf tissue of field isolate M1 served as template for the RT reaction, and the PCR protocol consisted of one cycle of 5 min at 94^0C, 1 min at 37^0C, and 1 min at 72^0C followed by 35 cycles of 30 sec at 94^0C, 2 min at 72^0C, followed by final extension time of 15 min at 72^0C. The PCR product was purified utilizing the rapid PCR purification system (Marlingen Biosciences, Ijamsville, MD) according to the manufacturer's instructions and incubated overnight with 100 units of NdeI and XhoI (New England Biolabs, Beverly, MA). The same digestion reaction was performed with 1μg of pET 21b plasmid (Novagen, Madison, WI). The digested plasmid and PCR product were gel purified using the RNAaid kit (Bio 101, Carlsbad CA) according to manufacturer's instructions. The ligation was performed using T4 DNA ligase (New England Biolabs, Beverly MA) according to the manufacturer's instructions. Transcription of the gene was controlled by the T7 promoter, and the recombinant protein contained six histidine residues at the C terminus, that were used for column purification. The final plasmid was transformed into *E. coli* (Epicurian Coli BL 21-CodonPlus cell line [Stratagene, La Jolla, CA]). A single colony was selected and the presence of the plasmid was verified after amplification of the insert using the PCR protocol described above with primers M13 forward and reverse. The construct was sequenced three times using primers M13 forward and reverse as well as primer CP R to confirm that the insert was in frame, and expression of the protein was performed according to the manufacturer's instructions. 100 μl of cell suspension were sonicated and subjected to electrophoresis through 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) followed by staining with Coomassie Brilliant Blue and Western blotting immunoassay according to standard procedures (Sambrook *et al.*, 1989), using monoclonal anti-histidine and goat anti-mouse antibodies conjugated with alkaline phoshatase for detection according to

manufacturer's instructions (Sigma Chemicals, St. Louis, MO).

Following verification of the expressed protein by detection of the six histidine epitope in whole cell extracts, the protein was purified using Talon Metal Affinity Resins, utilizing the native buffer extraction method, and the Talon CellThru columns (both from Clontech, Palo Alto, CA). The purified protein was subjected to SDS-PAGE and Western blotting (Fig. 2.4A). A New Zealand white rabbit was immunized by intramuscular injection with 200 μg of the recombinant protein in Freund's complete adjuvant followed by a booster injection after a two-week period. The final bleed was performed two weeks after the booster injection.

2.3.7 Immunoassays

Immunoglobulins were purified from the rabbit antiserum using sodium sulfate precipitation, diluted to a concentration of 1 mg/ml in PBS and conjugated with alkaline phosphatase (Sigma, St. Louis, MO) as described (Converse and Martin, 1990). Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was carried out as described previously (Converse and Martin, 1990). Leaf and petiole tissues were tested in ELISA at dilutions of 1:10 (w/v) in PBS containing 0.05% Tween-20, 2% PVP-10,000 and 0.2% nonfat skim milk powder. Positive samples included a cocktail of leaf or petiole sap of the two pallidosis isolates from the NCGR described above. A threshold of two times the average A_{405} readings from healthy tissue was used. Antibody coating and conjugate concentrations ranging from 0.5 μg/ml to 10 μg/ml in all combinations were used to test the antiserum in ELISA. Each of these tests was done twice. For tissue blots, cross sections of petioles were blotted to 0.45 μm nitrocellulose membrane (Biorad, Hercules, CA), that was then washed twice with PBS-Tween, and blocked by soaking in PBS-Tween containing 3% nonfat skim milk powder for 1 h. After a single wash with PBS-Tween, the membranes were transferred to antisera solutions (1:1000 to 1:25,000) diluted in PBS and incubated at room temperature for 1 h. The filters were then washed 3 times in PBS-Tween and goat anti-rabbit alkaline phosphatase conjugate (Sigma, St. Louis, MO) diluted 1:2000 in PBS containing 2% PVP-10,000 and 0.2% nonfat milk

powder was added and incubated for one h at room temperature. After washing as above, the filters were placed in buffer consisting of 0.1 M Tris, pH 9.5, 0.1M NaCl and 5 mM $MgCl_2$, containing precipitating substrate (NBT/BCIP, Sigma, St. Louis, MO). Reactions were stopped by transferring the filters to deionized water (Tzanetakis, 1998).

2.4 Results

2.4.1 Mechanical transmissions

None of the 24 indicator plant species (Table 2.1) developed visible symptoms when mechanically inoculated with SPaV infected tissue. Some plants had symptoms that were attributed to nicotine toxicity after testing the plants for the presence of SPaV by RT-PCR and inoculation of the plants with PBS without nicotine. We also tested one plant per treatment per species with RT-PCR for the presence of SPaV using the HSP70h primers without being able to acquire any amplicons.

2.4.2 Purification of virus and dsRNA

The virus purification gave low numbers of virions. The virions were 10-11 nm in diameter and ranged in length from 250-450 nm in length (data not shown), an indication that they were unstable and broke during purification. It was determined that the rods belonged to the virus by performing RT-PCR on fractions that contained rods in the electron microscope and on fractions where rods were absent. Amplicons were generated only from the fractions that contained rods. Twenty-two of the 38 isolates were tested for presence of dsRNA. All 22 gave similar band patterns, while all healthy plant material gave either low molecular weight (< 200 bps) or no visible dsRNA bands (Fig. 2.1). All isolates had three predominant bands at ~8.0, 2.8 and 0.8 Kbp (Fig. 2.1) with the exception of isolate M29 which has been verified to be *Beet pseudo-yellows virus* (BPYV) (Tzanetakis et al., 2003) and not SPaV infected. Additional bands were observed

ranging in size from 4 to ~1.3 Kbp, but the intensity of these bands varied, depending on the season.

Table 2.1. List of indicator plants used for mechanical transmission of *Strawberry pallidosis associated virus.*

Plant genus	**Family**
Antirrhinum majus	*Scrophulariaceae*
Beta vulgaris	*Chenopodiaceae*
Chenopodium giganteum	*Chenopodiaceae*
Chenopodium quinoa	*Chenopodiaceae*
Cucumis sativus	*Cucurbitaceae*
Datura stramonium	*Solanaceae*
Glycine max	*Fabaceae*
Gomphrena globosa	*Amaranthaceae*
Lathyrus odoratus	*Fabaceae*
Lettuca sativa	*Asteraceae*
Lycopersicon esculentum	*Solanaceae*
Medicago sativa	*Fabaceae*
Nicotiana benthamiana	*Solanaceae*
Nicotiana glutinosa	*Solanaceae*
Nicotiana rustica	*Solanaceae*
Nicotiana tabacum (cv. Samsun NN)	*Solanaceae*
Petunia x hybrida	*Solanaceae*
Phaseolus vulgaris	*Fabaceae*
Phlox drummondii	*Polemoniaceae*
Pisum sativum	*Fabaceae*
Spinacia oleracea	*Chenopodiaceae*
Tagetes patula	*Asteraceae*
Trifolium pratense	*Fabaceae*
Vigna unguiculata	*Fabaceae*

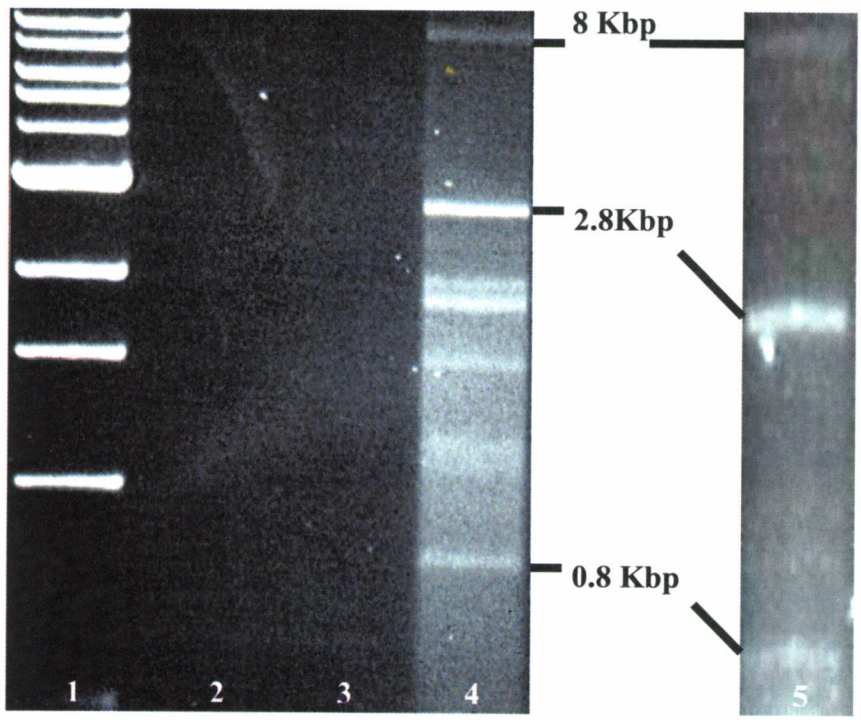

Fig. 2.1. DsRNA extracted from strawberry plants infected with the pallidosis agent. Lane 1: 1 Kilo base-pair DNA marker (BRL, Gaithersburg, MD); lane 2: Blank; lane 3: dsRNA extracted from healthy strawberry plant, lane 4: dsRNA extracted from *Strawberry pallidosis associated virus* infected plant in March, lane 5: dsRNA extracted from *Strawberry pallidosis associated virus* infected plant in August. Arrows show the 8.0, 2.8 and 0.9 Kbp bands present in all pallidosis isolates infected tissue regardless of time of year.

2.4.3 Cloning and sequence analysis

Preparation of cDNA from isolate M1 using random hexamer nucleotide primers and cloning as described above resulted in the production of multiple clones for analysis. BLAST searches showed that the putative pallidosis virus had sequence similarity to *Cucumber yellows virus* (CuYV), a strain of BPYV, *Cucurbit yellow stunt disorder virus* (CYSDV), *Sweet potato chlorotic stunting virus* (SPCSV) and *Lettuce infectious yellows virus* (LIYV), the type member of the *Crinivirus* genus (Fig. 2.2A).

The search indicated that sequences that flanked the HSP70h and the CP genes of the virus were present in the clones. Primers developed to these flanking regions allowed the amplification by RT-PCR of the complete HSP70h and CP sequences of the unknown

virus. The Genbank accession numbers are AY262158-AY262160 for the CP genes and AY262161- AY262163 for the HSP70h genes of M1, CFRA 9064 and C1 isolates, respectively.

2.4.4 Detection by RT-PCR

SPaV was detected in 37 out of the 38 isolates of pallidosis used in this study, while none of the 12 healthy plants gave any pallidosis specific amplicons while RT-PCR with the pectate lyase primers did result in 301 bp amplicons (Fig.2.3). Several additional sets of primers specific for parts of the HSP70h and CP genes of the SPaV genome were tested and all failed to amplify any regions using ds- or ssRNA from isolate M 29.

Table 2.2. List of oligonucleotide primers used for detection reverse transcription-polymerase chain reaction, sequencing and coat protein expression. For primers CPexp.F and CP exp.R underlined sequence show the NdeI and XhoI restriction sites respectively while bold indicates CP sequence.

Primer name	Nucleotide sequence (5'-3')
Detection primers	
SP 44F	GTGTCCAGTTATGCTAGTC
SP 44R	TAGCTGACTCATCAATAGTG
CP 5'	AGCTAGAACAAGGCAAGTC
CP n731 R	GCCAATTGACTGACATTGAAG
SPL F	TGCTAATGATGGAGACCTCG
SPL R	GGTGTCTAACTTGTCGTTCC
CP modification and sequencing primers	
CP exp. F	ACGCACAGTCATATGGCTGAAACAACCG
CP exp. R	GAGCTACTCGAGGTTTCCCGCCAATTGA
HSP 5'	GAGTCCGCTCTCCATGTGTT
HSP	AACGATCGGAATCAACTCTC
CP 5'	CAGCTAGAACAAGGCAAGTC
CP	TGGAACAGTGAGCTTGTCAG
SP 37	AGCGTTGGGTCGGTGTTGAT
CP R	CAACGGATTATTCACGCCAG

Fig. 2.2. A, Amino acids alignment of the HSP70h proteins and GenBank accession numbers of *Strawberry pallidosis associated virus* (SPaV, AAO92347), *Cucumber yellows virus* (CuYV, NP821143), *Cucurbit yellow stunt disorder virus* (CYSDV, NP 851572), *Sweet potato chlorotic stunting virus* (SPCSV, NP689401), *Tomato chlorosis virus* (ToCV, AAD01790) and *Lettuce infectious yellows virus* (LIYV, NP619695). B. Amino acids alignment of the coat proteins and GenBank accession numbers of SPaV (AAO92342), CuYV (NP821146) and CYSDV (NP851576). Asterisks indicate the identical amino acids in the aligned proteins.

A

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SPaV  MTEAKVGLDFGTTFSTISSYINNKMHVLKINDSPYIPTCLAISIDKDVIIGGAAQVLDSS 60
CuYV  -MQAKVGLDFGTTFSTISSFTNGEMKTLYVNNSPYIPTCLSISSEGDVIIGSAAQVIDES 59
CYSDV --MAKAGLDFGTTFSTISSYVNGVMKVLKLNETEFIPTCLAITSNNDVVVGGPAQVLSNS 58
SPCSV -MEAKAGLDFGTTFSTISAYVGGTMKVLRINGSEFIPTCLSVTATGDVVVGGAAQVLDSS 59
ToCV  -MSIKAGLDFGTTFSTISCFYNNKLFSLKLNGTEYIPTCLSITPNNEVIVGGPSQVLEAS 59
LIYV  MRDCKVGLDFGTTFSTVSTLVNNSMYVLRLGDSAYIPTCIAITPGGEAIIGGAAEVLSGD 60
          * ********** *         *       ****            *    *

SPaV  EVANCYFYDLKRWVGVDKVNFENIKAKINPQYVAKLVNDDVMLTGVDRGYSCTYTVKQLI 120
CuYV  EVKSCYFYDLKRWVGVDATNFLVIKEKIKPLYVVKLVGNDVYYTGVNKGFSCTYTVKQLI 119
CYSDV DMPNCYFYDLKRWVGVDSINYNVIKTKINPVYVTELRGNDVYITGIDRGYTCTYTVKQLI 118
SPCSV QLPHCYFYDLKRWVGVDRLSFEEIKRKISPQYTVRLEGNDVLITGISKGFSCTYTVKQLI 119
ToCV  ETPSCYFYDLKRWVGVTSVNYEVVKAKINPTYKTRLSNNKVYITGINKGFSTEFSVEQLI 119
LIYV  DTPHCFFYDLKRWVGVDDNTFKFAMNKIRPKYVAELVEGEVYLTGINKGFSIKLSVKQLI 120
          * **********          ** * *   *    *  **   *       * ***

SPaV  LLYIDTLVRLFSKTDNLNIISLNVSVPADYKCKQRMFMKSVCDSLNFSLRRIINEPSAAA 180
CuYV  LLFIDTMVRLFSKTNNLNIISLNVSVPADYKCKQRMFMKSVCDSLNFSLRRIINEPSAAA 179
CYSDV LLYIETLVRLFSKVESITITSLNVSVPADYKCKQRMFMKSVCDSLGFSLRRIINEPSAAA 178
SPCSV LLYVDTLVRLFSNVEKLKILSLNVSVPADYKTKQRMFMKSVCESLGFPLRRIINEPSAAA 179
ToCV  LHYVNTLVRLFSKTENLKITDLNVSVPADYKSGQRLFMQAVCSSLGFNLRRIVNEPSAAA 179
LIYV  KAYIETIVRLLASSYSLRVIDLNQSVPADYKNAQRLAARSVLKALSFPCRRIINEPSAAA 180
          * ***           ** *******  **     *   * *  *** *******

SPaV  IYSVSKYPNYKYFLMYDFGGGTFDTSLIVRDGKVVTVADTEGDSFLGGRDIDNAISRFIV 240
CuYV  IYSVSKYPQHNYFIMYDFGGGTFDTSLITRDGQYVTVADTEGDSFLGGRDIDNEIQQFIV 239
CYSDV IYFVSKYPQYNNFLMYDFGGGTFDSSLIVRDGKYVTVADTEGDSFLGGRDIDNAIADYIT 238
SPCSV IYSISKHPGFDYFLVYDFGGGTFDTSLIAKDGKFVTVADTLGDSFLGGRDIDRAILSHIM 239
ToCV  IYCVSKYPQYAYFYIYDFGGGTFDTSLIVRYGKFVTVADTQGDSFLGGRDIDKTISKFIM 239
LIYV  VYCVSRYPNYNYFLVYDFGGGTFDVSLIGKYKSYVTVIDTEGDSFLGGRDIDKSIEDYLV 240
       *  *  *    *  ********* ***      *** ** ***********  *

SPaV  EKHSLPRPLSSDFLASIKEEVNNSSKSNFIALDTKGNIVNVSFNKDDLATCIQPFSVKSI 300
CuYV  KSNNLSRPLPSDFLASIKEDCNTTGKSTFNVMDVDGKLLTIRFSREDLAACIEPYSKRSL 299
CYSDV TTYGMKGGLSADVLASIKEDCNSKGRENFNVIDSSGKLHNVKFTRQDLSRCIEPFSKKSI 298
SPCSV RTNSLQKPLSADSLAAIKEEVNSTGRSNFNVLDVDGNIIFVNFSGEELDKIVSKFTAKSL 299
ToCV  DKNALNAPLSADMLASIKEETNSTGRSSYNIISDDGSIINIQFTFDDLVKCVEPFARRSF 299
LIYV  GKYNIKKVIPATYLALIKEECNNTNKSIFTILFDDGSVQVVEFSKSELEKCVRPFVERSI 300
                  ** ***  *           *      *    *          *

SPaV  KILDNLVGRRKITNGALFLVGGSSLLKKIQQDVSSYARSKGLTCVIDEDLRCSVSFGCSM 360
CuYV  KILDNLVKRRKISSGALFLVGGSSLLSKVQQDVAAYASANNFECVIDKDLRCSVSFGCSM 359
CYSDV ALLDNMVVRNITKDSAVFMVGGSSLLKKVQHDVMNYCARTKLECIIDKDLRSAVSFGCSM 358
SPCSV KILKAIADRNKITSGALFLVGGSSLLRKVQLDVSNFAKSIGLTPIIDKDLRSAVSYGCSM 359
ToCV  SILRSLVSRNKTSNGALFLVGGSSLLRPIQNRADGFARNHGLALIIDPDLRAAVSFGCSM 359
LIYV  KLINDVVVRNKLTSGVIYMVGGSSLLQPVQDMVRSYASTKGLTLVADQDMRSAVSYGCSV 360
            *          *******   *                * * *  ** ***

SPaV  QHAQEDSGSMTYIDCNSHPLMDLLMYGNPKVVVRKPMPIPYTKYDTRTIRQHYNTVVNVY 420
CuYV  QHAQEDSGSMIYIDCNSHPLMDLTLFGNPKVVVRKPMSIPYSTSDSRTIRSHYSTAVNVY 419
CYSDV SHAQEDTKNMIYIDCNSHPLMDISYFCSPKIIVRKPMAIPYTGVREETLTRHYTTILNVY 418
SPCSV MHAQEDSGSMVYIDCNSHPLMDVSLFANPRVIIRKPMSVPFSYKTTRKVDRHMMTAVNVY 419
ToCV  LHAQEDSGNMTYIDCNSHPLMDLGLYCHPRIIIRKPMSVPYTHKIEREVTRFITTALNVY 419
LIYV  LHKLEDNKEIVYIDCNSHPLSDISFNCDPEPIIRKPMSIPYTHTVKMRHDRPLKTIVNIY 420
       *  **     ********* *      *    ****  *              *  * *
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Figure 2.2 (continued)

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SPaV  EGSDLFVLNNDWLVSAKVNTSDHANVGEDLTFVYKYTIDGILELYAKNEKTGVEKLLPNT 480
CuYV  EGSDIFTLNNDWLISANVRTSDHVNVGEDLVFIYKYNIDGILELYAKNGRTGVEKLLPNS 479
CYSDV EGSDPFVLNNDWLISANMQSNKYGEIGDTLQYLYKYNVDGILELVVRNKRTGKETVLPNS 478
SPCSV EGSSLFVLDNDWLVSANVNTQDFVDLGQELSYVYKYNVDGILDLFVRNESTGVESLLPNS 479
ToCV  EGSDLFVLNNDWLISADVDYSKYAKMGETLVSVYKYTIDGILELSMANKTTGKSWVLPNT 479
LIYV  EGSNLFMPENDWLISSNINTTDFAKVGEEYSKVYEYDIDGIITLKIRNEVTGKMFTLPNS 480
      ***  *   **** *           *      * *  ***  *   *  **    ***
SPaV  FSLTEKINKLDLQLTQLSTIDESATLISIMSYFDDNYTRLLSLLRTPTILERELLKITST 540
CuYV  FSLTEKIEKLNLKLTQLSNVDEIATILSILSAFDDSLTGLLKFVKTPSILEREVAKISTP 539
CYSDV FALTESIKKLDVNLTQLSNIDELATLVAIMSYYKPELKYLLTYVKTPTIFENEIKKFGSG 538
SPCSV FALTEKVKKLDLNLTQLSSVDELATVVSILSHFDSSFSSLLKLVNTPSIFETSAAKFGDL 539
ToCV  FARSEKIVISDLTLTQLSNVDELATIVSILSYFDTTFNYLISMFNTPSIFEREVGKISDA 539
LIYV  FTKSDNIKPITFKLTQLSNTDDLATLTSLLGYHDKNFERFYGLFNVPTILIKEIDKLGGF 540
      *           *****  *  **                        * *      *
SPaV  KKLYSALCDVNKNFNN 556
CuYV  KELLERLVKQNKNFS- 554
CYSDV EDLYKSLAALNKNFK- 553
SPCSV RSLYERLIFVNKNFS- 554
ToCV  KGLYNRLVEQNRNFS- 554
LIYV  KTLYRRLKSMNANF-- 554
        *   *   * **
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B

