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BIOTECHNOLOGICAL APPROACHES TO VIRUS AND PHYTOPLASMA DISEASES IN PLANTS

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ABSTRACT

Plant viruses and phytoplasmas represent a major constraint to crop production worldwide. Biotechnological approaches, utilising molecular tools, are employed to face the challenges and have opened a new avenue for research to study different strategies to contain their spread. Most of the time research efforts are centered on crop species, while "wild plants" gain attention only where they play a role as potential alternative host, harbouring a reservoir of inoculum threatening crop plants. PCR, RT-PCR and ELISA are currently the major tools employed for the identification of plant pathogens as well as for studies about plant-pathogen interactions. In this work different detection techniques were employed for several different cases of plant viruses, phytoplasmas and their vectors: 1) The vector nematode *Xiphinema index* was identified for the first time in an Austrian vineyard by PCR using species specific primers. This detection assay opens new approaches for epidemiological studies in Austria and for molecular investigations on the mechanism of *X. index*-mediated GFLV transmission. 2) The increasing importance of the ornamental industry together with the necessity of virus control, led to pursuit alternative ways to detect mixed virus infections. Since viruses have become a widespread problem in *Canna indica*, cultivars of an Austrian collection were selected to assess the viral status. Newly designed PCR primers allowed the first detection of *Canna yellow mottle virus* in Austria. 3) While attempting to detect the degree of phytoplasma infections in native plants in an Austrian forest by nested PCR and sequencing on 16S ribosomal gene, the presence of a new phytoplasma belonging to ribosomal group 16SrVI in *Vaccinium myrtillus* was revealed. Additionally phytoplasmas belonging to ribosomal group 16SrI-B in *Rubus fruticosus* and phytoplasmas of the subgroup 16SrXII-A infecting *Rubus idaeus* and *Fagus sylvatica* were identified. 4) The understanding of the complex plant-virus-virus interactions at a molecular and cellular level opens a new avenue to the defense, interfering with the interaction between, host and pathogen. The yeast two-hybrid assay of p20, p22 and CP proteins of *Pineapple mealybug wilt associated virus 2* was applied after the identification of the virus in Cuban pineapple fields. 5) The potential contribution of endogenous pararetroviral sequences (EPRVs) to plant pathogenicity and virus resistance is beginning to be explored. A family of EPRVs in cultivated tomato (*Solanum lycopersicum* L.) and a wild relative (*Solanum habrochaites*) was detected and characterized at the molecular and cellular level. The outcomes of this research might lead

to a reduction of the losses caused by virus and phytoplasma diseases in plants using the significant advances in plant biotechnology.

ZUSAMMENFASSUNG

Pflanzenviren und Phytoplasmen stellen weltweit ein wesentliches Hindernis für die Pflanzenproduktion dar. Biotechnologische Methoden unter Anwendung molekularer Arbeitstechniken ermöglichen eine effiziente Herangehensweise an diese Problemstellung und die Erarbeitung neuer Strategien zur Eindämmung einer weiteren Ausbreitung. Die Forschung konzentriert sich hauptsächlich auf Nutzpflanzen, während "Wildpflanzen" nur in jenen Fällen Aufmerksamkeit erhalten, wenn sie als möglicher und alternativer Virus-Wirt fungieren, indem sie einem Reservoir von Inokulum für die bedrohten Nutzpflanzen darstellen. PCR, RT-PCR und ELISA sind gegenwärtig die wichtigsten Arbeitstechniken, die sowohl bei der Identifizierung von Pflanzenpathogenen als auch bei der Erforschung von Pflanze-Pathogen Interaktionen, herangezogen werden. In dieser Arbeit wurden diese Verfahren mehrmals bei der Identifizierung von Pflanzenviren, Phytoplasmen und ihren Vektoren angewendet:1) Der Vektor für das *Grapevine fanleaf virus*, *Xiphinema index*, wurde erstmals in einem österreichischen Weingarten mit Hilfe der PCR unter Verwendung von speciesspezifischer Primer identifiziert. Dieses Verfahren eröffnet neue Perspektiven für epidemiologische Studien in Österreich und ermöglicht molekulare Untersuchungen der Übertragungsmethode des GFLV durch den *Xiphinema index*. 2) Die zunehmende Bedeutung des Zierpflanzenbaus zusammen mit der Notwendigkeit eines effizienten Virenschutzes erfordert Alternativen zur Erkennung gemischten Virusinfektionen. Da Viren in *Canna indica* ein weit verbreitetes Problem geworden sind, wurden Sorten von *Canna indica* einer österreichischen Sammlung für die Bewertung des viralen Kontaminationsstatus ausgewählt. Neuentwickelte PCR-Primer führten zum ersten Nachweis vom Gelbfleckenvirus von Canna in Österreich. 3) Bei der Feststellung des Infektionsgrades durch Phytoplasmen in einheimischen Pflanzen eines österreichischen Waldes mittels nested PCR und Sequenzierung des ribosomalen Gens 16S, wurde ein neues Phytoplasma in *Vaccinium myrtillus* entdeckt, das zur Ribosomalen Gruppe 16SrVI gehört. Zusätzlich wurden auch Phytoplasmen der ribosomalen Gruppe 16SrI-B in *Rubus fruticosus* und Phytoplasmen der Subgruppe 16SrXII-A in *Rubus idaeus* und *Fagus sylvatica* diagnostiziert. 4) Die Erkenntnisse der komplexen Interaktionen zwischen Pflanze und Virus auf molekularer und zellulärer Ebene ermöglichen neue Ansätze in der Pathogenabwehr durch das Eingreifen in die Interaktionen zwischen Wirtspflanze und Pathogen. Die Durchführung einer Hefe-Zwei-Hybrid-Untersuchung mit den Proteinen p20, p22 und CP des an den Ananas-Virus Mealybug wilt assoziierten Virus 2 wurde nach

der Identifizierung des obengenannten Virus in Ananasfeldern in Kuba angewandt. 5) Die mögliche Beteiligung Endogener Pararetroviraler Sequenzen (EPRVs) an einer pathogenen Wirkung auf Pflanzen bzw. an Resistenzen gegen Viren steht am Beginn der Aufklärung der Erforschung. In der Kulturtomate (*Solanum lycopersicum* L.) und einer verwandten Wildart (*Solanum habrochaites*) wurde eine Familie von EPRVs gefunden und auf molekularer und zellulärer Ebene charakterisiert. Bedingt durch die bedeutsamen Fortschritte in der Pflanzenbiotechnologie sind die Ergebnisse dieser Forschungsarbeit sehr vielversprechend vor allem hinsichtlich einer Reduzierung der durch Viren und Phytoplasmen verursachten Krankheiten und deren Folgen.

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INTRODUCTION

Plant diseases

Plant diseases can decrease the aesthetic and biological value of many crops. Damages to many plant organs, i.e. leaves, stems, roots, fruits, seed or flowers may cause economic losses by reduction in yield and quality of plant products. Viruses, bacteria, including phytoplasmas, affect plant health and have a high incidence in the fruit, ornamental and vegetable industry. Although fungal and bacterial pathogens are well estimated and more conspicuous, virus diseases are responsible for greater economic losses than generally recognized (HULL, 2002).

The impact of viral diseases in economically important fruit crops such as grapevine and pineapple has detrimental effects on the production. Grapevine is susceptible to as many as 58 viruses (MARTELLI and BOUDON-PADIEU 2006) and seven phytoplasmas associated to the grapevine yellows (GY) (MAIXNER, 2006; LAIMER et al., 2009) Among them *Nepoviruses* can cause more than 80% crop loss (FUCHS, 2006). Pineapple is also a representative case of a complex set of viruses that can decrease the yield of the fresh fruit market even up to 100 % (SETHER and HU, 2002).

The ability of viruses to induce alterations in the morphology and color of flowers and vegetative organs causes significant damages to the ornamental industry. Since commercial floriculture is an expanding activity, the early diagnosis of diseases of ornamental plants e.g. *Canna indica*, along with the accurate timely identification of their causal agents, is essential for establishing efficient management measures.

On the other hand the widespread presence of integrated viral sequences known as endogenous pararetroviruses (EPRVs) has a remarkable importance. They can be infectious, express viral particles and cause severe diseases in plants. Banana is the most prominent example of a plant species known to harbor infectious EPRVs (NDOWORA et al., 1999). The study of EPRVs in tomato opens a new avenue in terms of investigating the nature and the host control of these sequences, as well as the use of tomato offers a good model for other crop species whose fruit is also a fleshy berry.

Efficient molecular biological techniques for the detection of pathogens and their vectors and study of a potential contribution to plant pathogenicity or resistance to viruses and phytoplasmas can make considerable increases to the understanding and controlling of plant diseases.

Plant viruses

Viruses cause many important plant diseases and are responsible for huge losses in crop production and quality worldwide. Infected plants may show a range of symptoms depending on the disease, but often there is leaf yellowing (either of the whole leaf or in a pattern of stripes or blotches), leaf distortion (e.g. curling) and/or other growth distortions (e.g. stunting of the whole plant, abnormalities in flower or fruit formation).

In nature most plant viruses are transmitted from plant to plant by vectors such as nematodes, fungi, or insects (including leafhoppers, whiteflies, aphids, mealybugs, thrips, beetles and mites). Some plant viruses are mechanically transmitted and need wounds to enter a plant. Wounding disrupts the cuticle, the wall of epidermal cells and, the plasmalemma, thus giving access to the cytoplasm (VAN DER WANT and DIJKSTRA, 2006).

Also mechanical transmission is of great importance for many aspects of experimental plant virology, particularly the assay of viruses by local lesion production in the propagation of viruses for purification and in the study of the early events in the interaction between a virus and susceptible cells (HULL, 2002).

A virus is a set of one or more nucleic acid template molecules, normally encased in a protective coat or lipoprotein that is able to organize its own replication only within suitable host cells. Within such cells, virus replication is (1) dependent on the host's protein-synthesizing machinery, (2) organized from pools of the required materials, (3) located at sites that are not separated from the host cell contents by any lipoprotein bilayer membrane, and (4) continually giving rise to variants through various kinds of change in the viral nucleic acid (HULL, 2002).

In the current classification of plant viruses, there are 73 genera of plant viruses and 14 families recognized; some are also included in animal virus families (e.g. *Reoviridae* and *Rhabdoviridae*) (FAUQUET et al. 2005). Genera are named either after the type of species (e.g. *Caulimovirus* after cauliflower mosaic virus) or are given a descriptive name.

The properties used in classifying viruses are: a) structure of the virus particle, b) physicochemical properties of virus particles, c) properties of viral nucleic acids, d) viral proteins, e) serological relationship, f) activities in the plant and g) methods of transmission. A detailed descriptor list used in virus taxonomy can be found in Annex 1 (HULL, 2002).

The delineation of virus species is a practical necessity, especially for diagnostic purposes related to the control of virus diseases.

Control of viruses depends on good agronomic practices, e.g. use of virus-free propagules, rouging of infected individuals, elimination of alternative hosts and vectors and quarantine measures. Although some viral diseases can be diagnosed quickly by visual examination of symptoms, others require molecular tests for diagnosis because either they are symptomless or, because a number of different viruses cause similar symptoms in the same host plant, e.g. badnaviruses and ampeloviruses cause a similar decline in pineapple plantations (HU et al., 2009).

Immunological and DNA-based diagnostic tools play an increasingly important role in virus research. New molecular based diagnostic techniques, from RT-PCR to microarrays that have been developed, are more rapid, sensitive and accurate than traditional methods. Improved viral diagnostics in disease complexes will improve our understanding of these complexes, which may lead to more efficient control strategies.

Currently there exist many powerful molecular tools to study plant viruses and their biological and physical impacts. The choice should be made according to the question to be addressed. If the intention is to determine whether a plant is virus infected, for example for breeding or quarantine purposes, there is no need necessarily for a sophisticated technique to identify a virus strain. However, for each virus situation it is vital to compare different test systems to choose the most appropriated one. Nucleic acid tests may sometimes provide a better test across a range of related viruses. On the other hand, if the aim is to study the durability of a potential resistance virus gene (or transgene) (MAGHULY et al., 2006; MENDONÇA, 2005), interactions between virus components (Chapter 4, BORROTO et al., 2007a) or the mechanism by which virus induces a disease, advanced molecular biological techniques greatly facilitate the studies of viruses.

Phytoplasmas

Phytoplasmas are associated with diseases in about 1000 plant species (SEEMÜLLER et al., 2002). The study and description of molecular, biological and epidemiological characteristics of phytoplasmas are still indispensable in order to understand their evolution, to develop a valid taxonomy and finally to prevent further spreading of phytoplasma-associated diseases.

Phytoplasmas represent a major threat to the cultivation of economically important species, such as palms, fruit trees, and grapevines. However, the prompt identification of phytoplasma diseases is by no means trivial. Phytoplasmas induce symptoms that suggest an interference with plant growth regulators altering the development. Typical symptoms

include: witches' broom (clustering of branches) of developing tissues; phyllody (retrograde metamorphosis of the floral organs to the condition of leaves); virescence (green coloration of non-green flower parts); bolting (growth of elongated stalks); formation of bunchy fibrous secondary roots; reddening of leaves and stems; generalized yellowing, decline and stunting of plants and phloem necrosis. No control mesuares are available; therefore, sensitive and accurate diagnosis of these microorganisms is a prerequisite for the management of phytoplasma-associated diseases.

Phytoplasmas were discovered in the phloem of plants with yellows disease in 1967 (DOI et al., 1967). They are transmitted by phloem feeding insects of the order *Hemiptera*, mostly leafhoppers (*Cicadellidae*), planthoppers (*Fulgoromorpha*) and psyllids (*Psyllidae*) (WEINTRAUB and BEANLAND, 2006).

Phytoplasmas are bacteria with small genomes that are phylogenetically classified in the class *Mollicutes*. Their genome size ranges between 530 kb and 1350 kb, with a total of 671 and 754 genes for the Aster yellows witches´broom (AYWB) and the Onion yellows (OY) phytoplasma, respectively (OSHIMA et al., 2004; http://www.oardc.ohio-state.edu/phytoplasma/genome.htm). Recently the genome size sequence of '*Candidatus* Phytoplasma mali' (strain AT) was determined with a total length of 601 kb (KUBE et al., 2008). Compared to other mollicutes, phytoplasmas lack several genes that autonomous bacteria such as *E. coli* need for metabolism (OSHIMA et al., 2004). Being intracellular parasites, phytoplasmas have a more pronounced dependence on the host and apparently receive some metabolites which cannot be easily supplied with an artificial growth medium. The sequenced phytoplasmas have repeat-rich genomes, indicating that phytoplasmas have compensated for their constraints with horizontal gene transfer, rearrangement of DNA and recombination between the chromosome and the plasmids (NISHIGAWA et al., 2002; LIEFTING et al., 2004).

Phytoplasmas are genetically distinguishable from mycoplasmas infecting human and animal for the presence of a spacer region (about 300 bp) between 16S and 23S ribosomal regions, which codes isoleucine tRNA and part of the sequences for alanine tRNA. Sequencing of complete rRNA genes from two phytoplasma strains shows that tRNA coding for valine and asparagines are located downstream from the 5S rRNA gene, which is a unique feature of phytoplasmas (HO et al., 2001). Since in the case of the phytoplasmas sequencing represents the basis for taxonomic decisions, according to the recommendations of the International Committee of Systematic Bacteriology, the subcommittee on the Taxonomy of Mollicutes a new *Candidatus* species may be described

when a 16S rDNA sequence (longer than 1200 bp) has less than 97.5% identity with any previously described *Candidatus* species. Studies carried out on this gene in several strains led to the conclusion that phytoplasmas are a unique monophyletic group as indicated by the new name (GASPARICH et al., 2004). In fact two phytoplasmas sharing more than 97.5% homology in the 16S sequence can be designed as separate *Candidatus* species, when they meet the following three criteria: (1) they are transmitted by different vectors; (2) they have different natural plant host(s); and (3) there is evidence for molecular diversity between them (IRPCM, 2004).

DNA-specific dyes such as DAPI (SCHAPER and CONVERSE, 1985; HIRUKI and ROCHA, 1986) as well as electron microscopy (EM) (FIRRAO et al., 2007) represented an alternative approaches to the traditional indexing procedure for phytoplasmas diagnostic. However in the last years the applications of DNA-based technology allowed to preliminary distinguish different molecular clusters inside these prokaryotes (BERTACCINI, 2007).

The use of sensitive techniques such as PCR and nested-PCR is very important to study the phytoplasmas, but RFLP analyses of total genomic DNA provide additional evidences of differentiation (BERTACCINI, 2007). It should be noted, however, that the major limitation to the development of high throughput, robust diagnostic assays for phytoplasmas remains the difficulty in developing a rapid and cost/labour effective preparation of representative nucleic acids extracts. In plants, phytoplasmas remain mainly restricted to the phloem tissue (DOI et al., 1967; WHITCOMB and TULLY, 1989), where they spread throughout the plant by moving through the pores of the sieve plates that divide the phloem sieve tubes. The most reliable diagnostic protocols, therefore, include the collection of samples as pools of subsamples taken from different parts of the individual plant to be tested. In order to reduce the amount of material to be processed usually the samples are enriched for phytoplasma and/or phytoplasma containing tissues (i.e. leaf veins) before proceeding with nucleic acid extraction.

The diagnostic procedures developed in the past 15 years are used routinely and are adequate for detecting phytoplasma infection (HEINRICH et al., 2001, Chapter 3, BORROTO et al., 2007b) in plant propagation material and identifying insect vectors, thus preventing the spread by studing the role of some wild species as potential phytoplasma reservoirs.

Host plants

The simple, obligate nature of viruses requires them to usurp or divert cellular resources, including host factors, away from their normal functions. As viruses invade susceptible plants, they create conditions that favor systemic infections by suppressing multiple layers of innate host defenses. Therefore the management of virus diseases is difficult because cellular and viral functions are intimately connected leading to significant losses in agronomic, landscape, ornamental and floral crops.

Grapevines

Grapevine (*Vitis vinifera* L.) is one of the most valuable horticultural crops in the world. Given their nutritional and dietetic value, grapevines contribute considerably to an improved world food production and human nutrition. The Annual Production of grapes in 2007 reached 66,271,676 metric tons (FAOSTAT 2008), exceeding the production of any other temperate fruit crop. Furthermore, grapevine viruses have a great economic impact worldwide and the severe damage the viruses cause is considered second only behind losses due to fungal diseases (LAIMER, 2006). Among the viruses that attack grapevine, *Grapevine fanleaf virus* (GFLV) together with *Arabis mosaic virus* (ArMV), cause the grapevine fanleaf disease (BOVEY et al., 1980). This disease provokes a progressive decline of the plant vigor over several years combined with a reduction in productivity or the rapid death of young plants (ANDRET-LINK et al., 2004a).

GFLV

GFLV belongs to the genus *Nepovirus* in the *Comoviridae* family (WELLINK et al., 2000). Members of this family have isometric virions with icosahedral symmetry.

GFLV consists of polyhedral virus particles with a diameter of 28 nm. The GFLV capsid is composed of 60 subunits of a single protein determined by gene $2C^{CP}$ at the 3´end of RNA 2 (SERGHINI et al., 1990). In purified preparations three serologically indistinguishable density components called T (top), M (middle) and B (bottom) are present (QUACQUARELLI et al., 1976, MAYO and ROBINSON, 1996), being T component particles empty shells, M component particles containing RNA 2 and B component particles containing RNA 1 and RNA 2 (positive-sense ssRNA species). The RNAs are expressed as polyproteins that are processed post-translationally to yield the functional proteins (HULL, 2002).

GFLV is transmitted in a natural way by the soil-born nematode *Xiphinema index* (HEWITT et al., 1958). The nematode acquires and transmits GFLV while feeding on root

tips of actively growing grapevines (WYSS, 2000). The reproduction of *X. index* is parthenogenetic, males are scarce, and a single juvenile is capable of establishing a population (DALMASSO and YOUNES, 1969). The complete life cycle of *X. index* requires 2 to 14 months under controlled conditions, depending on biotic and abiotic factors (TAYLOR and RASKI, 1964; WYSS, 2000), but can require more than 14 months in the field where temperatures are more variable (WEISHER, 1975). Under adverse conditions, including low moisture, low temperature, and the absence of host plants, *X. index* undergoes a quiescent phase during which physiological functions are limited and development is interrupted (ANTONIOU, 1989). Transmission of GFLV by *X. index* is noncirculative and semipersistent (TAYLOR and BROWN, 1997; BROWN and WEISCHER, 1998; MC FARLANE, 2003). GFLV can be acquired from infected plants and inoculated to recipient plants within 1 to 10 min (WYSS, 2000). The virus does not replicate within the nematode and does not influence the reproduction of *X. index* (DAS and RASKI, 1969). Virions are retained by *X. index* up to 9 months but do not pass from females to eggs or juveniles (TAYLOR and RASKI, 1964). Indeed, juveniles lose their ability to transmit the virus after molting because the lining of the cuticle in the odontophore and the oesophagus, where GFLV particles are retained at specific sites (TAYLOR and BROWN, 1997), is shed. Therefore, after molting, the nematode must feed again on roots of GFLV-infected grapevines to acquire the virus and become viruliferous. Adults and all juvenile stages are able to acquire and transmit GFLV to healthy plants (TAYLOR and RASKI, 1964.). The transmission is highly specific (MARTELLI, 1975, CATALANO et al., 1992, ANDRET-LINK et al., 2004a, BROWN and WEISCHER, 1998). Recently, ANDRET-LINK et al., (2004b) showed that the coat protein (CP) is the sole viral determinant responsible for this specificity. Control strategies for GFLV in infected vineyards essentially are directed to the eradication of *X. index*. GFLV-infected grapevines are uprooted, soils disinfested with nematicides, and vineyards fallowed (LAMBERTI, 1981; RASKI et al., 1983; TAYLOR and BROWN, 1997; VUITTENEZ and LEGIN, 1964). These measures are of limited efficacy because nematicides have a poor penetration potential, especially in clay soils, and *X. index* can be present in deep layers up to 4 m (LAMBERTI, 1981). Furthermore, registered nematicides have a high acute toxicity and a detrimental effect on beneficial nontarget soil organisms (ABAWI and WIDMER, 2000), and the Common Agricultural Policy (CAP) of the EU prohibit some of them, like Aldicarb (LAIMER et al., 2009).

Finally, the recommended 10-year fallow (VUITTENEZ et al., 1969) is highly impractical and requires that viruliferous nematodes die and virus reservoirs disappear after

uprooting. Limited information is available on the ability of *X. index* to survive in soil and to retain GFLV over extended periods of time. *X. index* has been reported to survive for 4.5 years in a fallowed vineyard, although remaining grapevine roots were suspected to have maintained nematode viability by providing feeding sources (RASKI, et al., 1965). *X. index* also remains viruliferous for up to 9 months in moist soil in the absence of host plants (TAYLOR and RASKI, 1964).

Partial resistance to *X. index*, vectoring GFLV, has been discovered 25 years ago in Muscadine grapes and in *Vitis munsoniana* (BOUQUET, 1981; STAUDT and WEISCHER, 1992). However, nematode-tolerant grapevine hybrids can not totally impede nepoviruses to be transmitted to their rootlets. This just delays the infection process of GFLV in grapevine for a few years (BOUQUET et al., 2000). Nevertheless, several attempts have been made to achieve virus resistance in grapevine by expressing CP genes of GFLV (GAMBINO et al., 2005; GRIBAUDO et al., 2005; MAGHULY et al., 2006). These efforts have led the molecular characterization of transgenic grapevines carrying the entire or truncated GFLV-CP gene able to express GFLV CP gene in *Vitis vinifera* cv. Russalka (MAGHULY et al., 2006).

Nowadays in the field the only effective measure to control GFLV is preventing virus spread in the vineyard and nursery. Consequently the implementation of a sensitive diagnostic method and sanitation (GOUSSARD and WIID, 1992; LAIMER et al., 2003; GAMBINO et al., 2006) to produce virus-free grapes and rootstocks is essential.

Serology based diagnosis such as ELISA is an easy and inexpensive method that allows large scale screening of grapevines. The application of nucleic acid technology has a major impact especially when the virus titer is low and can affect reliability (LAIMER, 2003). The RT-PCR and PCR are extremely sensitive techniques used in GFLV detection (WETZEL et al., 2002). Along with the detection of the virus, the major advantage is the data information about strain types that can be provided from the amplicons sequence. Aditionally, *X. index* detection by PCR with conserved primers designed from the internal transcribed spacer 1 (ITS1) (WANG et al., 2003) has an increased importance. Identification and spatial distribution of this nematode is essential to efficiently control the disease (Chapter 1, LEOPOLD et al., 2007).

Canna

Canna is a plant genus of about ten species that originate from Central and South America including tender perennials with architectural foliage and flower at the end of

summer. They are cultivated as ornamentals worldwide, although their precise economic value to the nursery industry is not documented (MOMOL et al., 2004). In recent years cannas have enjoyed a renewed interest; but the growth in the popularity of cannas has been matched by an increase in the incidence of virus diseases (MONGER et al., 2007). This problem has been exacerbated as cannas are predominantly propagated through vegetative means and by frequent international exchange of untested material. As a result, viruses have now become a widespread problem in cultivated canna around the world. To date, four different viruses have been reported to infect canna, *Canna yellow mottle virus* (CaYMV) (YAMASHITA et al., 1985), *Bean yellow mosaic virus* (BYMV) (CASTILLO et al, 1956), *Cucumber mosaic virus* (CMV) (LOCKHART, 1988) and *Tomato aspermy virus* (TAV) (HOLLINGS and STONE, 1971).