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SPaV  MAETTG------DAPVINAETAPPRDQEVRNRSNEEFDEGFFSRAFNSVSKRDDVANDSH 54
CuYV  MGDNDDGKKSDDNVQLQNDVPAPVENKILDQKKLDEFSK--IDRMISSLGRRDDIVNQDI 58
CYSDV MASSSENKTSKDDTKIISEHVEDESDNETKGVTKKDIDG----DNKSTYNPRD-LITADH 55
      *                                                  **
SPaV  SDPNTFSDIKVTADRGDTLNEEQNKKYEVKLKEYCQTITKVDVDEKTFLAFYCSLIKMAK 114
CuYV  LEADVLKSIDVTADRGDVLNTQDSETFVRLCKSFCKSVVKAEVNEKQFTGFYLSFIQAAL 118
CYSDV MDPTKLKDIKVFSNRADVMSDQDEATFAKCMKDFATIVFGKEPDEKEFLTFYISLVQCWL 115
            * *   * *              *             ** *  ** *
SPaV  NQSTSIRNNNNPHLTNSFSVADKTFSYKTKDFLTFMAPHFTGVNNPLRRYMRKNEGRIKT 174
CuYV  NQSTSTKNLRNENLINTFKVDDQTYSWKTAHFIRFIKGHFPTIDNPIRQYLRGNENQVAI 178
CYSDV NQSTSMKNAKQMNLTNTLMHGDQKKTWRTADFINYVKGNLPHVPNPFRQYARAHEHEIEI 175
      *****  *     * *     *       *  *            ** * * *  *
SPaV  ISAAAGIDSDGHLAAKHGTTSQFWGATSDFTNGCETNISDDDLAANYMQREAATKNKARS 234
CuYV  LRATGKLKSDGHLAAKHGTTTQFWDSTSDFTNGCKVNISDDDLTANWLQRETATKGKNKK 238
CYSDV LKATGKVTVDHHLQAKHGVLPQFWNVPADYVNGSLMNISEDDLAANLLMKCQALKRNEKE 235
        *      * ** ****   ***    *  **   *** *** **      * *
SPaV  RTIFNVSQLAGNVQ-- 248
CuYV  NTIYNVSQLASYGN-- 252
CYSDV KKYYNVSQLAPGGCGN 251
        ******
```

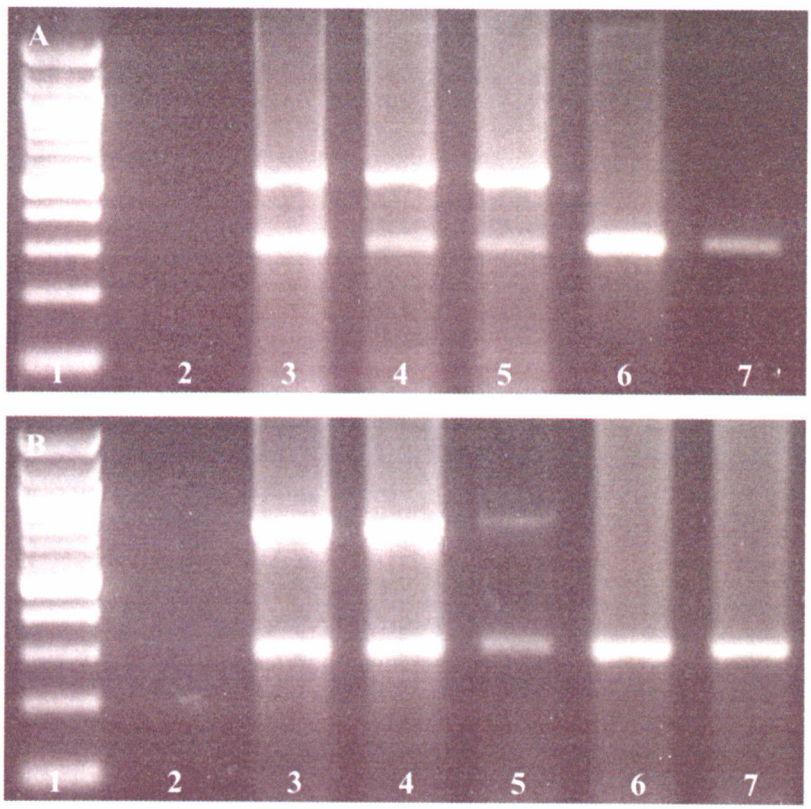

Fig. 2.3. Reverse transcription-polymerase chain reaction detection of *Strawberry pallidosis associated virus*. Ethidium bromide stained agarose gel with RT/PCR products of heat shock protein 70 homolog (3A) and coat protein (3B). Lane 1: 100bp DNA ladder (BRL, Gaithersburg, MD), lanes 3-5: SPaV isolates M1, M2 and CFRA 9037 respectively, lane 6: Isolate M29 – *Beet pseudo yellows virus* infected plant, lanes 7: healthy control, lane 2 is blank. The top band in both cases is an amplicon of the virus genome while the bottom band is an amplicon of the strawberry pectate lyase B gene used as an internal control.

2.4.5 CP expression and immunoassays

The recombinant major CP protein was expressed in *E. coli* and purified on a Cobalt column using the histidine tag (Fig 2.4A). Using the antibodies to SPaV the virus was detected in petiole tissue blots (Fig. 2.4B) from greenhouse material during the late autumn, winter and spring months but not during the summer and early autumn. The ELISA test was not sensitive enough to detect the virus at any time of the year, probably because of the low titer of the virus. Absorbance values in ELISA ranged from 0.1 to 0.2

depending on concentration of coating and conjugated antibodies with no separation between infected and healthy tissue. The tissue blot immunoassay is more sensitive than the ELISA procedure for SPaV detection in plants, and we were able to visualize the localization of the virus in the vascular tissue of infected plants (Fig. 2.4B).

Judging from the dsRNA band intensity after gel electrophoresis (Fig 2.1), the intensity of the bands of PCR products from the RT- PCR tests and the tissue blot results (data not shown) we believe that the virus reaches its maximum titer in late winter under greenhouse conditions while it reaches its lowest titer during the late summer months and the beginning of fall. Lane 5 in figure 2.1 represents the best dsRNA extraction obtained in late summer, generally no dsRNA was visible in gels at that time of the year. This fact makes greenhouse detection of SPaV extremely difficult during the summer and early fall using the tissue blot technique.

2.5 Discussion

By definition strawberry pallidosis is a disease caused by a graft transmissible agent(s) that induces visible symptoms in *F. virginiana* ('UC-10' or 'UC-11') but not in *F. vesca* indicators (Fulton, 1987). It is common to get false negatives in graft assays since symptom development requires exact environmental conditions (R.R. Martin, *personal observation*). Strawberry pallidosis disease is common in strawberry plantings of all ages in the mid-Atlantic states (Hokanson *et al.*, 2000), while the disease appears to spread slowly in eastern Canada but more rapidly in the southern USA (Fulton, 1987).

Inclusion bodies similar to those produced by BYV, the type member of the *Closteroviridae*, have been reported in strawberries infected with pallidosis disease (Henriques and Schlegel, 1975).

In the present study, we have identified SPaV as the putative causal agent of strawberry pallidosis. The virus belongs to the *Closteroviridae* family since it encodes the trademark gene of closteroviruses, the HSP70h gene. The alignment of the HSP70h and CP genes of SPaV shows multiple conserved residues with other viruses belonging to the *Crinivirus* genus (Fig. 2.2).

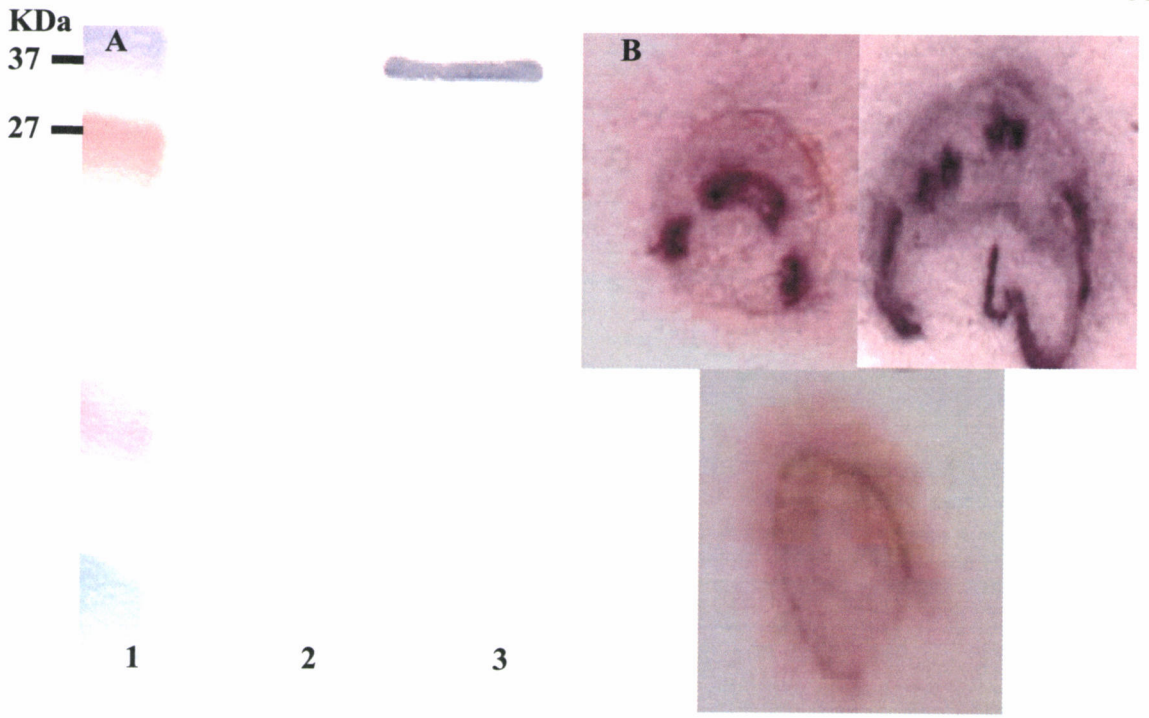

Fig. 2.4. A. Western blot of coat protein purified on a cobalt column and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed using anti-Histidine monoclonal antibodies. Lane 1: Kaleidoscope polypeptide standards (Biorad, Hercules, CA), lane 2: Protein purified from uninduced bacterial culture, lane 3: Protein purified from induced bacterial culture. B.Tissue blot immunoassay of petioles from pallidosis infected (top) and healthy strawberry (bottom) showing intense precipitation in the vascular tissue of the infected plants (top).

The HSP70h shares over 34% amino acid sequence identity with all fully sequenced crinivirus HSP70h genes found in the database (Fig. 2.2A), while the CP has 43% amino acid sequence identity with CuYV (BPYV) and 31% with CYSDV (Fig. 2.2B). The expectation (E) value is less than e^{-160} for the HSP70h gene and less than e^{-31} for the CP genes of CuYV and CYSDV. The two field isolates used for sequencing analysis originated from California (C1) and Maryland (M1) indicating some homogeneity in the virus populations since there are minimal differences between them, while the NCGR isolate (CFRA 9064) shows some diversity in the HSP70h gene. Unfortunately, we cannot draw more conclusions about the variability in the NCGR isolate because it has been maintained in a strawberry clone that has been propagated for

more than 30 years increasing the potential for accumulation of hypervariable genes (Little *et al.*, 2001).

A method for the detection of SPaV by RT-PCR was developed that allowed successful detection of the virus in 37 of the 38 isolates of strawberry pallidosis used in this study, while in the last isolate BPYV was present (Tzanetakis *et al.*, 2003). Strawberry has been an extremely difficult host for RNA extractions, and the procedure described here is the only one that has been reliable although the yield of the ssRNA may not be optimal. Using RT-PCR with the detection primers listed, a laboratory test is feasible for a virus that was previously only detected by grafting onto two different indicator plants. The detection by RT-PCR will benefit growers since it is believed that the virus is primarily introduced to the field from strawberry nurseries. In a survey in Maryland the number of strawberry plants with pallidosis was independent of how long the plants had been in the field, with infection rates approximately 70% (Hokanson *et al.*, 2000). The RT-PCR protocol was used to test 50 strawberry samples from southern California, and more than 75% of these samples tested positive for SPaV (I.E.Tzanetakis and W.M. Wintermantel, *unpublished data*). In California, infection may occur in the field since whiteflies are common in the strawberry plants there and whiteflies are the only known vector of *Criniviruses* although contamination of the nursery stock cannot be ruled out. It is also likely that since SPaV is most probably whitefly-vectored, field spread will be more of a problem in warmer regions where whiteflies are established in the field, than the cooler regions where whiteflies are less common. Testing of nursery and field plants will be required to determine the amount of virus in planting stock and the rate of spread of SPaV in the field.

Attempts to purify SPaV from strawberry gave very low virus yields and hence we expressed the recombinant CP gene in *E. coli*. The expressed protein was used to develop antibodies that allowed detection of the virus with tissue blot immunoassays in the vascular tissue of the plants as expected with viruses in the *Closteroviridae* family (Karasev, 2000; Wishler *et al.*, 1998). This detection procedure is season-dependent because of the low titer of the virus in strawberry during summer months. Based on the

tissue blots and the samples used for dsRNA analysis in this study, it appears that detection of SPaV may be season dependent, an effect not previously reported.

The detection of SPaV by tissue blots but not by ELISA even during optimal times for testing suggests that the antibodies produced to the expressed protein may not recognize intact virus or that ELISA is not as sensitive as tissue blotting for detection of this virus. Since the virus is phloem limited, local concentration on tissue blots at the vascular tissue may provide a concentration of virus high enough for detection that is not available with homogenized tissue used in ELISA. Alternatively, the virus particles are degraded during binding to the membrane such that some of the protein is conformationally similar to subunits and recognized by the antibodies that were made to expressed protein rather than intact virus. This seems like a less likely explanation since a direct coating of virus onto ELISA plates did not result in a differentiation between infected and healthy tissue samples.

The effects of the pallidosis disease, now associated with SPaV and BPYV, on yield and vigor of strawberry plants is not devastating though it has been shown in greenhouse studies using *F.* x *ananassa* cv. Northwest strawberry that pallidosis reduced runnering and root mass (Converse and Volk, 1990). Our future plans include the study of the effects of the disease on several other cultivars of strawberry plants and to study synergistic effects when it is present in complexes with aphid-borne strawberry viruses. We hope to obtain the complete sequence of SPaV, while studies are underway to identify a vector of SPaV among the whitefly species that transmit criniviruses. Seed and/or pollen transmission of pallidosis has been suggested, and will be examined in the future.

2.6 Acknowledgements

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Chapter 3

Nucleotide sequence, genome organization, phylogenetic analysis and epidemiology of *Strawberry pallidosis associated virus*

Ioannis E. Tzanetakis and Robert R. Martin

3.1 Abstract

The complete nucleotide sequence of *Strawberry pallidosis associated virus* (SPaV), a newly identified member of the genus *Crinivirus*, family *Closteroviridae* has been determined. RNA 1 is 8067 nucleotide long and encodes three open reading frames (ORFs). The first ORF (ORF 1a) is a multifunctional protein that has leader papain-like protease, methyltransferase, helicase domains. The sequence preceding the RNA-dependent-RNA polymerase (RdRp) ORF indicate that it probably is expressed by a +1 ribosomal frameshift and along with ORF 1a form the viral replicase. The 3'end ORF of RNA 1 encodes a small protein predicted to have two transmembrane domains. RNA 2 is 7979 nucleotides long and encodes 8 ORFs, including the heat shock 70 homolog (HSP70h), the trademark of the viruses that belong to the family *Closteroviridae*. All ORFs of RNA 2 are similar in amino acid sequence and arrangement with those of the other criniviruses that have been sequenced fully to date. SPaV encodes the largest structural protein of closteroviruses sequenced to date, as the minor coat protein of the virus is approximately 80 KDa. Unlike all criniviruses sequenced to date the first few nucleotides of both RNAs are not identical, while the 3' untranslated regions (UTRs) share nucleotide sequence identities of about 56% and the predicted folding of both RNA 3'UTR is similar although not identical . Phylogenetic analysis reveals that SPaV is related most closely to *Abutilon yellows virus* and *Beet pseudo-yellows virus*, another virus that has been identified recently and causes identical symptoms on strawberry indicator plants as SPaV. Epidemiological studies have revealed the greenhouse whitefly, *Trialeuroides vaporariorum* as a vector of the virus. The natural host range as well as pollen and seed transmission of the virus were also examined.

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession numbers AY488137 and AY488138 for RNA 1 and 2, respectively.

3.2 Introduction

Strawberry pallidosis is a disease that was first identified in 1957 (Frazier and Stubbs, 1967). Pallidosis disease (PD) by definition is caused by graft transmittable agent(s) that cause symptoms that include marginal chlorosis and epinasty on *Fragaria virginiana* indicator plants 'UC -10' and 'UC-11' while *F. vesca* plants remain asymptomatic. PD is asymptomatic in most commercial cultivars grown today but it is believed to act synergistically and exacerbate symptoms in plants infected with other viruses and especially *Strawberry mild yellow-edge virus* and *Strawberry mottle virus* (Fulton, 1987). PD recently has been associated with the presence of *Beet pseudo-yellows virus* (BPYV) and *Strawberry pallidosis associated virus* (SPaV) (Tzanetakis *et al.*, 2003b; Tzanetakis *et al.*, 2004), both members of the genus *Crinivirus*, family *Closteroviridae.*

Closteroviridae members have the largest genomes of all plant positive-strand RNA viruses and can reach up to 20 Kilobases in length (Martelli *et al.* 2002). The family is divided in three genera, *Closterovirus, Ampelovirus* and *Crinivirus.* The first two species are monocistronic while *Crinivirus* genome is segregated in two genomic RNAs. *Closterovirus* members are transmitted by aphids, *Ampelovirus* by mealybugs and the *Crinivirus* by whiteflies.

The genome of closteroviruses and ampeloviruses encode a replication-related polyprotein at the 5' proximal end of the genome or RNA 1 for criniviruses. The polyprotein has one or two leader papain-like proteases that are acting in cis and have been associated with accumulation of genomic RNA and long distance movement (Peng and Dolja, 2000; Peng *et al.*, 2003). Following the protease(s), methyltransferase and helicase motifs similar to those found in the Sindbis-like positive-strand RNA viruses are found in the polypeptide. A region without any obvious enzymatic activity links the methyltransferase domain with the helicase domain. The RNA-dependent-RNA polymerase (RdRp) is expressed by a +1 ribosomal frameshift (Agranovsky *et al.* 1994, Jelkmann *et al.* 1997) that is unique to the Closteroviridae among all positive-strand RNA plant viruses. The 3' proximal half of the closterovirus and ampelovirus genomes and

RNA 2 for criniviruses encode genes that are expressed via subgenomic RNAs. There is a five open reading frame (ORF) block that is conserved in all members of the family. The first of these ORFs is a small peptide with a strong transmembrane helix-motif followed by a heat shock protein 70 homolog (HSP70h) gene. Heat shock proteins are molecular chaparones with an ATPase domain located at the N' terminus of the protein and a substrate identification domain at the C' terminus and accommodate correct folding of cellular proteins (Bakau and Horwich, 1998). The closterovirus HSP70hs are found in plasmodesmata, associated with virions and are involved in virus movement (Tian *et al.*, 1999; Peremyslov *et al.*, 1999; Medina *et al.*, 1999; Napuli *et al.*, 2000; Satyanarayana *et. al.*, 2000). The next protein is also involved in virus movement and found as a structural protein in virions and in the case of *Beet yellows virus,* the type member for the genus *Closterovirus,* it has been shown to have homologies with coat proteins at its' C' terminus (Napuli *et al.*, 2003). The last two proteins that are involved in virus movement are the major and minor (CP and CPm, respectively) coat proteins of the viruses. CP protects 95% of the genome of the virus while CPm covers the remaining 5% at the 5' end of the genome (Zinovkin *et al.*, 1999; Satyanarayana *et al.*, 2004). Unlike other plant virus families which have set number and arrangement of proteins encoded in the genome, family *Closteroviridae* shows great diversity in the genome size and genes encoded by its members ranging from that of *Beet pseudo-yellows virus* (Hartono *et al.*, 2003; Tzanetakis and Martin, 2004) to that of *Citrus tristeza virus* (Karasev *et al.*, 1995). This diversity within the family makes it essential to study the genomes of closteroviruses in order to better understand the evolution of the family, including the genome segmentation and the different transmission modes of the three genera.

3.2 Materials and methods

3.2.1 RNA purification, cDNA synthesis and cloning

A field isolate (M1) from Maryland, now deposited in the National Clonal Germplasm Repository (Corvallis, Oregon) as CFRA 9089 was used for determination of

the complete genome of SPaV. Double-stranded (ds) and single-stranded (ss) RNA were extracted as described previously (Yoshikawa and Converse, 1990; Hughes and Galau, 1988).

For all reactions described hereafter enzymes, plasmids and kits from Invitrogen Corp. (Carlsbad, CA) were utilized according to the manufacturer's prorocols unless otherwise stated. For the genome cloning except the 5' and 3' termini, dsRNA was utilized as template for cDNA synthesis and reverse transcription-polymerase chain reaction (RT-PCR). Briefly, dried dsRNA extracted from the equivalent of 10 g of tissue was incubated with 40mM methyl mercuric hydroxide in the presence of 0.5-1 μg of random hexameric nucleotide primers for 30 min at room temperature. Reverse transcription was performed utilizing the Thermoscript® reverse transcriptase according to manufacturer's instructions except for the utilization of twice the volume of dithiothreitol in order to reduce the methyl mercuric hydroxide. Second-strand synthesis was performed as described previously (Jelkmann *et al.*, 1989) and cDNA was cloned into a pCR 2.1 vector. Recombinant plasmids were assayed for the present of inserts either by direct amplification of the fragments utilizing the M13 forward and reverse primers or by plasmid purification and digestion with restriction endonucleases.

3.2.2 Amplification of SPaV genome

All genome sequence of SPaV was acquired utilizing reverse transcription-polymerase chain reaction (RT-PCR). The original cloning inserts were utilized for development of oligonucleotide primers for PCR amplification. All primers were developed after the alignment of at least two clones. For amplification of all fragments *Platinum*® Taq polymerase was utilized. The PCR program consisted of a denaturation step at 94^0C for 5 min, followed by 40 cycles of 45 sec at 94^0C, 30 sec at 55^0C and 2-4 min (depending on the putative fragment length) at 68^0C. A final extension step of 10 min at 68^0C terminated the program. The 3' termini of both RNAs were determined by adding an oligo-adenosine tail (Sippel, 1973) onto purified dsRNA and performing reverse transcription as described above utilizing a forward primer near the 3' end of the

known sequence together with an oligo – thymidine primer for PCR amplification. Each of the 3' ends were amplified at least twice. For amplification of the 5' termini, the adenylated dsRNA was utilized and PCR amplification was achieved utilizing an oligo-thymidine primer as the forward primer and two individual oligonucleotide primers derived from near the 5' sequence of the non-coding strands as reverse primers. Commercially available 5' rapid amplification of cDNA ends (RACE) also was used according to the manufacturer's instructions using single-stranded RNA extracted according to the Hughes and Galau method (1988). Each of the 5' amplifications were performed twice except for that of RNA 2 utilizing adenylated dsRNA as template that was amplified once.

3.2.3 Sequencing, genome and phylogenetic analysis

All PCR fragments were amplified twice and each individual reaction was cloned into a pCR 2.1 vector. The PCR products and two or more clones obtained from each reaction were sequenced in both orientations. The consensus sequence was acquired utilizing the RT-PCR derived sequences and the original clones of the fragment where available. ClustalW (European Bioinformatics Institute http://www.ebi.ac.uk/clustalw) was used for the alignment of sequences and assembly of the consensus sequence. Sequencing reactions were carried out at the facilities of the Central Services Laboratory at Oregon State University (Corvallis, OR) and Macrogen Inc. (Seoul, South Korea) using an ABI 377 and 3700 DNA sequencers, respectively.

The predictions of the secondary structure of the untranslated regions of the virus were obtained using the mfold software (Zuker, 2003) at default settings. Phylogenetic analysis was performed utilizing maximum parsimony on the PAUP* 4.0b 10 software (Swofford 2001). Heuristic search applying ten replicates of random taxon sequence addition and the TBR (Tree Bisection Reconnection) swapping algorithm were the settings for the reconstruction of the phylograms. Bootstrap analysis consisted of 1000 replications utilizing the same parameters as above. Amino acid sequences of four genes of SPaV where chosen for the analysis: RdRp, HSP70h and CP and CPm. In all cases,

only genes with complete sequences in the database were utilized. Members of all three genera of the family were utilized for the reconstruction of the HSP 70h phylogram while for the other three phylograms, criniviruses genes were used. Analysis of the putative transmembrane domains of proteins and signal peptides were done using the respective tools at the CBS Prediction Servers (http://www.cbs.dtu.dk/services/). The identification of the putative ORFs were performed with both the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and gene finding in viruses at http://www.softberry.com. The conserved motifs of the proteins were identified applying the CDART (Conserved Domain Architecture Retrieval Tool) (Geer *et al.*, 2002) and CDD (Conserved Domain Database) (Marchler-Bauer *et al.*, 2003) programs.

3.2.4 Transmission studies

Three whitefly species that are known to be able to transmit *Crinivirus* species, were used to evaluate the potential of being vectors of SPaV. These species were *Trialeurodes vaporariorum* (greenhouse whitefly), *T. abutilonea* (banded-wing whitefly) and *Bemisia tabaci* biotype B or *B. argentifolii* (silver-leaf whitefly). One-hundred whiteflies were let feed on a SPaV-infected plant (Maryland isolate M 2) for 48 hours. The acquisition time followed a 48 hour feeding period on test plants. A total of three experiments were performed on the vector identification studies. The evaluation of the host range potential was performed similarly to the vector studies with the difference that 30 viruliferous whiteflies were applied onto the test plants. After feeding on the test plants, the whiteflies were killed with a contact insecticide. The presence of the virus in the test plants was confirmed by either RT-PCR (Tzanetakis *et al.*, 2004) or dot blot (Sambrook *et al.*, 2001).

3.3.5 Pollen and seed (achene) transmission

Pollen transmission studies were performed on strawberry cultivars 'Northeaster' and 'Hood'. Briefly, flowers were emasculated at the white tip stage and the receptacle

left to mature for 2-5 days before pollination. The flowers were pollinated with a mixture of pollen collected from strawberry plants infected with one of 20 SPaV isolates from Maryland and left to set fruit under greenhouse conditions. Three months after fruit maturation, plants were cold-treated for at least 30 days at 4^0 C at 12/12 h photoperiod. After the plants were removed from the vernalization treatment, they were grown for at least two months in a greenhouse prior to RT-PCR testing (Tzanetakis *et al.*, 2004).

Achenes from strawberries of the 20 isolates used for the pollen transmission studies were seeded and grown for at least three months under greenhouse conditions (16/8 h photoperiod). The seedlings were tested for the presence of the virus as described above utilizing RT-PCR.

3.3 Results

3.4.1 Genome organization

The complete nucleotide sequence of SPaV has been determined. RNA 1 encodes the replication-related proteins and consists of 8067 nucleotides while RNA 2 is 7979 nucleotides long and encodes movement and structural proteins in addition to proteins of unknown function. The 5' untranslated region (UTR) of RNA 1 is 265 nucleotides long while RNA 2 has a 5' UTR that is 241 nucleotides long. The nucleotide sequence identities between these two regions is 46% but there are no obvious similarities in the secondary structure of the RNA (data not shown). SPaV is the only member of the genus that does not have the first six to ten nucleotides of the two RNAs identical (Klaassen *et al.*, 1995; Kreuze *et al.*, 2002; Hartono *et al.*, 2003; Aguilar *et al.*, 2003). Both 5' termini have been determined by two tailing reactions, for a total of at least three PCR amplifications for each end and sequencing of more than eight cloned sequences in total. For RNA 2, the contig of the 5'teminus also included two additional clones that were obtained from the original shotgun cloning. The 3' UTR is 197 and 186 nucleotides long for RNA 1 and 2, respectively and they share 56% nucleotides sequence identity. The predicted secondary structure of the two regions are similar although not identical (Fig.

3.1), and may serve as a signal for initiation for the viral polymerase (Yi and Lemon, 2003).

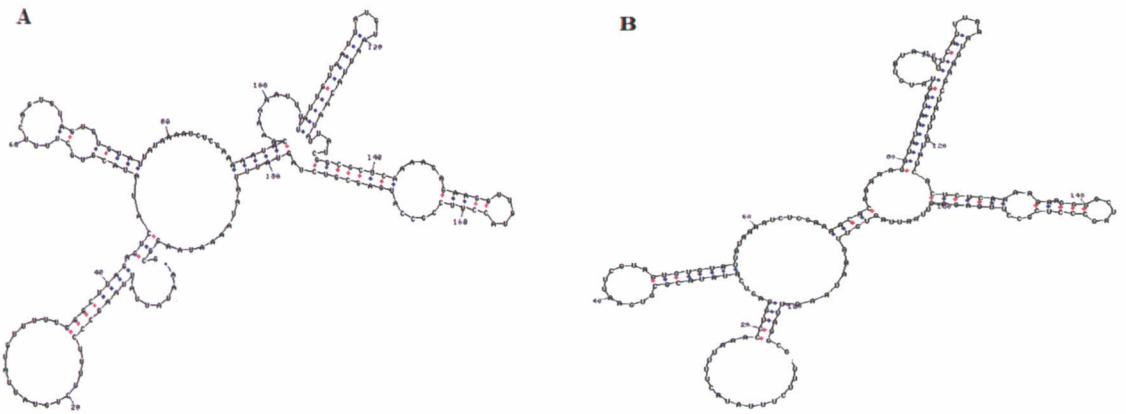

Fig. 3.1. Predicted folding of the 3' untranslated regions of RNA 1 and 2 of SPaV at 37^0C. A. RNA 1, B. RNA 2.

The gene arrangement of SPaV is presented in Figure 3.2. ORF 1a begins at nucleotide 266 and terminates at nucleotide 6106. The leader papain-like protease is found at the N' terminus of the polyprotein and the putative cleavage site is found between amino acids 404 (glycine) and 405 (isoleucine) and has the greatest similarity with the homologous domain of BPYV isolates exceeding 50%. The putative molecular weight of the peptide is estimated to be 47 KDa. The methyltransferase domain, similar to that encoded by all Alphavirus superfamily (Rozanov *et al.*, 1992; Marchler-Bauer *et al.*, 2003) is the most conserved domain of the protein among the genus. A region of more than 800 amino acids separate the methyltransferase from the helicase domain of the protein. This region is the least conserved among the genus, except for two conserved transmembrane helices that are found in all sequenced members of the family (data not shown). The two helices are found between amino acids 1195-1217 and 1311-1333 and their role is unknown. The helicase domain is located in the C' terminus of the peptide and has the characteristic glucine-rich A site that binds the phosphate group and the B site that binds Mg^{2+} (Gorbalenya and Koonin, 1989; Marchler-Bauer *et al.*, 2003). The termination signal of the virus is UUUGA (underlined sequence shows the amber stop codon). All criniviruses sequenced to date have the two uridines before the stop codon

that may indicate that they are involved in ribosomal slippage that leads to the +1 ribosomal frameshift. The +1 frameshift is unique to the closteroviruses and thought to be involved in expression of the polymerase gene (Agranovsky *et al.*, 1994, Karasev *et al.*, 1995) although no particular structures favoring the ribosomal slippage were identified utilizing the RNA folding software (data not shown). The putative RNA-dependent-RNA polymerase (RdRp) ORF (1b) is 505 amino acids and has molecular weight of 58 KDa. The protein shows high similarity with the sequenced RdRp genes of the other criniviruses reaching 68% amino acid sequence identity and more than 80% similarity with that of the BPYV isolates. The active site - motif VI (Koonin, 1991) of the enzyme is almost identical for all criniviruses with a 100% conserved motif of LVSGDDSLIFS (underlined GDD shows the triad Gly-Asp-Asp of the active site). The fusion 1a/1b ORFs terminate at nucleotide 7622 and its' size is 282 KDa.

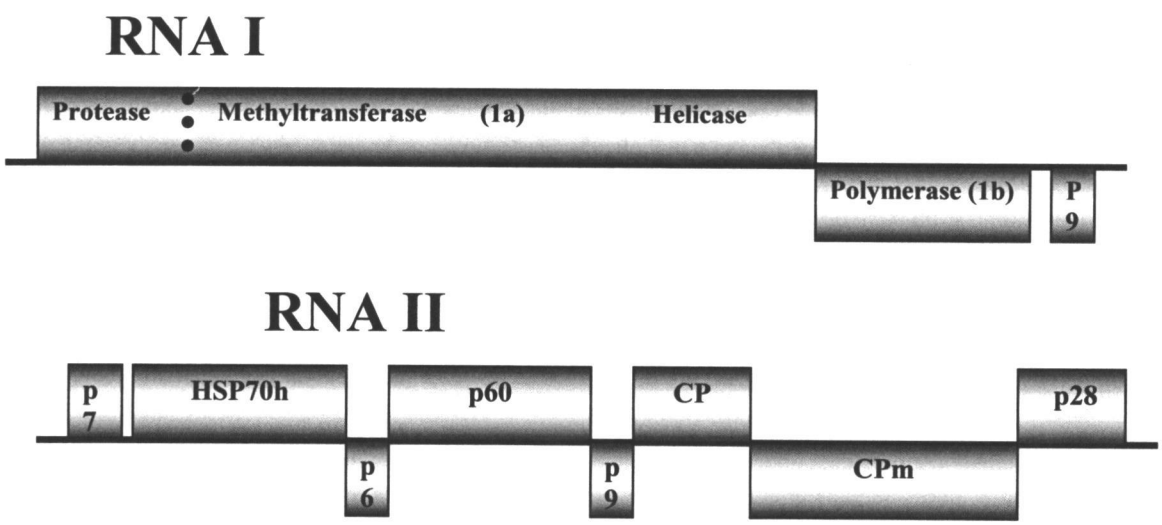

Fig 3.2. Schematic representation of the genome organization of SPaV. The sizes of the ORFs are not to scale.

Following the polymerase a small ORF that encode a putative 79 amino acid, 9 KDa protein is found. It starts at nucleotide 7631 and terminates at nucleotide 7870. This putative protein has two transmembrane domains that span from amino acids 4-26 and 52-74. When the peptide is examined for putative signal peptides, it was found that there is a potential cleavage between amino acids 21 and 22 (the program identifies signal

peptides for bacteria and eukaryotes). This is the first peptide with two transmembrane domains identified to date in all the closteroviridae and the first potential signal peptide encoded by a plant virus.

RNA 2 encodes eight ORFs. The first of these is a small hydrophobic protein similar in position and size with those encoded by all members of the closteroviridae. The putative peptide starts nucleotide 242 and terminates at nucleotide 430 and has molecular weight of 7 KDa. The transmembrane helix spans from amino acid 10-29. The second ORF is the HSP70h gene of the virus. The protein has 556 residues and a molecular weight of 62 KDa. All five motifs identified in the ATPase domain (Bork *et al.*, 1992) of HSP70 proteins are present in the HSP70h of SPaV. The alignment of the conserved regions of the HSP70h genes of SPaV and other criniviruses is presented in figure 3.3.

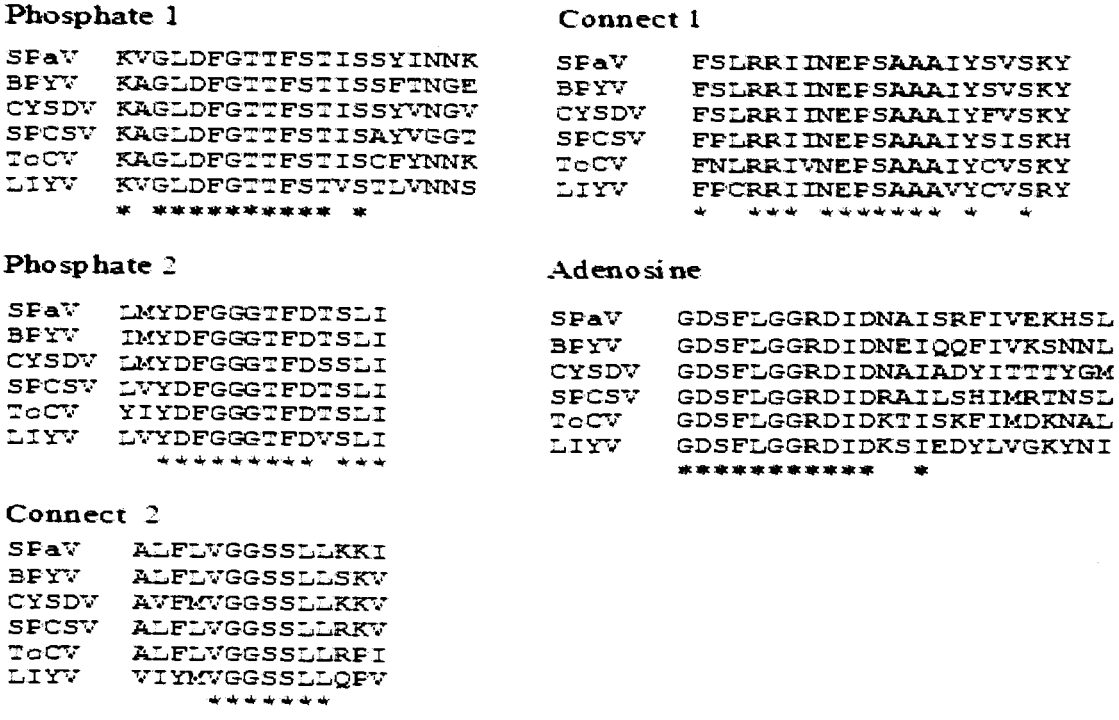

Figure. 3.3. Alignment of the five conserved motifs of HSP70h of criniviruses. Abreviations and Genbank accession numbers are as follows: *Strawberry pallidosis associated virus* (SPaV) AY 262163, *Beet pseudo-yellows virus* (BPYV) NC 005210, *Cucurbit yellow stunting disorder virus* (CYSDV) NC 004810, *Sweet potato chlorotic stunt virus* (SPCSV) NC 004124, *Tomato chlorosis virus* (ToCV) AF 024630, *Lettuce infectious yellows virus* (LIYV) NC 003618. Asterisks indicates identical amino acid residues.

The next ORF encodes for a small peptide of 6 KDa of unknown function. It begins after the termination signal of HSP70h at nucleotide 2347 and terminates at nucleotide 2508. The 53 amino acid peptide shows homology with homologous proteins in BPYV and *Cucurbit yellow stunting disorder virus* (CYSDV) and the C' terminus of *Little cherry virus-1* (LChV-1) HSP70h. The ORF has amino acid sequence identities that reach 40% with the homologous gene of BPYV, while the amino acid similarities exceed 50% among all three of these viruses for this ORF. An ORF of 518 amino acids and molecular weight of 60 KDa follows p6. The putative start codon is at nucleotide 2502, before the termination signal of p6 and it terminates at nucleotide 4058. The protein is most closely related to the homologous peptide of BPYV with 47% and 67% amino acid sequence identities and similarities, respectively. The conserved residues identified by Napuli *et al.* (2003) that are probably involved in stabilization of the structure of the protein by forming salt bridges (Dolja *et al.*, 1991) are also found in this protein at position 434 (arginine) and position 471 (aspartate). A small ORF of 9 KDa is after p60. The peptide is found only in criniviruses among the members of the family. The putative 85 amino acid peptide shows no significant homology with other proteins in the database other than the homologous proteins of the other criniviruses

The major CP of the virus spans from nucleotides 4287 to 5033 and encodes a protein of 248 amino acids and molecular weight of 28 KDa. The three conserved residues of all filamentous virus CP genes are found at positions 119 (serine), 166 (arginine) and 203 (aspartate) (Dolja *et al.*, 1991). The protein also bears conserved motifs at the C' terminus found in the coat proteins of members of the family *Closteroviridae* (Marchler-Bauer *et al.*, 2003). The minor coat protein start codon precedes the stop codon of the CP gene and the ORF starts at nucleotide 5002 and terminates at nucleotide 7071, coding for the largest structural protein of all closteroviruses sequenced to date, since it encodes a protein with 689 amino acids and has molecular weight of nearly 80 KDa. The conserved serine, arginine and aspartate are found at positions 536, 607 and 648, respectively in the minor CP.

The final ORF of SPaV RNA 2 could code for a 28 KDa protein that starts has its start codon at nucleotide 7074 and terminates at nucleotide 7793 encoding for 239 amino

acids. This protein is one of the most diverse of the crinivirus encoded proteins, and the SPaV protein shows the greatest similarity with that of the BPYV with 27% amino acid sequence identity and more than 57% similarity.

3.4.2 Phylogenetic analysis

The phylogenetic analysis of the HSP70h gene clearly places SPaV in the genus *Crinivirus* (Fig. 3.4). Within the genus SPaV is related most closely to BPYV. The relationship between the two viruses is also evident in the phylograms utilizing the RdRp and the CPm. The CP phylogram reveals a closer relationship of SPaV with *Abutilon yellows virus* (CP is the only completely sequenced gene of the virus) than BPYV, although these three viruses form a distinct cluster apart from all other criniviruses.

4.3.4 Epidemiological studies

Trialeuroides vaporariorum, the greenhouse whitefly has been identified as an efficient vector of SPaV while no transmission was detected when using *T. abutilonea* or *Bemisia tabaci* biotype B (Table 3.1).

Table 3.1. Experiments with the three whitefly species for identification of vectors of *Strawberry pallidosis associated virus*.

Host plant inoculated		**GWF**[1]	**BWWF**[2]	**SLWF**[3]
P. wrightii	(Experiment 1)	3/3*	0/3	0/3
N. benthamiana		3/3	0/3	0/3
P. wrightii	(Experiment 2)	2/3	0/2	0/6
N. benthamiana		3/5	0/4	0/12
N. benthamiana	(Experiment 3)	6/6	0/6	0/3
Total		17/20	0/18	0/27

[1] GW: Greenhouse whitefly (*Trialeurodes vaporariorum*)
[2] BWWF: banded-wing whitefly (*T. abutilonea)*
[3] SLWF: silver leaf whitefly (*Bemisia tabaci* biotype B or *B. argentifolii*)
* number of plants infected/ number of plants tested

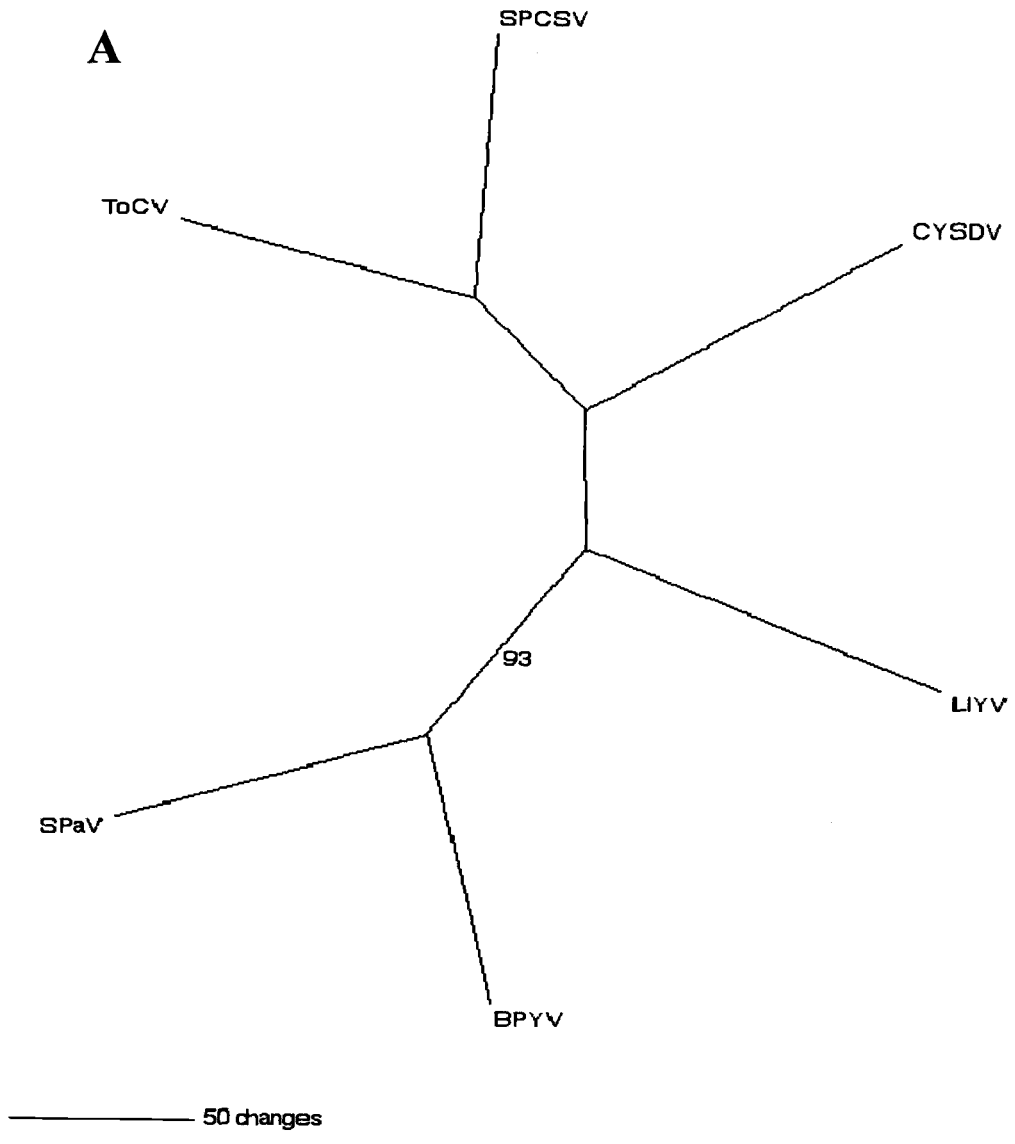

Figure. 3.4. A. Phylogram of the polymerase of SPaV and other criniviruses. Abreviations and GenBank accession numbers: SPaV, *Strawberry pallidosis associated virus*, AY488137; BPYV, *Beet pseudo-yellows virus*, NP940796; CYSDV, *Cucurbit yellow stunting disorder virus*, AAM73639; ToCV, *Tomato chlorosis virus*, (W.M. Wintermantel, personal communication); SPCSV, *Sweet potato chlorotic stunt virus*, NP733939; LIYV, *Lettuce infectious yellows virus*, AAA61798. Bootstrap values are shown as percentage value and only the nodes over 50% are labeled. The bar represents 50 amino acid changes over the length of the proteins.

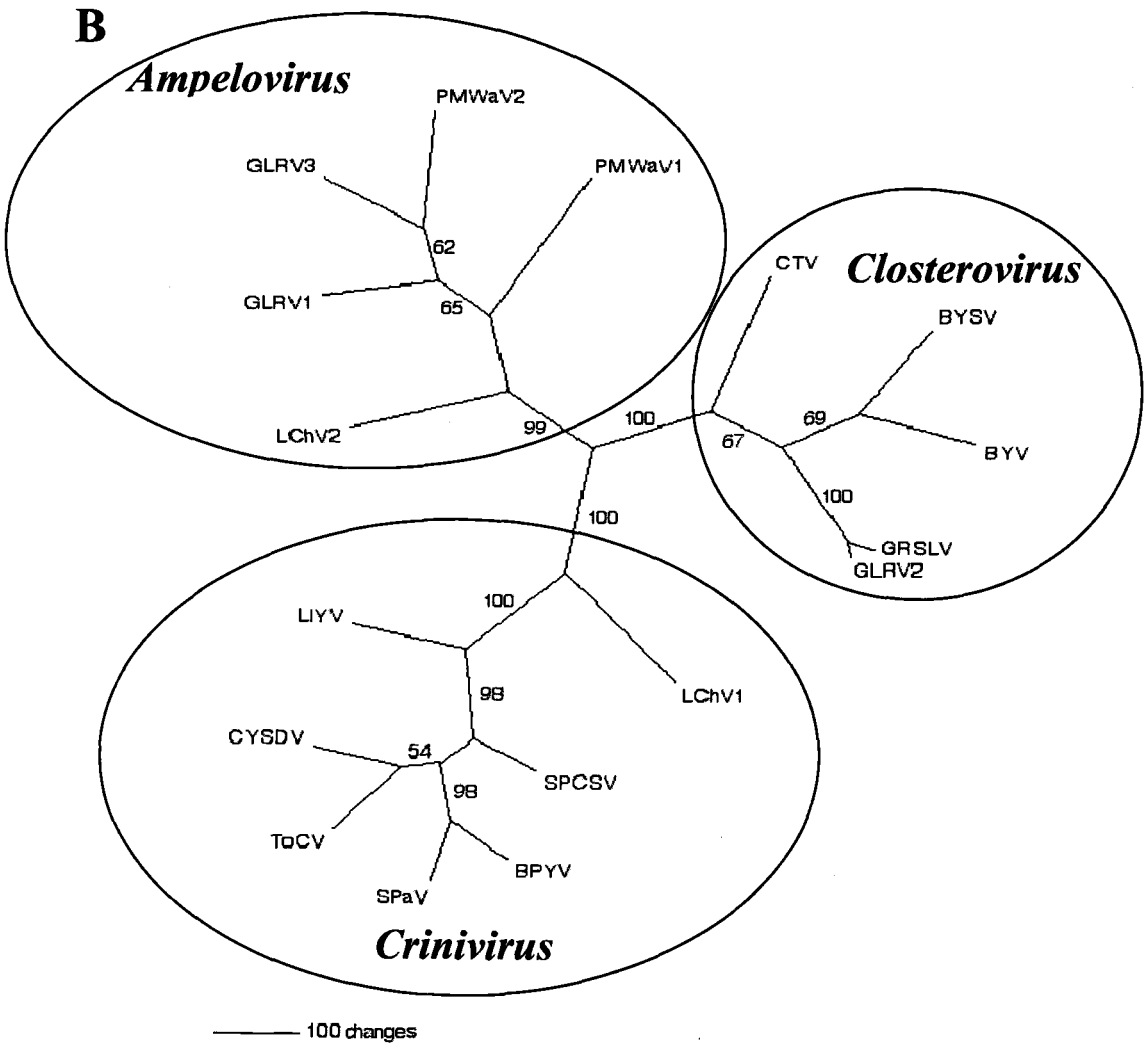

Fig. 3.4. B. Phylogram of heat shock protein 70 homolog of SPaV and other closteroviruses. Abreviations and GenBank accession numbers: SPaV, *Strawberry pallidosis associated virus*, AAO92347; BPYV, *Beet pseudo-yellows virus*, AAQ97386; CYSDV, *Cucurbit yellow stunting disorder virus*, NP851572; ToCV, *Tomato chlorosis virus*, AF024630; SPCSV, *Sweet potato chlorotic stunt virus*, NP689401; LIYV, *Lettuce infectious yellows virus*, NP619695; LChV-1, *Little cherry virus-1*, NP045004; CTV, *Citrus tristeza virus*, NP042864; BYSV, *Beet yellow stunt virus*, AAC55662; BYV, *Beet yellows vius*, NP041872; GRSLV, *Grapevine rootstock stem lesion associated virus* NP835247, GLRV2, *Grapevine leafroll associated virus-2*, AAR21242; LChV2, *Little cherry virus-2*, AF531505; PMWaV1, *Pineapple mealybug wilt-associated virus-1*, AAL66711; GLRV1, *Grapevine leafroll associated virus-1*, AAK38612; GLRV3, *Grapevine leafroll associated virus-3*, NP813799; PMWaV2, *Pineapple mealybug wilt-associated virus-2*, AAG13941. Bootstrap values are shown as percentage value and only the nodes over 50% are labeled. The bar represents 100 amino acid changes over the length of the proteins.

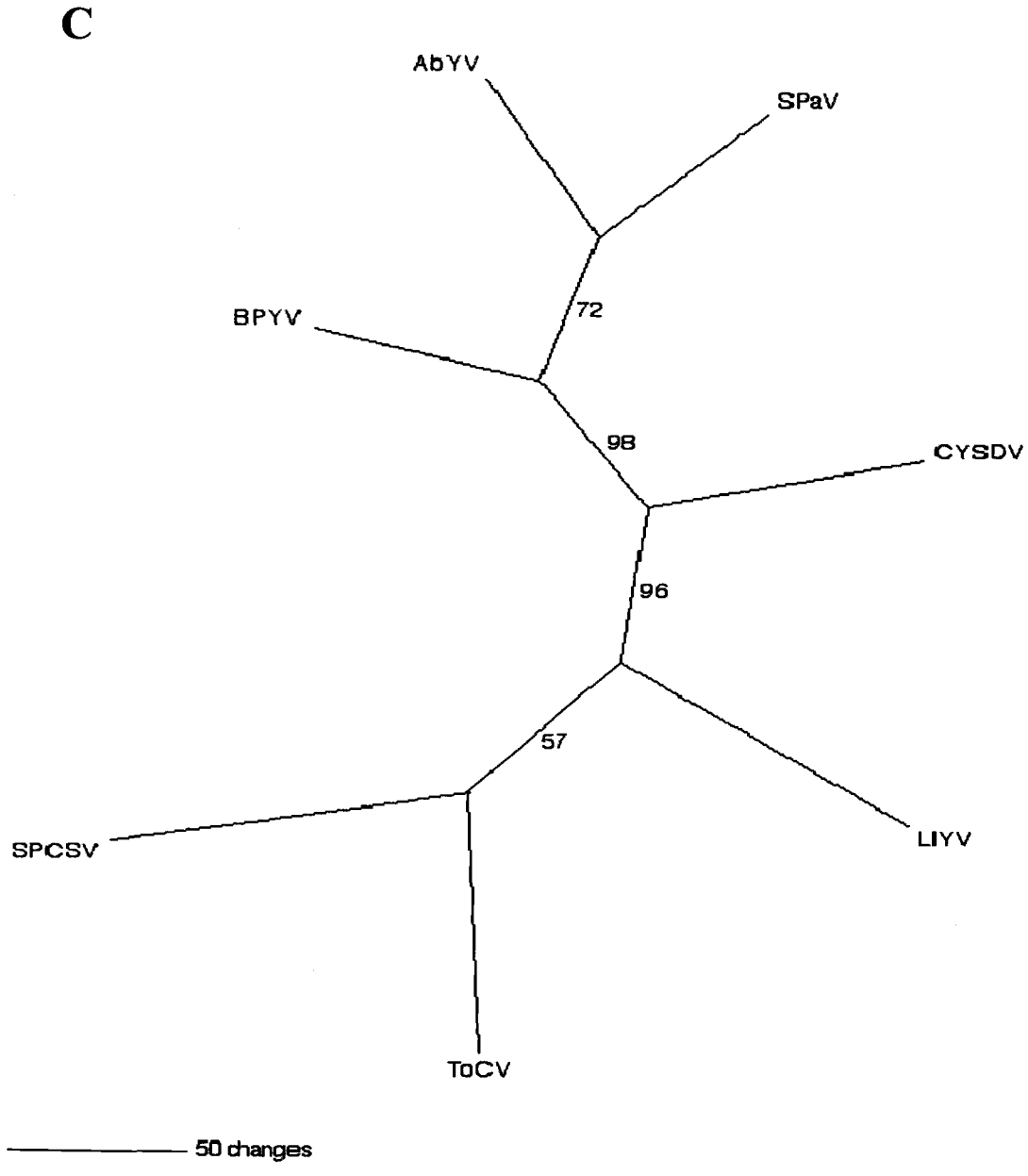

Fig. 3.4. C. Phylogram of the coat protein of SPaV and other criniviruses. Abreviations and GenBank accession numbers: SPaV, *Strawberry pallidosis associated virus*, AAO92342; AbYV, *Abutilon yellows* virus, AAR00224; BPYV, *Beet pseudo-yellows virus*, NP940792; CYSDV, *Cucurbit yellow stunting disorder virus*, NP851576; ToCV, *Tomato chlorosis virus*, AAR15080; SPCSV, *Sweet potato chlorotic stunt virus*, NP689404 ; LIYV, *Lettuce infectious yellows virus*, NP619697. Bootstrap values are shown as percentage value and only the nodes over 50% are labeled. The bar represents 50 amino acid changes over the length of the proteins.

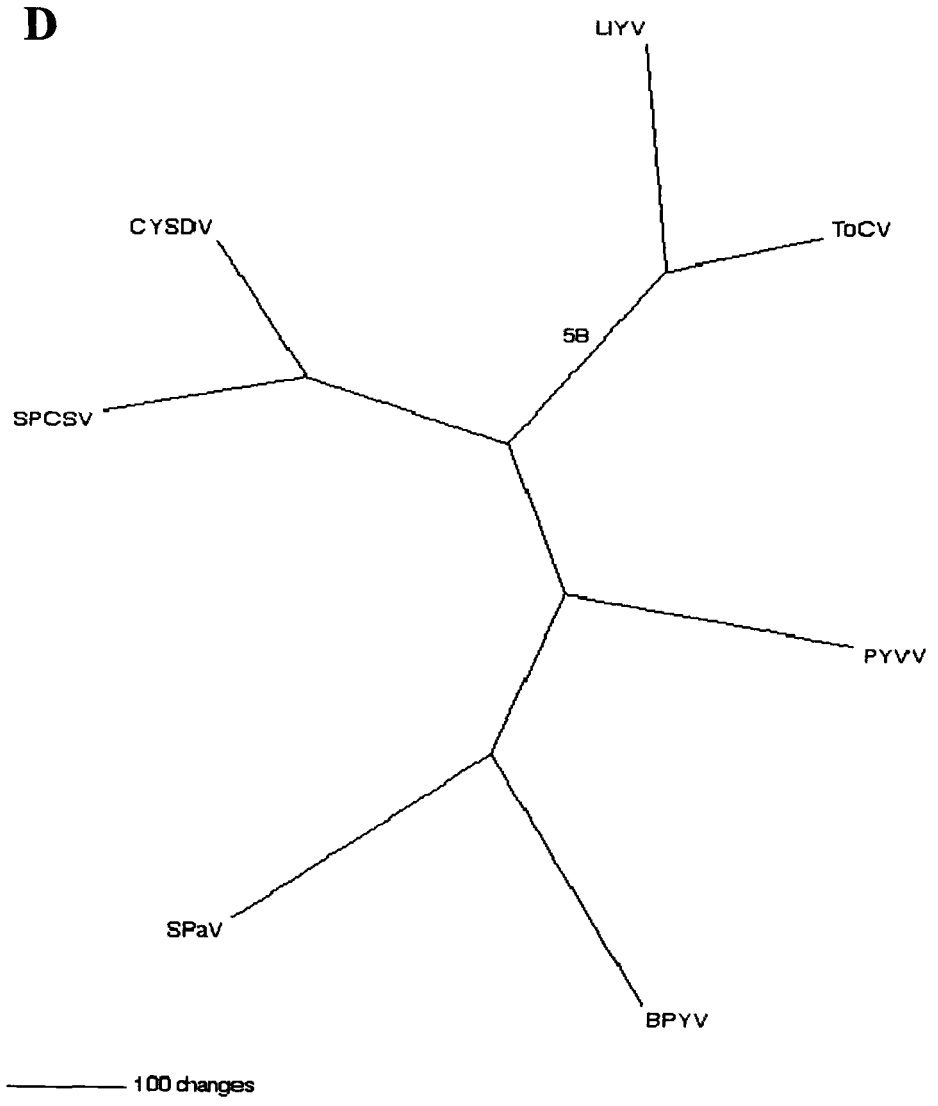

Fig. 3.4. D. Phylogram of the minor coat protein of SPaV and other criniviruses. Abreviations and GenBank accession numbers: SPaV, *Strawberry pallidosis associated virus*, AY488138; BPYV, *Beet pseudo-yellows virus*, NP940793; PYVV, *Potato yellow vein virus*, CAD48512; CYSDV, *Cucurbit yellow stunting disorder virus*, NP851577; ToCV, *Tomato chlorosis virus*, (W.M. Wintermantel, personal communication); SPCSV, *Sweet potato chlorotic stunt virus*, NP689405 ; LIYV, *Lettuce infectious yellows virus*, NP619698 . Bootstrap values are shown as percentage value and only the nodes over 50% are labeled. The bar represents 100 amino acid changes over the length of the proteins.

Four plants species were identified as experimental hosts of the virus utilizing whitefly transmissions (Table 3.2). All plants were inoculated with 30 viruliferous whiteflies except in the case of *Physalis wrightii* where 100 whiteflies were utilized as the original screen with 30 whiteflies failed to infect (0 out of 5) any of this plant infected with the virus. In a limited sampling of native plants near strawberry fields, nettle (*Urtica* spp.) has been identified as a host naturally host of SpaV.

Table 3.2. Host range studies of *Strawberry pallidosis associated virus* (SPaV) utilizing *Trialeuroides vaporariorum* for transmission.

Plant species	**SPaV**	**Mock**
Nicotiana benthamiana	6/10*	0/10
N. glutinosa	0/5	0/5
N. clevelandii	2/5	0/5
N. tabacum	0/5	0/5
Physalis wrightii	5/6	0/5
P. floridana	0/5	0/5
Malva parviflora	2/5	0/5
Citrullus sp.	0/5	0/5
Chenopodium murale	0/5	0/5
C. capitatum	0/5	0/5
C. amaranthicolor	0/5	0/5
Gomphrena globosa	0/5	0/5
Capsella Bursa-pastoris	0/5	0/5
Brassica oleracea var. *italica*	0/5	0/5
Lycopersicon esculentum	0/5	0/5

* number of plants infected/ number of plants tested

There have been unpublished studies that have indicated that the pallidosis agent may be pollen transmittable (Converse, 1992). Tests were carried out to determine if SPaV could be transmitted by pollen either horizontally to the pollinated plants or vertically through seed to seedlings. One hundred and seventy strawberry plants were pollinated with pollen from SPaV-infected plants. None of the pollinated plants tested positive for SPaV three months after the pollinated flowers had set fruit. Seed transmission was also tested as a means of transmission of the virus. Four hundred and ten seedlings acquired from achenes of SPaV-infected plants were tested for the presence of the virus. In this case, 40 seedlings were from seed where infected plants were

pollinated with pollen from healthy sources and 370 were seedlings where pollen from an infected plant was used to pollinate a healthy plant. None of the seedlings tested were positive for SPaV using the RT-PCR basesd assay described previously.

4.4 Discussion

The complete nucleotide sequence of SPaV, a newly identified member of the genus *Crinivirus,* family *Closteroviridae* has been determined. The virus encodes genes found in all members of the family and phylogenetic analysis demonstrated that the virus is a member of the criniviruses. SPaV has some unique features not found in other member of the genus. Multiple attempts have been done to identify identical nucleotides at the 5' terminus of the RNAs, a feature found in all the other members of the genus have at least the first six nucleotides identical. The termini were determined after sequencing more than eight clones per termini utilizing two different approaches. RNA 1 terminus was determined utilizing the four individual tailing reactions/amplifications while three were utilized for RNA 2. The original shotgun cloning had revealed two individual clones that were later identified as containing the 5' terminus of the molecule. Although unusual among viruses with segmented genomes there are examples where the nucleotides of the 5' end termini do no match (Ge *et al.*, 1997; Han *et al.*, 2002). The two transmembrane domains found in the middle ORF 1a is another feature that has been identified in SPaV and other members of the genus, while it was absent in the closteroviruses, *Beet yellows virus* and *Citrus tristeza virus* (CTV). However, CTV possesses two similar transmembrane domains in the first of the leader proteases. The role of the transmembrane domains in SPaV is unknown but it can be speculated that they may anchor the replication complex in cell membranes (den Boon *et al.*, 2001). The putative ribosomal frame shift by which the virus polymerase is expressed has the very similar sequence before the putative +1 frameshift that has been identified in all criniviruses sequenced to date. It should be noted that computer assisted analysis in the region surrounding the site did not reveal any distinct structures that may facilitate this function. In addition, a start codon is found 12 nucleotides downstream from the putative frameshift site, an additional indication that the virus may not express the protein

utilizing the frameshift if there were an internal ribosome entry site that could initiate translation of the RdRp. SPaV is the only *Crinivirus* that has been identified to encode a peptide with two transmembrane domains. This peptide also bears features similar to those of signal peptides with a putative cleavage site inside the domain that is found inside the membrane, making the possibility of actual cleavage less likely. RNA 2 of the criniviruses are more conserved than RNA 1. SPaV contains the seven genes found in all other criniviruses that have been fully sequenced. In addition, SPaV also contains a small ORF that follows the HSP70h and is found in BPYV and CYSDV. The similarity of the peptide with the C' terminus to the LChV-1 HSP70h suggests that the protein may have been part of a larger HSP70h gene and that has evolved to an individual gene. The C' terminus of heat shock proteins are involved in substrate recognition although in the case of *Beet yellows virus* such a function was not identified (Agranovsky *et al.*, 1997). The other genes of RNA 2 resemble homologous genes of the other criniviruses in size and location.

Whitefly transmission studies have identified an efficient vector, *T. vaporariorum*, among the whiteflies species known to transmit criniviruses. Two other species failed to transmit the virus indicating that they may not be vectors of the virus or the transmission rates are very low. It is also possible that there are other whitefly species in strawberry that could vector this virus in addition to the ones tested in this work. The hypothesis that the pallidosis agent is pollen transmissable was also tested. All seedlings from the pollen and seed transmission studies tested negative for the presence of SPaV. These results reveal that SPaV is either not pollen or seed transmitted or the efficiency of these modes of transmission is very low. The unpublished results of Dr. N. Frazier suggesting that the pallidosis disease agent is pollen transmitted likely is not referring to the SPaV described here. BPYV, the second virus that can cause pallidosis or another as yet undescribed virus that may cause this disease may be responsible for the transmission observed in his studies. Another possibility is that there were whiteflies in the greenhouse when the studies were carried out and they were dismissed as vectors since at that time whiteflies were not known as virus vectors.

Criniviruses have become an increasing problem in world agriculture due to the greater movement of plant materials and naturalization or increasing range of the the whitefly vectors. Small fruits are a new group of crops that have been identified to be infected with this group of viruses (Tzanetakis *et al.*, 2003a; b; Tzanetakis *et al.*, 2004). The greenhouse whitefly has recently become naturalized in many areas in southern U.S. and become a pest in strawberry production in California, the major strawberry producing area of the U.S. The ability to transmit both viruses that are associated with the pallidosis disease may be the reason this virus disease has become widespread in California. As the range of the vector continues to increase, this disease will become more important in strawberry production. In Maryland, it is suggested that SPaV has been transmitted through planting material as there appears to be little increase in field spread over several years (Hokanson *et al.*, 2000). Future plans include the determination of acquisition, transmission and retention times of the virus and continue the host range studies among small fruit crops and native vegetation.

3.6 Acknowledgments

We would like to thank William Wintermantel and Arturo Cortez (USDA-ARS, Salinas, CA) for performing the whitefly transmission studies. This project was funded by the North American Strawberry Growers' Association and the United States Department of Agriculture.

3.7 References

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Chapter 4

Complete nucleotide sequence and geographical distribution of *Beet pseudo-yellows virus* in strawberry

Ioannis E. Tzanetakis and Robert R. Martin

4.1 Abstract

In efforts to identify the causal agent(s) of strawberry pallidosis we found a single pallidosis positive strawberry plant that did not give any amplicons after reverse transcription-polymerase chain reaction using primer sets representing multiple regions of *Strawberry pallidosis associated virus* (SPaV) genome and failed to react with antibodies directed against the recombinant coat protein of SPaV. DsRNA extracted from this plant showed a similar pattern to that of SPaV indicating that another crinivirus may infect strawberry. Cloning of this dsRNA and sequence analysis of multiple cDNA clones corresponding to the heat shock 70 homolog gene (HSP70h) of the unknown virus indicated that it was *Beet pseudo-yellows virus* (BPYV). Analysis of the complete nucleotide sequence of BPYV-strawberry revealed that this isolate has several distinct features when compared to *Cucumber yellows virus* (CuYV), a cucumber strain of BPYV, including a 147 nucleotide insertion after the methyltransferase and an entire ORF at the 3' end of RNA 1 not found in CuYV. BPYV virus was identified in strawberry in the mid-Atlantic states, California, Oregon USA and in Ontario, Canada. In a limited survey in strawberry production fields in California, approximately 20% of the plants were infected with BPYV.

The nucleotide sequence data reported in this paper has been submitted to the GenBank nucleotide sequence database and has been assigned the accession numbers NC 005209 and NC 005210.

4.2 Introduction

Closteroviruses have the largest genome of all plant positive-strand RNA viruses, with genomes close to 20 Kbp in size (Martelli *et al.*, 2002). The viruses in the family are transmitted in a semi-persistent manner by aphids (genus *Closterovirus*), whiteflies (genus *Crinivirus*) or mealybugs (genus *Ampelovirus*) and are generally phloem-limited (Karasev, 2000). Viruses in the genera *Closterovirus* and *Ampelovirus* are monopartite while criniviruses have a divided genome. Members of the family *Closteroviridae* infect plants of agriculturally important families such as *Cucurbitaceae, Rosaceae, Solanaceae,*

Compositae, *Vitaceae* and *Rutaceae*, causing significant economical losses (Dolja *et al.*, 1994). Pallidosis is a disease of strawberry (*Fragaria* x *anannasa*) first identified in 1957 (Frazier and Stubbs, 1969) and may be the first disease described being caused by a *Crinivirus*. *Strawberry pallidosis associated virus* (SPaV), a putative causal agent of the pallidosis disease was found in all but one of the 38 plants used in studies to characterize that virus (Tzanetakis *et al.,* 2002). The thirty eighth plant (M29) failed to give any positive results in reverse transcription-polymerase chain reaction (RT-PCR) tests using oligonucleotide primers developed against the heat shock protein 70 homolog (HSP70h), coat protein (CP), minor coat protein (CPm) and p28 genes of SPaV (Tzanetakis and Martin, unpublished data). In addition, tissue blot immunoassay using polyclonal antibodies derived against the recombinant CP of the virus expressed in bacteria that gave positive results with other isolates failed to give a positive test with isolate M29. This isolate was re-grafted onto indicator plants *F. vesca* ('UC-4' or 'UC-5') and *Fragaria virginiana* ('UC-10' or 'UC-11'). *F. virginiana* plants showed typical pallidosis symptoms including leaf marginal chlorosis and cachexia (Fig. 4.1) while *F. vesca* plants remained asymptomatic. Thus, the plant was positive for pallidosis, based on the symptoms in indicator plants.

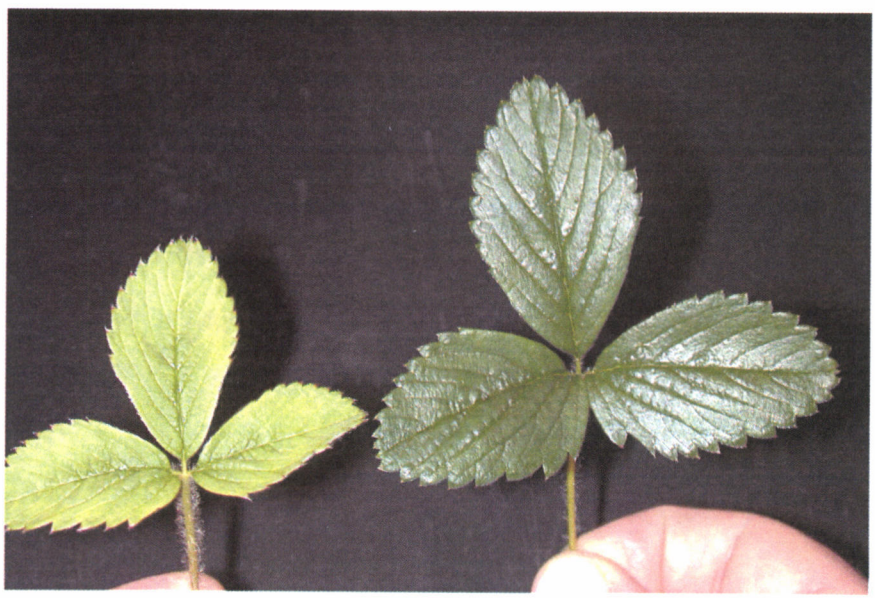

Fig. 4.1. Symptoms on Fragaria virginiana clone UC-10 grafted with pallidosis isolate M29. Left: Leaf from plant grafted with M29, right: leaf from healthy plant of 'UC-10'.

BPYV, the crinivirus with the largest host range known today (Wisler *et al.*, 1998), was first identified in 1965 (Duffus, 1965) and infects plants in the temperate and subtropical regions around the world (Coutts and Coffin, 1996). The virus causes yellowing diseases in plants belonging to the families *Cucurbitaceae, Solanaceae* and *Chenopodiaceae* among others (Duffus, 1965), and is solely transmitted by the greenhouse whitefly, *Trialeuroides vaporariorum,* with acquisition time of less than 1h. The whiteflies remain virulent for 6 days, making the insect an excellent disseminator of the virus (Wisler *et al.*, 1998).

Recently, the complete nucleotide sequence of *Cucumber yellows virus* (CuYV), a strain of BPYV was published (Hortono *et al.*, 2003). In this study, we present the complete nucleotide sequence of a strawberry isolate of BPYV (BPYV-strawberry) which bears unique features not found in CuYV, including an additional ORF.

4.3 Materials and Methods

4.3.1 Plant material

The plant material for the initial cloning was from Maryland, USA and designated M29. This plant had indexed positive for strawberry pallidosis disease on biological indicators but tested negative for *Strawberry pallidosis associated virus* in RT-PCR and tissue blot immuno-assays. After determining the presence of BPYV by cloning and sequencing from dsRNA template purified from isolate M29, the isolate was transferred to *Nicotiana benthamiana* using the whitefly vector of the virus, *Trialeuroides vaporariorum,* as a vector. The completion of the sequencing of the genome of BPYV was done with dsRNA isolated from *N. benthamiana* plants infected with isolate M29. The plant samples used for detection were field plants from Maryland, the Watsonville and Irvine areas of California, the north Willamette Valley of Oregon, Washington USA and British Columbia and Ontario, Canada. Many of these samples were sent to our laboratory by strawberry researchers at the distant locations.

4.3.2 cDNA synthesis and cloning

Initial dsRNA extraction was performed with tissue from the M29 isolate as described previously (Yoshikawa and Converse, 1990). Unless otherwise stated all enzymatic reactions described hereafter were performed using products from Invitrogen Corp., Carlsbad, CA, according to the manufacturer's recommendations. cDNA was synthesized as described previously (Jelkmann *et al.*, 1989) using the Thermoscript® reverse transcriptase and subsequently adenylated at the 3' ends using *Taq* polymerase (1 unit), for 15 min at 72^0C and purified utilizing the rapid PCR purification system (Marligen Biosciences, Ijamsville, MD) prior to cloning. The products were then ethanol-precipitated, resuspended in 8 µl water and cloned into the pCR4 TOPO vector. Plasmids were purified, digested with *Eco*RI (New England Biolabs, Beverly, MA) and visualized with a UV light after electrophoresis through agarose gels that were stained with ethidium bromide.

4.3.3 Amplification of BPYV genome

Templates for RT-PCR reactions were either dsRNA extracted as described above or ssRNA extracted using the Hughes and Galau method (Hughes and Galau, 1988). All the primers used for PCR amplification were developed after alignment of at least two cDNA clones. Reverse transcription was performed as described above while for PCR we used the *Platinum* Taq polymerase. The PCR program consisted of original denaturation for 5 min at 94^0C followed by 40 cycles of 30 sec at 94^0C, 30 sec at 50 or 55^0C annealing, depending on the primer melting temperature and extension of 1-2 min at 68^0C depending on the length of the amplified fragment. The reaction concluded after a final extension step at 68 ^{0}C for 15 min. Each fragment was amplified twice and cloned into the pCR 4 TOPO® vector.

Sequence contigs were assembled after sequencing the two PCR products; two clones in both directions using the M13 forward and reverse primers and clones of the original cDNA cloning reaction where applicable. For amplification of the 3' ends of the

virus, the dsRNA was poly-adenylated (Sippel, 1973) and reverse transcription performed as described previously (Jelkmann *et al.*, 1989) utilizing random nucleotide hexamers and an oligo thymidine primer. Amplification of the 3'ends was performed twice using the same parameters as above. For amplification of the 5' termini we used two approaches. We developed oligonucleotide primers from the minus-strand sequence of the viral dsRNA and utilized the poly-adenylated dsRNA as template and two individual primers with the oligo-thymidine primer. We also employed commercially available 5' rapid amplification of cDNA ends (RACE) using ssRNA extracted as above as the reaction template. Each of the 5' terminus amplification reactions was performed once with each template.

4.3.3 Nucleotide sequencing and genome analysis

All sequencing reactions were performed at the Macrogen Inc. facilities (Seoul, Korea) using an ABI 3700 DNA sequencer. Homology searches with other virus sequences were performed utilizing the BLAST database (Altschul *et al.*, 1997). The assembly of the virus genome was performed utilizing the ClustalW software (European Bioinformatics Institute http://www.ebi.ac.uk/ clustalw). The search for transmembrane domains in BPYV encoded proteins were performed using the TMHMM Server v. 2.0 for prediction of transmembrane helices in proteins (http://www.cbs.dtu.dk/services/ TMHMM/). The ORFs where verified using the NCBI ORF finder (http://www.ncbi.nlm. nih.gov/gorf/gorf.html) and the gene finder in viruses at http://www.softberry.com. The amino acid comparisons of the crinivirus ORFs were performed utilizing Vector NTI suite 6 (Informax, Bestheda, MD). For the RNA secondary structure prediction to investigate structures in the untranslated regions at both ends of the genomic RNAs, the mfold software at default settings was used (Zuker, 2003), while identification of conserved domains was done utilizing the CDART (Conserved Domain Architecture Retrieval Tool) software (Geer *et al.*, 2002).

4.3.4 Detection of BPYV in strawberry

For detection of the virus, RT-PCR was employed. Single-stranded RNA was extracted from 100 mg of tissue and used as template for reverse transcription as described above. Primers *BPYV CPm F* (5' TTCATATTAAGGATGCGCAGA 3') and *BPYV CPm R* (5' TGAAAGATGTCCRCTAATGATA 3') that amplify a 334 base pair (bp) fragment of the CPm gene were utilized. As an internal control for the reactions, primers originally described by Thompson *et al.* (2003) were tested. Due to the abundance of NADH dehydrogenase mRNA and the length of the fragment amplified, the product was consistently brighter than that corresponding to the CPm fragments of BPYV. In order to balance amplification of both fragments, the reverse primer was changed so the NADH dehydrogenase PCR product was larger than the 334 bp virus fragment. The control primers used were thus *NADH F* (5' GGACTCCTGACGTA ACGAAGGATC 3') and *NADH R* (5'AGTAGATGCTATCACACATACAAT 3') that amplify a 721 bp fragment of the gene (Fig. 4.6). The PCR program was identical with that described above with primer annealing temperature at 55^0C and template extension time for 1 min.

4.4 Results

The dsRNA pattern of this single isolate (M29) gave a large (~8 Kbp) band and several others that most probably represent subgenomic RNAs of the virus. The dsRNA pattern compared with those of other criniviruses (Fig. 4.2) suggested that a second crinivirus may be present in strawberry. The original cDNA synthesis cloning provided sequence data that contained more than 14 Kb of the viral genome but there were not a sufficient number of clones to build contigs other than nucleotide regions from approximately 1200 - 1800 and 4000 - 5600 of RNA 1 as well as nucleotide regions from about 60 - 1600 and 5000 – 7400 of RNA 2 that were assembled from clones derived from cDNA synthesis. These regions were represented by at least three clones per site.

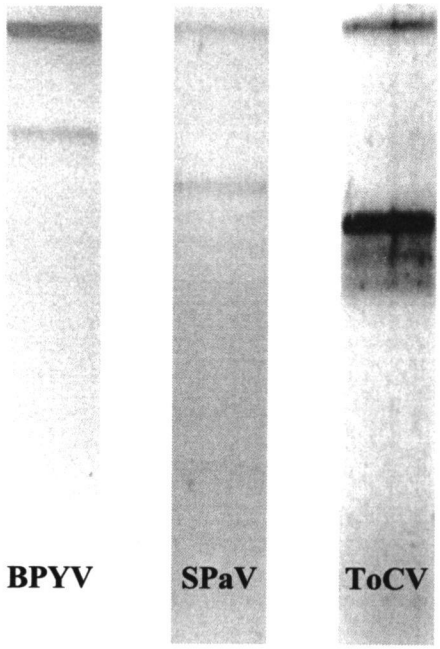

Fig. 4.2. DsRNA pattern of three criniviruses, *Beet pseudo-yellows virus* (BPYV), *Strawberry pallidosis associated virus* (SPaV) and *Tomato chlorosis virus* (ToCV). The genomic sizes (top band) represent RNA 1 and RNA 2 and are close to 8 kbp for BPYV and SPaV and for ToCV (both RNAs longer that 8300 base pairs, Wintermantel W.M., personal communication). The size of the subgenomics is similar but the abundance of the different subgenomics varies between the viruses.

The general organization of BPYV- strawberry resembles that of other sequenced criniviruses (Kreuze *et al.,* 2002; Livieratos and Coutts 2002; Klaassen *et al.*, 1995; Aguilar *et al.,* 2003). The overall nucleotide sequence identity to CuYV for RNA 1 was 91% and for RNA 2 it was 98%, while the amino acid sequence identities range from 94 to 100% (Table 4.1). Both RNAs of BPYV- strawberry have the same first 11 nucleotides but the 5' untranslated regions (UTR) are not predicted to fold to similar structures (data not shown). The 3' UTRs has 91% nucleotide identities in the last 156 nucleotides. The RNA 1 3' UTR is 203 nt long with predicted folding free energy is -41 kcal/mole. RNA 2 3'UTR is 185 nt long and is folded to a predicted structure similar to that of the 3' UTR of RNA 1 (Zuker, 2003) (Fig. 4.3). The similar folding into the conserved stem-loops may be a signal for the viral polymerase and facilitate initiation of replication (Yi and Lemon, 2003).

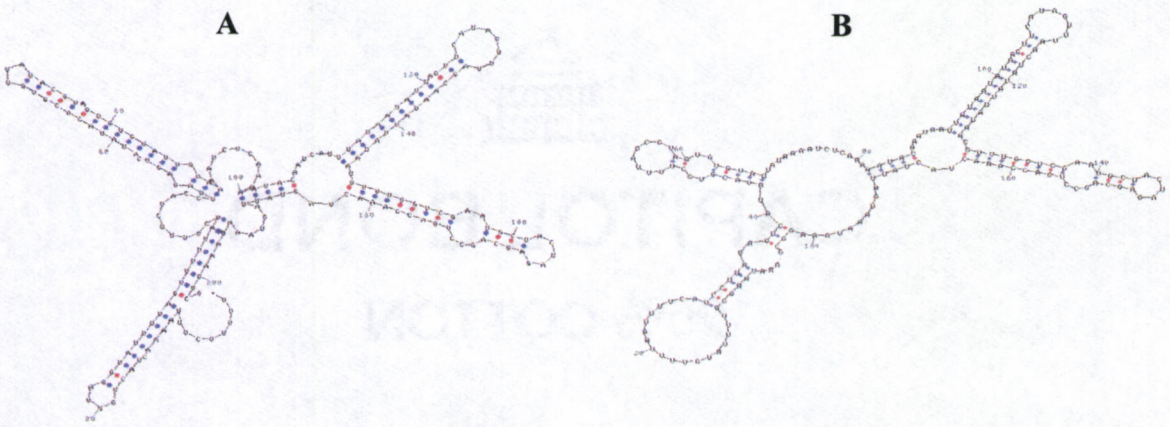

Fig. 4.3. Predicted folding of the 3' untranslated regions of RNA 1 and 2 of BPYV at 37^0C. A. RNA 1, B. RNA 2

RNA 1 of BPYV-strawberry consists of 8007 nucleotides and encodes three open reading frames (Fig 4.4). The first ORF of RNA 1 starts at nucleotide 244 and terminates at nucleotide 6054. It encodes a protein of 1936 amino acids and the estimated molecular weight is 221 KDa. The overall amino acid seqeunce identity with the CuYV strain is 94% (95% similarity). It encodes a multifunctional polyprotein with cysteine/papain protease, methyltransferase and helicase motifs found in all closteroviruses (Karasev, 2000). Protein database search revealed that the cysteine protease is encoded in the N' terminus of the polyprotein, followed by the methyltransferase domain while the helicase domain is encoded in the C' terminus of the protein.

The first major difference between BPYV-strawberry and CuYV is a 147 nucleotides insertion at nucleotide 3355, after the termination of the methyltransferase domain (Fig. 4.4). The nucleotide sequence identities between CuYV and BPYV-strawberry before the insertion is 86% while after it, it reaches 94%. The polyprotein has two transmembrane domains in the region between the methyltransferase and helicase (amino acids 1194 -1216 and 1298-1320) that could be the anchors for localization of the protein in vesicles as previously shown for *Beet yellows virus* (Erokhina *et al.*, 2001). The termination signal for the 1a ORF is UUUGA which is also found in *Lettuce infectious yellows virus, Sweet potato chlorotic stunt virus* and *Strawberry pallidosis associated virus* (Tzanetakis I.E., unpublished data), while *Cucurbit yellow stunting disorder virus* terminates with UUUAG (Aguilar *et al.,* 2003). In all cases, the three

uridines found near the stop codon of ORF 1a may accommodate the +1 ribosomal frameshift by which the viral polymerase is expressed, a novelty found only in closteroviruses among all plant viruses. ORF 1b, the viral polymerase, starts at nucleotide 6052 and terminates at nucleotide 7570. It contains the signature RNA polymerase active site GDD motif and its' molecular weight is 58 KDa. All criniviruses have the sequence: LVSGDDSLIFS - in the active site of the polymerase while most closteroviruses have the consensus VSGDDSLI.

Another major difference between CuYV and BPYV-strawberry is the presence of an additional ORF on RNA 1 (Fig. 4.4). This ORF encodes for a 54 amino acid peptide with hydrophobic residues found in transmembrane proteins and has a molecular weight of 6 KDa. This 6 KDa ORF does not show any homology with other viral proteins in the GenBank database when performing BLAST search (Altschul *et al.,* 1990) and has no significant homology to any proteins in the database. The transmembrane domain spans from amino acids 29-46. This is the first of two such peptides encoded by BPYV-strawberry.

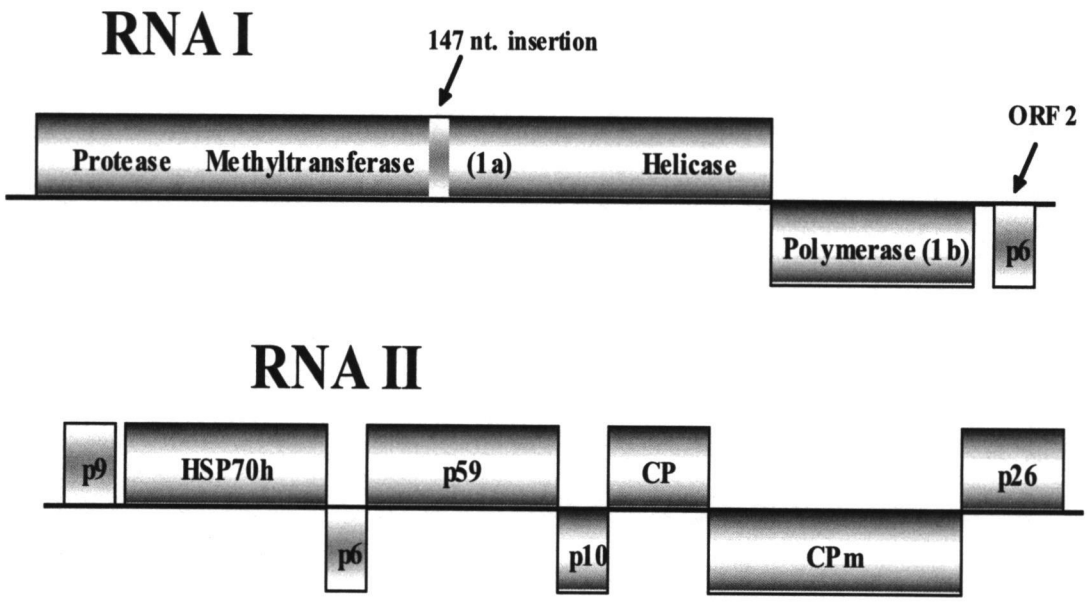

Fig. 4.4. Schematic representation of the genome organization of BPYV. The different shading indicates the main differences between BPYV, CuYV and other criniviruses. The sizes of the ORFs are not to scale.

RNA 2 of BPYV-strawberry is 7904 nucleotides in length and has 8 ORFs (Fig. 4.4) in contrast to most criniviruses sequenced to date that encode 7 ORFs. All viruses of the family encode an array of five genes that are thought to be involved in cell-to-cell and long distance movement, which are found in RNA 2 of criniviruses (Napuli *et al.*, 2003; Dolja, 2003). The first ORF of BPYV-strawberry RNA 2 and one of the genes involved in closterovirus movement is a small hydrophobic protein similar in position and motifs to small hydrophobic proteins that all closteroviruses encode (Karasev, 2000). The starting codon is found in position 250 and termination signal at nucleotide 480. This peptide has molecular weight of 9 KDa. The transmembrane helix starts at amino acid 37 and terminates at amino acid 57. This larger than CuYV ORF 1 of RNA 2 is due to an additional cytosine found at position 285. The BPYV-strawberry peptide has a very strong transmembrane motif in contrast to that of CuYV which does not possess such motifs according to the software utilized for the analysis. The next ORF which starts at nucleotide 697 encodes the 554 amino acid HSP70h gene of the virus. A consensus of HSP 70h genes of criniviruses is found in figure 4.5. The HSP70h gene has been shown to be associated with virions and plasmodesmata (Peremyslov et al., 1999; Medina et al. 1999; Napuli et al., 2000) for BYV, and is the second gene involved in viral movement.

Downstream of the HSP70h there is a 53 amino acid encoding ORF. This ORF is homologous to ORFs in *Cucurbit yellow stunting disorder virus* (GenBank Accession No NP 851573) and *Strawberry pallidosis associated virus* (Tzanetakis and Martin, unpublished data) with 49% amino acid sequence similarity in each case. This gene has homology to the C - terminus of *Little cherry virus –1* HSP70h with 28% sequence similarities. The C-terminus of the heat shock protein superfamily is involved in substrate recognition (Bakau and Horwich, 1998) and one can only speculate on the function of this ORF although that function has not been identified in *Beet yellows virus* HSP70h gene (Agranovsky et al., 1997). The fourth ORF starts at position 2536 and terminates at position 4089 encoding a gene of 59 KDa. This gene is also needed for virus movement and has recently been found associated with virions of BYV (Napuli *et al.*, 2003), being essential for assembly of the virions. RNA2 ORF 5 encodes a protein of 10 KDa and is a gene that is found only in criniviruses and whose function is still unknown. The next two

genes of RNA 2 of BPYV-strawberry encode the two coat proteins of the virus. CP encapsidates ~ 95% of the genome while CPm protects the remaining 5% (Argonovsky et al., 1995), tentatively the 5' end of the genome (Zinovkin et al., 1999, Satyanarayana *et al.*, 2004). CP starts at nucleotide 4309 and encodes a protein of 252 amino acids with molecular weight of 28 KDa. CPm begins at nucleotide 5057, ten nucleotides before the termination signal for the CP, and is the virus largest gene after the 1a ORF encoding a protein of 657 amino acids and with a molecular weight of 75 KDa. The last ORF of BPYV-strawberry RNA 2 encodes a protein of 230 amino acids and molecular weight of 26 KDa starting at 7027 and terminating at nucleotide 7719.

Table 4.1. Amino acid identities and similarities (in parenthesis) between the polypeptides encoded by the strawberry isolate of *Beet pseudo-yellows virus* (BPYV) compared to *Cucumber yellows virus* isolate of BPYV (CuYV), *Cucurbit yellow stunt disorder virus* (CYSDV), *Sweet potato chlorotic stunt virus* (SPCSV) and *Lettuce infectious yellows virus* (LIYV) ORFs. The comparison of ORF 2 with two transmembrane domain proteins present in the genome of CYSDV and SPCSV are not calculated because of the different position these ORFs have in the genome of the other viruses pointing to the fact that they may not be homologous genes to ORF 2.

Gene/Virus	CuYV	CYSDV	SPCSV	LIYV
1a	94 (96)	34 (50)	36 (51)	29 (45)
RNA1 1b (RdRp)	98 (98)	61 (74)	62 (78)	55 (71)
RNA1 (ORF2)	-	-	-	-
p9	97 (100)	12 (29)	16 (30)	31 (36)
HSP70h	99 (99)	62 (79)	63 (79)	49 (68)
p6	100	30 (43)	-	-
p59	98 (99)	39 (55)	38 (54)	32 (48)
p10	100	28 (42)	29 (39)	28 (29)
CP	99 (99)	40 (52)	29 (39)	26 (37)
CPm	97 (98)	26 (46)	23 (37)	19 (27)
p26	98 (98)	25 (46)	17 (35)	14 (29)

The virus was detected by RT-PCR in strawberry plants from Maryland, California and Oregon in the USA (Fig. 4.6). A limited number of samples were tested from New York, North Carolina, Washington USA and British Columbia, Canada and were negative for BPYV. The infection rate ranged from over 12 to 20% (Table 4.2) in the areas where the vector of the virus is most abundant in the field.

Fig. 4.5. Alignment of HSP70h proteins of BPYV-strawberry with the HSP70h of six other criniviruses. HSP70h accession nos; *Beet pseudo-yellows virus* (BPYV), NP 940788; *Cucumber yellows virus* (CuYV), NP 821143; *Strawberry pallidosis associated virus* (SPaV), AAO 92347; *Sweet potato chlorotic stunt virus* (SPCSV), NP 689401;, *Cucurbit yellow stunting disorder virus* (CYSDV), NP 851572; *Tomato chlorosis virus* (ToCV), AAD 01790; and *Lettuce infectious yellows virus*, LIYV, NP 619695.