Canna viruses are often spread by propagation from cuttings, on tools, or by human contact with infected plants then moving to a healthy plant. A screening of canna material using sensitive detection methods, based on PCR, demonstrates the definitive links between specific viruses and the expression of a particular symptoms, and therefore can be recommended to breeders as an efficient diagnostic tool to reduce the risk of spread of the viruses (MOMOL et al., 2004; Chapter 2, BORROTO et al., 2008).

CaYMV

CaYMV is a plant pararetrovirus of the *Caulimoviridae* family, genus *Badnavirus* (FAUQUET et al., 2005). The family *Caulimoviridae* contains six genera with two distinct virion morphologies: members of *Caulimoviruses*, *Soymoviruses*, *Cavemoviruses* and *Petuviruses* form isometric particles, whereas *Badnaviruses* and *Tungroviruses* have bacilliform virus particles (FAUQUET et al., 2005; HOHN and RICHERT-PÖGGELER, 2005). They are non enveloped viruses with a noncovalently closed circular double-stranded DNA of 7 to 8 kbp (FAUQUET et al., 2005). After infection, open circular viral DNA is released into the nucleus of the cell, where it is converted into supercoiled DNA and associates with histones to form a minichromosome. The viral DNA is then transcribed into mRNA, as well as pregenomic RNA, which is used for DNA replication in the cytoplasm via reverse transcription (HULL and COVEY, 1995). The genus *Badnavirus* groups several viruses of importance to tropical agriculture. Badnavirus have bacilliform particles not associated with cytoplasmatic inclusion bodies. CaYMV causes pale streaks along the veins of *Canna* plants, which in later stages turn necrotic. The disease was first reported by YAMASHITA et al., (1985), associated with a small non-enveloped bacilliform virus averaging 31X130 nm particles; however, no vector has been identified for this virus so far (LOCKHART, 1988).

The viruses have circular double-stranded DNA genomes with gaps or discontinuities at specific sites, one in one strand and between one and three in the other strand; these discontinuities represent priming sites for DNA synthesis during replication (HOHN, 1999; SCHOELZ and BOURQUE, 1999). The DNA genome encodes three open reading frames (ORFs) thought to be translated from the more-than-genome length RNA transcript. ORF III expresses a polyprotein processed to give, amongst other products, the virus coat protein, an aspartate protease, reverse transcriptase and RNaseH. Many members are transmitted in a semi-persistent manner by mealybugs (LOCKHART and OLSZEWSKI, 1999).

In the detection of the CaYM no immuno-diagnostic assay is available; however a PCR based test has been reported by MOMOL et al., (2004). Recently, an innovative analysis using highly specific PCR primers allow the detection of CaYMV in symptomatic and symptomless plant tissues (BORROTO et al., 2008).

CMV

CMV is a member of the *Bromoviridae* family, genus *Cucumovirus*. Viruses in this family have isometric particles, 26-35 nm in diameter or bacilliform particles whose symmetry is based upon the icosahedron. The viruses are divided into two subgroups (I and II) based on serology and nucleotide sequence identity (ROOSSINCK, 1999). The genomes of linear positive sense ssRNA are divided between three molecules with genome organizations. The subgenomic RNA for coat protein is often also encapsidated; several members also encapsidate satellite or defective interfering RNAs (FAUQUET et al., 2005).

CMV is commonly detected by ELISA.

BYMV

BYMV belongs to the genus *Potyvirus*, family *Potyviridae* (FAUQUET et al., 2005). BYMV virions are flexuous, filamentous particles, approximately 750 nm in length and 15 nm in diameter containing a single stranded, positive sense RNA genome of approximately 10 kb (GUYATT et al., 1996). Although the diagnostic of BYMV is performed in ornamental plants also by RT-PCR (KULSHRESTHA et al., 2006), ELISA is still very sensitive and useful test to detect it.

TAV

TAV is a member of the *Cucumovirus* genus, family *Bromoviridae*. The capsid is not enveloped and is isometric with a diameter of 29 nm (HOLLINGS and STONE, 1971). The genome is composed by a tripartite, positive-sense ssRNA genome.

The detection and identification of TAV can be achieved by ELISA as well as RT-PCR (VERMA et al., 2006).

Pineapple

Pineapple (*Ananas comosus* (L.) Merr) is the third most important tropical fruit crop, after bananas and mangoes. Approximately 70 % of the produced pineapple is consumed as fresh fruit in the country of origin due to its good nutritive value (DIAS-CARLIER et al., 2007). Although cultivated in all tropical and subtropical countries, minor plantations can be found beyond these latitudes areas with mild climates. According to the Food and Agricultural Organization (FAO) statistics (FAOSTAT 2008), world pineapple production was 18,873,577 metric tons in 2007.

One of the most devastating diseases of pineapple, worldwide, is mealybug wilt of pineapple (MWP). The disease is characterized by severe tip dieback, downward curving of the leaf margins, reddening, and wilting of the leaves that can cause total collapse of the plant (CARTER, 1945). MWP can cause up to a 100% fruit loss in a significant production area as Hawaii (SETHER and HU, 2002) or 40% of crop losses in areas with minor plantation as Cuba (ANONYMOUS, 1989).

Although MWP has been studied for more than 90 years, the etiology of this disorder has been in questioned. Serological and molecular data have revealed the presence of at least three distinct *Pineapple mealybug wilt-associated viruses* (PMWaVs), PMWaV-1, -2, and -3 (SETHER et al., 2001; 2005).

Despite the numerous viruses found to be involved in MWP, PMWaV-2 plays the decisive role in its etiology. The presence of PMWaV-2 in Hawaiian pineapple plants has shown a 99 to 100% correlation with MWP symptoms (SETHER et al., 2001).

The characterization of the ampeloviruses, the reliable assays to detect the viruses and the identification of the factor involved in the disease development have major implications for control strategies to manage MWP.

PMWaV-2

The mealybug-transmissible PMWaV-2 is placed in the genus *Ampelovirus* of the positive-strand RNA virus family *Closteroviridae*. Virions belonging to the genus *Ampelovirus* have a size greater than 1000 nm in length (usually 1400-2200 nm) (MARTELLI et al., 2002).

The family *Closteroviridae* includes positive-stranded RNA plant viruses with long, flexuous virions and elongated genomes of up to 20 kb. Members of the family

Closteroviridae are known for their long RNA genomes, the largest of the plant viruses (DOLJA et al., 2006). The viruses belonging to this family are usually phloem limited and cause yellowing-type symptoms or pitting or grooving of woody stems. Aphids, mealybugs and whiteflies are known to transmit these viruses (AGRANOVSKY, 1996; KARASEV, 2000).

GUNASINGHE and GERMAN, (1989) isolated flexuous, rod-shaped virus particles (12x1200 nm) and high molecular mass double-stranded (ds) RNA ($8{\cdot}35x10^6$ Da) from MWP-diseased plants, but not from healthy plants. The CP subunits have a molecular mass of 34 kDa and the genome was sequenced (MELZER et al., 2001).

In addition to mealybug transmissibility, members of the genus *Ampelovirus* are characterized by a genome length of 16.9–19.5 kb, a 35–37 kDa (CP), and an ORF encoding a duplicate of the CP (CPd), generally located immediately downstream of the CP ORF (MARTELLI et al., 2002).

Several attempts were made to develop rapid detection assays for the ampeloviruses such as the production of monoclonal antibodies for PMWaV-1 and PMWaV-2 used in tissue blot immunoassays (TBIA) (HU et al., 1997). RT-PCR assay, which can detect and differentiate PMWaV-1, PMWaV-2 (SETHER et al., 2001), and PMWaV-3 (SETHER et al., 2005) is extremely sensitive and suitable for screening young plants in tissue cultures, which are too small to be screened with TBIA, as well as allowed to probe that PMWaV-2 infection is consistently found in association with MWP, whereas PMWaV-1 is not (SETHER and HU, 2002).

The long-term approach to MWP still is understanding the interactions between viruses, vectors and host plants, and to use this information to develop strategies to manage this important disease. Recently the interactions of some viral components from PMWaV-2 was studied by two hybrid system (Chapter 4, BORROTO et al., 2007a,) and various gene constructs that were developed using RNA-mediated resistance technology were used to transform pineapple plants with PMWaV-2 genes (PEREZ et al., 2006).

Tomato

Tomato (*Solanum lycopersicum*) is the second most important vegetable crop next to potato. Present world production is about 126,246,708 metric tons fresh fruit produced on 4.6 million hectares (FAOSTAT, 2008).

S. lycopersicum can easily be crossed with a range of other *Solanum* species including *S. pimpinellifolium*, *S. neorickii*, *S. habrochaites*, *S. chmielewskii*, and *S. pennellii* sometimes collectively referred to as the *Solanum lycopersicum* complex. These

species display markedly different phenotypes from *S. lycopersicum* most notably with respect to their fruit but have been used successfully to introgress benefitial alleles from unadapted or wild germplasm into elite breeding lines (VIDAVSKY and CZOSNEK, 1998; KABELKA et al., 2002)

Traditional plant breeding has resulted in great progress in increasing yield, disease and pest resistance, environmental stress resistance, and quality and processing attributes. However, tomato plant breeding programs still strive to generate a better product.

In addition to its role as an important food crop, cultivated tomato, *S. lycopersicum* subsection lycopersicon, represents a model for other crop species whose fruit is also a fleshy berry (MEISSNER et al., 1997) and a large mutant collection is available (http://tgrc.ucdavis.edu).

The discovery of Endogenous pararetroviral sequences (EPRVs) has had a deep impact on the approaches for the diagnosis and management of diseases caused by pararetrovirus. Due to the fact, that the EPRVs can give rise to systemic virus infection, intense research efforts has been made in their diagnosis and study in economically important crops (HARPER et al., 2002).

Recently the presence of EPRVs has been studied in *S. lycopersicum* and a wild relative, *S. habrochaites*, which is exploited in crosses with *S. lycopersicum* to introgress favourable traits (Chapter 5, STAGINNUS et al., 2007).

EPRVs

For the replication of plant pararetroviruses belonging to the family *Caulimoviridae*, in contrast to animal retroviruses no integration of the viral genome into the host genome is required. Nevertheless, pararetroviral integrations within the host genome exist and are assumed to have originated from illegitimate recombination during the minichromosome phase (STAGINNUS and RICHERT-PÖGGELER, 2006). Such integrants, called EPRVs, range from small, incomplete fragments to larger sequences, and become part of the plant genome via integration in a germinal cell subsequently becoming fixed in the plant population by the evolutionary forces of natural selection and/or genetic drift.

EPRVs are widespread within the plant kingdom. The genomes of bitter orange (*Poncirus trifoliata*), potato (*Solanum tuberosum*), rice (*Oriza sativa*), tomato (*Solanum lycopersicum*), petunia (*Petunia* sp.), tobacco (*Nicotiana* sp.), and banana (*Musa* sp.) have been shown to harbor such integrants (HULL et al., 2000; STAGINNUS and RICHERT-PÖGGELER, 2006). Furthermore it has been found that sequences homologous to those of a non-retro RNA virus, *Potato virus Y* (PVY) are integrated into the genome of several

grapevine varieties most likely by recombination between viral RNA and the RNA of host cell retrotransposable element (TANNE and SELA, 2005). In 1999, JAKOWITSCH et al., described tobacco EPRVs as a novel class of dispersed repetitive elements, which can reach up to a 1,000 copies in tobacco (GREGOR et al., 2004; METTE et al., 2002). The widespread distribution of EPRVs among plants and their scattering within the host genome thus results in a discernible impact on host genome shape, plasticity, and evolution.

Some EPRVs could release virions. The data on the existence of these infectious EPRVs came from observations of spontaneous viral infection in petunia, tobacco, and banana by *Petunia vein clearing virus* (PVCV) (RICHERT-POGGELER and SHEPHERD, 1997), *Tobacco vein clearing virus* (TVCV) (LOCKHART et al., 2000), and *Banana streak virus* (BSV) (DALLOT et al., 2001), respectively.

The *de novo* appearance of these viruses is thought to be linked to epigenetic modifications that occur during genome hybridization (RICHERT-PÖGGELER, et al., 2003; METTE et al., 2002); an asymmetric ratio of EPRV copies between parental genomes is considered to be a prerequisite for this (HARPER et al., 2002; LOCKHART et al., 2000; LHEUREUX et al., 2003). Epigenetic control is weakened, for example, via transient hypomethylation of EPRVs, which is probably also responsible for activation after wounding (RICHERT-PÖGGELER, et al., 2003) and during tissue culture (NDOWORA et al., 1999; DALLOT et al., 2001). Furthermore, EPRV activation after exposure to abiotic stresses such as drought or heat stress (RICHERT-PÖGGELER, et al., 2003) and changes in day-length (LOCKHART et al., 2000) have been reported. Even the expression of a single genomic EPRV locus is sufficient to result in viral infection.

It is important to note that EPRVs, just like their exogenous counterparts, can lead to epidemics and therefore be of considerable economic importance. This has severe economic consequences, particularly for banana and plantain, which are propagated vegetatively, and hampers the creation of new breeding lines based on interspecific hybridization (LHEUREUX et al., 2003, DAHAL et al., 1999; MEINS et al., 2005).

Understanding EPRV control is therefore essential, particularly with respect to RNA silencing that might help to prevent disease outbreak of activatable copies. Given that the RNA interference-based plant defense system is sequence-specific, suitable conserved regions should allow targeting of a wide selection of different EPRV variants in the respective genome. Increase the knowledge of endogenous pararetroviral sequences in economically relevant crops as well as understanding its activation and control for the host

have important meanings for breeders for designing strategies to prevent viral diseases caused by EPRVs in newly generated cultivars and model plants (Chapter 5, STAGINNUS et al., 2007).

AIM OF THE WORK

Since plant diseases cause huge direct and indirect losses every year, thus limiting food supply, the advances in molecular biology over the past 20 years have had a major impact by contributing valuable methods. The application of the new findings in phylogeny of pathogens and their vectors, genetics and epidemiology has aided the development of powerful diagnostic tools that have improved the ability to manage diseases, induced by viruses and phytoplasmas, pathogens against which no treatment exist. Viruses and phytoplasma, as intracellular plant pathogens, cannot spread independently, but need either biological vectors or mechanical means of transmission. Sap-sucking insects, nematodes, plant sap carried on tools and vegetative propagation are possible modes of spread. For instance, the nematode vector *Xiphinema index* is responsible for the spread of *Grapevine fanleaf virus*. In the case of vegetatively propagated crops as canna and pineapple, infected by *Canna yellow mottle virus* and *Pineapple mealybug wilt associated virus* respectively, vectors are still speculative. Therefore a strategy to control viruses and phytoplasma diseases is to limit their spread, which requires indispensably precise detection tools.

Studies aiming at the characterization of causal agents of diseases induced by plant viruses and phytoplasmas were predominant at the beginning of plant pathology. Gradually the emphasis moved to analyses of the plant-virus, virus-virus and virus-vector interactions at a molecular, cellular and genetic level. Phytoplasmas as important insect transmitted pathogenic agents, which cause many lethal diseases, in hundreds of plant species, have gained attention. Since they cannot be cultivated *in vitro*, phytoplasmas remain the most poorly understood plant pathogens (FIRRAO et al., 2007).

Important components in the fight against viruses and phytoplasmas is their rapid detection and identification and, in some cases, the interference with their transmission vectors. Diagnostics that can discriminate pathogens will improve our understanding of disease complexes, which may lead to more efficient control strategies. Virus identification continues to be heavily dependent on the immunoassays, EM visualisation of virus particles and bioassays on specific indicator host plants. The low-cost, high through-put nucleic acid-based assays provide screening procedures that yield a rapid and complete picture of crop plant health. Further development and deployment of these technologies might allow coverage of more plant pathogens detection as well as clarify the disease cycle.

Consequently, as many pathogenic viruses and phytoplasmas remain latent and in low numbers in planting material, preventive measures to avoid planting of contaminated material by developing accurated diagnosis are of highest importance in the context of an integrated approach to control virus and phytoplasmas. Besides the sensitive and specific diagnosis, a more completed picture of the life cycle of plant pathogens is provided by the understanding of their relationships, structure and functions. Therefore the milestones of the thesis are summarized in:

1. The identification of phytoplasmas, viruses and their way of spreading, either by known or by unknown vectors, or by vegetative propagation.

2. The evaluation of plant-virus interactions as an approach of how they recognize each other and differentiate to establish either a successful or an unsuccessful relationship.

The first topic involves the identification of *Xiphinema index*, the vector of *Grapevine fanleaf virus*, in a new geographic area, as well as the study of the virus status of a valuable *Canna´s* collection with special emphasis on symptomless tissue. On the other hand, the evaluation of phytoplasma-infected plants in the forest, and their role as a source of inoculum in wild plants, due to the crucial importance of epidemiological studies in order to prevent the further spreading of phytoplasma-associated diseases, was stated.These studies counted with the developing of improved tools for their contribution to the understanding and controlling the spread of phytoplasmas and viruses.

The second topic intending to give a deeper insight and to explore in protein-protein interactions of some viral component of *Pineapple mealybug wilt associated virus 2,* to gain in the understanding of ampeloviruses proteins with still unknown function. In addition, a family of endogenous plant pararetrovirus (EPRVs) in cultivated tomato (*Solanum lycopersicum* L.) and a wild relative (*S. habrochaites*) as a model plant was investigated. This work may help to a better understand mechanisms that could lead to virus resistance.

CHAPTER No. 1

Identification of *Xiphinema index* in a vineyard in Austria

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Introduction

The ectoparasitic Longidorid nematode *Xiphinema index* has a worldwide economic importance because of its ubiquitous presence and ability to transmit *Grapevine fanleaf virus* (GFLV). Infection with GFLV leads to low yield, reduced fruit quality and a shorter lifespan of vines (ANDRET- DINK et al., 2004). Although GFLV was detected in various Austrian viticultural regions (GANGL et al., 2000, 2001, 2003), and despite the fact that the soil-borne nature of fanleaf disease had been observed in Austria well over a century ago (RATHAY 1882, 1883), its nematode vector *Xiphinema index* has never been encountered, contrary to other species of the *Longidoridae* family, predominantly *Xiphinema vuittenezi*, *X. pachtaicum* and other species. In this study soil samples from a vineyard in Burgenland, where vines displayed typical symptoms of GFLV infection, were analyzed for the presence of *X. index* by morphological and molecular analyses, using species specific PCR primers.

Material and Methods

For the isolation of nematodes in a vineyard in Burgenland soil samples including fine roots were collected in the rhizosphere of two *Vitis vinifera* cvs 'Neuburger' (2 and 25 years old) and 'Zweigelt' (5 years old), showing symptoms of GFLV infection. Roots were visually analyzed for nematode feeding symptoms. Leaf samples of the corresponding host plants were collected for GFLV testing by ELISA, using a commercially available kit (Bioreba, Switzerland). Nematodes were extracted from 1.5 kg of soil by a modification of Cobb´s method (SHURTLEFF and AVERRE 2000) using a sieve with 200 µm meshes. The genus of nematodes was determined initially by morphological characteristics (THORNE and ALLEN 1950). An authentic population of *X. index* was used as a positive control. Species identification within the genus *Xiphinema* was performed by multiplex PCR analysis of pooled samples. DNA isolation was carried out by placing 15 nematodes in 25 µl of lysis buffer (1x Taq DNA polymerase buffer and 60 µg of proteinase K ml-1) between two glass slides and crushed gently. The homogenate was taken up carefully with a pipette, transferred to a 0.5 ml Eppendorf tube and frozen at -80 °C for 15 min followed by subsequent incubation at 60 °C for 1 h and at 95 °C for 15 min. PCR was carried out with the following cycles: 95 °C for 3 min, followed by 39 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min, ending with 1 cycle at 72 °C for 5 min. The primers A-ITS1, I27, D24, V18, ITA26 were used (WANG et al., 2003). Amplification products were separated on a 1.5 % agarose gel in 1 x TAE buffer.

Results and Discussion

Although GFLV is present in various Austrian viticultural regions (GANGL et al., 2000, 2001, 2003), surveys for nematodes of the *Longidoridae* family detected predominantly *X. vuittenezi*, and *X. pachtaicum* as well as other species, which are not vectors for GFLV. This left unanswered the question, how GFLV spreads in Austrian vineyards. In fact, *X. index* was never identified, probably due to morphological similarity to *X. vuittenezi* and perhaps due to a lower abundance of *X. index*.

Grapevines with characteristic fanleaf symptoms tested positive for GFLV in ELISA. Examination of fine roots showed signs typical for nematode feeding. Analyses of soil samples resulted in 10-14 individual nematodes of different developmental stages per kg soil, with a head-region and other characteristics typical for the genus *Xiphinema.* Species definition, however, was not possible, because morphological and morphometric diagnostic characteristics of female adults, as currently used for *Xiphinema* ssp. identification, exhibit extensive plasticity. The availability of molecular markers specific for the most important *Xiphinema* species is of a major advantage. Molecular differentiation according to WANG et al., (2003) should result in a 349 bp band for *X. index*, a 813 bp band for *X. diversicaudatum*, a 591 bp band for *X. vuittenezi*, and a 414 bp band for *X. italiae*. Indeed, the species specificity, sensitivity and reliability of the primers were confirmed by HÜBSCHEN et al., (2004).

All nematode populations analysed by PCR consisted of *X. vuittenezi*, except for one which also contained *X. index* (*Figure 1*). Bands specific for *X. diversicaudatum* and *X. italiae* could not be detected. These results prove, for the first time, that *X. index,* the recognized vector for the detrimental grapevine fanleaf disease, is present in the vineyards of a specific Austrian region. It can be expected that the use of species specific primers, as described here for samples from other viticultural regions in Austria, will reveal a more widespread occurrence of *X. index*. As nematicides are banned by the Common Agricultural Policy (CAP) of the EU, these results emphasize the importance of alternative defence strategies in modern viticulture, such as genetically improved rootstocks exhibiting virus resistance (MAGHULY et al., 2005).

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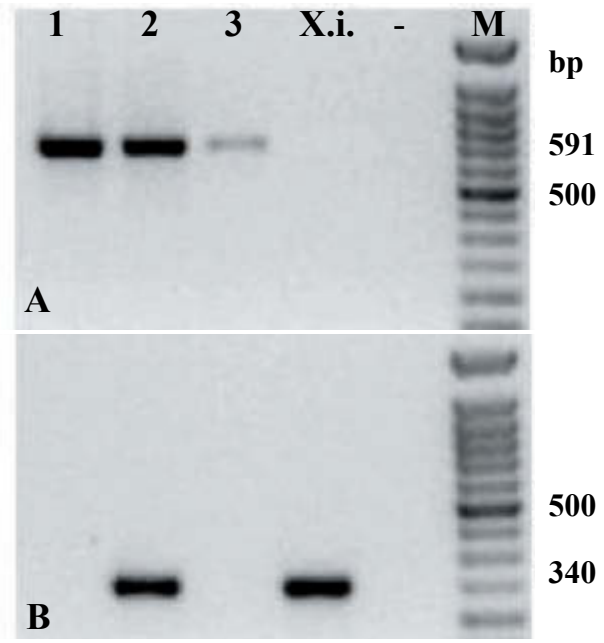
Figure 1. Molecular analysis of nematode populations (1-3) from the rhizosphere of three GFLV-infected grapevines using species specific primers for **A**) *X. vuittenezi* and **B**) *X. index. X. index* was identified in a mixture with *X. vuittenezi* in sample No. 2. X.i. = *X. index*, - = negative control, M = molecular size marker, bp = basepairs.



CHAPTER No .2

Determination of viral infections in an Austrian collection of *Canna indica*

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***Key words*:** *Canna indica,* virus detection, ELISA, PCR, TAV, BYMV, CMV and CaYMV

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Abstract

The viral status of a cultivar collection of *Canna indica* in Austria, which could be of major interest for breeders of ornamental plants, has been determined by ELISA and PCR methods.

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Zusammenfassung

Der Virusbefall einer Sortensammlung von *Canna indica* in Österreich, die für Züchter von Zierpflanzen bedeutend sein könnte, wurde mittels ELISA und PCR festgestellt.

Canna indica L., known as Indian shot or canna lily, is an upright, perennial, rhizomatous herb species of the family *Cannaceae*. Originating from tropical and subtropical areas, *Canna* cultivars are widely cultivated as ornamental plants, because of their spectacular habit of the yellow or red flowers, but also for their eye-catching leaves, which may be simply green, or – in the case of bi- or tricolored cultivars –with yellow or red streaks. This trait might have contributed to the unintended spread of viral pathogens. C*anna indica* rhizomes also showed antitumor (PORNSIRIPRASERT et al., 1987), antibacterial (AVIRUTNANT and PONGPAN 1983) and anti-HIV-reverse transcriptase activity (WORADULAYAPINIJ et al., 2005).