```
BPYV    -MQAKAGLDFGTTFSTISSFTNGEMKTLYVSNSPYIPTCLSISSEGDVIIGSAAQVIDES 59
CuYV    -MQAKVGLDFGTTFSTISSFTNGEMKTLYVNNSPYIPTCLSISSEGDVIIGSAAQVIDES 59
SPaV    MTEAKVGLDFGTTFSTISSYINNKMHVLKINDSPYIPTCLAISIDKDVIIGGAAQVLDSS 60
CYSDV   --MAKAGLDFGTTFSTISSYVNGVMKVLKLNETEFIPTCLAITSNNDVVVGGPAQVLSNS 58
SPCSV   -MEAKAGLDFGTTFSTISAYVGGTMKVLRINGSEFIPTCLSVTATGDVVVGGAAQVLDSS 59
ToCV    -MSIKAGLDFGTTFSTISCFYNNKLFSLKLNGTEYIPTCLSITPNNEVIVGGPSQVLEAS 59
LIYV    MRDCKVGLDFGTTFSTVSTLVNNSMYVLRLGDSAYIPTCIAITPGGEAIIGGAAEVLSGD 60
            * ********** *          *       ****           *    *

BPYV    EVKSCYFYDLKRWVGVDATNFLVIKEKIKPLYVVKLVGNDVYLTGVNKGFSCTYTVKQLI 119
CuYV    EVKSCYFYDLKRWVGVDATNFLVIKEKIKPLYVVKLVGNDVYYTGVNKGFSCTYTVKQLI 119
SPaV    EVANCYFYDLKRWVGVDKVNFENIKAKINPQYVAKLVNDDVMLTGVDRGYSCTYTVKQLI 120
CYSDV   DMPNCYFYDLKRWVGVDSINYNVIKTKINPVYVTELRGNDVYITGIDRGYTCTYTVKQLI 118
SPCSV   QLPHCYFYDLKRWVGVDRLSFEEIKRKISPQYTVRLEGNDVLITGISKGFSCTYTVKQLI 119
ToCV    ETPSCYFYDLKRWVGVTSVNYEVVKAKINPTYKTRLSNNKVYITGINKGFSTEFSVEQLI 119
LIYV    DTPHCFFYDLKRWVGVDDNTFKFAMNKIRPKYVAELVEGEVYLTGINKGFSIKLSVKQLI 120
            * **********          ** * *   *    *  **   *      * ***