Since vegetatively propagated plant material can contribute to virus dissemination (GRAY and GRANT 2003), currently the destruction of infected plants is recommended to prevent the spread of pathogens. *Canna indica* can be propagated (KROMER and KUKULCZANKA, 1985) and sanitized by *in vitro* methods i.e. meristem preparation combined with thermotherapy (LAIMER, 2003).

The most common virus diseases in *Canna* are caused by *Bean yellow mosaic virus* (BYMV) (SKELTON et al., 2006) *Tomato aspermy virus* (TAV) and *Canna yellow mottle virus* (CaYMV) (Plant Viruses Online http://image.fs.uidaho.edu/vide/famly033.htm#Canna). Also *Cucumber mosaic virus* (CMV) has been found in mixed infections with CaYMV in *Canna* (LOCKHART, 1988). While they can be detected by commercial ELISA kits, Polymerase chain reaction (PCR) is a sensitive and reliable test used for badnaviruses and CaYMV detection (BRAITHWAITE et al., 1995; TAKAHASHI et al., 1993; MOMOL et al., 2004; MARINO et al., 2007).

CaYMV belongs to the *Caulimoviridae* family, genus *Badnavirus* (FAUQUET et al., 2005); causing pale streaks along the veins of *Canna* plants, which in later stages turn necrotic (*Figure 1*). The disease was first reported in Japan (YAMASHITA et al., 1985) and associated with a small non-enveloped bacilliform virus; however, no vector has been identified for this virus so far (LOCKHART, 1988).

This study was undertaken to determine the virus status of a valuable Austrian *Canna* collection. In autumn 2005, typical virus symptoms were observed on *Canna* cultivars and breeding lines. Symptomatic and symptomless tissues from *Canna* plants (Lucifer; Perkeo; Opera La Boheme; V3-2004; V17; Thai IV; Ra 051104; Durban; Prince Charmant; Prof. Lorenz) were analyzed by ELISA and PCR, using a newly developed primer pair yielding a short-sized product (315 bp).

DAS- ELISA was performed to determine the presence/absence of TAV, BYMV and CMV using commercial kits according to the manufacturer's recommendations (Loewe, Germany). The ELISA results did not indicate the presence of any of these three viruses (*Table 1*).

Initially, for the molecular detection of CaYMV, total genomic DNA was extracted from 100 mg of leaf tissue of the 10 *Canna* cultivars and breeding lines by DNeasy® Plant Mini Kit (Qiagen) following the supplier's instructions. DNA quality and concentration were determined by gel electrophoresis and spectrophotometry. Primers CaYMV-3 (5'-GACTTCCTGGGTGCAACAAT-3') and CaYMV-4 (5'-TCTGTGCAATCTTGGCGTAG-3') (MOMOL et al., 2004) were used for the first amplification from all samples, yielding a 565 bp fragment of CaYMV in some infected samples. PCR amplifications were conducted in a total volume of 25 µl using 1x PCR Buffer (Qiagen), 2 mM $MgCl_2$, 0.2 mM dNTPs, 4 pmol of each Primer, 0.6 units HotStarTaq Polymerase (Qiagen HotStarTaq Plus TM PCR), 20-30 ng of total genomic DNA. PCR-cycling conditions (Biometra), after optimization with gradient PCR, consisted of an initial denaturation step of 95°C for 5 min followed by 35 cycles of 60 s at 95°C, 60 s at 60°C annealing temperature (AT) and 2 min at 72°C. A final step of 10 min at 72°C ended the cycle.

The PCR products were analyzed by electrophoresis in a 1% agarose gel, and subsequently purified using NucleoSpin® Extract II Gel Extraction Kit (Macherey-Nagel) according to manufacture's instructions. The extracted DNA was sequenced (VBC-BIOTECH Services GmbH, Vienna) and analyzed with DNASTAR (DNAstar software package). Sequences from four PCR positive samples (Perkeo, Lucifer, Opera La Boheme and V17) were compared with the sequence of CaYMV, detected in Florida (LOCKHART, personal communication) and the high homology detected allowed to conclude that CaYMV was present in the Austrian *Canna* plants (Accession no. EF189147, EF189148. EF189149, EF189150).

The consensus sequences obtained from the Austrian isolates were compared with related *Badnavirus* sequences in the NCBI Genbank and used to design specific primers for the Austrian isolates. The primers pCan1 (5´-ATCCATACATCCGTCTGTT-3´) and pCan2 (5´-ATAGGGCAGTCAAGGATTA-3´) yield a 315 bp product, when tested on 31 samples, taken from the yellow or red streaks veins (symptomatic) as well as from the green (asymptomatic) lamina of leaves (*Figure 1*) (data not shown). This PCR analysis allows the detection of CaYMV even in symptomless plant tissues and thus allows to avoid the purchase and propagation of infected plants. Nucleotide sequences analysis revealed a

high degree of homology (>98%) between the isolates from Perkeo, Lucifer, Opera La Boheme and V17. The incomplete sequences correspond to the region of ORF 3 for the *Badnavirus* genus, while BLAST analyses revealed only 6% of query coverage to *Canna streak virus* (Accession no. AJ810080, AJ810079) and 98% with CaYMV found in Italy and Netherlands (EF156357 to EF156364).

Conclusion

Since no mixed infections were found with TAV, BYMV and CMV, the etiology of the viral disease in the Austrian *Canna* cultivars studied might be associated mainly with CaYMV. This investigation reports for the first time the presence of CaYMV–infected *Canna* plants in Austria. The newly developed, highly specific PCR primers, yielding a short-sized product of 315 bp product at 55°C AT, allow the detection of the pathogen in symptomatic and symptomless plant tissues, with the exclusion of false negatives for the Austrian *Canna* accessions used in this study. This is important, since no commercial ELISA kit is available. These PCR primers can therefore be recommended to breeders of *Canna indica* as an efficient, specific early warning diagnostic tool to reduce the risk of spread of the disease.

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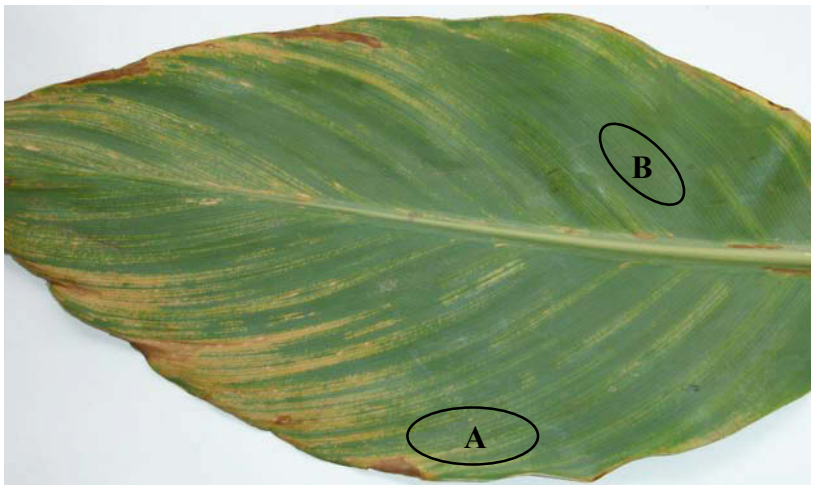
Table 1. Viral detection in a cultivar collection of *Canna indica* in Austria. TAV, BYMV and CMV were determined by DAS-ELISA and CaYMV by PCR using newly developed primers (pCan1/ pCan2).

	DAS-ELISA			PCR
Canna Accessions	TAV	BYMV	CMV	CaYMV
Lucifer Nr.1	-	-	-	+
Perkeo Nr.1	-	-	-	+
Opera La Boheme Nr.10	-	-	-	+
V 3 2004	-	-	-	-
V17	-	-	-	+
Thai IV	-	-	-	-
Ra 051104	-	-	-	-
Durban Nr. 10	-	-	-	-
Prince Charmant	-	-	-	-
Prof. Lorenz Nr. 10	-	-	-	-

Figure 1. Localization of samples taken from *Canna* indica cv. V17 (infected with CaYMV) leaves with vein streaking symptoms on (**A**) symptomatic (**B**) asymptomatic area.



CHAPTER No. 3

Phytoplasma infected plants in Austrian forests: role as a reservoir?

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***Key words*:** Vaccinium witches' broom, forest plants, phytoplasmas, molecular detection, micropropagation.

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Abstract

Reports on phytoplasma diseases in plant wild species are rare. Particularly interesting is the case in an Austrian forest, in the region Rosalia, Lower Austria, where a high number of plants, including *Euonymus europaea*, *Sorbus aucuparia*, *Fraxinus excelsior*, *Fagus sylvatica*, *Betula alba*, *Sambucus nigra*, *Pyrus* sp. and *Picea abies*, appeared with typical symptoms attributed normally to phytoplasma infection. Plants of *Rubus ideaus*, *Rubus fruticosus* and *Vaccinium myrtillus*, which represent small fruit species, showed clear symptoms. In preliminary test to verify phytoplasma presence in *V. myrtillus* the presence of phytoplasmas belonging to ribosomal group 16SrVI were identified after nested PCR on 16S ribosomal gene and restriction digestion with the appropriate enzymes. These plants were introduced *in vitro* to assure the conservation of the phytoplasma isolate for further analysis. Since in this area only a few home gardens are present in the neighbourhood of the forest land, the way of introduction of the phytoplasmas remains obscure.

Introduction

Phytoplasma diseases in wild plant species have been described sporadically, but in the majority of cases are overlooked or attributed to other causes, e.g. herbicide treatments. Disease development and the inoculum build-up in woody species however deserve particular attention, since overlooked foci of infection may develop disproportionate consequences.

Similarly as in studies on the genetic diversity of wild relatives of domesticated species also the migration of pathogens from and into the forest might represent a more complex pattern than the simple assumption that the diseases originate from the natural flora. Detection and identification of phytoplasmas is necessary for accurate disease diagnosis in both host systems, i.e. in plants, where phytoplasmas are localized in phloem cells, and in insect vectors. The choice of a particular method will vary according to the intention/goal of research, e.g. the determination of the degree of infection of an area, the distribution of different strains. Of extraordinary value is therefore *in vitro* reference material, which provides control samples independently of the season and prolongs the availability of strains, and has been attempted by several laboratories (BERTACCINI et al., 1992; 2000; JARAUSCH et al., 1996; LAIMER, 2003).

In an Austrian forest, in the region Rosalia, Lower Austria, a high number of plants appeared with typical symptoms attributed normally to phytoplasma infection, including *Euonymus europaea*, *Sorbus aucuparia*, *Fraxinus excelsior*, *Fagus sylvatica*, *Betula alba*, *Sambucus nigra*, *Pyrus* sp., and *Picea abies*. Plants of *Rubus ideaus*, *Rubus fruticosus* and *Vaccinium myrtillus*, (*Figure 1*) which represent small fruit species, showed also clear symptoms.

To verify phytoplasma presence molecular analyses were carried out.

Material and Methods

Symptomatic plant samples from the above described species were collected in the forest and preparation of midribs, or bark scrapings was carried out immediately from fresh material, before freezing 1 g samples in liquid nitrogen. For wild European blueberry (*Vaccinium myrtillus* L.) a plant exhibiting symptoms of shoot proliferation and small leaf was potted in the greenhouse (*Figure 1A*) to serve as donor material for tissue culture experiments. Tissue cultures were established according to standard procedures (STENICZKA et al., 2006).

DNA was extracted from plant samples using either the phenol/chloroform method (PRINCE et al., 1993) or with the commercial kit DNeasy-Qiagen according to the manufacturer's instructions.

To verify phytoplasma association with the described symptoms, molecular identification by PCR/RFLP analyses was carried out using general primers located in the 16S rDNA or at the beginning of spacer regions of the phytoplasma genome. Nested PCR experiments were carried out using in direct PCR R16mF2/mR2 (GUNDERSEN and LEE, 1996) and PA2F/PA2R followed by R16F2/R2 (LEE et al., 1995) or NPA2-F/NPA2-R respectively. The latter primer combination was designed to amplify in direct PCR a product of 1 187 bp between nucleotide 482 and nucleotide 1,669. Nested NPA2F/R primers amplifies a product from nucleotide 1 182 to nucleotide 1 667 (HEINRICH et al., 2001). A second nested PCR amplification on R16F2/R2 amplicons was also carried out using primers R16(I)F1/R1 (LEE et al., 1995).

RFLP analysis of PCR-amplified rDNA was employed using frequently cutting restriction endonucleases, such as *TaqI*, *TruI*, *TaiI*, and *Tsp509I* (Fermentas, Vilnius, Lithuania).

The PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to manufacture's instructions. The extracted DNA was sequenced by VBC-BIOTECH Service GmbH and analyzed with DNASTAR (DNAstar software package).

Results and Discussion

Using PA2R/PA2F followed by NPA2-F/NPA2-R in nested PCR assays, a product of 489 bp was amplified from DNA of blueberry samples from a plant with witches' broom symptoms, indicating that the plant was phytoplasma infected.

Nested PCR on P1/P7 amplicons with PA2R/PA2F primers produce the expected length DNA fragments only from symptomatic blueberry samples. Using RFLP analyses with *TruI* and *Tsp509I* it was possible to preliminary identify the phytoplasmas as belonging to 16SrVI ribosomal group (clover proliferation, sensu HEINRICH et al., 2001; CALARI et al., unpublished).

Sequence analyses confirmed the presence of a phytoplasma of the 16SrVI group in *V. myrtillus*, which so far was only reported to contain phytoplasmas belonging to the 16SrIII group (PALTRINIERI et al., 2000). The infected plant was successfully micropropagated (*Figure 1B*).

Phytoplasma presence was also detected after a second nested PCR amplifications with primers R16(I F1/R1 on R16F2/R2 amplicons in symptomatic samples from *R. fruticosus*, *R. ideaus* and *Fagus* spp. RFLP characterization allow to verify that *R. fruticosus* was infected by phytoplasmas belonging to ribosomal group 16SrI-B while in *R. idaeus* and *Fagus* samples phytoplasmas belonging to ribosomal subgroup 16SrXII-A were identified. Aspecific amplification was obtained with some of the other samples, molecular tests to further verify phytoplasma presence in these samples are in progress to evaluate the role of these phytoplasmas as reservoirs for epidemic outbreaks.

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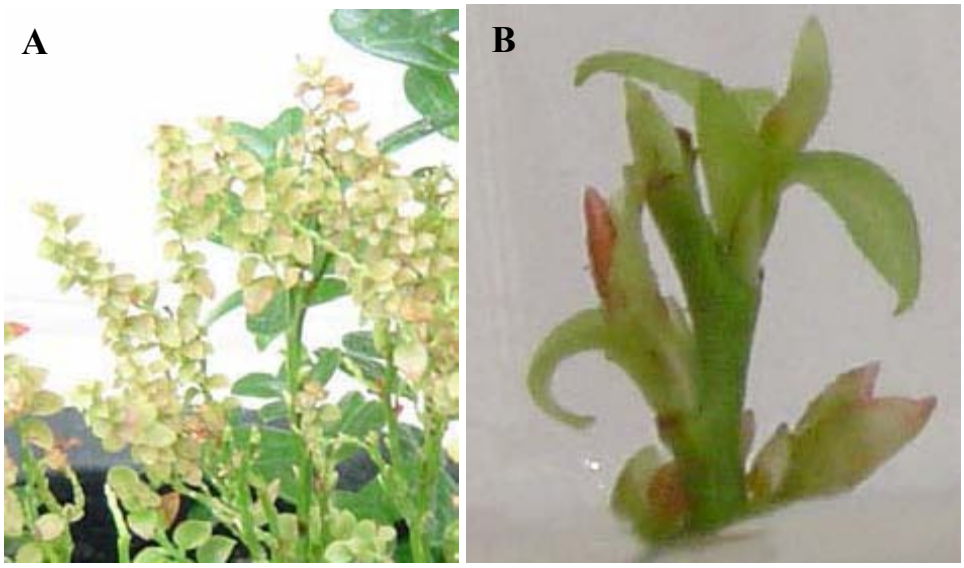
Figure 1. *Vaccinium myrtillus* with typical symptoms due to phytoplasma infection. **A**. *in vivo* **B.** *in vitro*.



CHAPTER No. 4

RT-PCR detection and protein-protein interaction of viral components of *Pineapple mealybug wilt associated virus 2* in Cuba

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***Key words**: Ampelovirus*, electron microscopy, *Pineapple mealybug wilt associated closterovirus-2*, yeast two-hybrid system

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Abstract

During a search for the causal agent of symptoms of mealybug wilt in Cuban pineapple plants, *Pineapple mealybug wilt associated virus 2* (PMWaV-2) was detected. A reverse transcription polymerase chain reaction (RT-PCR) test was developed to amplify seven ORFs, using primers designed on the RNA sequence of a Hawaiian isolate of PMWaV-2. Cuban and Hawaiian PMWaV-2 isolates shared high homology. Yeast two-hybrid assays showed a homodimeric nature for the 20 kDa protein (p20) and the 22 kDa protein (p22), but not for the 34 kDa coat protein (CP). This is the first report of protein-protein interactions between isolated protein components of PMWaV-2.

Mealybug wilt of pineapple (MWP) was first described in Hawaii and since then has been reported as a severe disease exclusively of pineapple crops worldwide (CARTER, 1942; ROHRBACH et al., 1988; HU et al., 1993; BORROTO et al., 1998; HUGHES and SAMITA, 1998). Leaves of MWP affected plants turn bronze-red colour, loose turgidity and show severe tip necrosis (ROHRBACH et al., 1988), although symptoms are cultivar dependent and in some cases plants recover (SETHER and HU, 2002). The metabolic changes induced in pineapple (Smooth Cayenne) with strong MWP symptoms include the appearance of high levels of abscisic acid, soluble proteins, free proline and phenols, coupled with peroxidase and acid invertase activities (NIEVES et al., 1996).

Although the involvement of viral agents and insect vectors is now accepted, the etiology of MWP was controversial. The association of *Ampelovirus* particles and mealybug species with the disease is long known (HU et al., 1996; SETHER and HU, 2002). GUNASHINGE and GERMAN (1989) identified long, flexuous, rod-shaped virus-like particles and isolated double-stranded RNA from MWP-diseased plants. Members of the *Closteroviridae* family have been associated with MWP in Hawaii, Australia and Cuba (BORROTO et al., 1998; GUNASHINGE and GERMAN, 1989; HU et al., 1993; WAKMAN et al., 1995). In accessions around the world, at least three serotypes of PMWaVs, members of the genus *Ampelovirus*, family *Closteroviridae* exist (MARTELLI et al., 2005, MELZER et al., 2001). As partially characterized, genome sizes were reported of 10.7 Kb for PMWaV-1 (seven ORFs; accession no. AF414119) and 14.8 kb for PMWaV-2 (ten ORFs; accession no. AF283103) were reported, respectively (MELZER et al., 2001). Among ampeloviruses, PMWaV-2 shares the highest sequence identity with *Grapevine leafroll-associated virus-3* (MELZER et al., 2001).

In Cuba, MWP disease represents an important economic problem for pineapple production, causing crop looses of up to 40% (ANONYMOUS, 1989). Initial work undertaken to develop efficient detection tools confirmed the presence of closterovirus-like particles in MWP-affected pineapple plants (*Ananas comosus* cv (L) Merr.) of cv Smooth Cayenne by electron microscopy (EM) (BORROTO et al., 1998).

The molecular analysis of Cuban isolates of pineapple ampeloviruses was done in order to further characterize the presence of PMWaV-2 in Cuba. Virus purification and EM were used to provide information about the size and shape of virus particles as well as to select infected plants for subsequent molecular characterization. Thus, RT-PCR technique in combination with sequencing was used to identify genomic sequences of PMWaV-2 and

protein-protein interactions of some viral components were studied to gain a better insight of fundamental mechanisms of virus functions.

Pineapple plants of cv Smooth Cayenne originating from tissue culture propagation were cultivated in open field conditions at the "Tomás Roig" Experimental station at Ciego de Avila, Cuba. Leaf tissue of mealybug wilt-affected plants was macerated in a mortar in the presence of extraction buffer (0.5 M Tris-HCl pH 8.4, containing 4% (v/v) Triton X-100 and 0.2% 2-mercaptoethanol) at a 1:2 (w/v) ratio. The stirring period for the slurry was 1.5 h and the filtrate clarified by centrifugation at 8 000 rpm for 15 min in a RPR 16-8 rotor (Hitachi, Tokyo, Japan). The supernatant was collected and layered over 5 ml of 20% (w/v) sucrose in TM buffer (100 mM Tris-HCl pH 8.5, 10 mM $MgCl_2$) and centrifuged at 30 000 rpm for 2 h and 35 min in Ti 70 rotor (Beckman). The pellet was dissolved in 500 µl TM buffer with 100 g of starting material. The suspension was stirred overnight at 4^{o}C, then centrifuged at 10 000 rpm for 5 min. Samples were negatively stained with 2% aqueous uranyl acetate and observed in a JEOL (JEM-200 EX) TEM at X30 000 magnification with 80 kV.

In order to process large amounts of MWP affected pineapple leaves and to increase the virus titre minor, modifications of a purification protocol for a pineapple ampelovirus (GUNASHINGE and GERMAN, 1989) were necessary. Highest yields were obtained by increasing the stirring period for the first supernatant, by reducing the time of differential centrifugation and by prolonging density gradient centrifugation.

Electron microscopy of the purified virus showed the presence of long, flexuous, rod-shaped virus particle strongly resembling PMWaV (*Figure 1A*). No such particles were seen in symptomless greenhouse plants derived from plants sanitized by meristem tip culture. Purified preparations of PMWaV from Hawaii and Australia were reported to have modal length of 1 200 nm and 1 200-1 600 nm in purified preparations, respectively (GUNASHINGE and GERMAN, 1989; WAKMAN et al., 1995). However in Australia, particles of 1 700-1 900 nm were also trapped from the sap of affected plants (WAKMAN et al., 1995). The Cuban PMWaVs were isolated from 16 MWP affected pineapple plants and 75% (36 out of 48) of particles measured had an estimated size of 1 200-1 450 nm and a diameter of 12 nm (*Figure 1A*). These values are similar to those described from Hawaii (GUNASHINGE and GERMAN, 1989), but different from those reported in Australia (WAKMAN et al., 1995). However the possibility of particle fragmentation during purification may not be ruled out.

The presence of putative PMWaVs in Cuban pineapple crops prompted us to test the potential association between MWP and the presence of these viruses. At the time of our analysis a nearly full-length sequence of the PMWaV-2 genome was available (MELZER et al., 2001; accession no. AF283103). Using this information, combined with our electron microscope (EM) observations for diagnosis of PMWaV, specific oligonucleotide primers were designed for use in RT-PCR to confirm the presence of this virus in Cuban MWP-affected pineapple crops (*Table 1*). Total RNA was isolated from 90 mg of leaf tissue of three individual, EM-positive plants, using an RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA quality and concentration were determined by gel electrophoresis and spectrophotometry, respectively. Five micrograms of total RNA was used as a template for reverse transcription using the Superscript RT II kit (Invitrogen, Carlsbad, CA, USA). Two microliters of cDNA was used as PCR template for optimizating the use of different primer sets (*Figure 1B*). Proofreading polymerase (*pfu*, Stratagene, La Jolla, CA, USA) was used to ensure sequence fidelity. PCR amplicons corresponding to the predicted product sizes were cloned into pGEM-T (Promega, Madison, WI, USA) and five clones of each were sequenced and analyzed.

The resulting DNA sequences were almost identical (99.4% identity CP and p20 and 97.5% identity for the p22) to those of seven ORFs predicted from the sequence of PMWaV-2 and corresponded to: ORF2 (p5, 144 bp), ORF4 (p46, 1209 bp), ORF5 (CP, 906 bp; accession no. DQ225114), ORF6 (CPm, 1473 bp), ORF7 (p20, 503 bp; accession no. DQ228819), ORF8 (p22, 575 bp; accession no. DQ228818) and ORF9 (p6, 150 bp). This procedure allowed specific detection of nucleotide fragments of PMWaV-2 by RT-PCR within 8 hours and conclusively confirmed the presence of PMWaV-2 in Cuban pineapples.

The extracts observed under EM certainly contain PMWaV-2 particles but, dual infection with PMWaV-1 or *Pineapple mealybug wilt associated virus-3* (PMWaV-3), as observed in pineapple plants from Hawaii (MELZER et al., 2001; SETHER et al., 2005) cannot be excluded.

The genome organization of PMWaV-2 resembles that of members of the *Closteroviridae* family (MARTELLI et al., 2005). The 21-kDa protein (p21) of *Beet yellows virus* (BYV), is a suppressor of RNA silencing and it is known that proteins from other viruses of the genus *Closterovirus* also possess similar suppressor activity (CHIBA et al., 2006; REED et al., 2003). *Citrus tristeza virus* (CTV) is known to have evolved a strategy involving three different silencing suppressors: p20 and CP, which interfere with the

systemic spread of silencing, and p23, which suppresses the local silencing (LU et al., 2004).

The CP gene of PMWaV-2 shows a 42.2% amino acid identity with GRLaV-3 CP and a moderate homology to CPs of other closteroviruses (MELZER et al., 2001). The same authors proposed that p20 or p22 in PMWaV-2 might have a similar activity, but with a different mode of action. QU and MORRIS (2005), mentioned that the majority of characterized plant virus suppressors do not share any obvious sequence or structural similarity across viral families and groups.

Yeast two-hybrid technology has been successfully applied to investigate protein-protein interactions in closteroviruses (GOWDA et al., 2000). To investigate protein-protein interaction of PMWaV-2 components, potentially involved in the regulation of virus pathogenesis, p20, p22 and CP were cloned into yeast two-hybrid vectors and co-transformed into yeast cells (*Figure 2*). The DNA fragments of p20, p22 and CP genes were obtained by digestion of the pGEM-p20, pGEM-p22 and pGEM-CP plasmids with EcoRI and BamHI enzymes and subcloned in pGBT9 and pGAD424 vectors (Clontech. San Jose, CA, USA). The yeast HF7c reporter strain (Clontech, San Jose, CA, USA) was co-transformed with pGAD-p20, pGAD-p22, pGAD-CP and empty pGAD424 against the pGTB9-p20, pGTB9-p22, pGTB9-CP and empty pGTB9 using the lithium acetate method (GIETZ et al., 1992). After incubation for two days on medium without Leu and Trp at 30^{o}C, colonies were plated on His-lacking SC (Synthetic Complete) medium. Homo-dimerization was shown by p20 and p22, as indicated by the ability to grow in autotrophic medium of the co-transformed yeast with both genes in both orientations (prey and bait). This result suggests that p20 and p22 play a preferential role *in vivo* rather as homodimeric than as heterodimeric complexes (*Figure 2*) and suggests that these three proteins (p20, p22 and CP) apparently do not have any overlapping functions. Analysis of additional viral components will yield more information on their mechanisms and functions.

Our analyses confirm the presence of PMWaV-2 in Cuban pineapple plants. Results obtained from the yeast two-hybrid assays contribute to the knowledge of ampelovirus proteins with as yet unknown functions. Constructs involving the genes coding for p20 and p22 will be considered as candidates for the production of transgenic pineapple plants to study their possible protection against PMWaV-2, an approach that has been successfully used for potyviruses and closteorviruses (DI NICOLA-NEGRI et al., 2005; FAGOAGA et al., 2006).

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Table 1. Primer sequences used for the detection of seven ORFs of PMWaV-2 in pineapple

ORF	Primers (5' to 3')
p5 (5 kDa protein)	CCGAATTCATGTTAGACGCTTTCACAGCC
	GGGGATCCTTACGCCGCTCCATACGATCGC
p46 (46 kDa protein)	CCGAATTCATGCATCGCGAGTCCGCCTTGAC
	CCGGATCCTTAAGTATTCGAACCATACTCTCCGCC
CP (34 kDa coat protein)	GGGAATTCATGGCTCAGAATTACGTAGCCG
	GGGGATCCCTACCCTGAAACAGCTCCCTGG
CPm (56 kDa minor coat protein)	GGGAATTCCTATGTGGCTTTAAGCTTAATCG
	CCGAATTCATGGAATTTCAGCGGATACCTGC
p20 (20 kDa protein)	CCGAATTCATGGAGTTTAGACCGATAGAAG
	GGGGATTCCTTGGGTAACAGAATAGTTGCG
p22 (22 kDa protein)	CCGAATTCATGAGTGAGGAGATCCTGAAGTCGGC
	GGGGATCCCGACAGTTTCGGGTATATAACTC
p6 (6 kDa protein)	CCGAATTCATGAACACGAATGCTAAAAAATATC
	GGGGATCCTTAATATTCATTTATATCTTTTATTATC

Underlined sequences correspond to enzyme restriction sites overhang (*BamHI* and *EcoRI*).

Figure 1. Molecular identification of viral particles and RT-PCR analysis of PMWaV-2 in pineapple (Smooth Cayenne) tissue from Cuba. (**A**) Electron micrograph of PMWaV particle. Arrows indicate a viral-like particle (Bar= 200 nm); (**B**) Agarose gel electrophoretic analysis of RT-PCR products. Dashes indicate the correct band of the expected sizes. Numbers below each lane represent the annealing temperature used in the PCR program. Asterisk mark the correct band when more than one product was present.

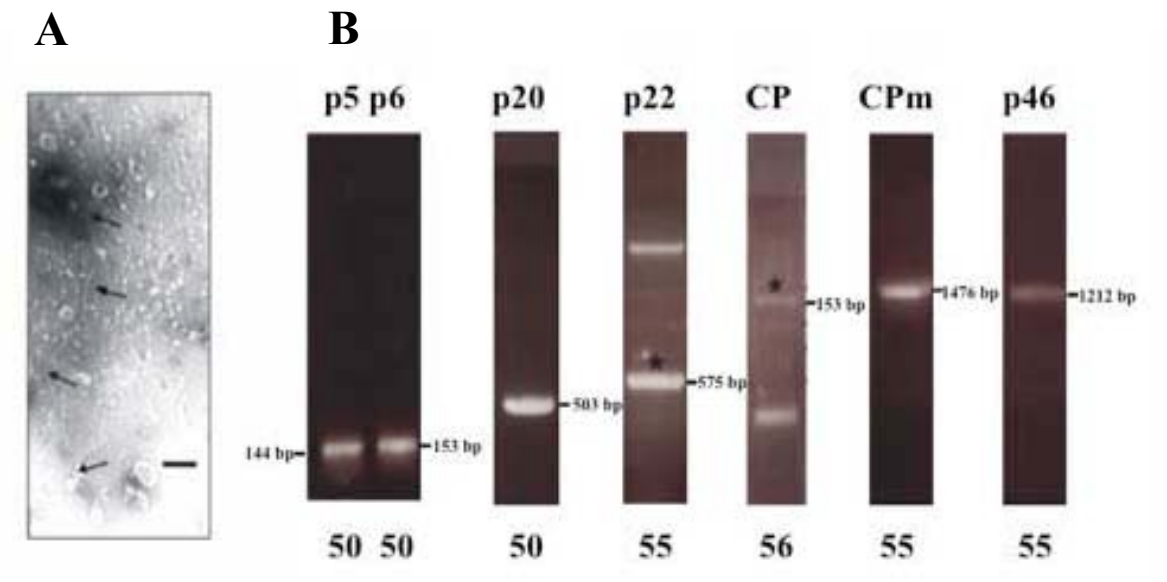
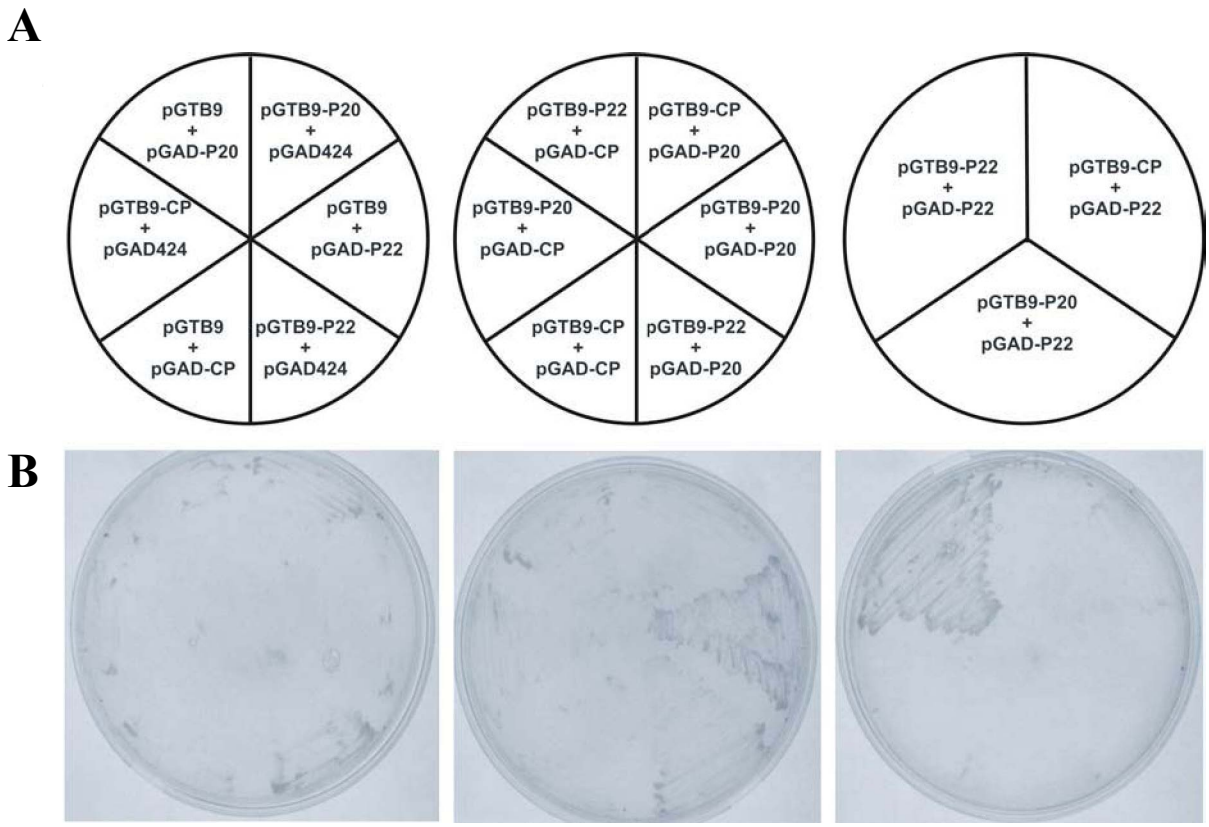


Figure 2. Yeast two-hybrid assays for the protein-protein interaction analysis of three PMWaV-2 proteins. (**A**) Diagram of arrangement of yeast strains on each set of plates; (**B**) Growth of yeast on His-lacking SC medium.



CHAPTER No. 5

Endogenous pararetroviral sequences in tomato (*Solanum lycopersicum*) and related species

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Abstract

Endogenous pararetroviral sequences (EPRVs) are a recently discovered class of repetitive sequences that is broadly distributed in the plant kingdom. The potential contribution of EPRVs to plant pathogenicity or, conversely, to virus resistance is just beginning to be explored. Some members of the family Solanaceae are particularly rich in EPRVs. In previous work, EPRVs have been characterized molecularly in various species of *Nicotiana* including *N. tabacum* (tobacco) and *Solanum tuberosum* (potato). Here we describe a family of EPRVs in cultivated tomato (*Solanum lycopersicum* L.) and a wild relative (*S. habrochaites*). Molecular cloning and DNA sequence analysis revealed that tomato EPRVs (named *Lyc*EPRVs) are most closely related to those in tobacco. The sequence similarity of *Lyc*EPRVs in *S. lycopersicum* and *S. habrochaites* indicates they are potentially derived from the same pararetrovirus. DNA blot analysis revealed a similar genomic organization in the two species, but also some independent excision or insertion events after species separation, or flanking sequence divergence. *Lyc*EPRVs share with the tobacco elements a disrupted genomic structure and frequent association with retrotransposons. Fluorescence *in situ* hybridization revealed that copies of *Lyc*EPRV are dispersed on all chromosomes in predominantly heterochromatic regions. Methylation of *Lyc*EPRVs was detected in CHG and asymmetric CHH nucleotide groups. Although normally quiescent EPRVs can be reactivated and produce symptoms of infection in some *Nicotiana* interspecific hybrids, a similar pathogenicity of *Lyc*EPRVs could not be demonstrated in *Solanum* L. section *Lycopersicon* [Mill.] hybrids. Even in healthy plants, however, transcripts derived from multiple *Lyc*EPRV loci and short RNAs complementary to *Lyc*EPRVs were detected and were elevated upon infection with heterologous viruses encoding suppressors of PTGS. The analysis of *Lyc*EPRVs provides further evidence for the extensive invasion of pararetroviral sequences into the genomes of solanaceous plants. The detection of asymmetric CHH methylation and short RNAs, which are hallmarks of RNAi in plants, suggests that *Lyc*EPRVs are controlled by an RNA-mediated silencing mechanism.

Introduction

Plant pararetroviruses (*Caulimoviridae*) have double-stranded DNA genomes and are considered retroelements because they use reverse transcription for replication. Unlike other retroelements, such as retroviruses and retrotransposons, integration into the host genome is not essential during their replication cycle. Nevertheless, in recent years there have been accumulating reports of endogenous pararetroviral sequences (EPRVs) in the nuclear genomes of many plants including tobacco (*Nicotiana tabacum*) and other *Nicotiana* species (JAKOWITSCH et al., 1999; LOCKHART et al., 2000; GREGOR et al., 2004), potato (HANSEN et al., 2005), banana (HARPER et al., 1999b, NDOWORA et al., 1999, GEERING et al., 2005), petunia (RICHERT-PÖGGELER et al., 2003) and rice (KUNII et al., 2004). EPRVs are assumed to integrate by illegitimate recombination into the host genome, where they may accumulate to high copy numbers (JAKOWITSCH et al., 1999; HARPER et al., 2002). Although EPRVs are being detected in an increasing number of plant species, the detailed structure of individual EPRV integrants and flanking regions has been analysed only in a few families (GREGOR et al., 2004; KUUNI et al., 2004; RICHERT-PÖGGELER et al., 2003; NDOWORA et al., 1999; JAKOWITSCH et al., 1999).

The role of EPRVs in plant-virus interactions is not yet fully understood. Current information suggests that EPRVs are not always neutral components of plant genomes but can potentially contribute to either pathogenicity or virus resistance in the host. Indeed, integrated sequences of *Banana streak virus* (BSV), *Tobacco vein clearing virus* (TVCV) and *Petunia vein clearing virus* (PVCV) (RICHERT-PÖGGELER et al., 2003; LOCKHART et al., 2000; HARPER et al., 1999b; NDOWORA et al., 1999) can be reactivated in response to abiotic or genomic stress. Episomal copies are probably formed by transcription from tandemly arranged integrants or recombination from fragmented integrants (NDOWORA et al., 1999; RICHERT-PÖGGELER et al., 2003), which leads to the assembly of virus particles and symptoms of virus infection. Interspecific crosses and *in vitro* propagation can induce EPRV reactivation, which has been shown to be economically detrimental in banana breeding (L`HEUREUX et al., 2003; DALLOT et al., 2001; LOCKHART et al., 2000; HARPER et al., 1999a; NDOWORA et al., 1999).

Under different conditions or in other genome constitutions, EPRVs remain silent and might even have beneficial effects for their hosts by providing virus resistance via homology-dependent transcriptional or posttranscriptional gene silencing (JAKOWITSCH et al., 1999; HULL et al., 2000). Consistent with this proposal, EPRV-derived enhancer-promotor sequences integrated as transgenes into tobacco chromosomes became silenced

and methylated in the presence of homologous EPRVs (METTE et al., 2002). Homology-dependent silencing can be induced by several interrelated pathways (MEINS et al., 2005) that involve aberrant or double stranded RNA that is processed to short RNAs by RNAseIII-like enzymes (Dicer). Post-transcriptional gene silencing (PTGS), which is the plant equivalent of RNAi, is able to counteract RNA and DNA viruses at the mRNA level (COVEY and AL KAFF, 2000; VOINNET et al., 1999). In addition, RNA-mediated epigenetic modifications, such as RNA-directed DNA or histone methylation (MATZKE and BIRCHLER, 2005), could transcriptionally repress DNA viruses at the chromatin level. Further elucidation of host control over EPRVs will not only faciliate assessment and the prevention of EPRV reactivation but may also suggest strategies for genetically engineering pathogen resistance in agriculturally important plants.

Studies so far indicate that EPRVs are abundant in some members of the family *Solanaceae*, an economically important taxon that includes tobacco, petunia, potato, (bell) pepper (*Capsicum annuum*) and tomato. In addition to its role as an important food crop, cultivated tomato, *S. lycopersicum* subsection lycopersicon, represents a model plant within this family with a small diploid genome (2n=24, size 953 Mb; ARUMUGANATHAN and EARLE, 1991) with a high-density genetic map (TANKSLEY et al., 1992), and large mutant collection (http://tgrc.ucdavis.edu). Recently, it has been chosen for sequencing by an international consortium (MUELLER et al., 2005). Repetitive sequences comprise wide blocks of pericentromeric heterochromatin in the tomato genome (BUDIMAN et al., 2000; PETERSON et al., 1996) that nevertheless also harbour a considerable share of genic sequences (GUYOT et al., 2005; PETERSON et al., 1998). In an *S. lycopersicum* (HEINZ 1706) BAC library (BUDIMAN et al., 2000), 194 of the 1205 sequenced-tagged connectors (STCs) were similar to retrotransposons and four were similar to tobacco EPRVs, although these sequences were not characterized further.

To increase our understanding of endogenous pararetroviral sequences in economically relevant, genetically tractable crops, we have characterized a family of EPRVs in *S. lycopersicum* and a wild relative, *S. habrochaites* which is exploited in crosses with *S. lycopersicum* to introgress favourable traits (KABELKA et al., 2002; VIDAVSKY and CZOSNEK, 1998) with respect to sequence and structure of a number of integrated copies, as well as to chromosomal localization. In addition, we have analysed the methylation status of the EPRV integrants and their transcriptional activity in *S. lycopersicum*, *S. habrochaites* and interspecific hybrids to investigate the nature of host control of these sequences.

Material and Methods

Plant material and DNA isolation

Seeds of *Solanum lycopersicum* L. (syn. *Lycopersicon esculentum* Mill.) "MicroTom" were provided by Dr. A.A. Levy, Rehovot, Israel. *S. pimpinellifolium* L. (syn. *Lycopersicon pimpinellifolium* (L.) Mill.) IPK genebank accession LYC 1835, *S. cheesmaniae* (L.Riley) Fosberg (syn. *Lycopersicon cheesmaniae* L.Riley) IPK genebank accession T 675, S.peruvianum L. (syn. Lycopersicon peruvianum (L.) Mill.) IPK genebank accession T 353 and *S. habrochaites* S. Knapp & D.M. Spooner (syn. *Lycopersicon hirsutum* Dunal) IPK genebank accession T 436 were procured from the „Institut für Pflanzengenetik und Kulturpflanzenforschung"(IPK) in Gatersleben, Germany. *S. lycopersicum* ("Moneymaker") lines were obtained from D. Scharf, Frankfurt University. Plants were grown in the greenhouse. Genomic DNA was isolated from leaves with the DNeasy Plant Maxi kit (Qiagen) following the manufacturer's instructions.

λ-library and sequencing

Two genomic DNA libraries were prepared from *Solanum lycopersicum* ("MicroTom") and *S. habrochaites* using the λ-FIX II system (Stratagene) according to the protocols provided by the supplier. The libraries were screened with a subcloned 5.5 kb *Not*I-*Hin*dIII fragment of NsEPRV clone V6 corresponding to the approximate NsEPRV nucleotide positions 2-7.5 kb (JAKOWITSCH et al., 1999). Λ-DNA was isolated using the Lambda Midi Kit (Qiagen) and sequenced with fluorescent chain terminators (ABI PRISM 3100 system). For analysis of DNA sequences the software programs BLAST (ALTSCHUL et al., 1997) and CLUSTAL (THOMPSON et al., 1997; THOMPSON et al., 1994) were used, homology searches employed public domain sequence databases (GenBank, EMBL, DDBJ, SwissProt, PDB, PIR, PRF). GenBank/EMBL/DDBJ accession numbers for sequences reported in this paper are DQ273220-DQ273264.

Southern hybridization

For Southern hybridization 1 to 2 µg of genomic DNA was sequentially digested with *Xba*I and an additional enzyme of the appropriate isoschizomer pair, fractionated on 1.5% agarose gels and transferred onto nylon membranes (Hybond N, Amersham) using standard techniques. Fragments amplified from clone Le1 with primers Le1-L: 5´GGAGGTATGACCA CGGATATAA 3´/ Le1-R: 5´CCTGGTGCTAACTCTATTCCTG 3` (probe E1) and from clone Lh7 with primers Lh7-L: 5`GCAAGATATATCAGAAAGATTCC 3´/ Lh7-R: 5´CCTTAGGATGGCATAGTCTG 3` (probe H7), respectively, were radiolabelled with α-[P^{32}]dATP (Amersham) by random

priming and hybridized onto Southern blots at 65°C in 6xSSC overnight and washed at 65 °C in 1xSSC (saline sodium citrate)/1%(w/v) SDS (sodium dodecyl sulphate).

RT-PCR and cDNA cloning

Total RNA was isolated from leaf material using the RNeasy Plant Mini kit (Qiagen) and enriched for polyA^+ RNA using the Oligotex mRNA Mini kit (Qiagen). First strand DNA was produced by Revert Aid H Minus M-MuLV Reverse transcriptase (Fermentas) according to standard protocols and used in PCR reactions with the following primer pairs: CP: 5`CWTGTTAYAAYTGYGGAAARWTAGGAC 3`/ MP: 5`TTTCWATRGGNGTATCTATTCCTTCTC3` and TAV1: 5`RMWDNTANHAGTCAGCAGCATGAC3`/TAV2:5`CATHRHYTGATCTCKTDHAT ARTA3` for the coding region (annealing temperature: 50°C) and IGR1: 5`CWYTTAAGWTYATGAGTAGCTAWATTAATTTATTCCTG 3`/ IGR2: 5` CCTCAAMTYTGTTTAMTCCCCTAAACGG 3` (annealing temperature 56°C) for the intergenic region (*Figure 5B*). An actin sequence spanning an intron was amplified in parallel to detect genomic DNA contaminations using the primer pair ActL: 5`GTTGCTATTCAGGCTGTGCT 3`/ ActR: 5`TCTTTTCAATGGAGGA GCTG 3` (annealing temperature: 50°C). Reactions (50µl) contained 50 pmol of each primer, 1.5 mM $MgCl_2$, 150 µM dNTPs, 0.25U *Taq* Polymerase and ~ 50ng 1st strand DNA. PCR products were gel purified and cloned into the pGemT vector (Promega). In order to discriminate between different copies, cloned fragments were *Hin*fI restricted and separated on agarose gels. Fragments producing different restriction patterns were sequenced.

Short RNA extraction and hybridization

RNA enriched for the low-molecular weight fraction (10 to 100nt) was isolated from leaves and flowers, samples of 50µg per lane were separated on a 15% polyacrylamide gel containing 7M urea and transferred onto nylon membranes (Hybond N^+, Amersham) following the protocols described in METTE et al., (2005). The blots were hybridized with RNA probes of both orientations derived from the cloned cDNA fragments of IGRcLe-8 (DQ273223) for the intergenic region and from pooled TAVcLe-4, TAVcLe-8, TAVcLe–19 (DQ273225, DQ273229, DQ273228) for the TAV region. Hybridization conditions and probe preparation were following METTE et al., (2005), omitting the probe fragmentation step.

Heterologous virus infection

For mechanical transmission trials, plants at the six leaf stage were inoculated with leaf extracts from *S. lycopersicum* infected plants with *Potato virus Y* (PVY) strain PVY-NTN (BECZNER et al., 1984) or with *Tomato bushy stunt virus* (TBSV) strain TBSV-type (MARTELLI et al., 1988), respectively. The virus strains were obtained from the Department of Plant Protection Virology, University of Bari, Italy. Infected leaves were ground in 0.1 M phosphate buffer (pH 7.2) with 0.2% DIECA and the extract was rubbed on celite-dusted plants. The virus spread to younger leaves after 4–6 weeks post inoculation was verified by ELISA using TBSV and PVY detection kits (Loewe, Germany). An ELISA sample was taken as positive when its OD value was at least three times higher than the negative control values. All determinations were run in duplicate.

Fluorescent in situ hybridization (FISH)

Root tips from seedlings or plants growing in pots were treated with 0.02M 8-hydroxyquinoline, fixed in ethanol: glacial acetic acid (3:1), digested with proteolytic enzymes, and dissected in 60% (v/v) acetic acid. Chromosome preparations were either made by squashing (SCHWARZACHER and HESLOP-HARRISON, 2000) or spreading (ZHONG et al., 1996). Flower buds were fixed untreated and anthers were dissected and the stage of meiosis determined to be pachytene, before they were processed as above.

The ribosomal probe (clone pTa71), contains a 9 kb *Eco*RI fragment of the repeat unit of 25S-5.8S-18S rDNA from *T. aestivum* (GERLACH and BEDBROCK, 1979). Part of the dispersed middle repetitive tomato sequence U30 (VOSMAN and ARENS, 1997) was amplified and cloned from *S. lycopersicum* (DQ273250). Mixtures of four probes each for *S. lycopersicum* and *S. habrochaites* (LycEPRV-Le, LycEPRV-Lh) were selected (*Table 2*). PCR amplified inserts of clones were labelled with biotin α16-dUTP (Roche) or digoxigenin α11-dUTP (Roche) by random priming (Bioprime & Random primer kit; Invitrogen).