BPYV    LLFIDTMVRLFSKTNNLNIISLNVSVPADYKCKQRMFMKSVCDSLNFSLRRIINEPSAAA 179
CuYV    LLFIDTMVRLFSKTNNLNIISLNVSVPADYKCKQRMFMKSVCDSLNFSLRRIINEPSAAA 179
SPaV    LLYIDTLVRLFSKTDNLNIISLNVSVPADYKCKQRMFMKSVCDSLNFSLRRIINEPSAAA 180
CYSDV   LLYIETLVRLFSKVESITITSLNVSVPADYKCKQRMFMKSVCDSLGFSLRRIINEPSAAA 178
SPCSV   LLYVDTLVRLFSNVEKLKILSLNVSVPADYKTKQRMFMKSVCESLGFPLRRIINEPSAAA 179
ToCV    LHYVNTLVRLFSKTENLKITDLNVSVPADYKSGQRLFMQAVCSSLGFNLRRIVNEPSAAA 179
LIYV    KAYIETIVRLLASSYSLRVIDLNQSVPADYKNAQRLAARSVLKALSFPCRRIINEPSAAA 180
             * ***           ** *******  **     *   * *  *** *******

BPYV    IYSVSKYPQHNYFIMYDFGGGTFDTSLITRDGQYVTVADTEGDSFLGGRDIDNEIQQFIV 239
CuYV    IYSVSKYPQHNYFIMYDFGGGTFDTSLITRDGQYVTVADTEGDSFLGGRDIDNEIQQFIV 239
SPaV    IYSVSKYPNYKYFLMYDFGGGTFDTSLIVRDGKVVTVADTEGDSFLGGRDIDNAISRFIV 240
CYSDV   IYFVSKYPQYNNFLMYDFGGGTFDSSLIVRDGKYVTVADTEGDSFLGGRDIDNAIADYIT 238
SPCSV   IYSISKHPGFDYFLVYDFGGGTFDTSLIAKDGKFVTVADTLGDSFLGGRDIDRAILSHIM 239
ToCV    IYCVSKYPQYAYFYIYDFGGGTFDTSLIVRYGKFVTVADTQGDSFLGGRDIDKTISKFIM 239
LIYV    VYCVSRYPNYNYFLVYDFGGGTFDVSLIGKYKSYVTVIDTEGDSFLGGRDIDKSIEDYLV 240
         *  *  *    *  ********* ***      *** ** ***********  *

BPYV    KSNNLSRPLPSDFLASIKEDCNTTGKSTFNVMDVDGKLLTIRFSREDLAACIEPYSKRSL 299
CuYV    KSNNLSRPLPSDFLASIKEDCNTTGKSTFNVMDVDGKLLTIRFSREDLAACIEPYSKRSL 299
SPaV    EKHSLPRPLSSDFLASIKEEVNNSSKSNFIALDTKGNIVNVSFNKDDLATCIQPFSVKSI 300
CYSDV   TTYGMKGGLSADVLASIKEDCNSKGRENFNVIDSSGKLHNVKFTRQDLSRCIEPFSKKSI 298
SPCSV   RTNSLQKPLSADSLAAIKEEVNSTGRSNFNVLDVDGNIIFVNFSGEELDKIVSKFTAKSL 299
ToCV    DKNALNAPLSADMLASIKEETNSTGRSSYNIISDDGSIINIQFTFDDLVKCVEPFARRSF 299
LIYV    GKYNIKKVIPATYLALIKEECNNTNKSIFTILFDDGSVQVVEFSKSELEKCVRPFVERSI 300
                   ** ***  *            *      *    *          *

BPYV    KILDNLVKRRKISSGALFLVGGSSLLSKVQQDVAAYASANNFECVIDKDLRCSVSFGCSM 359
CuYV    KILDNLVKRRKISSGALFLVGGSSLLSKVQQDVAAYASANNFECVIDKDLRCSVSFGCSM 359
SPaV    KILDNLVGRRKITNGALFLVGGSSLLKKIQQDVSSYARSKGLTCVIDEDLRCSVSFGCSM 360
CYSDV   ALLDNMVVRNITKDSAVFMVGGSSLLKKVQHDVMNYCARTKLECIIDKDLRSAVSFGCSM 358
SPCSV   KILKAIADRNKITSGALFLVGGSSLLRKVQLDVSNFAKSIGLTPIIDKDLRSAVSYGCSM 359
ToCV    SILRSLVSRNKTSNGALFLVGGSSLLRPIQNRADGFARNHGLALIIDPDLRAAVSFGCSM 359
LIYV    KLINDVVVRNKLTSGVIYMVGGSSLLQPVQDMVRSYASTKGLTLVADQDMRSAVSYGCSV 360
              *          *******   *                 * * *  ** ***
```

Figure 4.6 (continued)

```
BPYV    QHAQEDSGSMIYIDCNSHPLMDLTLFGNPKVVVRKPMSIPYSTSDSRTIRSHYSTAVNVY 419
CuYV    QHAQEDSGSMIYIDCNSHPLMDLTLFGNPKVVVRKPMSIPYSTSDSRTIRSHYSTAVNVY 419
SPaV    QHAQEDSGSMTYIDCNSHPLMDLLMYGNPKVVVRKPMPIPYTKYDTRTIRQHYNTVVNVY 420
CYSDV   SHAQEDTKNMIYIDCNSHPLMDISYFCSPKIIVRKPMAIPYTGVREETLTRHYTTILNVY 418
SPCSV   MHAQEDSGSMVYIDCNSHPLMDVSLFANPRVIIRKPMSVPFSYKTTRKVDRHMMTAVNVY 419
ToCV    LHAQEDSGNMTYIDCNSHPLMDLGLYCHPRIIIRKPMSVPYTHKIEREVTRFITTALNVY 419
LIYV    LHKLEDNKEIVYIDCNSHPLSDISFNCDPEPIIRKPMSIPYTHTVKMRHDRPLKTIVNIY 420
         *  **     ********* *      *    ****  *              *  * *

BPYV    EGSDIFTLNNDWLISANVRTSDHVNVGEDLVFIYKYNIDGILELYAKNGRTGAEKLLPNS 479
CuYV    EGSDIFTLNNDWLISANVRTSDHVNVGEDLVFIYKYNIDGILELYAKNGRTGVEKLLPNS 479
SPaV    EGSDLFVLNNDWLVSAKVNTSDHANVGEDLTFVYKYTIDGILELYAKNEKTGVEKLLPNT 480
CYSDV   EGSDPFVLNNDWLISANMQSNKYGEIGDTLQYLYKYNVDGILELVVRNKRTGKETVLPNS 478
SPCSV   EGSSLFVLDNDWLVSANVNTQDFVDLGQELSYVYKYNVDGILDLFVRNESTGVESLLPNS 479
ToCV    EGSDLFVLNNDWLISADVDYSKYAKMGETLVSVYKYTIDGILELSMANKTTGKSWVLPNT 479
LIYV    EGSNLFMPENDWLISSNINTTDFAKVGEEYSKVYEYDIDGIITLKIRNEVTGKMFTLPNS 480
        ***  *   **** *           *      * *  ***  *   *  **    ***

BPYV    FSLTEKIEKLNLKLTQLSNVDEIATILSILSAFDDSLTGLLKFVKTPSILEREVAKISTP 539
CuYV    FSLTEKIEKLNLKLTQLSNVDEIATILSILSAFDDSLTGLLKFVKTPSILEREVAKISTP 539
SPaV    FSLTEKINKLDLQLTQLSTIDESATLISIMSYFDDNYTRLLSLLRTPTILERELLKITST 540
CYSDV   FALTESIKKLDVNLTQLSNIDELATLVAIMSYYKPELKYLLTYVKTPTIFENEIKKFGSG 538
SPCSV   FALTEKVKKLDLNLTQLSSVDELATVVSILSHFDSSFSSLLKLVNTPSIFETSAAKFGDL 539
ToCV    FARSEKIVISDLTLTQLSNVDELATIVSILSYFDTTFNYLISMFNTPSIFEREVGKISDA 539
LIYV    FTKSDNIKPITFKLTQLSNTDDLATLTSLLGYHDKNFERFYGLFNVPTILIKEIDKLGGF 540
        *            *****  *  **                        * *      *

BPYV    KELLERLVKQNKNFS- 554
CuYV    KELLERLVKQNKNFS- 554
SPaV    KKLYSALCDVNKNFNN 556
CYSDV   EDLYKSLAALNKNFK- 553
SPCSV   RSLYERLIFVNKNFS- 554
ToCV    KGLYNRLVEQNRNFS- 554
LIYV    KTLYRRLKSMNANF-- 554
          *   *   * **
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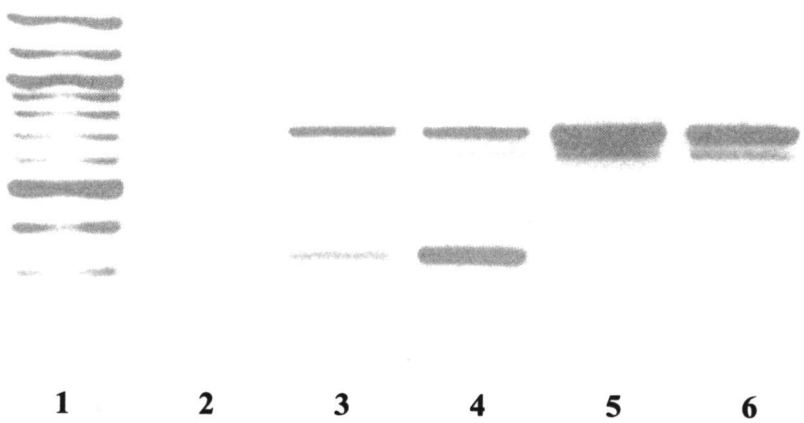

Fig. 4.3. Detection of *Beet pseudo-yellows virus* (BPYV). Reverse transcription-polymerase chain reaction products were resolved on 2% agarose gel stained with ethidium bromide and visualized with UV-light.. Lane 1: 100 base pair molecular size marker (New England Biolabs, Beverly, MA), lane 2: Blank, lane 3: BPYV infected strawberry, lane 4: BPYV infected *Nicotiana benthamiana,* lane 5: *Strawberry pallidosis associated virus* infected strawberry, lane 6: healthy strawberry seedling. The top bands in the samples correspond to NADH dehydrogenase fragment while the lower bands correspond to BPYV minor coat protein fragment.

Table 4.2. Strawberry samples tested for the presence of BPYV.

Sampling area	Samples tested	BPYV infected samples
California	63	12
Maryland	30	6
Oregon	24	3
Washington	6	-
New York	6	-
North Carolina	8	-
British Columbia	6	-
Ontario	10	2

4.5 Discussion

The *Closteroviridae* is a very diverse virus family. The genome size and the genes encoded by members of the family varies greatly from that of *Citrus tristeza virus* (Karasev *et al.,* 1995) to *Cucumber yellows virus* (Hortono *et al.*, 2003). The diversity between BPYV strains is striking with the insertion of 147 nucleotides in ORF 1a and the additional ORF in RNA1 found in the strawberry strain reported here compared to

CuYV. This may be an indication that the genus is evolving or there are plant species specific strains that differ in genome size and organization since CuYV was isolated from cucumber and the isolate presented in this study from strawberry. Future plans include further investigation on the diversity of BPYV to determine if the the strawberry isolate reported here or the CuYV isolate is more typical for the species.

The presence of the virus was examined in three strawberry producing states on the east and west coast of the U.S. The virus was present in all three states at high infection rates. The naturalization of the greenhouse whitefly, the vector for BPYV and SPaV, in the southern U.S. the last few years may be the cause for the high incidence of the virus in production fields in California where the whiteflies are common. In California, whiteflies have become a serious problem for the strawberry industry as they reach high populations and insecticides are applied multiple times in a growing region for to reduce the impact of the pest on production. BPYV is increasing in severity in fruit and vegetable crops in the southern U.S. In the last few months, two additional hosts of the virus have been identified (Wintermantel, 2004; Tzanetakis and Martin, 2004) and there may be other asymptomatic hosts that serve as a virus reservoir for field infections. The role of BPYV in the decline symptoms of California strawberry the last two years (Martin R.R., personal observation) is not clear but since the virus is able to cause pallidosis disease it needs to be considered as a possible component in the complex that is responsible for the decline. Pallidosis often acts synergistically with other viruses that infect strawberry (Fulton, 1987). Further studies to evaluate the impact of BPYV in single infections and its' role when found in complexes with other strawberry viruses need to be performed in order to determine the possible role of the virus in yield losses and plant vigor.

The finding of BPYV in Oregon and Maryland suggest that the virus is not being detected reliably with the current indexing system that relies on grafting onto *F. virginiana* and *F. vesca*. In previous work with strawberry pallidosis, approximately 70% of field samples from Maryland were found to be infected (Hokanson *et al.*, 2000). They did not find a relationship between the incidence of pallidosis and the length of time plants were in the field. Plants that were in the field for 4-5 years had the same level of

pallidosis as did strawberry plants in the planting year. A comparison of detection efficiency between graft indexing and RT-PCR using the protocols describe here will determine if the problem is in the detection with biological indicators. Another possible entry for infection in areas where the whitefly vector is not present in the field is in the early stages of propagation where strawberries are multiplied in greenhouses for the first cycle of increase after the indexing is completed. Previously, the vector of pallidosis was unknown and little attention was paid to whiteflies in the greenhouses. It is possible that the "Elite" or "Nuclear" stock plants were infected after they were indexed but before they were planted in certification blocks for increase under field conditions.

4.6 Acknowledgments

We would like to thank Karen Keller for her excellent technical assistance. We would also like to thank William Wintermantel and Arturo Cortez (USDA-ARS, Salinas, CA) for transferring the virus isolate from strawberry to *N. benthamiana* and infected tissue of ToCV for dsRNA extraction. This project was funded by the North American Strawberry Growers' Association and the United States Department of Agriculture.

4.7 References

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Chapter 5

Strawberry necrotic shock virus is a distinct virus and not a strain of *Tobacco streak virus*

Ioannis E. Tzanetakis and Robert R. Martin

5.1 Abstract

Fragaria (strawberry) and *Rubus* species (blackberry, wild blackberry, red raspberry and black raspberry) were thought to be infected with distinct isolates of *Tobacco streak virus* (TSV). Employing serology and nucleic acid hybridization it has been shown that these isolates form a cluster distinct from other strains of TSV. In this study we have cloned and sequenced the complete RNA 3 of an isolate of TSV from strawberry as well as the coat protein (CP) gene of 14 additional isolates of TSV originating from *Fragaria* and *Rubus* species. Our data suggest that the isolates of TSV that infect *Fragaria* and *Rubus* belong to a distinct virus for which we propose the name Strawberry necrotic shock virus (SNSV). The RNA 3 of SNSV contains 2248 nucleotides, 43 more than the type isolate of TSV from white clover (TSV-WC), with a CP gene that is 669 nucleotides long, in contrast to the 714-7 nucleotides of the TSV CP sequences found in the database. The movement protein gene of SNSV is 897 nucleotides in length, 27 more than that of the TSV-WC isolate of TSV. The CP genes of the 15 *Fragaria* and *Rubus* isolates that we studied form two distinct phylogenetic clusters that share about 95% amino acid sequence identity, while they only share 60-65% amino acid sequence identity with TSV-WC.

5.2 Introduction

Tobacco streak virus (TSV) was first identified in 1936 (Johnson, 1936) and is known to infect more than 80 plant species belonging to the families *Asteraceae, Brassicaceae, Cucurbitaceae, Rosaceae, Solanaceae*, and others (Fulton, 1948). TSV is the type member of the *Ilarvirus* genus of the *Bromoviridae* family. Ilarviruses form the largest genus in the family with more than 15 members and seven or eight subgroups (Hull, 2002). TSV is naturally transmitted vertically through seed and horizontally by pollen and thrips (Sdoodee and Teakle, 1987). The genome of TSV consists of three RNAs. RNA 1 encodes the viral replicase that has methyltransferase and helicase motifs. RNA 2 encodes the RNA dependent RNA polymerase (RdRp) and a putative 2b protein

of 22 KDa (Scott *et al.*, 1998). The movement (MP) and coat (CP) proteins of the virus are encoded in RNA 3 (Cornelissen *et al.*, 1984). The latter is expressed via a subgenomic RNA (RNA 4).

Strawberry necrotic shock virus (SNSV) was identified in strawberry in 1956 (Frazier *et al.*, 1962). The virus can infect strawberries and many *Rubus* species (Converse, 1972; Frazier, 1966). Symptoms rarely are seen in either strawberry cultivars or *Rubus* species, but the virus can reduce strawberry yield by more than 15% and runner production by 75% (Johnson et al., 1984). Johnson *et al.* (1984) and Converse (1979) reported seed transmission as high as 35% in strawberry and 25% in black raspberry, respectively. Grafting of infected tissue on strawberry indicator plants (*Fragaria vesca* var. *semperflorens* 'Alpine') causes a transient shock reaction (necrosis) after which plants recover without showing any apparent symptoms (Stace-Smith *et al.*, 1987).

It has been shown previously that two isolates of TSV from *Rubus* are related to (using host range, symptomatology or superinfection [Fulton, 1978]), but distinct from other strains of TSV tested (Frazier *et al.*, 1962). In agar gel double diffusion immunological studies several isolates from *Rubus* reacted most strongly with homologous antiserum but formed spurs with heterologous antisera against a tobacco isolate (HF, also known as T) (Fulton, 1967), the bean red node strain, and a British TSV isolate from dahlia. Stenger *et al.* (1987) performed Northern blot analysis on five TSV isolates (from strawberry, blackberry, tobacco, bean and white clover) and confirmed a relationship between the blackberry and strawberry isolates and showed that probes developed from the strawberry isolate did not hybridize with isolates from white clover, bean or tobacco, indicating that these two isolates were very distinct.

Reverse transcription-polymerase chain reaction (RT-PCR) with total RNA extracted from *Fragaria* and *Rubus* species utilizing primers developed from sequences of the TSV white clover (TSV-WC) isolate (described in Genbank, accession number NC 003845) failed to generate any amplicons although, these plants gave positive reactions in enzyme linked immunosorbent assay (ELISA) using an antiserum prepared against a red raspberry isolate of TSV. cDNA from one of these isolates was cloned and sequenced. It was determined that the strawberry necrotic shock isolate of TSV is a distinct virus

designated here as Strawberry necrotic shock virus, a name originally used by Frazier et al. (1962) and derived from the symptoms developed on strawberry indicator plants (*Fragaria vesca*) when grafted with infected material. In this study, the complete sequence of RNA 3 of the virus from a strawberry isolate is presented in addition to the sequences of the CP gene of nine additional *Fragaria* isolates from the United States and Japan as well as five *Rubus* isolates from the United States.

5.3 Materials and methods

5.3.1 Virus and plant material

All SNSV isolates used in this study were obtained from the United States Department of Agriculture, Agricultural Research Service National Clonal Germplasm Repository (NCGR) in Corvallis, OR, except for the isolate in red raspberry, which was from our virus collection and an isolate from strawberry, which was a field isolate from Maryland that was utilized to determine the complete sequence of RNA 3. The NCGR sources of the infected plants used in this study are listed in Table 5.1.

5.3.2 cDNA cloning and analysis

DsRNA from the Maryland isolate was extracted as described previously (Yoshikawa and Converse, 1990), ethanol precipitated and air-dried. Unless otherwise stated, all enzymatic reactions described hereafter were performed using products of Invitrogen Corp., Carlsbad, CA according to the manufacturer's instructions. The dsRNA pellet from the equivalent of 8 g of tissue was incubated with 20 mM methyl mercuric hydroxide and 1 μg of random nucleotide hexamers for 30 minutes at room temperature. Reverse transcription was performed using Thermoscript® reverse transcriptase according to the manufacturer's instructions, except for the fact that the amount of dithiothreitol (DTT) was doubled to reduce the methyl mercuric hydroxide. The cDNA was ethanol precipitated and used for second strand synthesis as described previously

(Jelkmann et al., 1989). The reaction was heat-inactivated and incubated with 1 unit of Taq polymerase for 15 min at 72^0C in order to adenylate the 3' ends of the products.

Table 5.1. Source of virus isolates including host plant, cultivar, NCGR identification No.and region of origin.

Species and cultivar	NCGR No.	Region
Fragaria X ananassa, Strawberry, US-70	1189	Mississippi
Fragaria X ananassa, Strawberry, US-159	1191	Mississippi
Fragaria X ananassa, Strawberry, Melcher	1196	Louisiana
Fragaria X ananassa, Strawberry, Koro	1268	Japan
Fragaria X ananassa, Strawberry, Ooishi-shikinari	1291	Japan
Fragaria X ananassa, Strawberry, Sokusei	1294	Japan
Fragaria X ananassa, Strawberry, Sweet Charlie	1314	North Carolina
Fragaria X ananassa, Strawberry, G980	9034	California
Fragaria X ananassa, Strawberry, MS-US-540	9078	North Carolina
Fragaria X ananassa, Strawberry	(MD)	Maryland
Rubus ursinus, Blackberry, Dyke	139	PNW*
Rubus occidentalis, Black Raspberry, Munger	9003	PNW
Rubus occidentalis, Black Raspberry, New Logan	9012#	PNW
Rubus ursinus, Wild Blackberry	9025	PNW
Rubus idaeus, Red Raspberry, Willamette	N/A (RR)	PNW
Phaseolus vulgaris, Bean**	(WC)	N/A
Nicotiana tabacum, Tobacco**	Tob	N/A
Phaseolus vulgaris, Bean**	N/A	N/A

Black raspberry latent virus
PNW* = Pacific Northwest
** = TSV isolates, (WC) = White Clover, Tob = tobacco

The dsDNA products were purified using the Rapid PCR Purification System® (Marligen Biosciences, Ijamsville, MD). The purified product was concentrated to 8 µl, and half the volume was used for ligation into the pCR4-TOPO® vector according to the manufacturer's instructions. The recombinant plasmids were digested with EcoRI (New England Biolabs, Beverly, MA) and analyzed using agarose gel electrophoresis. Clones were sequenced (Macrogen Inc., Seoul, Korea using an ABI 3700 DNA sequencer) and several were determined to contain sequences of the new virus after comparison with sequences of other Ilarviruses found in GenBank.

5.3.3 Sequencing and RT-PCR detection

Virus-specific oligonucleotide primers were developed after aligning at least three clones of the same region of the genome, and were used for amplification of the CP genes of all *Rubus* and *Fragaria* isolates. RNA from 100 mg tissue was extracted according to the method of Hughes and Galau (1988). The RNA pellet was resuspended in 40 µl of RNAse-free water and subjected to reverse transcription as described above with the Superscript III® reverse transcriptase using the RNA sample as 1/10 of the total RT reaction volume. The reaction was heat inactivated and digested with RNAse H (1 unit), for 30 min at 37^0C. Primers SNSV MPbeg F/ SNSV CPbeg R and SNSV CPbeg F/ SNSV CPend R (Table 5.2) were developed to amplify the complete MP and CP genes, respectively, as well as the intergenic region between the two genes. To determine the 3' end of the molecule, RNA was extracted as described above and poly A polymerase used as previously described (Sippel, 1973) to introduce an oligo adenosine tail at the 3' end of the molecule. Reverse transcription was performed as described above using the Adapter primer (Table 5.2) instead of random oligonucleotide hexamers. For PCR amplification, primers SNSV CPend F and Adapter primer were utilized (Table 5.2). The reaction was performed twice. For verification of the 5' end of the RNA we performed 5' RACE using primers SNSV MPbeg R and Abridged Anchor Primer (AAP) (Table 5.2). All amplification reactions were performed using the Taq polymerase. The PCR program for amplification of all fragments, except for the first cycle of the 3' end amplification reaction where the annealing step was at 42^0C, consisted of an original denaturation step of 5 min at 94^0C followed by 40 cycles of 30 sec denaturation at 94^0C, 30 sec annealing at 50^0C or 55^0C and 1.5 min extension at 72^0C and a final extension step for 10 min at 72^0C. For routine detection of SNSV, primers SNSV CPbeg F/ SNSV CPend R were used. Amplification of the TSV isolates were performed utilizing oligonucleotide primers TSV CP F / TSV CP R that were developed from sequences of the TSV-WC isolate (GenBank Accession number NC 003845) (Table 5.2). The PCR program was identical to the one described above.

Table 5.2. Oligonucleotide sequence used for detection of SNSV and TSV and determination of the sequence of RNA 3 of SNSV.

Primer Name	Nucleotide sequence (5'-3')
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
SNSV MPbeg R	CAGTGTTTACGGCTGCGAAG
SNSV MPbeg F	GGGATCGATTGGTTAGGACCGTCAT
SNSV CPbeg R	ACACCACCATTGCGCATACATCTC
SNSV CPbeg F	GAGTATTTCTGTAGTGAATTCTTGGA
SNSV CPend R	ATTATTCTTAATGTGAGGCAACTCG
SNSV CPend F	GACCTAATCCGTTGATGCCTCCAGA
Adapter Primer	GGCCA CGCGT CGACT AGTAC$(T)_{18}$
TSV CP F	ACGAGTATTAAGTGGATGAATTCT
TSV CP R	ACTTACAATACGTCGAGGTGTG

5.3.4 Sequence alignment and phylogenetic analysis

The consensus sequence of each isolate was achieved after cloning the PCR products into the pCR4-TOPO® vector and sequencing of the PCR product and four individual clones in both directions using M13 forward and reverse primers. Alignments of the sequences were performed employing the ClustalW program (European Bioinformatics Institute, http://www.ebi.ac.uk/clustalw). For the phylogenetic analysis maximum parsimony was performed utilizing the PAUP* 4.0b 10 software (Swofford, 2001). The amino acid sequences of the CP genes were aligned and backtranslated (Wernersson and Pedersen, 2003). Heuristic search with ten replicates of random taxon sequence addition and the tree bisection reconnection swapping algorithm were applied for the reconstruction of the phylogram. The bootstrap analysis consisted of 1000 replications utilizing the same parameters as above. The accession numbers for RNA 3 of the virus and all CP sequences of the isolates from *Fragaria* and *Rubus* deposited in GenBank are AY 363228-363242.

5.4 Results

The complete nucleotide sequence of RNA 3 of SNSV was determined. The length of the molecule is 2248 nucleotides. The first open reading frame (ORF) is 897 nucleotides long and encodes a protein of 32.3 KDa that is the putative MP of the virus.

The CP gene is 669 nucleotides long and has a predicted product of 24.4 KDa. Between the two genes there is a small ORF of 102 nucleotides without significant similarity to any gene in the database and this was predicted not to be a virus-encoding gene sequence (Virus gene identification, www.softberry.com). The MP of SNSV shares 74% and 63% amino acid sequence identity with TSV and *Parietaria mottle virus* (PMoV) (GenBank Accession number U35145) (Fig. 5.1A), respectively.

The amino acid sequence identity of the CP of all SNSV isolates range from 60-65% to the TSV-WC, while the identities to PMoV were 45-46% (Fig 5.1B). The overall nucleotide sequence identity of the Maryland strawberry SNSV isolate and the TSV-WC RNA 3 are 70%. Sequence data of more than 1 Kb at the 5' end of the polymerase gene in RNA 2 of SNSV shows similar homology to TSV as in the case of RNA 3, with 70% nucleotide and 72% amino acid identity to polymerase gene of the TSV-WC (Tzanetakis I.E. and Martin R.R., unpublished data). Three isolates of TSV from non Rosaceous hosts were used to confirm that the variation between the SNSV isolates sequenced and TSV were not artifacts of our cloning and sequencing procedures. PCR products and clones for the TSV-WC CP as well as one isolate from bean and one from tobacco from our own virus collection were sequenced. The sequence obtained for the TSV-WC isolate was 100% identical to the published sequence demonstrating that the differences between TSV-WC and SNSV observed in this work were not due to errors in sequencing (data not shown).

Primers developed for amplification of the coat protein (CP) of TSV-WC failed to generate any amplicons when applied to the detection of isolates of SNSV from either *Fragaria* or *Rubus* species (Fig. 5.2). The reverse was also true in that primers developed to amplify the CP of SNSV was used successfully to amplify fragments from each of the isolates listed in Table 5.1, but failed to yield amplicons when RNA from TSV-WC infected plants was used as template for RT-PCR (Fig. 5.2).

Fig. 5.1 A. Amino acids alignment of the movement proteins of *Strawberry necrotic shock virus* (SNSV) (Maryland strawberry isolate, GenBank Accession number AAQ 76575), *Tobacco streak virus* (TSV-WC) (White clover isolate GenBank Accession number CAA 25132) and *Parietaria mottle virus* (PMoV) (GenBank Accession number AAA80344. **B.** Amino acids alignment of the coat proteins of SNSV, Maryland strawberry isolate, GenBank Accession number AAQ 76576; TSV-WC, GenBank Accession number CAA 25133; and PMoV, GenBank Accession number AAA80345. Asterisks indicate the identical amino acids in all three proteins.