In situ hybridization followed SCHWARZACHER and HESLOP-HARRISON (2000). The hybridization mixture consisted of 50 to 100 ng/slide of each probe, 50% (v/v) formamide, 2x SSC, 10% (v/v) dextran sulphate, 0.12% (w/v) SDS, 0.12mM EDTA (ethylene-diamine-tetra-acetic acid) and 1µg/µl salmon sperm DNA. After overnight hybridization, slides were washed in 20% (v/v) formamide/0.1x SSC at 42°C, giving a hybridization stringency of 85%. Hybridization sites were detected by streptavidin conjugated to Alexa 594 (Molecular Probes) or FITC (fluorescein isothiocyanate) conjugated anti-digoxigenin antibody (Roche) in 4xSSC, 0.1% (v/v) Tween-20, 5% (w/v) BSA (bovine serum

albumin). Preparations were stained with DAPI (4'-6-diamidino-2-phenylindole) and analysed on an Axioplan 2 epifluorescence microscope (Zeiss) with single band pass filters equipped with a cooled colour CCD camera (Optronics, model S97790). FISH and DAPI images were overlaid using the RGB channels of Adobe Photoshop CS and CS2 software; DAPI images were sharpened using the Gaussian deblur function and colour balance and processing of the FISH signal was achieved using only those function that treat all pixels equally. For the pachytene overlay figures (*Figures 3E and F*) the captured colour images were converted to gray image, enhanced and overlaid: DAPI images were left B&W and the FISH signals were falsely coloured red and green, respecively. Each hybridization experiment was at least carried out twice and for each probe eight to twenty cells were analysed.

Results

Lyc*EPRV identification, isolation and sequence analysis*

Tomato EPRVs were originally detected by DNA blot analysis using a 5.5 kb DNA fragment of *Ns*EPRV (*Nicotiana sylvestris* EPRV), one of three EPRV families in tobacco; LOCKHART et al., 2000; JAKOWITSCH et al., 1999) to probe DNA prepared from various species of *Solanum*. The resulting banding pattern was complex, with numerous strong and weak bands superimposed on a background smear (*Figure 1*). This pattern is reminiscent of that observed with *Nicotiana* species (JAKOWITSCH et al., 1999) and suggests a dispersed organization of multiple copies of a related EPRV family. Judging from the hybridization intensity, the relative copy number of the elements detected by the *Ns*EPRV probe was similar in all five *Solanum* species tested. The banding pattern in *S. lycopersicum* strongly resembled that in *S. cheesmaniae* and *S. pimpinellifolium*, whereas notable differences were observed in *S. habrochaites* and *S. peruvianum* (*Figure 1*).

To analyze the tomato EPRV sequences in more detail, a genomic λ-library was constructed from cultivated tomato (*S. lycopersicum* "MicroTom"; SCOTT and HARBAUGH, 1989) and the wild relative *S. habrochaites*. Both λ-libraries were screened with the 5.5 kb fragment of *Ns*EPRV. Five positive clones were isolated and partly sequenced for *S. lycopersicum* and nine for *S. habrochaites*. Each clone contained EPRV-like DNA and flanking plant genomic sequences (*Figure 2A, Table 1*).

EPRV-like sequences from both species were AT-rich (65.4-78.4%) and were most similar to EPRVs in *Nicotiana*, revealing up to 83% sequence identity to endogenous *Tobacco vein clearing virus* (TVCV; LOCKHART et al., 2000), *Ns*EPRV (JAKOWITSCH et

al., 1999), and *Nto*EPRV (*N. tomentosiformis* EPRV; the second EPRV family in tobacco; GREGOR et al., 2004). Similar to the *Nicotiana* EPRVs, four open reading frames (ORFs) were identified (*Figure 2A*): coat protein (CP), cell-to-cell movement protein (MP), polyprotein (POL) and transactivator protein (TAV). The POL domain revealed 80 to 90% identical nucleotides, compared to MP (75 to 91%) and TAV (63 to 95%). Only one clone contained a full CP sequence that showed 65 to 94% sequence identity to fragments of CP sequences from other clones. The identity between DNA sequences derived from the same species (*S. lycopersicum* or *S. habrochaites*) was generally not higher than between species. Thus, in the subset of clones analyzed, no species-specific clusters of identity were identified and sequences within one species are as divergent as between species. We therefore assigned these sequences to a single family termed *Lyc*EPRV (Lycopersion endogenous pararetrovirus).

The putative amino acid sequence identities of the coding regions ranged from 60 to 87% identity for MP, 72 to 89% for POL and 48 to 91% for TAV (CP shares 39 to 85% identity to various fragments). However, all of the cloned protein-coding regions are either truncated or harbour several frameshifts and stop codons and can therefore be considered translationally defective, a feature also found with *Nicotiana* EPRVs. Nine of the clones contained parts of the putative non-coding intergenic region (IGR) of the virus. The IGR was less conserved compared to the ORFs except for a 272 to 282 bp box (*Figure 2B*) which revealed 80 to 92% sequence identity on the nucleotide level. The conserved 272 to 282 bp box has an overall identity of up to 70% with its counterpart in *S. tuberosum*, SoTu (HANSEN et al., 2005) and 80% to the IGR of *Nicotiana* EPRVs with several highly conserved motives. Some IGR sequences contained short (27 to 104 bp) AT-rich structures of low complexity (Lh2, Lh5, Le4, Le5) while others revealed short (12 to 24 bp) direct repeats which were not conserved between the different IGRs (Lh2, Lh5, Lh7, Le5). Some clones (Lh7, Lh2, Lh3) contain a conserved 12 bp motif complementary to the 3´end of the $tRNA^{Met}$ (5´-TGGTATCAGAT/GC-3´) 50 to 60 bp upstream of this box as well as a putative polyadenylation signal (5´-AATAAA-3´) and a putative TATA box (5`-TATAAA-3`) at a distance of 130 to 140 bp and 150 to 160 bp upstream, respectively.

All of the cloned *Lyc*EPRV sequences were truncated and flanked either by plant DNA unrelated to EPRVs or by rearranged (fragmented, inverted or otherwise partly duplicated) EPRV regions that appeared to be out of context when compared to the TVCV-like consensus structure (Le4, Le5, Lh3, Lh7; *Figure 2A*). Nearly all *Lyc*EPRV junctions analysed adjoin transposable elements, most frequently retrotransposon LTRs or related

sequences (see *Table 1* and *Figure 2A*). Clones from *S. habrochaites* revealed homologies to members of the PCRT1 family, a *Ty3-gypsy* (*Metaviridae*) element that is dispersed throughout the pericentric heterochromatin of *S. lycopersicum* (AY850394; YANG et al., 2005). The LTRs of PCRT1 partly correspond to the repetitive families TGRII and U30, the latter of which comprises more than 4000 copies in the *S. lycopersicum* genome (YANG et al., 2005; VOSMAN and ARENS, 1997). The junctions between EPRV and PCRT1 sequences were verified for three clones by PCR amplification from genomic DNA (Lh2, Lh4 and Lh7, data not shown), confirming that the *Lyc*EPRV sequences are indeed physically joined to plant DNA while these sequences could not be amplyfied in *S. lycopersicum*.

We reconstructed a general structure from the alignments of several incomplete sequences (upper bar in *Figure 2A*). The coding region closely resembles that of the tobacco elements (*Ns*EPRV, *Nto*EPRV) in size with 1779 bp for CP, 1293 bp for MP, 1933 bp for POL which overlaps with TAV (1279 bp) forming a coding region of 6221 bp. The intergenic region varies between 1606 to 1680 bp for different clones, summing up to a total length of approx. 7900 bp (7827 to 7901 bp) for a putative full copy of *Lyc*EPRV. The 140 kb sequence of a BAC clone (AC171732) that was submitted only recently (November 2006, note added in revision) revealed a single *Lyc*EPRV copy. A single stretch of 6125 bp of this sequence corresponds to the putative *Lyc*EPRV coding region and reveals the same order of the four ORFs as reconstructed from the λ-clones. The coding region is flanked by altogether 1542 bp homologous to the IGR on both sides and reveals only one internal stop codon. The nucleotide sequence of this copy contains 84–96% identical nucleotides compared with the λ-clones and 76–92% homology to TVCV. Approximately 2.7 kb upstream of this LycEPRV copy sequences homologous to the LTR of PCRT1a could be identified

Fluorescent in situ hybridization (FISH)

To analyze the chromosomal distribution of *Lyc*EPRVs, we performed FISH on root tip metaphase chromosomes and pollen mother cells at meiotic prophase of *S. lycopersicum* and *S. habrochaites*. By mixing several probes covering most of the *Lyc*EPRV (*Lyc*EPRV-Se; *Table 2*), we were able to observe several weak *Lyc*EPRV-SI hybridization sites with signal strength of several magnitudes lower than that observed with the control 45S rDNA probe. Sites were visible in varying number near the centromeres of most *S. lycopersicum* chromosomes (*Figure 3A, B*): there were four to six chromosomes with a stronger signal, four chromosomes showing very weak signals

(arrows) and no signal in the NOR region. Similar results were obtained with extended pachytene chromosomes demonstrating that the EPRV signals were located mainly in the DAPI positive pericentromeric heterochromatin or intercalary chromocentres (*Figure 3D, E* arrowheads), but rarly in the euchroamtin. The weak, but in cases distinct signals of varying size and arrangements indicate that probably only few copies of *Lyc*EPRV-Sl are integrated in each cluster, that they might not contain all parts of the probe used or that sequences are only partly conserved. The FISH data (*Figure 3A-C*) support the results from Southern hybridization (*Figure 1*) and cloning as well as sequencing data derived from λ-clones (*Figure 2*) and the BAC clone AC171732 indicating that *Lyc*EPRV-Sl are probably not arranged in perfect tandem arrays, are truncated and frequently degenerated.

FISH of *Lyc*EPRV-Sl in combination with the retroelement sequence U30 on metaphases (*Figure 3C*) and pachytene chromosomes (see Additional file 1) showed signal from both sequences near the centromeres. The signal of the U30 probe covered a larger area of the centromeric heterochromatin while the *Lyc*EPRV-Sl hybridization signal appeared to be nested within the U30 hybridizing regions. The U30 signal, as the *Lyc*EPRV-Sl signal, was absent from the NOR regions (*Figure 3C*) as has been previously reported (CHANG 2004). FISH of *Lyc*EPRV-Sh (*Table 2*) on metaphase chromosomes of *S. habrochaites* showed similar, but not identical hybridization patterns to *Lyc*EPRV-Sl on *S. lycopersicum* in the pericentromeric region of most chromosomes (*Figure 3G-I*). However, the signal strength seemed to be more variable between chromosomes (*Figure 3I*); again, there was no hybridization detected to the NOR region (*Figure 3G*).

DNA methylation analysis

Cytosine methylation of *Lyc*ERPVs in *S. lycopersicum* and *S. habrochaites* was investigated using methylation-sensitive restriction enzymes and DNA blot analysis. Previous work on EPRVs in *Nicotiana* has shown that the isoschizomer pair *Hpa*II/*Msp*I (recognition sequence CCGG), which is normally used to study CG methylation in animals, is not informative because of frequent CHG methylation in plants, that inhibits both *Hpa*II and *Msp*I, in these sequences (METTE et al., 2002). We therefore focused on enzymes sensitive to CHG and CHH methylation: *Scr*FI-*Bst*NI (C^mCNGG or CCWGG, respectively) reports on CHG methylation while *Sau*3AI-*Nde*I (GAT^mC) reports on methylation in potentially non-symmetrical cytosines, depending on the sequence context. The first enzyme in each isoschizomer pair is methylation-sensitive. Following a predigestion with *Xba*I, an additional digest was performed with either the methylation-sensitive or -insensitive enzyme from a particular isochizomer pair. Southern blots of

electrophoretically separated DNA were hybridized to two different probes each (*Figure 4*). One was the 1.3 kb fragment (probe E1) of the CP/MP reading frame of a cloned *S. lycopersicum* EPRV copy (Le1), the other one was derived from a *S. hirsutum* clone (Lh7) and comprises 580 bp of the IGR including most of the 273 bp box (probe H7).

For both species, the methylation-sensitive *Scr*FI cleaved little beyond the *Xba*I predigest whereas methylation-insensitive *Bst*NI digested substantially more, indicating the presence of CHG methylation of *Lyc*EPRV sequences (*Figure 4 A, B*). Little difference between coding regions and IGRs was observed. Hybridization of both the *Sau*3AI-and *Nde*I digested DNA with the CP/MP probe (E1) revealed substantial cleavage compared to the *Xba*I predigestion, suggesting little asymmetrical CHH methylation within the coding EPRV sequences (*Figure 4C*). Reprobing of the same blot with the IGR probe (H7) revealed a similar pattern, although smaller bands in the *Nde*I digests were more emphasized (*Figure 4D*). This suggests that asymmetrical methylation of the intergenic region is low but slightly stronger than in coding regions. The sequence of the cloned *Lyc*EPRV sequences did not reveal striking differences in the relative number of CHG and CHH residues between IGR and coding regions.

Expression analysis

Even though the *Lyc*EPRVs sequenced are defective and unable to encode intact viral proteins, one or more full-length copies could exist and potentially be pathogenic if activated under stress conditions. To test this possibility, we made inter-specific crosses with the aim of provoking a genome stress and then examined the hybrids for symptoms of virus infection. Four different interspecific crosses were made between different wild species (*S. pimpinellifolium*, *S. habrochaites*, *S. cheesmaniae* and, *S. peruvianum*) and *S. lycopersicum* ("MicroTom"). The phenotype of these plants resembled the phenotype of the wild parent rather than the dwarf cultivar of *S. lycopersicum* ("MicroTom"). Their hybrid nature was confirmed by SSR marker analysis (LE 20592; SMULDERS et al., 1997) to exclude selfed offspring.

No typical symptoms of virus-induced diseases could be detected at any time during the development of the hybrids that were grown in a greenhouse for a full year and trimmed frequently. In addition, hybridization of undigested, genomic DNA of selected individuals to probe E1 and H7 (coding region and IGR, respectively) failed to demonstrate episomal virus DNA since all individuals lacked the expected three bands for the linear, circular or supercoiled episomal DNA species (*Figure 4*, first lane each).

The cytosine methylation of the interspecific hybrids was analysed in comparison to parental genomes of each cross. In all cases the methylation pattern of the hybrid individuals resembled that of their parents: CHG and CHH methylation in the *Lyc*EPRV coding regions as well as in the IGRs could be observed (*Figure 4*). The unchanged methylation pattern and the absence of any virus-induced disease symptoms in the interspecific hybrids suggest that active virus was not produced by endogenous virus sequences under the conditions tested.

Interestingly, despite the inability to induce active virus in hybrids and the presence of cytosine methylation *Lyc*EPRVs appeared to be transcribed to some extent in healthy plants. The NCBI EST sequence databases contain transcripts from *S. lycopersicum, S. habrochaites* and *S. pennellii* with high similarity to our sequenced *Lyc*EPRVs from *S. lycopersicum* and *S. habrochaites*. More than 30 EST homologies were distributed over all four EPRV ORFs and the intergenic region. The cDNAs were derived from different tissues including flowers, red or green fruits, seeds, trichomes and shoot meristems as well as from suspension culture, callus tissue or crown galls (*Figure 5A, Table 3*). This suggests widespread transcription of sequences closely related to *Lyc*EPRVs in healthy tomato plants and related wild *Solanum* species not only under stress but also under normal growing conditions.

To further study the transcriptional activity of *Lyc*EPRVs in *S. lycopersicum*, *S. habrochaites* and an inter-specific hybrid, RT-PCR was performed using the conserved primer pairs CP/MP and TAV1/TAV2 amplifying parts of the coding region and IGR1/IGR2 for the conserved box within the intergenic region (*Figure 5A, B*). Fragments of the expected size were amplified in all individuals (*Figure 5B*) and DNA sequence analysis revealed high sequence similarities to the respective *Lyc*EPRV regions. Twenty-one cDNA sequences and six genomic sequences of the TAV region comprising 761 to 806 bp each were aligned. Many turned out to be identical or nearly identical (>98% sequence identity) on the nucleotide level whereas others diverged up to 30 to 37% (63 to 70% identity, *Figure 5C*). Taking into account the error-prone activity of reverse transcriptase, highly similar or identical transcripts appear to be derived from identical or corresponding EPRV copies present in both species. Nevertheless the transcripts are generally derived from more than one copy in each genome since diverging sequences are falling into at least five different clusters in *S. lycopersicum,* into four in *S. habrochaites* and six in the hybrid. None of the cloned genomic fragments of the corresponding region was matched with 100% sequence identity (97 to 99%). Many (62%) of the cDNA

sequences are translationally defective, i.e. contain frameshifts and stop codons in their putative amino acid sequence. Similarly nine cDNA sequences and one genomic fragment of the IGR were analysed, which revealed higher homogeneity, but still fall into more than one cluster (*Figure 5D*).

Short RNA analysis

Given the absence of viral disease symptoms in plants constitutively expressing *Lyc*EPRV transcripts, we tested whether homologous short RNAs – which might be indicative of RNA-mediated silencing - were present in healthy plants. Northern blots containing short RNA fractions from leaf material of *S. lycopersicum, S. habrochaites* and an interspecies hybrid as well as flowers of *S. lycopersicum* were hybridized to RNA probes derived from the *Lyc*EPRV intergenic region and the TAV region, respectively. For the IGR probe a cDNA sequence homologous to the conserved 272 bp box served as a template. A mix of three different clones was chosen for TAV since this region is more heterogeneous. Signals could be detected in the two parental species and the hybrid with both probes and in both sense and antisenses orientations. A distinct band of ~ 21 nucleotides in length and several bands ranging from 22-25 nucleotides in length were detected in all samples analysed. Generally, the flower-derived fraction produced the strongest signals (*Figure 6*).

To assess whether the short RNAs were derived from an RNAi/Post Translational Gene Silencing (PTGS) pathway, and hence might contribute to viral defense, we analyzed short RNAs in plants infected with heterologous RNA viruses -exploiting their ability to counteract RNA silencing by encoded proteins that suppress PTGS (VOINNET et al., 1999; CARRINGTON et al., 2001; BAULCOMBE, 2002). *Potato virus Y* (PVY, *Potyvirus*) expresses HCPro, which is known to prevent the maintenance of RNA silencing and binds to siRNAs preventing the formation of the siRNA-initiated RISC assembly (BUCHER and PRINS 2006, LAKATOS et al., 2006). *Tomato bushy stunt virus* (TBSV, *Tombusvirus*) encodes p19, which forms homodimers and prevents the strand separation of 20–22 nt siRNA duplexes. This is a prerequisite for their integration into the RNA induced silencing complex (RISC; CHAPMAN et al., 2004; VARGASON et al., 2003), rev. in (BAULCOMBE and MOLNAR, 2004). Plants infected with either PVY or TBSV revealed increased amounts of the 21–22nt *Lyc*EPRV short RNA fraction compared to mock infected individuals and plants harvested before starting the infection procedure (*Figure 7*). The accumulation of the smaller sized short RNAs homologous to both the intergenic region of *Lyc*EPRVs (IGR) and part of the coding region (TAV) could be observed in the cultivars "MicroTom" as well as in

"Moneymaker". The phenomenon is consistent with a formation of the *Lyc*EPRV short RNAs in the RNAi/PTGS pathway.

Discussion

In this study, we have characterized members of a new endogenous pararetrovirus family, *Lyc*EPRV, from cultivated tomato (*Solanum lycopersicum*) and a wild relative (*S. habrochaites*). Sequence homologies in cloned fragments of genomic *Lyc*EPRV from both species lead us to conclude that they are probably derived from the same pararetrovirus. A corresponding exogenous counterpart of *Lyc*EPRV has not yet been detected, probably because the virus has not been found yet, is extinct, or has not been identified as the virus sequence could be diverged due to faster evolution of an episomal form. Hence we could also postulate TVCV as a possible origin. As shown by the DNA blot hybridization patterns (*Figure 1*), *S. lycopersicum* and *S. habrochaites* share similarities in *Lyc*EPRV sequence organization, but each species also has unique restriction fragments, indicating independent insertions or excisions after species separation or flanking sequence divergence. Junctions that could be amplified by PCR from *S. habrochaites* but not from *S. lycopersicum* support such species-specific insertions. The other two wild relatives tested, *S. cheesmaniae* and *S. pimpinellifolium*, have hybridization patterns strongly resembling the pattern found in *S. lycopersicum*, indicating they harbor the same organisation of *Lyc*EPRVs, although this has not been confirmed by sequence analysis. Another wild relative, *S. peruvianum* also has sequences related to *Lyc*EPRVs, but with hybridization patterns distinct from the other species investigated. The patterns of EPRV hybridization bands reflect the morphology-based taxonomy with *S. lycopersicum*, *S. pimpinellifolium* and *S. cheesmaniae* most closely related and *S. habrochaites* and *S. peruvianum* as more distant relatives (PERALTA and SPOONER, 2001). Our results demonstrate that *Lyc*EPRVs and related sequences are common in many species of *Solanum* subsection lycopersicon. Given that tomato and potato are in the same genus, *Lyc*EPRVs are more similar to known EPRVs from *Nicotiana* than to the SoTu EPRV family (HANSEN et al., 2005) from potato.

All *Lyc*EPRV clones differed in sequence and revealed junctions between *Lyc*EPRV sequences and non-viral plant genomic sequences, indicating that the cloned sequences are indeed derived from endogenous EPRV copies in the nuclear genome, rather than from extra-genomic viral DNA. Alignment of the 14 clones of different nuclear EPRV fragments with overlapping homologous domains allowed reconstruction of a hypothetical full length *Lyc*EPRV sequence (*Figure 2A*) that contains all the components of a typical

pararetrovirus with a structure intermediate to that of *Caulimoviruses* and *Badnaviruses* (HARPER et al., 2002; HULL 2002). The coding region includes four ORFs and resembles the structure of TVCV (*Tobacco vein clearing virus*) and CsVMV (*Cassava vein mosaic virus*), two members of *Cavemoviruses*, but differs from typical *Caulimoviruses* with six ORFs (LOCKHART et al., 2000; DE KOCHKO et al., 1998). This structure was confirmed in a complete coding region sequenced from BAC AC171732 recently. EPRVs from tomato, potato and tobacco share structural features including putative signals for transcription initiation and termination, and significant sequence homology, of both DNA and hypothetical proteins in the ORFs. By contrast, another endogenous pararetrovirus sequence, ePVCV from *Petunia* (also *Solanaceae*), differs in sequence and genomic structure (RICHERT-PÖGGELER et al., 2003; RICHERT-PÖGGELER and SHEPHERD, 1997).

Sequences complementary to $tRNA^{Met}$ as a priming site for the minus-DNA strand synthesis in the intergenic region (IGR) were detected in at least a subset of copies and could be expected, since the replication of pararetroviruses is driven by transcription via RNA polymerase II and reverse transcription. Parts of the IGR with high conservation are notable, such as the 272 to 282 bp box (*Figure 2B*) found in all published EPRVs from tobacco, tomato and potato. Though lacking the direct repeats reported for *Ns*EPRV (JAKOWITSCH et al., 1999), the 272 to 282 bp conserved box of *Lyc*EPRV makes up part of the B1 box that has proved functionality as a promoter-enhancer element for *Ns*EPRV driving GUS expression in apical meristems of *A. thaliana* (METTE et al., 2002) which suggests a function, possibly as a regulatory element.

Individual *Lyc*EPRV sequences showed substantial divergence (e.g. with as little as 75% homology in the second ORF and a less conservation in the intergenic region) but no sequence motifs specific to either *S. lycopersicum* or *S. habrochaites* were evident in the clones examined. Consistent with the related but distinct hybridization patterns on DNA blots, *Lyc*EPRV sequences in *S. lycopersicum* and *S. habrochaites* have a similar, although not identical, dispersed chromosomal distribution with sites scattered in paracentromeric and some intercalary heterochromatic regions, while being largely excluded from euchromatin and the NOR region (*Figure 3*). Individual chromosomes of both species showed characteristic stronger or weaker hybridization indicating that sequence amplification or degeneration has occurred at specific integration sites.

All EPRV containing clones revealed sequence truncations and rearrangments when compared to the TVCV-like consensus structure (*Figure 2A*). Inverted, duplicated and truncated EPRV fragments adjacent to plant genomic DNA without viral homology have

been reported for *Ns*EPRV in tobacco (JAKOWITSCH et al., 1999), rice EPRVs (KUNII et al., 2004) and endogenous *Banana streak virus* (BSOEV) copies in banana (NDOWORA et al., 1999). Homologous recombination between new viral integrants, pre-existing EPRVs and perhaps retroelements could be responsible for the variable and complex genomic structures (GEERING et al., 2005; HARPER et al., 2002).