A

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SNSV MALTPSFKALTFSADDESSLEKAVTEALSGSVELNMGIRRCAAFAAVNTEAFLCELTTKE  60
TSV  MALAPTMKALTFSADDETSLEKAVTEALSGSVDLNMGLRRCAAFPAENTGAFLCELTTKE  60
PMoV MAFVPTKTALTFSAEDESGLEKAVSDALSGCVELNMGIRRCAAFPAINQDAFLCELTTKE  60
     **  *   ****** **  *****  **** * **** ****** * *  **********
SNSV TKGFVSKFTDKVRGRVFVDHAVVHLMYIPVILNTTHAIADLKIKNLATGDELYGGTKVNL  120
TSV  TKSFIGKFSDKVRGRVFIDHAVIHMMYIPVILNTTHAIAELKLKNLATGDELYGGTKVNL  120
PMoV AKGVLRSLADKVRGRMFVDHAVIHLMYIPVILDTTHAVADLKLKNLATGDELYGGTKVNL  120
      *       ****** * **** * ******* **** * ** *****************
SNSV NEAFILTMTWPRSLFSDAVATHRGLYLGGTVSCASSVPKGAKIGMWYPMWTEKVSNKQLY  180
TSV  NKAFILTMTWPRSLFAEAVHNHKGLYLGGTVSCASSVPAHAKIGMWYPIWSEKVSIKQLY  180
PMoV NEAFILTMTWPRSLFADAVNEHKGLYLGGTISCSSSVPRHAKVGMWYPLWTEKVSNKQLY  180
     * ************  **  * ******* ** ****  ** ***** * **** ****
SNSV QQTTPIVNTKALETFTRTMISSDKEMRSLLRSRASIDIAAKTKENPVICSPSVNLLDNQT  240
TSV  QNTIDIHKTEAIETFTPTMISSDKEMRSLLRSRASIDVAAKTREKPVICSERVSLLDQHT  240
PMoV QKTVALTNTRAIETFARTMIHSDKEMRSLLRSRASTDIASKRYENPVLCSSRIGLMDNAT  240
     * *     * * ***  *** ************** * * *  * ** **    * *  *
SNSV SGVDFTVKRVSIPTVAAIQNNDDVEASGTSVLESKMIPIEHSDGNVGSNSAGRNLLTV---  298
TSV  QGVDFTVTEIEP------EKDDD---AGTSILGPKMVPIEQVPSVKLSSEAGRNLLTA---  289
PMoV AGIDFTVNRLEE------TAVDDTDPTGSDVLVPRST--ISTDSKLRVSETLPTLGSDESN  293
      * ****              **    *   *                        *
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B

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SNSV ----------MSVNRGKNVLNNNSNGCPTCFDKLDAAATNCARCMRNGGVSNRQRRNAR  49
TSV  MNTLIQGPDHPSNAMSSRANNRSNNSRCPTCIDELDAMARNCPAHNTVNTVSRRQRRNAA  60
PMoV ----------------------MYELFNHSLQPNQLTPCLRTRANGGQVSNRQSRNVR  36
                                                    ** ** **
SNSV RAANFRNQ--------RPVPVVPVSAPT-TSKSLKLPGNQVWVTRKPSDWAAKTVDTNDA  100
TSV  RAAAYRNAN---ARVPLPLPVVSVSRPQ-AKASLRLPNNQVWVTRKASEWSAKTVDTNDA  116
PMoV RAAAFRNSQQTSARIPVPVPVIPVSRPNGPKASLKLPNNQVWVYKVASELAAKTSDANDA  96
     ***  **          * **  ** *     ** ** *****    *   *** * ***
SNSV ITLKTIFNGIPEINDDTKIFRVLIGFVAVSDGTFGLVDG--VVGGSIPDPPIIGRLGFKK  158
TSV  IPFKTIVEGIPEIGAETKFFRLLIGFVAVSDGTFGMVDG--VTGDVIPDPPVVGRLGFKK  174
PMoV ISLTTMLSGISDVKPETKLYRLVFGFVAESDGSFGVVEDENVSGNVVPDPPVVGRAGFKK  156
     *   *   **       **  *   **** *** ** *    * *   **** ** ****
SNSV NTYRSRDFRFDGKLTPQVADKAIVWCLDSNRRDAKRVRLADFWIAISRPNPLMPPDEFLS  218
TSV  NTYRSRDFDLGGKLLNQLDDRAVVWCLDERRREAKRVQLAGYWIAISKPAPLMPPEDFLV  234
PMoV HTNKCRDVNLEGKTPDELKNMAVVWCLDENRKVAKRIAFTHFWFAISRPSPLMPPENILV  216
      *   **    **        * *****  *  ***      * *** * *****   *
SNSV GDSD  222
TSV  NQD-  237
PMoV DGNQ  220
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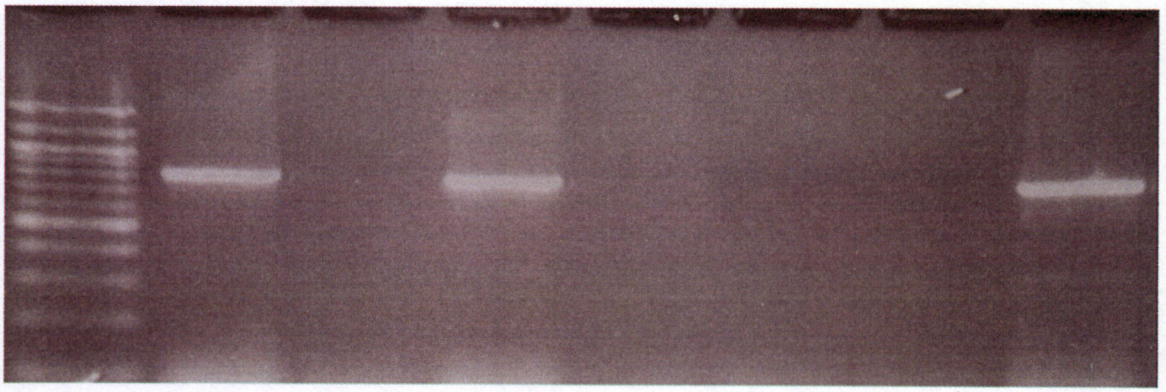

Fig. 5.2. Reverse transcription-polymerase chain reaction (RT-PCR) for amplification of the coat protein gene of *Strawberry necrotic shock virus* (SNSV) and *Tobacco streak virus* (TSV). Lane 1: 100 base pair DNA marker (Gibco-BRL, Gaithersburg, MD), lanes 2-5: RT-PCR using SNSV specific primers - lane 2: Isolate 9034 from strawberry, lane 3: TSV white clover (TSV-WC) isolate, lane 4: BRLV isolate 9012, lane 5: TSV-WC isolate. Lanes 6-8: RT-PCR using TSV specific primers - lane 6: Isolate 9034 from strawberry, lane 7: BRLV isolate 9012, lane 8: TSV-WC isolate.

The CP of CRUB 9012 (Table 5.1), a plant in which Black raspberry latent virus (BRLV) was first identified was amplified and sequenced utilizing the SNSV primers, while no amplicons were obtained from this source using the TSV specific oligonucleotide primers (Fig. 5.2, and 9012 in Fig. 5.3). This confirms the grouping of BRLV as an isolate of SNSV (Brunt and Stace-Smith, 1976; Jones and Mayo, 1975) rather than a distinct virus (Converse and Lister, 1969), or a strain of TSV.

Phylogenetic analysis showed that of all the *Fragaria* and *Rubus* isolates formed two distinct but closely related clusters that were distinct from TSV (Fig. 5.3). One *Fragaria* isolate (MD) clustered with the *Rubus* isolates. It can also be seen in this phylogram that *Spinach latent virus* and *Citrus variegation virus* are two distinct viruses.

5.5 Discussion

Strawberry necrotic shock virus (hereafter referred to as SNSV) has been a chronic disease problem in strawberry, blackberry, and black and red raspberry

production. In addition to the yield losses the virus causes in single infections (Johnson *et al.*, 1984), it has synergistic effects in double infections (Stace-Smith *et al.*, 1987). Since

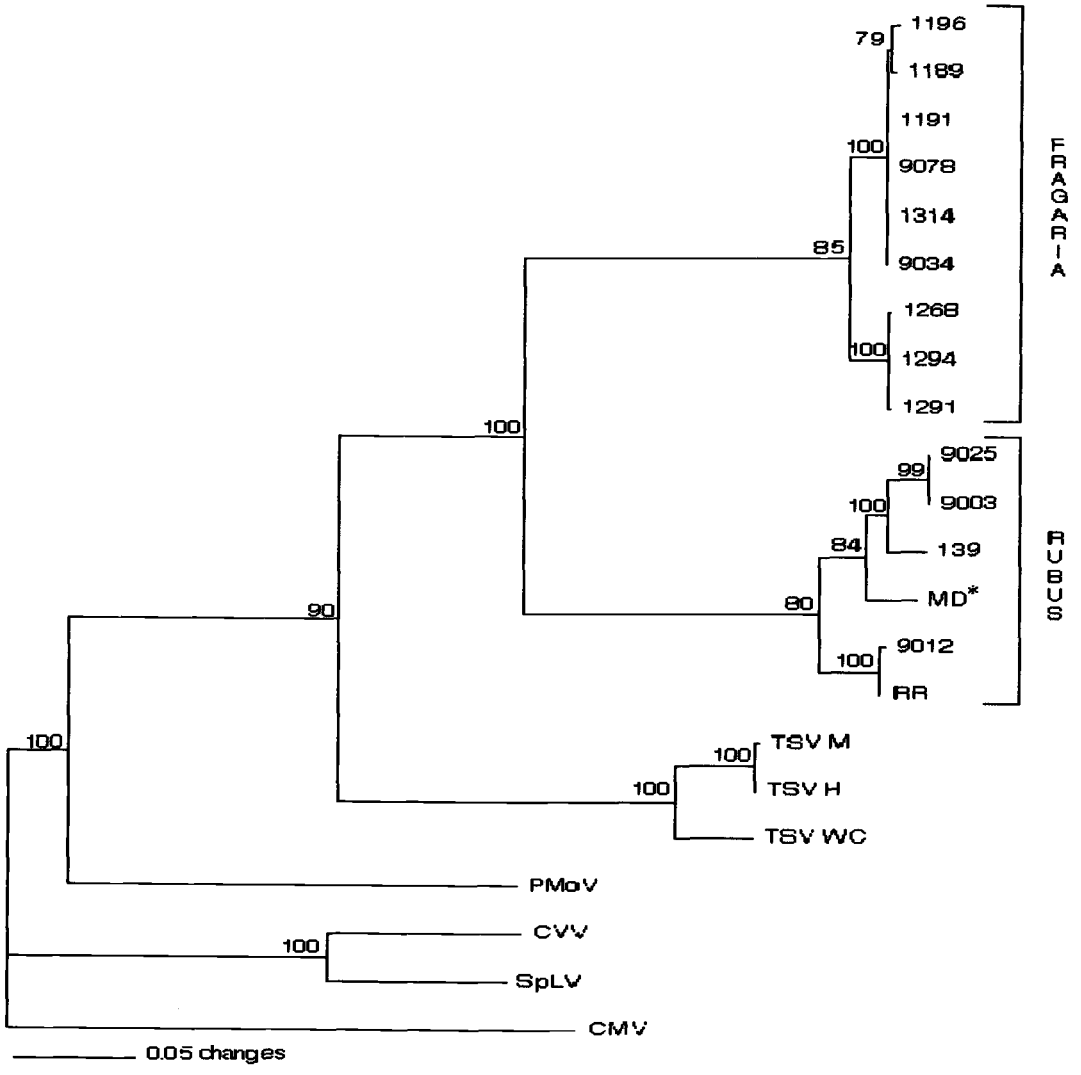

Fig. 5.3. Phylogram of the coat protein gene of 15 *Strawberry necrotic shock virus* isolates and other Bromoviruses. The strawberry and *Rubus* isolates are identified in Table 5.1. Abbreviations and GenBank Accession numbers of the other viruses: TSV-WC, *Tobacco steak virus* (TSV) white clover isolate, NC 003845; TSV-M, TSV Mungbean isolate, AF 515823; TSV-H, TSV Sunn-hemp isolate, AF515825; PMoV, *Parietaria mottle virus,* U 35145; CVV - *Citrus variegation virus,* AF 434912; SpLV, *Spinach latent virus*, NC 003810; CMV, *Cucumber mosaic virus*, AF 523352. The asterisk indicates the strawberry field isolate from Maryland, USA, that clusters with the *Rubus* isolates. The bootstrap values are given in percentage values. The bar is normalized to represent 0.05 nucleotide changes per site. CMV is used as the outgroup.

the virus is pollen- and seed-transmitted, it is also of concern in small fruit breeding programs.

The similarities of the CP of the SNSV isolates of this study and the published TSV isolates confirm the results that immunological methods have previously given (Brunt and Stace-Smith1976; Fulton 1967). Superinfection using red and black raspberry isolates failed to protect plants against other isolates of TSV (Fulton, 1978). Strawberry and blackberry isolates of SNSV (formerly identified as TSV isolates) failed to hybridize in Northern blots with three TSV isolates (Stenger *et al.*, 1987), we were unable to detect TSV (as distinct from SNSV) in any of the *Fragaria* or *Rubus* isolates (SNSV) used in this study. This may be an indication that *Fragaria* and *Rubus* are hosts of SNSV but not of TSV. Mechanical transmission of viruses to small fruit crops is a challenging task, and what were previously considered TSV isolates of small fruits (SNSV) are not readily mechanically transmitted to *Fragaria* or *Rubus* (Martin R.R., personal observation). For this reason we did not attempt to mechanically transmit our TSV isolates to small fruit crops to verify whether TSV is able to infect small fruits.

We were originally unsure whether SNSV was a distinct virus from TSV, but the molecular data revealed major differences in the MP and CP of the two viruses. SNSV encodes an additional nine amino acids at the C' terminus of the MP while it lacks the first 12 amino acids in the CP gene indicating that SNSV is distinct from the four TSV isolates found in GenBank from white clover, mungbean, cotton, and sunn-hemp (GenBank accession numbers NC 003845, AF515823, AF515824, AF 515825 respectively). The phylogenetic analysis (Fig. 5.3) revealed clustering of all strawberry and *Rubus* isolates. The Maryland field isolate used to determine the complete sequence of RNA 3 is the only strawberry isolate that clusters with the *Rubus* isolates. Since all the *Rubus* isolates came from the U.S. Pacific Northwest (PNW), there are two possible explanations for this exception. The clustering is due to geographical isolation and the Maryland source was infected with a PNW isolate from strawberry or *Rubus*. This explanation is less likely since the North Carolina (1314) and California (9034) isolates are identical. If we assume that strawberry and *Rubus* isolates do indeed form two distinct clusters, the explanation for the Maryland isolate clustering would involve an intraspecies

transmission between *Rubus* and strawberry, possibly by thrips (Sdoodee and Teakle, 1993).

The use of primers designed against TSV sequence likely would not be useful in detection of SNSV. This was observed with a number of primers that were developed for TSV or SNSV, where the primer pairs designed for SNSV were useful for detection of SNSV but not TSV (Fig. 5.2). The reverse was also true in that TSV primers were not useful for the detection of SNSV. It should be noted that these two viruses do cross react serologically with polyclonal antisera (Fulton, 1967). However, spurs do form suggesting differences in epitopes on the two viruses and thus caution should be used if one uses monoclonal antibodies specific for either of these viruses since they may not detect the other virus. This is important since most certification programs use serology as a means of detection for these viruses

This study identifies a new member of the *Ilarvirus* genus that was characterized previously as a cluster of isolates of the highly heterogeneous TSV species. It may be that what we think of today as a single virus species, TSV, is a cluster of related but distinct viruses as appears to be the case with the viruses in subgroup 2 of the genus *Ilarvirus* (Scott *et al.*, 2003). At the nucleotide level the CP of the viruses in subgroup 2 of the genus *Ilarvirus* have identities ranging from 62-84% which is similar to the identities observed between CP gene of TSV and SNSV. The amino acid sequence identities of the CP of SNSV isolates range from 60-65% to the published sequence of TSV-WC. This is in the same range as observed between different viruses in subgroup 2 of the genus *Ilarviruses* that ranges from 58-79%. As an example, the CP of *Citrus variegation virus* (CVV, Genbank Accession number AF 434912) has 67% amino acid sequence identity with *Spinach latent virus* (SpLV, Genbank Accession number NC 003810), both shown in the phylogram in figure 5.3. *Elm mottle virus* and CVV, both members of subgroup 2, have 79% sequence identity at the amino acid level. If one compares the similarity between the viruses in subgroup 2 with that observed between TSV and SNSV it is clear that SNSV should be considered a distinct virus rather than a strain of TSV.

5.6 Acknowledgments

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5.7 References

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Chapter 6

Molecular detection, phylogenetic analysis and geographic distribution of *Fragaria chiloensis latent virus* in North America

Ioannis E. Tzanetakis and Robert R. Martin

6.1 Abstract

Fragaria chiloensis latent virus (FClLV), was first identified in the early 1990's. The virus was found only in *F. chiloensis* plants from South America and while large scale immunological testing took place on material from North America, the virus was not found in this part of the world. DsRNA from FClLV infected *Fragaria chiloensis* plants from Chile was extracted, cloned and sequenced. Sequences belonging to the polymerase and coat protein (CP) genes have been identified. A sensitive reverse transcription-polymerase chain reaction (RT-PCR) test for the detection of the virus has been developed, amplifying a 333 base pairs fragment of the polymerase gene. Applying the RT-PCR detection method we were able to detect the virus in North America, a region previously thought to be free of the virus. Phylogenetic analysis based on the CP gene sequence reveals that FClLV is related most closely to *Prune dwarf virus*, the type member of subgroup 4 of the *Ilarvirus* group.

6.2 Introduction

Fragaria chiloensis latent virus (FClLV) was first identified in 1993 (Spiegel *et al.*, 1993) and only was detected in *Fragaria chiloensis* (Chilean strawberry) plants from Chile. In that study, over 2000 *F. chiloensis* plants from along the coast from California to Alaska tested negative for FClLV. *F. chiloensis* is distributed along the west coast of the Americas from Alaska to the southern regions of Chile, except for the tropics (Daubeny, 2003). In North America, *F. chiloensis* is referred to as the 'beach strawberry' since it is only found very close to the ocean, whereas, in Chile this species is found inland and is grown for its' fruit. FClLV is a member of the *Ilarvirus* genus of the family *Bromoviridae* (Spiegel *et al.*, 1993). Ilarviruses are positive-strand tripartite RNA viruses and encode four or five proteins (Bol, 1999). RNA 1 is monocistronic encoding for the virus replicase. The gene has signature methyltransferase and helicase motifs and in the case *Brome mosaic virus*, the type member of the family, it has been shown to be directly involved in the replication complex not only as an enzyme but also by anchoring the

replication complex onto cell membranes (den Boon *et al.,* 2001). RNA 2 encodes the RNA-dependent-RNA polymerase (RdRp) of the virus. Some members of the family sequenced to date also contain a second ORF on RNA 2 that encodes a gene product that may be involved in suppression of RNA interference (RNA silencing) (Brigneti *et al.,* 1998). The movement and coat proteins of the viruses are encoded in RNA 3. The coat protein is expressed via RNA 4, which is a subgenomic produced from RNA 3, and, in addition to protecting the genome and being involved in virus movement, it is also required for genome activation (Jaspars, 1999; Neeleman *et al.*, 2004).

More than 15 viruses have benn identified to infect strawberry (Spiegel and Martin 1998, Tzanetakis *et al.*, 2003; Tzanetakis *et al.,* 2004). Strawberry plants are generally asymptomatic when infected with a single virus, while infection with multiple viruses can result in a serious decline symptom and loss of production (Converse, 1987). This latency in single infections has made virus detection a complex undertaking in strawberry. Until recently, reliable detection was based on grafting of suspicious material onto *F. virginiana* and *F. vesca* clones (Spiegel and Martin, 1998) that developed symptoms characteristic for many of the viruses. In recent years, strawberry virus detection has improved dramatically with the application of reverse transcription-polymerase chain reaction (RT-PCR) (Thompson *et al.*, 2003; Tzanetakis *et al.*, 2003; Tzanetakis *et al.*, 2004). Although FClLV can be detected using monoclonal and polyclonal antibodies, there may be strains of the virus that are not detected using the available antibodies. Also, since the virus is seed transmitted readily and *F. chiloensis* is used in breeding programs there is a need for the development of a more sensitive detection test. These facts along with recent problems of decline in strawberry in California, and the Pacific Northwest contributed to the decision to clone cDNA derived from dsRNA from FClLV infected plants. An RT-PCR test that amplifies a fragment of the polymerase (RdRp) gene of RNA 2 of the virus has been developed and used to detect the virus in commercial strawberry fields (*Fragaria x anannasa*) in California, Oregon, Washington and British Columbia, regions previously thought to be free of this virus. Phylogenetic analysis utilizing the coat protein gene (CP) places the virus in subgroup 4 of the *Ilarvirus* genus along with *Prune dwarf virus* (PDV).

6.3 Materials and methods

6.3.1 DsRNA extraction and cloning

DsRNA was extracted as described previously (Yoshikawa and Converse, 1990) from National Clonal Germplasm Repository (Corvallis, OR) virus collection clone *Fragaria chiloensis* CFRA 9087. First- and second-strand cDNA synthesis were performed as described previously (Tzanetakis *et al.*, 2004) employing dsRNA extracted from five grams of tissue. The DNA fragments were cloned into a pCR4 TOPO® vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Polymerase chain reaction (PCR) was performed on the recombinant plasmids utilizing primers M 13 forward and reverse to identify those with the largest inserts. Plasmids were sequenced utilizing an ABI 3700 DNA sequencer (Macrogen Inc., Seoul, Korea).

6.3.2 Sequence and phylogenetic analysis

The acquired sequences were compared using BLAST (blastn and blastp) (Altschul *et al.*, 1997) to identify the regions of the genome that were cloned. The clones of the polymerase and CP genes were aligned utilizing the ClustalW software (European Bioinformatics Institute http://www.ebi.ac.uk/clustalw/). Oligonucleotide primers were developed after aligning at least two cDNA clones and were used in PCR in which the template consisted of RT reactions using total RNA from FClLV infected strawberry as template and random primers. The consensus of the sequence presented here was acquired by aligning the sequences of the original clones and the products of two individual RT-PCR reactions for each. The GenBank accesion numbers are AY451391 for the polymerase fragment and AY562497 for the CP gene. Maximum parsimony was performed utilizing the PAUP* 4.0b 10 software (Swofford, 2001) utilizing heuristic search with ten random addition replications and the tree bisection reconnection swapping algorithm. The bootstrap analysis consisted of 1000 replications utilizing the same parameters as above.

6.3.3 Molecular and immunological detection

RNA of plants from southern California, Oregon, Washington and British Columbia was extracted and reverse transcribed as described previously (Tzanetakis *et al.*, 2004). PCR was performed utilizing the Platinum Taq ® polymerase (Invitrogen, Carlsbad, CA) and primers FC F (5' ACCACTTCACCACCAGATCG 3') and FC R (5' CAAGCCAACTCACCATGACC 3'). The PCR program consisted of original denaturation for 5 min at 94^{0}C followed by 40 cycles of 45 sec at 94^{0}C, annealing for 30 sec at 55^{0}C and extension for 1 min at 68^{0}C. The final step included incubation of the reaction at 68^{0}C for 10 min. The same PCR program, with the exception that the extension time of 1.5 min, was used for amplification of the polymerase region and 1 min for the CP region.

ELISA was performed as described previously (Converse and Martin, 1990). Polyclonal and monoclonal antibodies and polyclonal goat anti-mouse alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) were applied in triple antibody sandwich (TAS) enzyme-linked immunosorbent assay (ELISA). For double antibody sandwich (DAS) ELISA, purified IgG of the polyclonal antibody was used for coating and the same IgG conjugated with alkaline phosphatase was used as the detection antibody. Immunocapture RT-PCR (IC-RT-PCR) was performed as described previously (Wetzel *et al.*, 1992). ELISA plates were coated with antibodies specific to *Strawberry necrotic shock virus* (SNSV), *Prunus necrotic ringspot virus* (PNRSV), *Apple mosaic virus* (ApMV) and FCILV for 2-4 hours at room temp. The plates were then washed and leaf sap added before incubation overnight at 4^{0}C. Leaf sap also was added directly to ELISA plates without pre-coating with antibodies. The plates were then washed with phosphate buffer saline-Tween-20 (PBS-T) and 50 µl of RT-mix added to the plates and incubated at 37^{0}C for 50 min. The RT reaction then was used as template for PCR as described above. All the immunological tests were performed utilizing FCILV antibodies developed in the original study of the virus (Spiegel *et al.*, 1993).

6.4 Results

DsRNA extractions from FClLV gave similar patterns to the RNA pattern of the original study of FClLV (Spiegel *et al.*, 1993; Fig. 6.1).

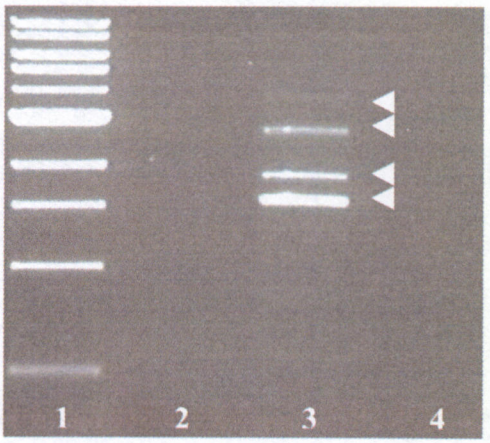

Fig. 6.1. DsRNA pattern of *Fragaria chiloensis latent virus* (FClLV). Lane 1. One kilo base-pair marker (BRL, Gaithersburg, MD); lane 2: Blank; lane 3: dsRNA extracted from clone CFRA 9087; lane 4: dsRNA extracted from healthy strawberry. Arrowheads indicate the dsRNA bands associated with FClLV.

In the IC-RT-PCR assays amplicons were acquired only from plates that were coated with the anti-FClLV antibodies and not with any of the other three anti-*ilarvirus* antibodies used suggesting that they failed to capture FClLV particles (Fig.6.2). The direct coating with the plant sap may have failed to give any amplicons with IC-RT-PCR probably because of the instability of the virions that may have broken upon attachment to the plastic matrix of the plate. RT-PCR was used to detect FClLV in strawberry samples from the U.S. and Canada. More than 10 PCR products were sequenced to verify that the polymerase region of the virus was amplified. All of the sequenced amplicons showed greater than 98% nucleotide identity with the sequence of the Chilean isolate (CFRA 9087). Both DAS-ELISA with polyclonal antibodies and TAS-ELISA with polyclonal and monoclonal antibodies, failed to detect the virus in plants other than four *F. chiloensis* plants from NCGR (accession numbers CFRA 9087-9090) that were FClLV positive. The RT-PCR protocol used did not give quantifiable results, but all four positive controls gave consistently more intense amplicons resolved on agarose gel

electrophoresis than any of the positive *F. x ananassa* plants. That may be an indication that *F. chiloensis* is a better host for the virus than *F. x ananassa*.