Half of the *Lyc*EPRV elements isolated are flanked on one or both sides by retrotransposon sequences (*Table 1*). Some 60% are represented by LTR sequences characteristic of the *Metaviridae* (*Ty3-gypsy*-like) elements, PCRT1 and 2 that are dispersed throughout the centromere region (YANG et al., 2005) and evidenced by the interspersed FISH signal with the LTR-homologous probe U30 (*Figure 3C*). In *S. habrochaites*, two of the nine *Lyc*EPRV loci were actually flanked on both sides by PCRT1. Also the tomato BAC clone AC171732 revealed PCRT1 sequences in the region flanking the *Lyc*EPRV copy. Associations of tobacco, petunia and banana EPRV sequences with *Metaviridae* elements have also been noted (JAKOWITSCH et al., 1999; GREGOR et al., 2004; RICHERT-PÖGGELER et al., 2003; NDOWORA et al., 1999). These associations may be random, due to preferential integration of either element in the other, or due to co-amplification of both elements. If retroelements constitute some 50% of the genome (HESLOP-HARRISON, 2000; DEVOS et al., 2002; BUDIMAN et al., 2000), then the association is little different from random, particularly if there is a preference for EPRVs and metaviridae elements to cluster in the genomic regions such as the centromere (see *Figure 3* and RICHERT-PÖGGELER et al., 2003, HANSEN et al., 2005 for petunia and potato). Nevertheless, it is tempting to suggest functional associations: pararetroviruses do not encode an integrase, so intact retrotransposons may supply this function in trans and related structural sites (HULL et al., 2000). Pararetroviruses that insert into retrotransposon structures may be coamplified as chimerical structures or by template switches of RT to viral transcripts (MATZKE et al., 2004), in addition to other mechanism of repetitive sequence amplification (see SCHWARZACHER 2003), such as unequal and illegimate crossing over or replication slippage of conserved short repeats as are found within the L*yc*EPRVs and related sequences.

Cytosine methylation within *Lyc*EPRV sequences was observed in both CHG and asymmetrical CHH contexts (*Figure 4*). CHH methylation in particular is a hallmark of RNA-directed DNA methylation in plants (MATZKE and BIRCHLER, 2005). Similar patterns of EPRV methylation have been observed in *Petunia* (NOREEN, 2005) and *N. tabacum* (METTE et al., 2002). There is evidence that cytosine methylation subdues EPRVs in

different species. In petunia, endogenous *Petunia vein clearing virus* looses methylation upon reactivation in *Petunia* hybrida (RICHERT-PÖGGELER et al., 2003). In tobacco, regulatory IGR sequences of *Ns*EPRV introduced stably into tobacco became a target of methylation and were transcriptionally silenced (METTE et al., 2002). In rice, the copy number of endogenous *Rice tungro bacilliform virus* (RTBV) in different strains was directly proportional to the degree of DNA methylation and virus resistance (KUNII et al., 2004). Whether the observed cytosine methylation is responsible for transcriptionally silencing copies of *Lyc*EPRV is not known. Clearly, at least some copies of *Lyc*EPRV are transcribed, as demonstrated by the detection of transcripts derived from the *Lyc*EPRV sequences in healthy plants and homologous ESTs in databases (*Figure 5*). Whether these transcripts are initiated from a promoter within an EPRV sequence or from a flanking plant promoter is not known. Most ESTs correspond to the TAV region and sequence heterogeneity, including frameshifts and stop codons, suggests that the transcripts are probably non-functional and derived from more than one locus in the genome. The absence of copies with a full-length coding sequence or a functional promoter region in the genomic library does not exclude the existence of a full copy elsewhere in the genome since the cDNAs were not identical to the genomic copies sequenced. EPRV-like EST matches from normal and stressed tissue respectively were also reported for the EPRV family SoTu in the potato genome (HANSEN et al., 2005).

Activation of EPRVs to form virus particles that produce symptoms of infection has been reported for BSV in banana (HARPER et al., 1999a; NDWORA et al., 1999, LHEUREUX et al., 2003), TVCV in *Nicotiana edwardsonii* (LOCKHART et al., 2000), and PVCV in Petunia (RICHERT-PÖGGLER et al., 2003). In most cases, activation occurred exclusively in interspecific hybrids and was enhanced by an additional abiotic stress (such as *in vitro* propagation/tissue culture, changes in the light regime, or frequent wounding) (DALLOT et al., 2001; LHEUREUX et al., 2003; LOCKHART et al., 2000; RICHERT-PÖGGLER et al., 2003). By contrast, symptoms of virus infection due to activation of latent *Lyc*EPRV were not observed in new interspecific hybrids grown under greenhouse conditions and stressed by frequent trimming. Whether this is due to stable silencing of as-yet-unidentified non-defective copies of *Lyc*EPRV in hybrids or to the general lack of potentially reactivatable copies is not known. Additionally also the absence of an asymmetric ratio of EPRV copies between parental genomes may have prevented a reactivation as this seems to enable the formation of episomal virus from integrated copies in other hybrid genomes (LOCKHART et al., 2000; HARPER et al., 2002; LHEUREUX et al., 2003). The function of the LycEPRV

transcripts in asymptomatic plants is unclear but it is tempting to speculate that they repress the pathogenicity of endogenous pararetroviruses, perhaps by an RNA-based gene silencing mechanism(s) (METTE et al., 2002). This idea is supported by the detection of at least some CHH methylation in *Lyc*EPRVs and the presence of short RNAs with homology to *Lyc*EPRVs in healthy plants (*Figure 6*). Moreover the increased level of 21-22nt short RNAs in plants infected with a heterologous virus encoding suppressors of PTGS suggests a role in a constitutive RNAi/PTGS pathway. By contrast, significant amounts of short RNAs could be detected in petunia only in symptomatic tissue after activation of endogenous PVCV sequence(s) or after infection with PVCV by inocculation (NOREEN, AKBERGENOV, HOHN, RICHERT-POEGGELER in preparation).

The presence of two size classes of short RNA, which have been implicated previously in triggering either PTGS (21 nt) or Translational Gene Silencing and RNA-directed chromatin modifications (24 nt) (HAMILTON et al., 2002), could provide a multi-pronged defense against endogenous or exogenous forms of the virus. The accumulation of 21-22nt LycEPRV short RNAs after heterologous virus infection with two different points of interactions in the silencing process supports the involvement of PTGS for such a defence. Given the complex and interconnected nature of RNA-mediated silencing pathways (MEINS et al., 2005; WASSENEGGER, 2005; BAULCOMBE, 2004, ALMEIDA and ALLSHIRE, 2005), and the fitness advantage of suppressing viral infection, RNA-mediated silencing of EPRVs might involve several species of short RNAs, RNA-directed DNA methylation, and both PTGS and TGS pathways.

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Table 1. Description of identified sequences flanking the *Lyc*EPRVs. Numbers correspond to those shown in *Figure 2A*.

No.	Clone	Description	Position in clone
1.	Le1	Integrase ORF, *Solanum demissum* retrotransposon (AAT39954); polyprotein ORF, *S. lycopersicum* retrotransposon (AAD13304); PCRT2-2, *S. lycopersicum* pericentromeric retrotransposon (AY850394); PCRT1c-1 *S. lycopersicum* pericentromeric retrotransposon (AY850394).	5431-5846 5800-5968, 5477-5498
2.	Le3	Putative solo-LTR, UTRT3, tandem repeat (AC139840; AY850394).	1-797
3.	Le3	Putative DEAD/DEAH box, RNA helicase protein ORF, *A. thaliana* (AAO00880), *O. sativa* (AAN62787).	1237-1992
4.	Lh1	putative coding region, PCRT1b-3 *S. lycopersicum* pericentromeric retrotransposon (AY850394); putative coding region, Caterina-2 *S. lycopersicum Ty3-gypsy* retrotransposon (AY678298).	3879-4211, 3901-4212
5.	Lh2	putative LTR, PCR1b *S. lycopersicum* pericentromeric retrotransposon (AY850394).	98-332
6.	Lh3	TNP2 like transposon protein(Tpase), class II transposon, *O. sativa* (AAM18727).	5925-5990
7.(a,b)	Lh4	LTR ends, inverted, PCRT1a *S. lycopersicum* pericentromeric retrotransposon (AY850394).	131-1076; 4868-6132
8.	Lh5	51 bp repeat motif; unclassified transposable element XC (AY678298); unclassified transposable element XB (AY678298).	5870-6206 6156-6171 6218-6252
9.	Lh7	LTR end, PCRT1a (as in Lh4) *S. lycopersicum* pericentromeric retrotransposon (AY850394).	11-1257
10.	Lh7	not annotated pericentromeric repeat; PCRT1b *S. lycopersicum* pericentromeric retrotransposon (AY850394).	8132-8988
11.	Lh8	PCTR1a *S. lycopersicum* pericentromeric retrotransposon (AY850394); TGRII dispersed rep. sequence (AY880062).	49-1000
12.	Lh8	Solo-LTR, PCRT1d & PCRT1g *S. lycopersicum* pericentromeric retrotransposon (AY850394); not annotated sequences from pericentric heterochromatin; root knot nematode resistence marker (DQ090954).	6863-8656 8108-8320
13.	Lh9	root knot nematode resistence marker (DQ090954); *S. habrochaites* RGA marker sequence (AF534327).	414-716

Table 2. Origin of fragments mixed for pooled FISH probes (LycEPRV-Se and LycEPRV-Sh) covering most of the EPRV.

Pooled probe	Derived from region	Derived from λ-clone	Length
LycEPRV-Se	CP/MP	Le4 (position: 2882 - 4182)	1300 bp
	MP	Le3 (position: 2365 - 3185)	820 bp
	TAV	Le2 (position: 1260 - 2365)	1100 bp
	IGR (box)	Le5 (position: 2203 - 3207)	1000 bp
LycEPRV-Sh	CP	Lh7 (position: 1576 - 2455)	880 bp
	MP	Lh6 (position: 1186 - 1862)	680 bp
	TAV	Lh4 (position: 3617 - 4706)	1090 bp
	IGR (box)	Lh7 (position: 7133 - 7716)	600 bp

Table 3. Selection of ESTs from the genus *Solanum* subsection lycopersicon with homology to cloned LycEPRV sequences as shown in *Figure 5A*.

No.	EST	species	tissue
1	473899	*S. lycopersicum*	shoot, meristem
2	512316	*S. lycopersicum*	shoot, meristem
3	311489	*S. lycopersicum*	tomato red fruit
4	322740	*S. habrochaites*	trichome
5	247583	*S. lycopersicum*	carpel
6	511691	*S. lycopersicum*	shoot, meristem
7	465904	*S. lycopersicum*	crown gall
8	465989	*S. lycopersicum*	crown gall
9	531912	*S. lycopersicum*	callus
10	277245	*S. lycopersicum*	callus
11	414362	*S. lycopersicum*	green fruit
12	281120, 542315	*S. lycopersicum*	callus
13	245240	*S. lycopersicum*	carpel

Figure 1. Genomic organization of EPRV sequences in the genus *Solanum* subsection *Lycopersicon*. DNA preparations from five species of the *Solanum* subsection *Lycopersicon* and *Solanum tuberosum* were restricted with *Xba*I and hybridized to a 5.5 fragment of *Ns*EPRV covering ORF 2 to 4 and the IGR. Similar data (lanes 1 to 5) have been shown previously (MATZKE et al., 2004).

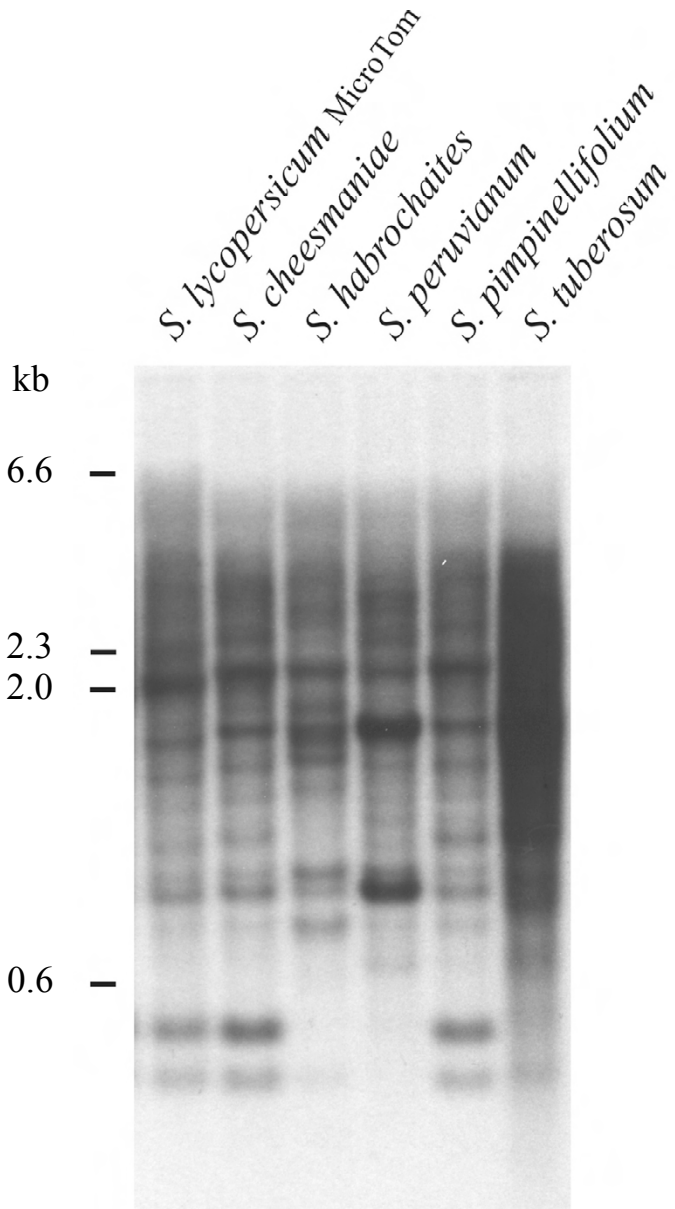
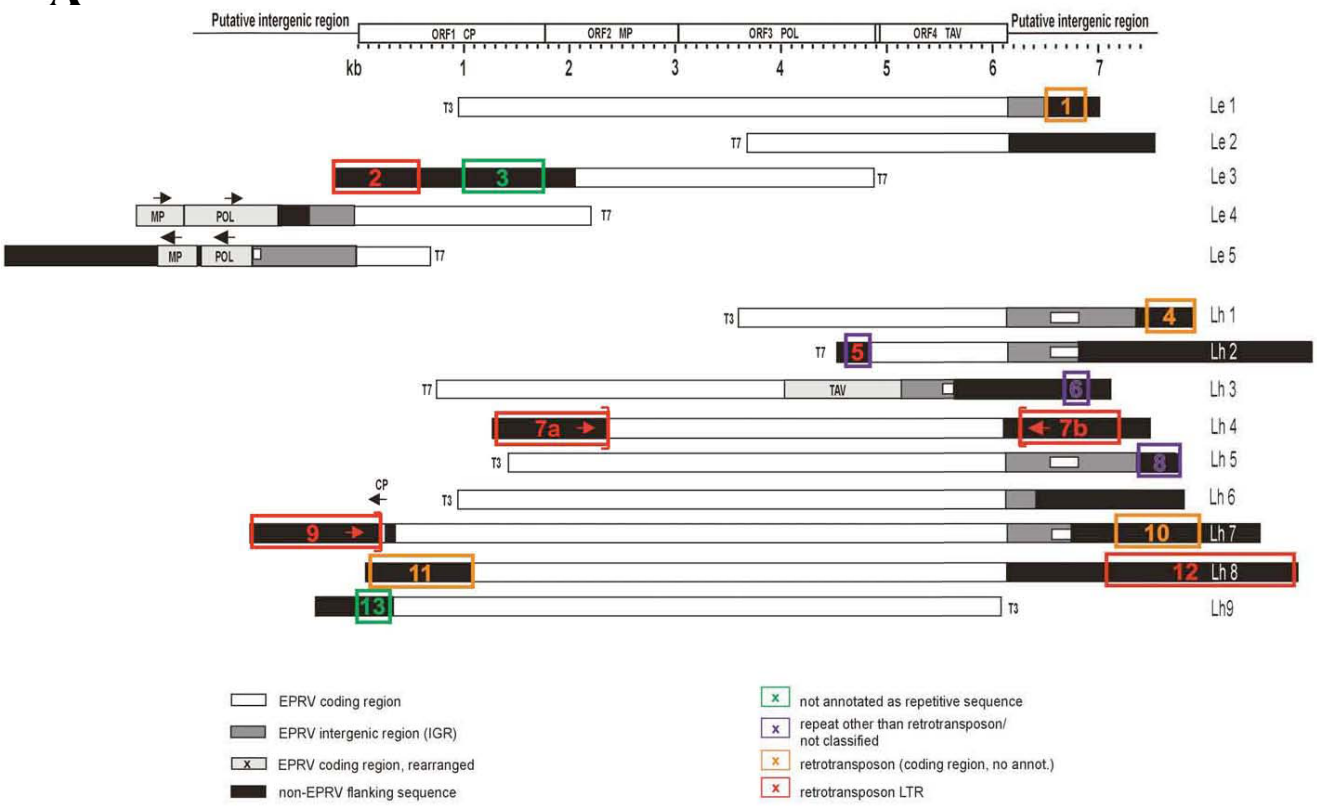


Figure 2 **A.** Analysis of cloned LycEPRV sequences and flanking sequences. Alignment of cloned EPRV sequences from *S. lycopersicum* (Le1-5) and *S. habrochaites* (Lh1-9) to the structure of TVCV-like EPRVs comprising four ORFs (upper bar): coat protein (CP), cell-to-cell movement protein (MP), polyprotein (POL) and transactivator domain (TAV). Rearranged coding regions are indicated by extra boxes and arrows for a deviating orientation of the reading frames. Nine clones contain parts of the intergenic region (IGR) marked by grey boxes with a white square for the position of the conserved 272 to 282 bp-box. Black bars indicate flanking sequences unrelated to EPRVs. Survey of sequences flanking the EPRVs in *S. lycopersicum* and *S. habrochaites* is given by coloured boxes. The majority represents repetitve elements (orange, red and blue boxes) most of which belong to retrotransposons (orange and red boxes), especially the LTR regions (red boxes). Arrows point towards the end of similar LTRs which is marked by a bracket. **A** description of the flanking sequences is listed in *Table 1* according to the numbers. **B**. Sequence conservation within a 272 to 282 bp box of the IGR from different *Solanaceae* EPRVs. Alignment of the respective region of three LycEPRVs (Lh1, Lh2, Lh5) to three different tobacco EPRVs (TVCV, (LOCKHART et al., 2000); NsEPRV, (JAKOWITSCH et al., 1999); NtoEPRV, (GREGOR et al., 2004) and to two *Solanum tuberosum* EPRV copies (SoTuI-2, SoTuI-10; AJ564214, AJ564220; (HANSEN et al., 2005). Next to a remarkable overall sequence homogeneity within the IGR region several shorter motives are highly conserved between EPRVs from all three species (red frames).

A


Putative intergenic region
ORF1 CP
ORF2 MP
ORF3 POL
ORF4 TAV
Putative intergenic region
kb
1
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Le 1
Le 2
Le 3
Le 4
Le 5
Lh 1
Lh 2
Lh 3
Lh 4
Lh 5
Lh 6
Lh 7
Lh 8
Lh9
MP
POL
TAV
CP
T3
T7
7a
7b
EPRV coding region
EPRV intergenic region (IGR)
EPRV coding region, rearranged
non-EPRV flanking sequence
not annotated as repetitive sequence
repeat other than retrotransposon/ not classified
retrotransposon (coding region, no annot.)
retrotransposon LTR

B

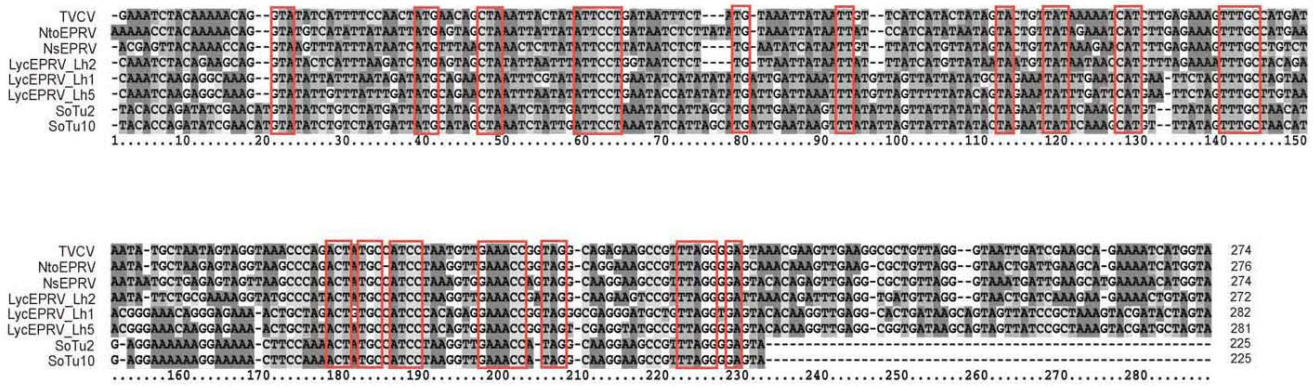
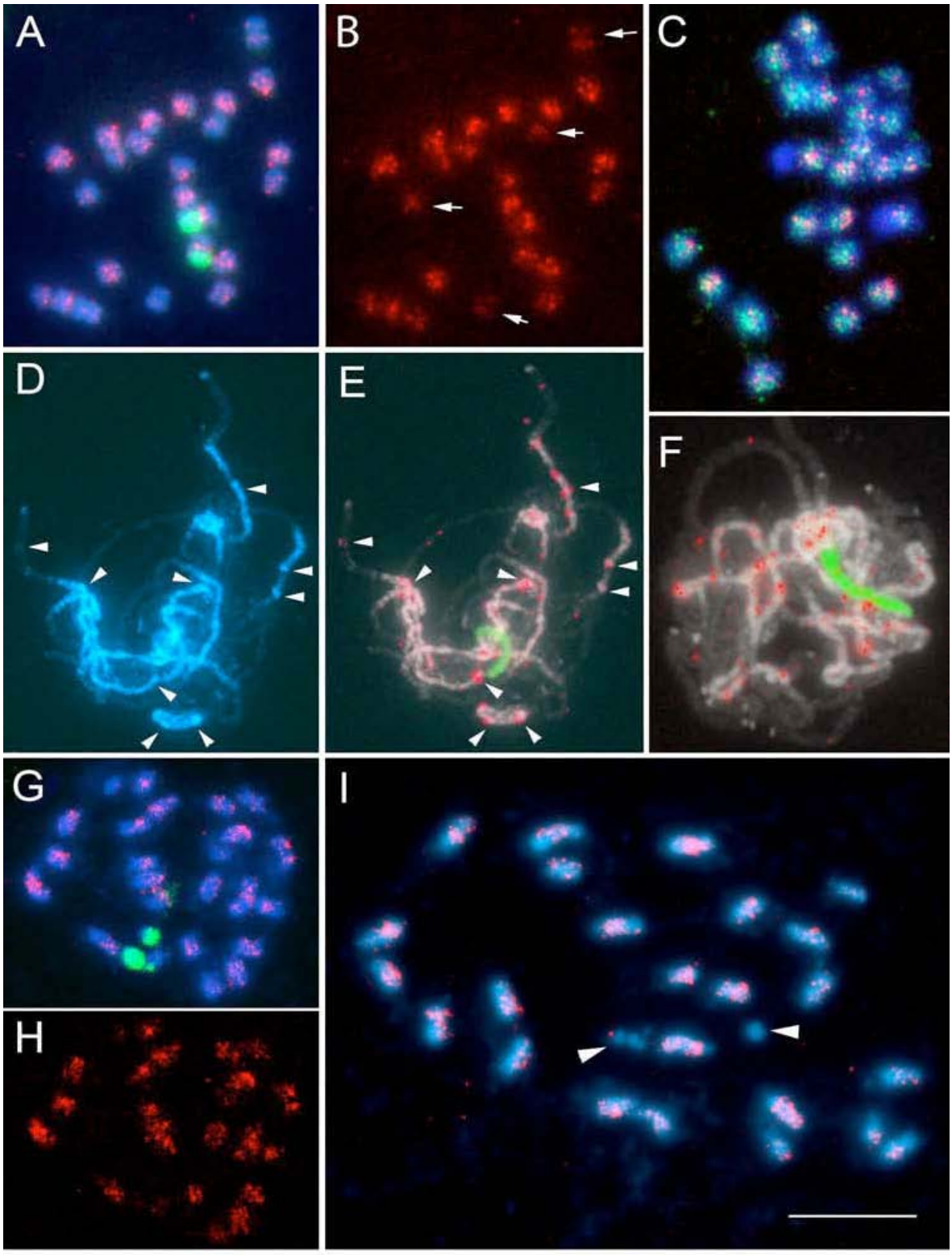

TVCV
NtoEPRV
NsEPRV
LycEPRV_Lh2
LycEPRV_Lh1
LycEPRV_Lh5
SoTu2
SoTu10
1.....10.....20.....30.....40.....50.....60.....70.....80.....90.....100.....110.....120.....130.....140.....150
160.....170.....180.....190.....200.....210.....220.....230.....240.....250.....260.....270.....280.....

Figure 3. Chromosomal localization of ***Lyc*EPRVs.** Double target fluorescent *in situ* hybridization was carried out on root tip metaphases and male meiotic pachytene cells of *S. lycopersicum* (**A-F**) and *S. habrochaites* (**G-I**). Biotin labelled pooled probes of *Lyc*EPRVs from *S. lycopersicum* (*Lyc*EPRV-Sl, A-F) and *S. habrochaites* (*Lyc*EPRV-Sh, G-I), respectively, that cover most of *Lyc*EPRV sequence (for clone combinations see Table 2) were detected by red Alexa-594 fluorescence and hybridized together with digoxigenin labelled repeated DNA probes detected by green FITC fluorescence. Chromosomes were counterstained with DAPI (blue fluorescence). **A-C.** Metaphase chromosomes of *S. lycopersicum* (2n = 24). *Lyc*EPRV-Sl sequences (red in B and magenta in the overlay with blue DAPI staining in A) are located at the centromeres of most chromosomes with variable intensity, but are absent from the NOR region (green rDNA probe in A) and reduced on four chromosomes (arrows in B). In C the *Lyc*EPRVs are shown to co-localize with the retroelement sequence U30 from *S. lycopersicum* (green) that shows dispersed signals on all chromosomes. **D-F.** Pachytene chromosomes of *S. lycopersicum* are much more extended than metaphase chromosomes and show differentiation with DAPI into strongly stained heterochromatin and weakly stained euchromatin (D). The red *Lyc*EPRV signal is almost exclusively seen in the pericentromeric heterochromatic regions and intercalary chromocentre (arrowheads in D and E), but not at the NOR region (green in E, F; DAPI is shown as grey image with the probe signal falsely coloured red and green, respectively). **G-I.** Metaphase chromosomes of *S. habrochaites* (2n = 24). *Lyc*EPRV-Sh sequences (red in H, magenta in the overlay with blue DAPI staining in G, I) are located near the centromeres of most chromosomes showing stronger signal in some. No signal is visible in the NOR regions (green rDNA probe in G, arrow heads in I). Bar 10 μm.


A
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H
I

Figure 4. Analysis of cytosine methylation in *Lyc*EPRV sequences. DNA of parental plants (flanking) and interspecific hybrids (central) was restricted with *Xba*I (lane 2 to 4 each) and either *Scr*FI (S) and *Bst*NI (B) to detect CHG methylation (A, B) or *Sau*3aI (Sa) and *Nde*I (N) for asymmetric cytosine sites (C, D), the first enzyme of each pair being methylation sensitive. The first lane each contains undigested DNA (un). **A, C.** DNAs were hybridized to a 1.3 kb fragment of the CP/MP reading frame (E1) of a *S. lycopersicum* EPRV copy (Le1) and **B, D.** to a 580 bp fragment of the IGR (H7) of a *S. habrochaites* clone (Lh7).

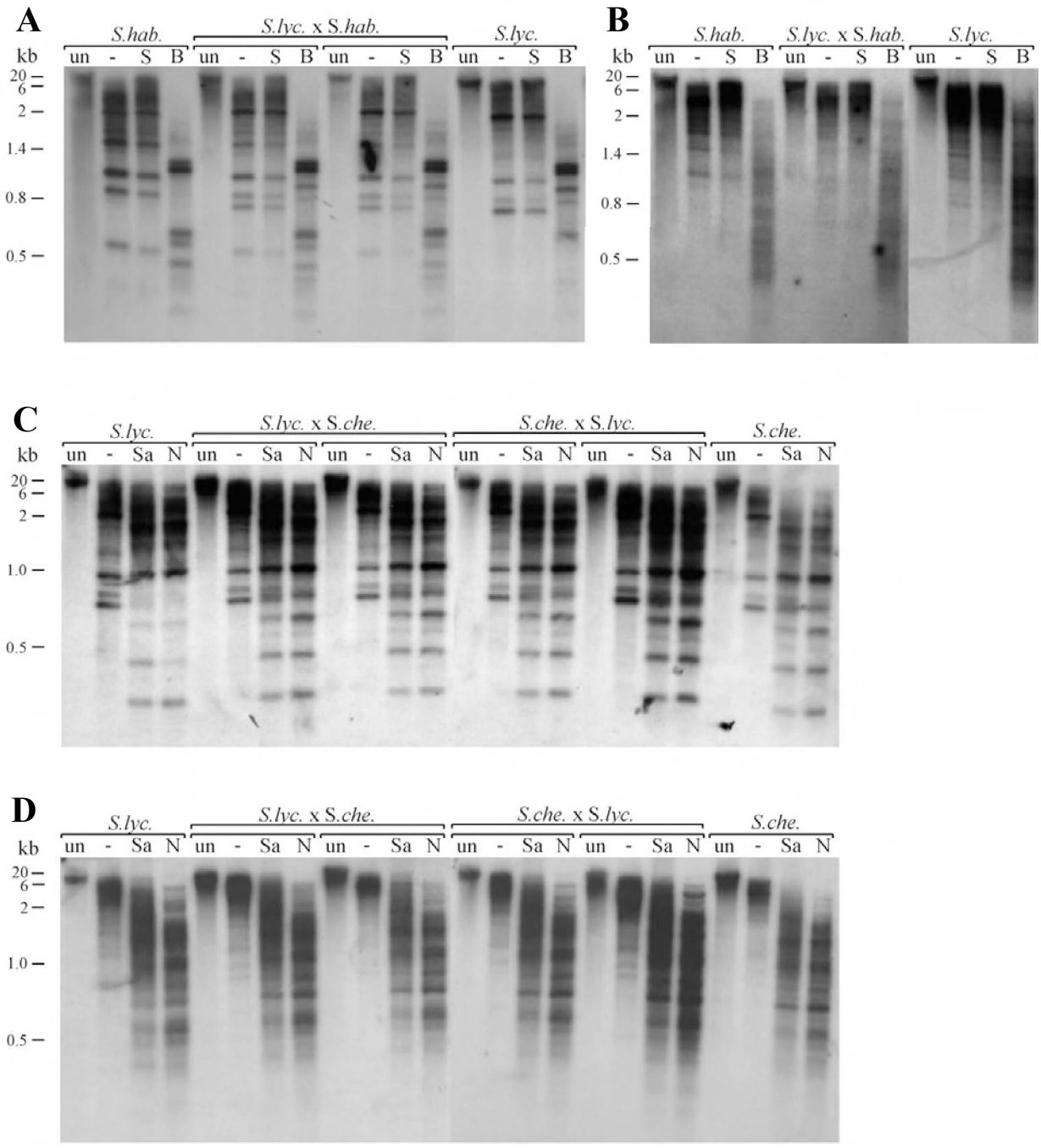
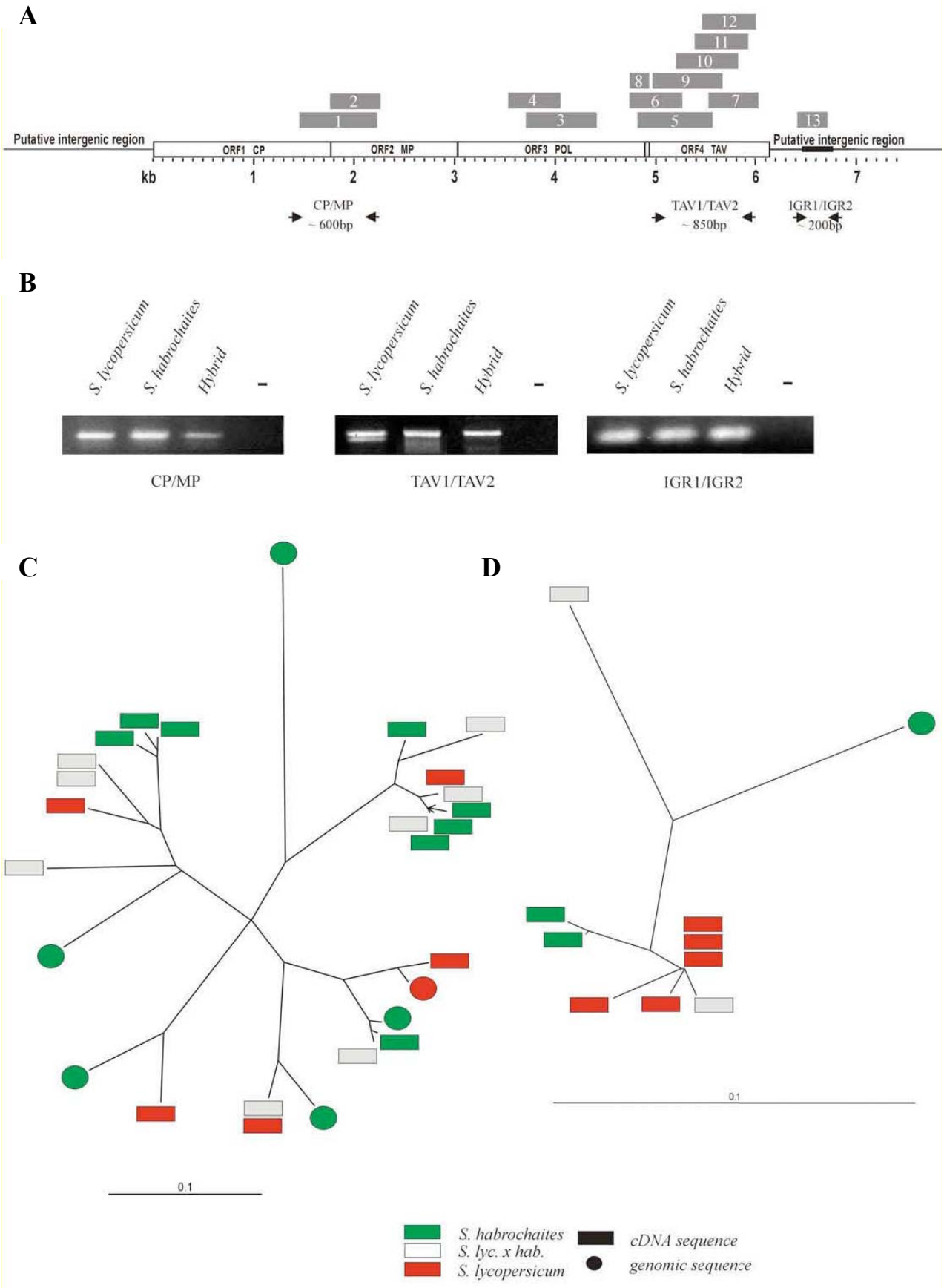


Figure 5. Identification of transcripts homologous to *Lycopersicon* EPRVs. **A.** Survey over a selection of homologous ESTs of the genus *Solanum* subsection *Lycopersicon* and their position (grey boxes) in relation to the *Lyc*EPRV structure. Details about the ESTs (according to the numbers) are given in Table 3. Arrows mark the position of primers used for RT-PCR. **B.** PolyA$^+$-enriched RNA of *S. lycopersicum, S. habrochaites* and an interspecific hybrid was used for RT-PCR with primer pairs of the CP/MP and TAV ORFs and the IGR as indicated in (A). The first strand DNA template was prepared from polyA$^+$ enriched RNA from leaves of *S. lycopersicum, S. habrochaites* and an interspecific hybrid (lane 1–3 each). To detect possible genomic DNA contaminations an actin sequence spanning an intron was amplified in parallel. Water controls are indicated by a dash (lane 4 each). **C, D.** Unrooted dendrograms showing the genetic distance between genomic and cDNA sequences of the TAV region (C) and the IGR (D) of *S. lycopersicum* (red boxes), *S. habrochaites* (green boxes) and an interspecific hybrid (white boxes). cDNA sequences are indicated by a square, circles mark genomic sequences. The horizontal bar represents percent divergence (/100).


A
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Putative intergenic region
ORF1 CP
ORF2 MP
ORF3 POL
ORF4 TAV
Putative intergenic region
kb
1
2
3
4
5
6
7
CP/MP
~ 600bp
TAV1/TAV2
~ 850bp
IGR1/IGR2
~ 200bp
B
S. lycopersicum
S. habrochaites
Hybrid
-
CP/MP
TAV1/TAV2
IGR1/IGR2
C
D
0.1
0.1
S. habrochaites
S. lyc. x hab.
S. lycopersicum
cDNA sequence
genomic sequence

Figure 6. Analysis of short RNAs homologous to *Lyc*EPRV. The short RNA fraction of *S. lycopersicum* leaves (1, 2), *S. lycopersicum* flowers (3), *S. habrochaites* leaves (4) and leaves of an interspecific hybrid (5) was hybridized to riboprobes derived from three different TAV clones (top) and from a clone carrying the conserved part of the IGR (bottom). Ethidium bromide staining of the major RNA on the gel is shown as a loading control below each blot.

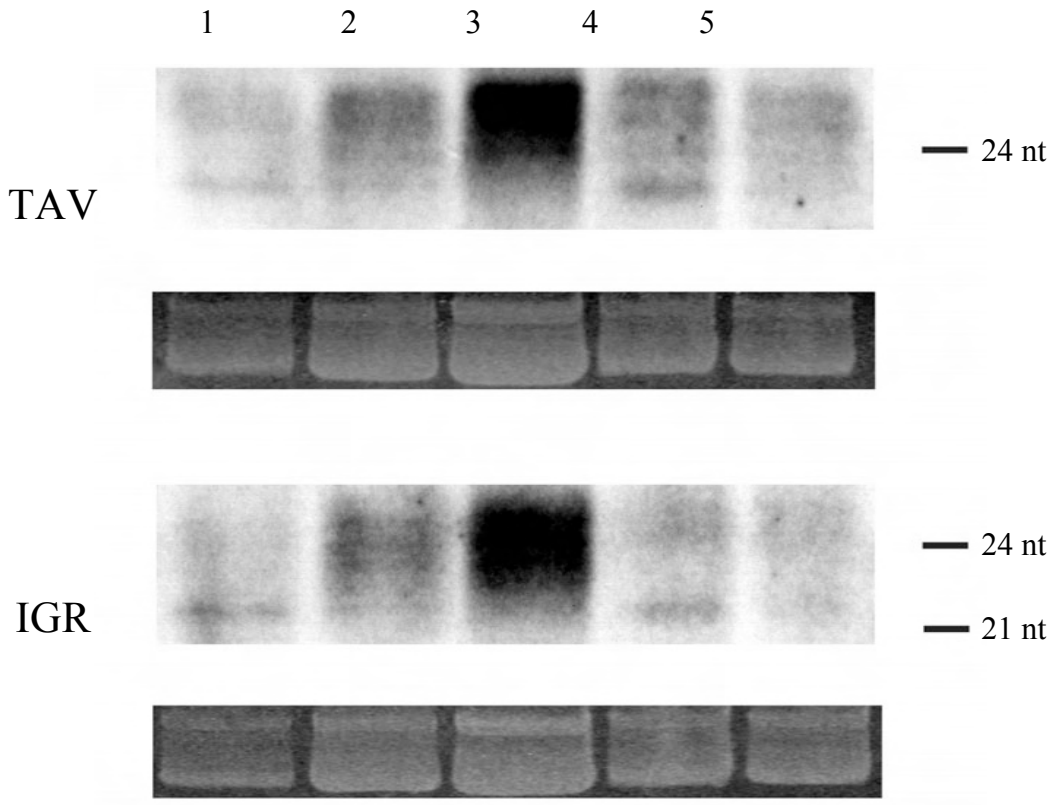
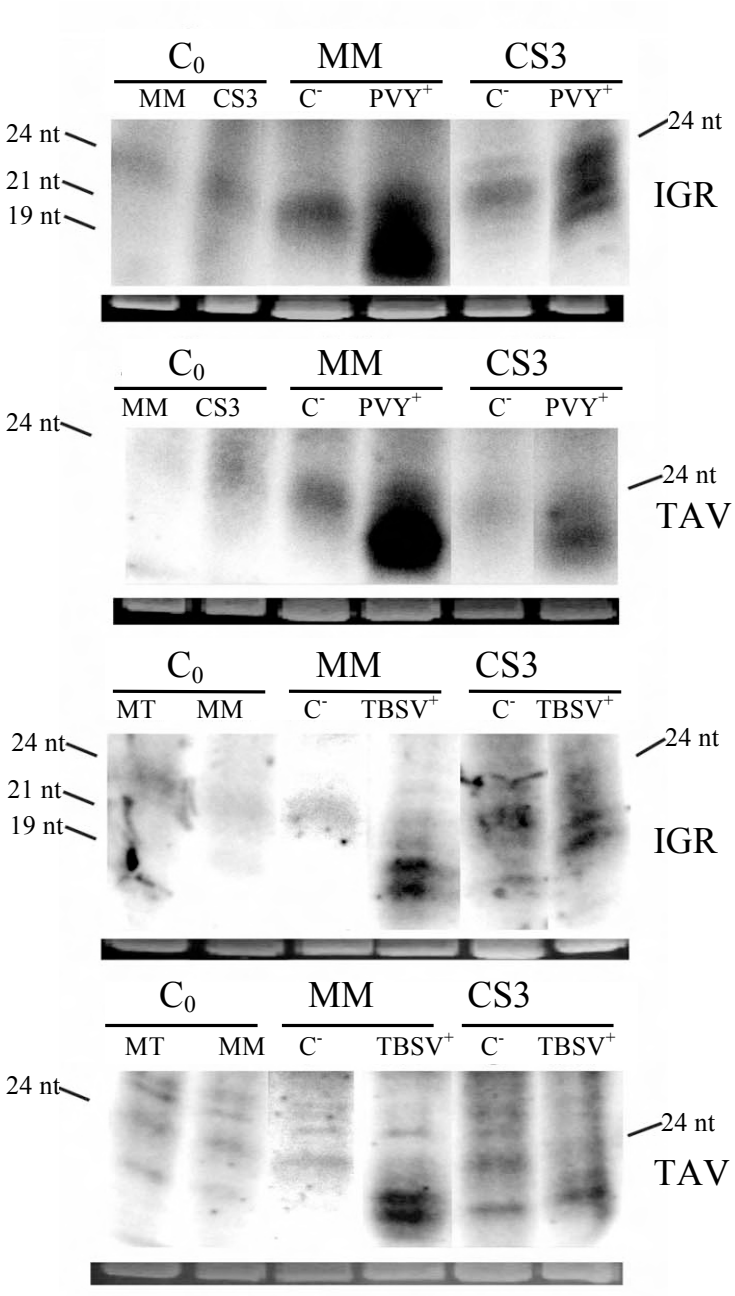


Figure 7. Short *Lyc*EPRV RNAs after heterologous virus infection. The short RNA fraction of *S. lycopersicum* leaves derived from the cultivar "Moneymaker" (MM), a transgenic line of "Moneymaker" (CS3, MISHRA et al., 2002) and the cultivar "MicroTom" (MT) was hybridized to TAV (B, D) and IGR (A, C) riboprobes after infecting the plants with PVY (*Potyvirus Y*; in A, B) or TBSV (*Tomato bushy stunt virus*; in C, D) that express suppressors of PTGS. Individual plants may show different reactions to virus infection therefore several individuals were infected in each assay. Since a general trend became visible only one representative plant is shown here. C_0: bulked leaves harvested before infection; C^-: mock infection; $TBSV^+$: infected with *Tomato bushy stunt virus*; PVY^+: infected with *Potyvirus Y*. Ethidium bromide staining of the major RNA on the gel is shown as a loading control below each blot.



GENERAL CONCLUSIONS

Plant diseases caused by viruses and phytoplasmas seriously affect yield and quality of agricultural production. Therefore effective control strategies are required to minimize their negative impacts and ensure food security at a global level. Much less certain is the precise nature of the threat from these important pathogens in the face of expanding global trade, changing agricultural practices, food security demands of an expanding population, demand for bioenergy, climate change and the genetic plasticity of viruses themselves. Since viruses are transmitted by animal, arthropod or fungal vectors, each of these factors will also bring a unique set of challenges relating to the biology of each of the vectors.

A principal component in the fight against plant viruses and phytoplasmas is their rapid detection and identification and, in some cases, the control of coincidently present transmitting vectors. Diagnostics that can discriminate pathogens in disease complexes will improve our understanding of these complexes. The low-cost, high through-put nucleic acid-based assays provide screening procedures that yield a rapid and complete picture of crop plant health. In adition to time benefits, there is a great advantage in terms of specifity, when using PCR with specific primers, as they allow the detection of plant pathogenic phytoplasmas and viruses even camouflaged by a high number of other microorganisms. Further development and deployment of these technologies might allow more plants pathogens detection as well as shed light on the disease cycle.

Key elements in understanding virus pathology in horticultural crops and ornamentals, such as *Vitis vinifera*, *Ananas comosus* and *Canna indica*, are the diagnosis of the infecting agents and the interpretation of the virus-vector-plant relationships.

The ecology and epidemiology of viruses with nematodes vectors have their own characteristics. Vector nematodes do not have a resistant resting stage, but can survive adverse soil conditions by movement through the soil profile. As soils become dry in summer or cold in winter, they move to the subsoil and return when conditions are favorable (HULL, 2002). Agricultural practices will increase distribution of the vectors during cultivation and probably in drainage or floodwater. Farm's workers footwear and machinery contaminated with infested soil may transmit nematodes and their associated viruses over either short or long distances (BOAG, 1985). During the past decade there has been significant progress in the molecular characterization of plant-nematode (WILLIAMSON and KUMAR, 2006). Rapid progress in genomics and genetics of both plants

and nematodes will improve our understanding of plant-nematode interactions and therefore contribute to minimize the risk of spreading.

Molecular identification methods using species specific primers (WANG et al., 2003) confirmed, for the first time, that *X. index,* the recognized vector for the detrimental grapevine fanleaf disease, is present in the vineyards of a specific Austrian region (Chapter 1, LEOPOLD et al., 2007). It can be expected that the use of species specific primers, as described here, if applied to samples from other viticultural regions in Austria, will reveal a more widespread occurrence of *X. index*. These results emphasize the importance of alternative defence strategies in modern viticulture, such as genetically improved rootstocks exhibiting virus resistance, as nematode-tolerant gapevine hybrids have been obtained but do not totally block transmission of nepoviruses to their rootlets (BOUQUET et al., 2000). It will provide the opportunity for developing novel resistance strategies for durable resistance in grapevine plants as reported for GAMBINO et al., (2005) and MAGHULY et al., (2006) by expressing sequences of the GFLV CP gene in transgenic grapevine. On the other hand, the detection of *X. index* by multiplex PCR can be adapted in the screening of transgenic rootstocks growing in nepovirus-longidorid infested soils, under controlled conditions.

Vegetative propagation is an important horticultural practice, but is unfortunately a very effective method for perpetuating and spreading viruses. Economically important viruses spread systemically through most vegetative parts of the plant. A plant once systematically infected with a virus usually remains infected for its life time. Thus, any vegetative parts taken for propagation, such as tuber, bulbs corms, runners and cuttings will normally be infected (HULL, 2002). This is that the case of *Canna indica* (GRAY and GRANT 2003) where vegetatively propagated plant material can contribute to virus dissemination.

Hence, of special interest for breeders of *C. indica* is the determination of the status of their collections. Therefore, to assess the degree of virus infection of a valuable Canna collection symptomatic and asymptomatic tissue of selected plants were evaluated by ELISA and PCR. *Canna indica* can be propagated (KROMER and KUKULCZANKA 1985) and sanitized by *in vitro* methods i.e. meristem preparation combined with thermotherapy (LAIMER, 2003).

Since no commercial ELISA test is available for CaYMV, a PCR procedure, using newly developed primers (Chapter 2, BORROTO et al., 2008) was established. This confirmed for the first time the presence of CaYMV–infected *Canna* plants in Austria.

The newly developed, highly specific PCR primers, yielding a short-sized product of 315 bp product at 55°C AT, allow the detection of the pathogen in symptomatic and symptomless plant tissues, with the exclusion of false negatives for the Austrian *Canna* accessions used in this study. The methodology developed for the identification of CaYMV is recommended to breeders, as an efficient and early diagnostic tool to reduce the risk of spreading of the viral disease (Chapter 2, BORROTO et al., 2008).

The results obtained in the Austrian Canna collection emphasize the importance of certification programs to avoid the introduction of virus diseases in ornamental plants as a cultural practice to limit the virus spread. The future will bring novel tests for a rapid, sensitive and specific diagnosis which contributes to select the most cost-effective diagnostic strategies.

Among plant pathogens, phytoplasmas are unique, and the molecular detection and their biological cycle have stimulated interest and investigations. Due to the limitation of not being culturable *in vitro*, attention mostly focused to their role as pathogens with special remarks on the epidemiology, spread, diagnosis and economic impact. Sensitive and accurate diagnosis for these microorganisms is therefore a prerequisite for the management of phytoplasma-associated diseases. In the last few years, several procedures have been proposed for the analysis of the PCR amplification products from phytoplasma infected plants, including PCR-ELISA (POGGI-POLLINI et al., 1997), PCR-dot blot (BERTIN et al., 2004), heteroduplex mobility assay (WANG and HIRUKI, 2000, 2001), 16S-23S spacer length polymorphism (PALMANO and FIRRAO, 2000), real time PCR (JARAUSCH et al., 2004), microarray (FROSINI et al., 2002) and nanobiotransducer hybridization (FIRRAO et al., 2005). Nevertheless, routine diagnostic and classification protocols are mainly based on the study of the variability of 16S rRNA sequences using nested PCR and RFLP (GARCIA-CHAPA and MARZACHI, 2007; LAIMER and BERTACCINI, 2008).