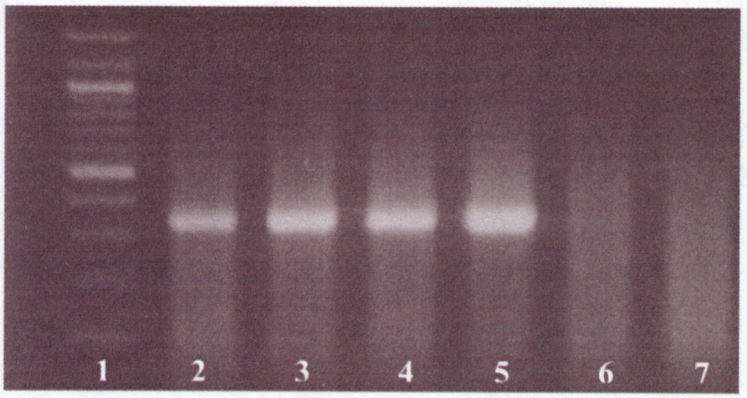

Fig. 6.2. Immunocapture RT-PCR for detection of *Fragaria chiloensis latent virus*. Lane 1: 100 base pair DNA marker (Gibco-BRL, Gaithersburg, MD); lanes 2-5: FClLV National Clonal Germplasm Repository accession numbers CFRA 9087-9090; lanes 6-7: Virus-free plants

The highest percentage of infection was found in Washington where 30% of the plants tested were infected with the virus while the virus was absent in the few samples tested from New York, Maryland, North Carolina and Florida (Table 6.1).

Table 6.1. Strawberry samples tested for the presence of *Fragaria chiloensis latent virus* (FClLV).

Sampling area	Samples tested	FClLV infected samples
California	112	25
Maryland	15	-
Oregon	24	3
Washington	34	10
Florida	17	-
New York	6	-
North Carolina	8	-
Canada	34	4

The phylogenetic analysis disclosed that FClLV is related most closesly to *Prune dwarf virus* (PDV), the type and sole member of subgroup 4 of the Ilarvirus genus (Fig. 6.3).

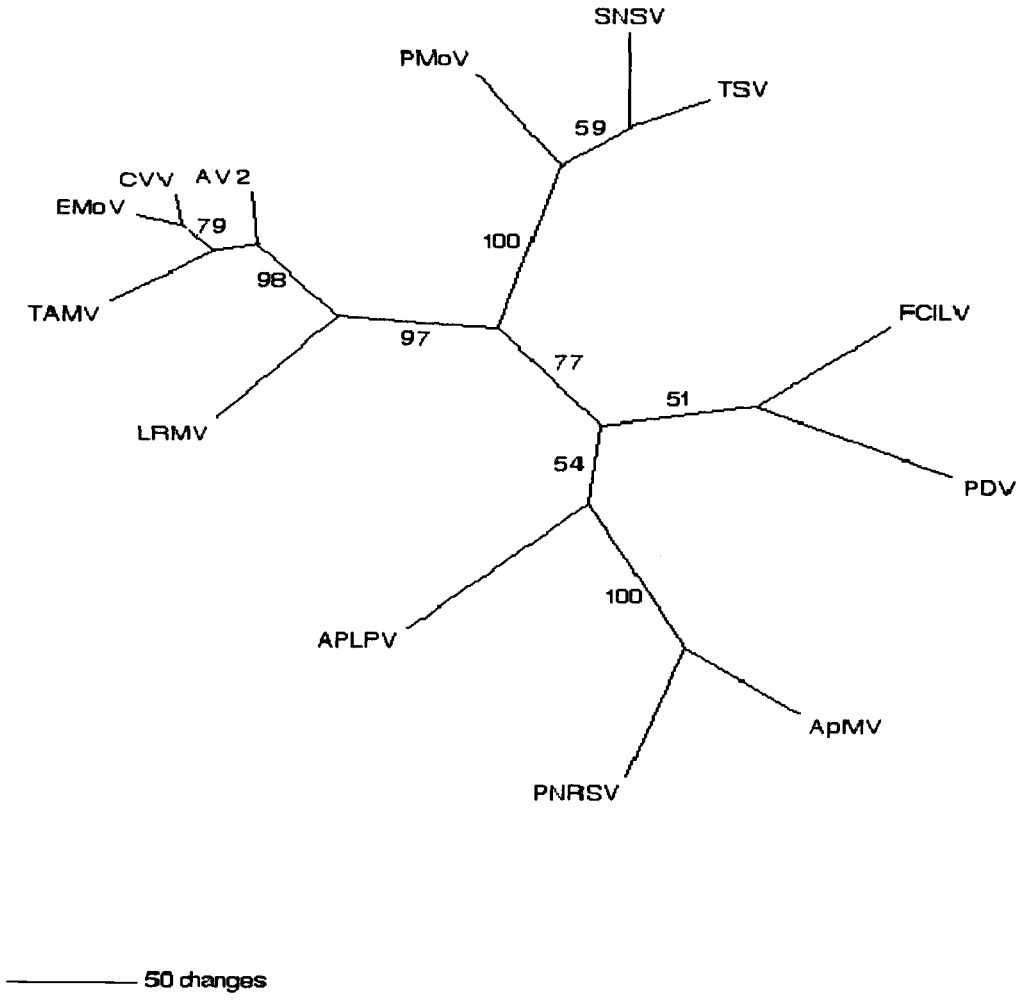

Fig. 6.3. Phylogram of the coat protein of *Fragaria chiloensis latent virus* and other ilarviruses. Abbreviations and GenBank accession numbers: FClLV, *Fragaria chiloensis latent virus,* AY562497; PDV, *Prune dwarf virus*, AF208740; ApMV, *Apple mosaic virus,* S78319; PNRSV, *Prunus necrotic ringspot virus,* AY037790; APLPV *American plum line pattern virus,* NC003453; SNSV, *Strawberry necrotic shock virus,*AY363228; TSV, *Tobacco streak virus*, NC003845; AV-2. Asparagus virus – 2, X86352; TAMV, Tulane apple mosaic virus, NC003835; EmoV, *Elm mottle virus*, NC003570; CVV, *Citrus variegation virus*, AF434911; LRMV, *Lilac ring mottle virus,* U17391. Bootstrap values are shown as percentage value and only the nodes over 50% are labeled. The bar represents 50 amino acid changes over the length of the proteins.

6.5 Discussion

This study demonstrated the presence of FCILV in North America, a region previously thought to be free of this virus. Utilizing IC-RT-PCR it was verified that the ilarvirus identified in strawberries from North America was the same as that previously reported from Chile (Spiegel *et. al.*, 1993). Ilarviruses are known to be transmitted horizontally by pollen and thrips and vertically by seed (Sdoodee and Teakle, 1987). The lack or the very mild symptoms that this virus causes on indicator plants may explain the failure to detect this virus previously in graft indexing programs and explain the presence of this virus in most regions tested. Since the isolates of the virus from North America were captured by polyclonal antibodies and detected in IC-RT-PCR it suggests that the monoclonal antibodies used for routine testing may be specific for strains from Chile. This would have explained the negative ELISA results when testing the strawberries that were positive for FCILV by PCR or IC-RT-PCR. However, when direct conjugates were made with the polyclonal antibodies and used in DAS-ELISA we still failed to detect FCILV in the plants that were positive by RT-PCR. This suggests that the virus titer in *F. x ananassa* may be below the level of detection by ELISA whereas in *F. chiloensis* the titers are higher and detectable by ELISA.

Strawberry breeders often use *Fragaria* species including *F. chiloensis* in their programs to incorporate the positive trait of the species. The pollen transmission of the virus exceeds 50% (Spiegel *et al.*, 1993) and may be the primary method of dissemination of the virus in North America. There is always the possibility that the virus is present in the native populations of *F. chiloensis* of the North America west coast, although this possibility less likely since plants grown inland were found infected.

During the original characterization of the virus the authors used trapping of particles on electron microscope grids as a means to identify serological relationships between FCILV with other ilarviruses. Applying this technique, the virus was reported to be most closely related to *Lilac ring mottle virus* and *Asparagus virus -2,* viruses belonging to *Ilarvirus* subgroups 7 and 2, respectively. Based on sequence information

reported here FCILV is most closely related to PDV, these differences may be the result of the quality of the antisera used in the initial study.

In this study based on amino acid sequence comparisons of the CP gene, FCILV is related most closely to *Prune dwarf, Prunus necrotic ringspot, Apple mosaic,* and *American plum line pattern viruses,* ilarviruses that primarily infect plants belonging to the family *Rosaceae*, and FCILV fits this grouping (Fig. 6.3) as strawberry is a member, of the *Rosaceae.*

The presence of FCILV in North America in addition to that of *Strawberry latent ringspot virus* (Martin *et al.*, 2004) emphasizes the need of further survey studies to determine if other viruses of strawberry not known to infect strawberry in North America may also be present in North America strawberry (namely *Arabis mosaic*, *Tomato black ring* and *Raspberry ringspot* viruses). This study also points to the need of better detection tools for strawberry viruses that would make detection not only more sensitive but also faster. Although immunological methods are straight-forward and many samples can be processed in relatively short period of time the detection limits make them insufficient for detection of some strawberry viruses (this study, Tzanetakis *et al.*, 2004). The use of RT-PCR has greatly improved detection but it is time consuming and expensive when testing for many viruses as in the case of strawberry. Lately, the utilization of microarrays has improved detection of viral pathogens (Wang *et al.*, 2002). Since strawberry is propagated vegetatively and plants are grown for several years in the field during plant increase to get the number of plants required for production, the need for sensitive rapid virus detection is important to monitor the virus status in the production of certified strawberry plants. Since there is sequence information currently available for the majority of strawberry viruses, the use of microarrays or other similar technologies would be possible. This would greatly improve detection and help identify where viruses are being introduced into the strawberry plant production system pinpointing where the control measures need to be applied to minimize viruses in strawberry nurseries.

The aim for the future is to complete the sequence of FCILV and study the variability of the virus in the different regions that it occurs.

6.6 Acknowledgments

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Chapter 7

Conclusion

Ioannis E. Tzanetakis

7.1 Criniviruses in strawberry

Whitefly transmitted viruses have become an immense problem in world agriculture, due to migration and naturalization of the whitefly vectors of the viruses and movement of plant germplasm. Whitefly-transmitted geminiviruses belonging to the genus *Begomovirus* (Thompson, 2002) and closteroviruses belonging to the genus *Crinivirus* are the predominant virus genera that have emerged in the last decade (Wisler *et al.*, 1998; Rubio *et al.*, 1999). The losses caused by these viruses and discovery of new viruses in these genera are increasing by the year (Abou-Jawdah *et al.*, 2000; Tomassoli *et al.*, 2003) making their study crucial in order to understand the etiology and epidemiology of the causal agents, develop detection tools and strategies to minimize their impact in agricultural production.

Prior to the work presented in this dissertation there were no whitefly-transmitted viruses known to infect strawberry. This work identified and characterized two criniviruses infecting strawberry, *Strawberry pallidosis associated virus* (SPaV) and *Beet pseudo-yellows virus* (BPYV) and associated both with symptoms typical of the pallidosis disease. The complete nucleotide sequence of both viruses has been determined and molecular detection tests have been developed for both viruses. The epidemiology of SPaV was further studied, including the identification of a whitefly vector, host range, as well as pollen and seed transmission of the virus.

A survey for the presence of the viruses in the major strawberry producing areas of the U.S. revealed that these two viruses are present at higher incidences than other strawberry viruses in most production areas of southern U.S. (Table 7.1). The abundance of the viruses in the south and their absence or limited incidence in northern latitudes coincides with the presence of greenhouse whitefly, the vector of the viruses, in strawberry fields. Presence in northern latitudes may suggest that there is limited transmission of this virus in nurseries. All plants for strawberry production in the Pacific Northwest are grown in California, in the same nurseries that grow the plants for California strawberry growers. This suggests that most of the infection in California is

occurring in the production fields rather than in nurseries, though limited spread may be happening in the nurseries.

7. 2 Ilarviruses in strawberry

Tobacco streak virus (TSV) (Johnson, 1936; Frazier *et al.*, 1962) and *Fragaria chiloensis latent virus* (FClLV) (Spiegel *et al.*, 1993) were two viruses previously identified in strawberry that are transmitted through pollen and seed and potentially by thrips (Sdoodee and Teakle, 1987). It was determined that strawberry isolates of TSV form a distinct phylogenetic cluster and were characterized as a new virus in strawberry designated as *Strawberry necrotic shock virus* (SNSV). It also was shown that TSV isolates from *Rubus* species clustered with the SNSV from strawberry. Sequence data revealed that all "TSV" isolates from *Rubus* and strawberry in this study were SNSV. The complete nucleotide sequence of RNA 3 of the virus was determined and a molecular test developed. Phylogenetic analysis revealed the potential of grouping the virus in strains according to the plant species the virus infects. The lack of identification of any true TSV isolates may indicate that TSV does not infect small fruits.

The finding of SNSV in native *R. ursinus* in remote locations in the coastal range (Finn and Martin, 1996) Suggests that SNSV may be an indigenous virus to the native blackberry in the Pacific Northwest that has migrated to commercial production rather than a transmission in the reverse direction as suspected previously, (Stace-Smith, 1987; Finn and Martin, 1996). Also, the differentiation of SNSV from TSV emphasizes the importance of antiserum source when indexing these small fruit crops for viruses. Testing for "TSV" in these crops has often been done using antisera prepared against isolates of TSV from tobacco and other sources.

FClLV was identified in the 1990's and a single study published on the virus, identifying it as a member of the *Ilarvirus* genus. Immunological tests were performed on plants from South and North America and the virus was only identified in *Fragaria chiloensis* plants from South America. The development of a molecular detection test for the virus that was used to test plants for the presence of the virus in North America

demonstrated that the virus was widespread in strawberry production areas. Phylogenetic analysis of the coat protein gene of the virus placed the virus in subgroup 4 of the genus along with *Prune dwarf virus*. The monoclonal antibodies used for FClLV detection in the 1990's do not react with isolates of the virus found in North America. Thus, the virus may have been present in North America for quite some time and gone undetected. Graft analysis for this virus causes subtle symptoms in only some *F. vesca* indicator clones (Speigel *et al.*, 1993) and the failure of antibodies developed previously to detect this virus suggests earlier tests likely have missed this virus completely. This example points to the necessity for the continued use of broad spectrum virus testing, such as mechanical transmissions, dsRNA and electron microscopy for plant material that enters certification programs. This additional testing is especially important for plant material collected in the wild and added to germplasm collections since this is a potential source for introducing exotic viruses into a region or cropping system. It will be interesting to determine if there are isolates of FClLV in Chile that do not react with the monoclonal antibodies developed. This can be done by testing strawberry material in Chile with both the RT-PCR based test described here and the serological test described previously (Spiegel *et al.*, 1993).

With the finding of BPYV and FClLV in strawberry, primers were developed for all viruses of strawberry for which sequenced information is available and RT-PCR based detection methods developed. These tests were used in studying strawberry decline in production fields. In this work an additional virus not discussed in this publication, *Strawberry latent ringspot virus* (SLRSV) was detected. This virus was identified in strawberry from California and British Columbia (Martin *et. al.*, 2004). This virus is reported to be nematode transmitted, so the presence in California strawberry fields was quite surprising as most of these fields are fumigated prior to planting and one would not expect a nematode transmitted virus to be present especially where the crop is grown in an annual system.

7.3 Strawberry decline

Many fields in southern California (Oxnard area) and the central coast (Watsonville area), as well as northern Washington and British Columbia have exhibited unusual symptoms that included leaf reddening, plant decline and yield losses and in some cases plant death (Fig. 7.1).

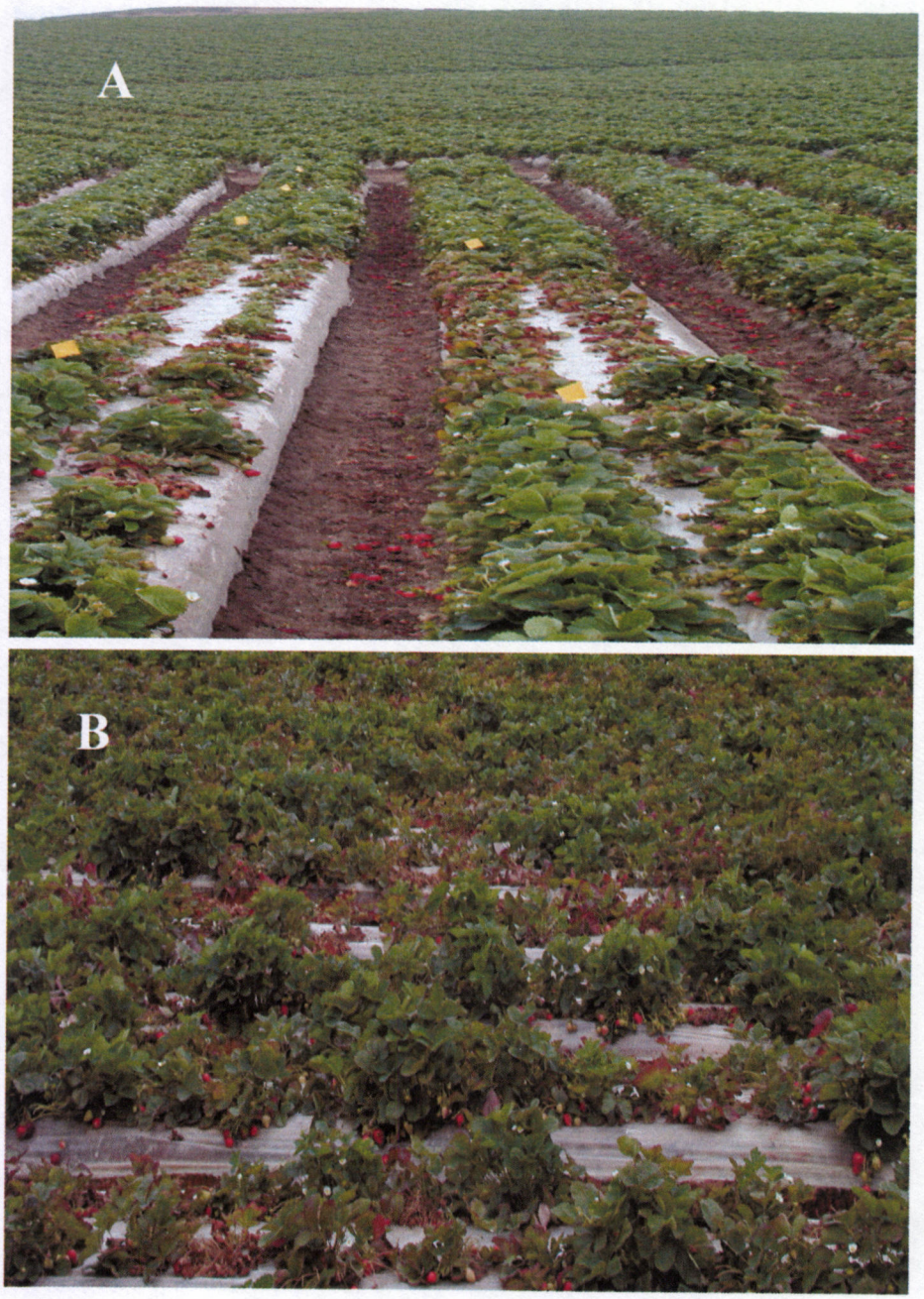

Fig. 7.1. Symptoms typical of strawberry decline in California. A. Strawberry (*Fragaria x ananassa*) 'Camarosa'; B. Strawberry (*F. x ananassa*) 'Ventana'.

A limited survey from California strawberry fields revealed the presence of mixed virus infections in all the plants that showed these unusual symptoms (Table 7.1). The majority of the plants were infected with more than two viruses and as many as five viruses identified in some of the declining plants. The majority of the plants (exceeding 75%) where infected with SPaV while BPYV, FClLV, *Strawberry mild yellow edge* (SMYE), *Strawberry crinkle* (SCV) and *Strawberry mottle viruses* (SMoV) were each present in 15 - 25% of the plants tested. This was the first time that the presence of FClLV was verified in North America. In British Columbia, unlike California, no samples tested positive for the presence of SPaV and BPYV. Instead, the aphid-borne viruses were predominant with SMEYV infecting 100% of the plants tested. The symptoms overall were similar with those observed in southern California (Fig. 7.2).

Table 7.1. Results of virus infectetion in strawberry. A.Virus infestation in plants showing decline symptoms. B. Virus infection in asymptomatic plants.

A

Virus	Region	
	California	B.C. Canada
SPaV	18/24*	0/5
BPYV	9/24	0/5
SMEYV	22/107	5/5
SCV	5/19	3/5
SMoV	N/A	3/5
SNSV	0/24	0/5
SLRSV	1/24	3/5
FClLV	6/19	N/A

B

Virus	Region				
	California	Oregon	Florida	Washington	Canada
SPaV	4/65	1/24	0/17	0/36	0/22
BPYV	6/65	2/24	3/17	0/36	2/22*
SMEYV	1/134	N/A	1/17	N/A	N/A
TomRSV	1/142	N/A	0/17	N/A	N/A
SNSV	0/134	N/A	0/17	0/36	N/A
SLRSV	6/47	0/24	0/17	0/36	N/A
FClLV	19/93	3/24	0/17	10/36	0/22

* number of plant tested positive/total plants tested; ** Plants acquired from certification program

Fig. 7.2. Symptoms associated with virus infestation in strawberry (*Fragaria x ananassa*) cultivar 'Totem' from British Columbia, Canada.

The lack of SPaV and BPYV in B.C. is not unexpected since whiteflies were not observed in strawberry plants there. Also, the high incidence of aphid-borne viruses in B.C. is consistent with high aphid populations on strawberry in that area. This was the first finding of SCV in strawberry in British Columbia.

Strawberry is propagated asexually and thus the need for virus-free stock is essential for good yield in production fields. The normal protocol in strawberry plant certification programs is for the industry to obtain virus-free plants developed by heat treatment and excision of virus-free apical meristems. This method although efficient does not assure 100% success of virus elimination and virus testing is performed after the virus elimination steps have been completed.

The development of a new strawberry cultivar takes at least five years with ten years being more typical. During this time the plants are grown in the field in order for

the plant breeders to evaluate the material for fruit quality, yield and disease reaction. This allows enough time that there is a high probability of plants acquiring one or more viruses by the time a cultivar is ready for release. Most modern strawberry cultivars appear symptomless when infected with one or two viruses which makes visual inspection of plants ineffective. Thus, the normal procedure for certification programs is to have a selection put through the "virus clean up" program prior to release of the new cultivar. While the procedure is typical in most breeding programs, there are cases were plant breeders omit this step in order to meet the demand for new cultivars. This makes it possible for some cultivars to be universally infected with one or more viruses when they are released. These plants grown in commercial fields can acquire more viruses and develop severe symptoms as the ones seen in southern California and British Columbia or alternatively be a reservoir for infection of surrounding plantations with the viruses they carry causing significant yield losses.

Plants showing decline symptoms were infected with up to five different viruses (probably plants infected with more viruses did not survive to be tested). In the Pacific Northwest, British Columbia, Oregon and Washington strawberries are grown in the field for more than one growing season (usually three or four) and have the potential to accumulate viruses over that period of time. In California, strawberries are grown primarily in the annual hill system and the plants are in production fields for less than a year. These fields are under intense programs for pest and disease management. However, in recent years the management has moved to minimize chemical applications and insecticides are used on more of an as needed basis rather than by a fixed schedule. In most cases this works well, however, when vectors and viruses are both present this can result in rapid virus dissemination. The recent occurrence of whiteflies as a pest in California strawberry fields has led to the development of programs to mitigate the damage caused by whiteflies. Now with the presence of SPaV and BPYV in strawberries the mitigation needs to be reevaluated in terms of virus control rather than whitefly damage.

Further studies need to be conducted to determine the origin of the infected plants. It may be that strawberry plants are infected with some viruses at the nursery level,

perhaps at low incidences such that the plants are symptomless when planted. After planting in the fields and further transmission of viruses within fields but also into fields from other crops or native vegetation resulting in mixed virus infections and producing the symptoms that are seen as strawberry decline. The other possibility would be that the plants are under tremendous disease pressure in the fields where vectors and viruses are abundant. As noted above that possibility is less likely in California where fields are constantly monitored and sprayed for pests and diseases.

It has to be noted that the majority of the plants grown in North America are originally propagated in California nurseries and shipped to their end destinations for planting. Before 2002,only tests for the nematode-borne viruses, TSV (now SNSV), SMYEV and *Strawberry vein banding virus* were available, while there was no testing performed for the presence of FClLV. In the last few years new detection protocols have been developed (Klerks *et al.*, 2004; Thompson *et al.* 2003) for the aphid-borne viruses, in addition to those for SPaV and BPYV. Most of the strawberry viruses give subtle symptoms on indicator plants making their detection through the laborious grafting procedures difficult. The newly developed detection protocols will help better evaluate the extent of virus infestation in the nuclear material used for most of the growing producing areas in North America. Strawberry decline was prominent in the 2002 and 2003 growing seasons giving minimal time for studies on the epidemiology of the disease complex. Further studies are needed to identify the exact time that the viruses are introduced in the strawberry propagation scheme and develop appropriate plans to minimize virus disease impact in strawberry production.

7.4 References

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