Addressing the question of the possible association of phytoplasma, with wild species in Austria, *Euonymus europaea*, *Sorbus aucuparia*, *Fraxinus excelsior*, *Fagus sylvatica*, *Betula alba*, *Sambucus nigra*, *Pyrus* sp., *Picea abies, Rubus ideaus*, *Rubus fruticosus* and *Vaccinium myrtillus* were analyzed by PCR/RFLP using general primers located in the 16S rDNA (LEE et al., 1995; GUNDERSEN and LEE, 1996; HEINRICH et al., 2001; BORROTO et al., 2007b). The analyses of sequences confirmed the presence of a phytoplasma of the 16SrVI group in *V. myrtillus*, which so far was only reported to contain phytoplasmas belonging to the 16SrIII group (PALTRINIERI et al., 2000). The presence of phytoplasma was also detected after a second nested PCR amplifications with primers

R16(I)F1/R1 on R16F2/R2 amplicons in symptomatic samples from *R. fruticosus*, *R. ideaus* and *Fagus* spp. RFLP characterization allowed to verify that *R. fruticosus* was infected by phytoplasmas belonging to ribosomal group 16SrI-B, while in *R. idaeus* and *Fagus* samples phytoplasmas belonging to ribosomal subgroup 16SrXII-A were identified.

This work also stressed the relevance to make forestry nurseries aware of the quality issue and also reconsider the bewildering approach with more care, since these areas might constitute large sources of inoculum of undesired pathogens (Chapter 3, BORROTO et al., 2007b). Consequently, more appropriate sampling methodologies in the forest will be set up for efficient detection of hidden phytoplasmas.

The phytoplasma investigations related with the molecular characterization are still in their beginning stages, several tasks could be fulfilled in order to acquire a clear knowledge for controlling diseases spreading. The sequencing of complete phytoplasma genomes, (e.g. 'Candidatus Phytoplasma asteris (strain AYWB) (BAI et al., 2006) and 'Candidatus Phytoplasma mali' (strain AT) (KUBE et al., 2008), will provide more a precise basis for taxonomy, but it will be necessary to do it for several other phytoplasmas, in order to achieve comparative genomic analysis that could allow a deeper understanding about physiology of these organisms. It appears the most promising approach to phytoplasma classification and identification, since it is still difficult to fulfil the minimal requirements for a formal taxonomy, not only because they can not grow *in vitro*, but also because of their low titre in infected plants.

In conclusion the early detection of new hosts and vectors for phytoplasmas and viruses using reliable and effective diagnostic test support the success of future eradication programmes. Alternatively, sequence information of viruses and phytoplasmas could also be applied to develop and validate tests to identify them under local conditions.

The developments in plant virology have spurred the increased use of molecular diagnostics and this trend will undoubtedly continue with increasing momentum over the years to come (HULL, 2002). In contrast to the practical questions of virus diagnosis, our understanding of the factors that regulate the population dynamics of virus pathogens, particularly in the context of multi-trophic interactions between virus, plant, vector, environment and agronomic practice, is still in its infancy. A challenge for future plant virus research would, therefore, be to shed light upon the molecular interactions between virus and host that eventually result in a diseased plant. All these efforts have a social mission for scientists in the field of plant pathology as an active service and contribution to an active control of plant diseases.

Different interactions are generated between the plant and the virus during each stage of the viral cycle. If the viral particle is not recognized by the host plant, a compatible interaction between the plant and the virus is established. This interaction may be favorable for the virus (HAMMOND-KOSACK and JONES, 2000). However, if the plant recognizes the viral particle, an incompatible interaction, unfavorable for the virus, is established. It is known that plants can recognize the virus, limiting it to the site of the infection. A cascade of complex defense reactions can be induced, limiting virus replication and virus movement within the host plant. Plant-virus interactions are extremely complex and have been studied in depth for more than half a century. As a consequence, the mechanisms linked to viral accumulation inside host cells, virus-virus interactions as well as the plant defense mechanisms, have been partially elucidated. Many recent insights into virus-virus interactions have emerged from studies using the yeast *Saccharomyces cerevisiae* (BOONE et al., 2007), which reveal novel interacting components and key properties in which they participate. Yeast two-hybrid technology has been successfully applied to investigate interaction in virus such as closteroviruses (GOWDA et al., 2000).

Mealybug wilt of pineapple (MWP) was first described in Hawaii and since then has been reported as a severe disease exclusively of pineapple crops worldwide (CARTER, 1942; ROHRBACH et al., 1988; HU et al., 1993; BORROTO et al., 1998; HUGHES and SAMITA, 1998). Leaves of MWP affected plants turn bronze-red colour, loose turgidity and show severe tip necrosis (ROHRBACH et al., 1988), although symptoms are cultivar dependent and in some cases plants recover (SETHER and HU, 2002).

In searching for the causal agent of MWP in Cuba, 16 MWP-affected pineapple plants were used for virus purification and EM. The presence of virus particles strongly resembling PMWaVs in pineapples crops assisted the work to design specific primers for use in RT-PCR and confirm the presence of PMWaV-2. This procedure allowed the specific detection of nucleotide fragments of PMWaV-2 by RT-PCR and conclusively confirmed the presence of PMWaV-2 and the high homology between Cuban and Hawaiian PMWaV-2 isolates (Chapter 4, BORROTO et al., 2007a). On the other hand, to investigate protein-protein interaction of PMWaV-2 components, potentially involved in the regulation of virus pathogenesis (MELZER et al., 2001; QU and MORRIS 2005), p20, p22 and CP were analyzed by yeast two-hybrid. The study showed the homodimeric nature for the p20 and the p22, but not for the CP and suggests that these three proteins (p20, p22 and CP) apparently do not have any overlapping functions (Chapter 4, BORROTO et al., 2007a). The results obtained from the yeast two-hybrid assays contribute to the knowledge of

ampelovirus proteins with as yet unknown functions. This information could provide insight into novel genetic manipulations of pineapple that could lead to virus resistance. Constructs involving the genes coding for p20 and p22 can be candidates for the production of transgenic pineapple plants to induce protection against PMWaV-2, an approach that has been successfully used for potyviruses and closteroviruses (DI NICOLA-NEGRI et al., 2005; FAGOAGA et al., 2006).

Due to studies of model virus-plant interactions, more is known about how viruses work, how they exploit their hosts and how they overcome the layers of host defence responses mounted against them (HULL, 2002). A systematic investigation in the genetics of the interacting components in plant viruses will provide tools to better understand their mechanisms and to utilize non-transgenic and transgenic approaches for virus control in plants. It is timely to see the focus of research shifting towards pathogens and crops of significant economic importance to the agriculture, and to the translation of our fundamental advances in plant virology to processes and products in crops (MAULE et al., 2007).

The genomes of plant DNA viruses and pararetroviruses do not actively integrate into their host's chromatin, as integration is not required for viral replication (HOHN et al., 2008). Nevertheless, integrated geminivirus and PRV-related sequences have been detected in the genomes of several plant species and have been termed geminivirus-related DNA (BEJARANO et al. 1996) and EPRVs (MATZKE et al. 2000; HARPER et al. 2002; STAGINNUS and RICHERT-PÖGGELER 2006), respectively.

EPRVs are unusual pathogens, they can be regarded either as a specific type of repetitive component in the plant genome or as 'incorporated viruses' – 'natural transgenes' (STAGINNUS and RICHERT-PÖGGELER, 2006) that have maintained infectivity depending on the extent of their conservation. Because the assembly of complete and intact pararetroviral sequences and structures is a limiting factor, the possibility of EPRV activation seems to be low in most genomes. Nevertheless, it remains a serious obstacle for breeders of specific genera, e.g. *Musa*. Achieving EPRV control is therefore essential, particularly with respect to RNA silencing that might help to prevent disease outbreak of activatable copies. Given the fact, that the RNA interference-based plant defense system is sequence-specific, suitable conserved regions should allow targeting of a wide selection of different EPRV variants in the respective genome (STAGINNUS and RICHERT-PÖGGELER, 2006).

Probably in response to their potential harmful effects, natural selection has favored several host defenses against EPRV activation. Despite the ever-increasing amount of data on EPRVs, many questions still remain unanswered. The pathways of EPRV integration require further clarifications using knowledge about DNA repair in plants (RICHERT-PÖGGELER and SHEPERD, 1997; HESLOP-HARRISON, 2000). The question of whether only representatives of a single genus of the family *Caulimoviridae* invades a specific host genome or whether this is because of limited research also needs to be clarified. It seems particularly interesting, whether this might be caused by host suppression mechanisms inhibiting virus invasion. Searching for viral suppressors of gene silencing should be helpful in determining the answer to this question. Furthermore, putative interference between EPRVs and *Metaviridae* that would affect replication, integration or the structure of EPRVs needs to be investigated further. Therefore to increase our understanding of endogenous pararetroviral sequences *S. lycopersicum* and a wild relative, *S. habrochaites* were studied with respect to sequence and structure of a number of integrated copies, as well as to chromosomal localization. In addition, the methylation status of the EPRV integrants and their transcriptional activity was investigated to gain understanding about the nature of host control of these sequences (Chapter 5, STAGINNUS et al., 2007).

The sequence similarity of *Lyc*EPRVs in *S. lycopersicum* and *S. habrochaites* indicated that they are potentially derived from the same pararetrovirus. DNA blot analysis revealed a similar genomic organization in the two species, but also some independent excision or insertion events that must have occurred after species separation, or flanking sequence divergence. *Lyc*EPRVs share with the tobacco elements a disrupted genomic structure and frequent association with retrotransposons. Fluorescence *in situ* hybridization revealed that copies of *Lyc*EPRV are dispersed on all chromosomes in predominantly heterochromatic regions. Methylation of *Lyc*EPRVs was detected in CHG and asymmetric CHH nucleotide groups. Although normally quiescent EPRVs can be reactivated and produce symptoms of infection in some *Nicotiana* interspecific hybrids, a similar pathogenicity of *Lyc*EPRVs could not be demonstrated in *Solanum* L. section *Lycopersicon* [Mill.] hybrids. Even in healthy plants, however, transcripts derived from multiple *Lyc*EPRV loci and short RNAs complementary to *Lyc*EPRVs were detected and were increased upon infection with heterologous viruses encoding suppressors of PTGS. The analysis of *Lyc*EPRVs provided further evidence for the extensive invasion of pararetroviral sequences into the genomes of solanaceous species. The detection of asymmetric CHH methylation and short RNAs, which are hallmarks of RNAi in plants,

suggested that *Lyc*EPRVs are controlled by an RNA-mediated silencing mechanism (Chapter 5, STAGINNUS et al., 2007). The impact of EPRVs on genome evolution and shape is becoming evident and indicates a close association between virus and host (HOHN et al., 2008; STAGINNUS et al., 2009).

There is clear evidence that plant DNA viruses can integrate into a host genome, and some of these integrations become fixed. These viruses are natural genetic engineers and may have been altering plant genomes since the origin of the plant kingdom (HOHN et al., 2008). This is not a passive situation; the plant host has to cope with powerful regulatory sequences present within the viral genome and uses mechanisms similar to those controlling transposable elements that are parasites of any genome. Future research is necessary to provide more precise insights to unravel possible functional roles of EPRVs in the regulatory pathways of the host.

Within this work was possible to confirm that virus and phytoplasma detection, diagnosis and epidemiological studies are important factors since they are integral to predicting the demands for improved resistance and its durability. With the assistance of a multitude of diagnostic methods the detection has contributed to enhance the efficient control of pathogens in the agriculture. On the other hand, virus and phytoplasma characterization, including genome analysis and deeper studies of the molecular processes that support their multiplication and capacity to infect their hosts, is also a widespread and necessary activity especially when it relates to newly emerging viruses. The results about the plant-virus relationships have provided valuable information to understand the function of viral genomes as well as the strategies adopted by viruses in their replication and expression. These examples might lead to significant advances in designing new and more efficient strategies towards resistance against pathogens in the form of transgenic plants.

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APPENDICES

Annex 1. Descriptor used in virus taxonomy (HULL, 2002).

I. Virions properties

A. Morphology properties of the virions

1. Size
2. Shape
3. Presence or absence of an envelope or peplomers
4. Capsomeric symmetry and structure

B. Physical properties of virions

1. Molecular mass
2. Buoyant density
3. Sedimentation coefficient
4. pH stability
5. Thermal stability
6. Cation (Mg^{2+}, Mn^{2+}, Ca^{2+}) stability
7. Solvent stability
8. Detergent stability
9. Radiation stability

C. Properties of the genome

1.Type of nucleic acid, DNA or RNA
2. Strandedness: single-stranded or double stranded
3. Linear or circular
4. Sense: positive, negative or ambisense
5. Number of segments
6. Size of genome or genome segments
7. Presence or absence and type of 5 terminal cap
8. Presence or absence of 5 terminal covalently-linked polypeptide
9. Presence or absence of 3 terminal poly (A) tract (or other specific tract)
10. Nucleotide sequences comparisons

D. Properties of proteins

1. Number
2. Size
3. Functional activities (especially virions transcriptase, virion reverse transcrptase, virion hemagglutinin, virion neuraminidase, virion fusion protein)
4. Amino acid sequence comparisons

E. Lipids

1. Presence or absence
2. Nature

F. Carbohydrates

1. Presence or absence
2. Nature

II. Genome organization and replication

1. Genome organization
2. Strategy of replication of nucleic acid
3. Characteristics of transcription
4. Characteristics of translation and post-translational processing
5. Sites of accumulation of virion proteins, site of assembly, site of maturation an release
6. Cytopathology, inclusion body formation

III. Antigenic properties

1. Serological reletionshps
2. Mapping epitopes

IV. Biological properties

1. Host range, natural and experimental
2. Pathogenecity, association with disease
3. Tissue tropisms, pathology, histopathology
4. Mode of transmission in nature
5. Vector relationships
6. Geographical distribution

Annex 2. Table of abbreviations.

A. thaliana	*Arabidopsis thaliana*
ArMV	*Arabis mosaic virus*
AT	Annealing temperature
AYWB	Aster yellows witches´broom
B	bottom
bp	Base pair
BSA	bovine serum albumin
BSOEV	endogenous *Banana streak virus*
BSV	*Banana streak virus*
BYMV	*Bean yellow mosaic virus*
BYV	*Beet yellows virus*
CAP	Common Agricultural Policy
CaYMV	*Canna yellow mottle virus*
cDNA	Complementary deoxyribonucleic acid
CMV	*Cucumber mosaic virus*
CP	Coat protein
CPd	Coat protein duplicate
CsVMV	*Cassava vein mosaic virus*
CTV	*Citrus tristeza virus*
DAPI	4',6-diamidino-2-phenylindole
Dicer	RNAseIII-like enzymes
DNA	Deoxyribonucleic acid
DNASTAR	DNAstar software package
dNTPs	Deoxyribonucleotides triphosphate
dsDNA	Double stranded DNA
ELISA	Enzyme Linked Immunosorbent Assay
EM	Electron microscopy
EPRVs	Endogenous pararetroviral sequences
ePVCV	*Endogenous Petunia vein clearing virus*
EST	Expressed sequence tag
FAO	Food and Agricultural Organization

FISH	Fluorescent *in situ* hybridization
FITC	Fluorescein isothiocyanate
g	Gram
GFLV	*Grapevine fanleaf virus*
HcPro	helper component-proteinase
IC–PCR	Immunocapture PCR
IGR	Intergenic region
kbp	Kilo base pair
kDa	Kilodaltons
kV	Kilovolts
LTR	Long terminal repeat
*Lyc*EPRV	Lycopersion endogenous pararetrovirus
M	middle
MAbs	Monoclonal antibodies
Mb	Megabase
mM	millimolar
MP	cell-to-cell movement protein
mRNA	Messenger ribonucleic acid
MT	Metric tons
MWP	Mealybug wilt of pineapple
N. tabacum	*Nicotiana tabacum*
ng	Nanogram
nm	Nanometer
NOR	Nucleolus organizer region
*Ns*EPRV	*N. sylvestris* EPRV
nt	Nucleotide
*Nto*EPRV	*N. tomentosiformis* EPRV
OD	Optical density
ORFs	Open reading frames
OY	Onion yellows
p20	20 kDa protein
p21	21 kDa protein

p22	22 kDa protein
p23	23 kDa protein
P^{32}	Radioactive isotopes of phosphorus
PCR	Polymerase chain reaction
pmol	picomole
PMWaV-1	*Pineapple mealybug wilt associated virus-1*
PMWaV-2	*Pineapple mealybug wilt associated virus 2*
PMWaV-3	*Pineapple mealybug wilt associated virus-3*
POL	Polyprotein
PRVs	Pararetroviruses
PTGS	Post-transcriptional gene silencing
PVCV	*Petunia vein clearing virus*
PVY	*Potato virus Y*
R. fruticosus	*Rubus fruticosus*
R. ideaus	*Rubus ideaus*
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase H	Ribonuclease H
rpm	Revolutions Per Minute
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RTBV	*Rice tungro bacilliform virus*
RT-PCR	Reverse transcription polymerase chain reaction
S. cheesmaniae	*Solanum cheesmaniae*
S. habrochaites	*Solanum habrochaites*
S. pennellii	*Solanum pennellii*
S. peruvianum	*Solanum peruvianum*
S. pimpinellifolium	*Solanum pimpinellifolium*
S. tuberosum	*Solanum tuberosum*

S. lycopersicum	*Solanum lycopersicum*
SC	Synthetic Complete medium
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SNP	single-nucleotide polymorphism
sRNA	Short RNA
SSC	Saline sodium citrate
ssDNA	Single stranded DNA
SSR	Single Sequence Repeat
STCs	Sequenced-tagged connectors
T	top
T. aestivum	*Triticum aestivum*
TAE	Tris-acetate-EDTA
TATA box	5`-TATAAA-3`
TAV	*Tomato aspermy virus*
TAV	Transactivator protein
TBIA	Tissue blot immunoassay
TBSV	*Tomato bushy stunt virus*
TCA	Tricarboxylic acid
tRNA	Transfer RNA
TSWV	*Tomato spotted wilt virus*
TVCV	*Tobacco vein clearing virus*
V. myrtillus	*Vaccinium myrtillus*
v/v	Volume/volume
w/v	Weight/volume
X. diversicaudatum	*Xiphinema diversicaudatum*
X. index	*Xiphinema index*
X. italiae	*Xiphinema italiae*
X. pachtaicum	*Xiphinema pachtaicum*
X. vuittenezi	*Xiphinema vuittenezi*
Λ,λ	Lambda
µg	Microgram

2n	Diploid

Curriculum vitae

Name: Eduviges Glenda Borroto-Fernández

Date of birth: April 7, 1970

Sex: Female

Citizenship: Cuban

Birthplace: Camagüey. Cuba

EDUCATION

- **2004-2010** PhD-studies, Institute of Applied Microbiology, Plant Biotechnology Unit University of Natural Resources and Applied Life Sciences, Vienna, Austria
- **1999** 2-D Electrophoresis European Seminar (Amersham-Pharmacia-Biotech), Leuven, Belgium (2 days)
- **1999** Two-dimensional gel electrophoresis (carrier ampholines and Immobilized pH-gradient) and immunoblotting. Practical training, University of Gent, Department of Plant Genetic, Belgium (2 months)
- **1995** Viral diseases in plants and their diagnosis. Practical course, National Center on Plant Health (CNSV), Cuba by Prof. Phil Jones from Plant Pathogen Interactions Division, Rothamsted Research, Harpenden, Hertfordshire, UK (5 days)
- **1994** Somaclonals variant in sugar cane. Molecular characterization. Practical course, Bioplant Center, University of Ciego de Avila, Cuba (10 days)
- **1988-1993** Engineer in agricultural sciences. University of Ciego de Avila, Cuba. Diploma-thesis: Chemical composition of Cleopatra mandarin mature seeds and biochemical changes during their germination.

RESEARCH EXPERIENCE

- **2000-2001** Isolation of *Pineapple mealybug wilt virus-2* from Cuba by RT-PCR techniques and protein-protein interactions of its viral components
- **2000-2000** Studies about the function of motor proteins during cell cycle. University of Gent, Department of Plant Genetic, Belgium
- **2000-2000** Replication analysis of *African cassava mosaic virus* (ACMV) in Cassava cell suspensions system and in transgenic tobacco plants. ETH Zurich, Institute of Plant Sciences, Switzerland
- **1999-1999** Molecular markers during somatic embryogenesis in *Saccharum officinarum* L., Bioplant Center, University of Ciego de Avila, Cuba

- **1997-1999** Monoclonal antibody production against closterovirus-like particle associated with Mealybug wilt of pineapple. Center for Genetic Engineering and Biotechnology (CIGB), Cuba
- **1996-1997** Isolation of closterovirus-like particles associated with Mealybug wilt of pineapple. Center for Genetic Engineering and Biotechnology (CIGB), Cuba
- **1994-1996** Metabolic changes related with Mealybug wilt of pineapple. Bioplant Center, University of Ciego de Avila, Cuba
- **1993-1995** Chemical composition of Cleopatra mandarin mature seeds and biochemical changes during their germination. Bioplant Center, University of Ciego de Avila, Cuba

TEACHING ACTIVITIES

- **2006-2009** Molecular Phytopathology (954.309). Practical course at BOKU University under the supervision of Ao.Univ.Prof. Dr.phil. Margit Laimer

PUBLICATION AND PRESENTATIONS

Peer reviewed papers

- **Borroto-Fernandez, E.G.**, Sommerbauer, T., Popowich, E., Schartl, A., Laimer, M. (2009) Somatic embryogenesis from anthers of the autochthonous *Vitis vinifera* cv. Domina leads to *Arabis mosaic virus*-free plants. European Journal of Plant pathology 124(1):171-174.
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- **Borroto-Fernández, E.G.**, Maghuly, F., Fellner, A., Laimer, M. (2008) Determination of viral infections in an Austrian collection of Canna indica. Journal of Plant Diseases and Protection. 115(3):102-103.
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- **Borroto-Fernández, E.G.**, Torres-Acosta, J.A., Laimer, M. (2007) RT-PCR detection and protein-protein interaction of viral components of *Pineapple mealybug wilt associated virus-2* in Cuba. Journal of Plant Pathology 89(3):435-439.
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- Staginnus, C., Gregor, W., Mette, M-F., Teo, C.H, **Borroto-Fernandez, E.G.**, Laimer da Camara Machado, M., Matzke, M., Schwarzacher, T. (2007):

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Oral presentations

- Khan, M., Mendonca, D., **Borroto-Fernandez, E.G.**, Maghuly, F., Marzban, G., Katinger, H. Laimer, M.(2007) New views on pathogen derived resistance (PDR).European Meeting On Plum Pox. 24-28 September, pp 31, Pula, Croatia, Abstract book, 31.
- Maghuly, F., Leopold, S., **Borroto-Fernandez, E.G.**, Khan, M.A., Gambino, G., Gribaudo, I., Laimer da Câmara Machado, M. (2006) What do we learn from the molecular characterization of grapevine plants transformed with GFLV resistance genes? 15th Meeting of the ICVG, Stellenbosch, Extended Abstract book, 62-64.
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- Maghuly, F., **Borroto-Fernandez, E.G**., Leopold, S., Pedryc, A., Katinger, H., Laimer, M. (2006) Simple sequence repeat (SSR) analysis for assessment of genetic variability in apricot cultivars. In: IAPTC & B: Regionale wissenschaftliche Konferenz Pflanzenbiotechnologie IAPTC&B – Sektionen Österreichs, Deutschlands und der Schweiz, 22–24. March 2006, Vienna, Austria, Abstract book, 58.
- Maghuly, F., Leopold, S., Khan, M.A., **Borroto-Fernandez, E.G.**, Drumonde Neves, J., Laimer, M. (2005) Molecular charcterization of transgenic grapevines carrying nepovirus genes. 5. Symp. Phytomedizin und Pflanzenschutz im Gartenbau, Vienna, Abstract book, 29-30.
- **Borroto-Fernandez, E.G.**, Torres-Acosta, J.A., Reytor-Saavedra, E., Laimer, M. (2005) Pineapple mealybug wilt associated virus in Cuba. 5. Symp. Phytomedizin und Pflanzenschutz im Gartenbau, Vienna, Abstract book, 53-54.
- Laimer, M., Pejic, I., **Borroto-Fernandez, E.G.**, Hanzer, V., Fenino, E., Katinger H. (2005) In vitro sanitation of an old Croatian grapevine cultivar.. In: EHS: 5. Symp. Phytomedizin und Pflanzenschutz im Gartenbau, Sept. 2005, Vienna, Abstract book, 27-28.
- Laimer, M., **Borroto-Fernandez, E.G.**, Hanzer, V., Balla, I., Katinger, H. (2005): In vitro collection and sanitation of valuable germplasm of temperate fruit trees. In: IBC: XVII IBC, Vienna; Abstract Book, 128.

Poster Presentations

- Maghuly, F., **Borroto-Fernandez**, **E.G.**, Khan, M.A., Herndl, A., Marzban, G., Laimer, M. (2009) Calmodulin and lipid transfer protein gene expression in *Prunus incise x serrula* (PIS) vary independently under different stress conditions. In: Plant & Animal Genomes XVII Conference, 10-14 January, San Diego, USA.
- Laimer, M., Maghuly, F., Khan, M.A., **Borroto-Fernandez, E.G.**, Hanzer V., Balla I., Katinger, H. (2008) Biotechnological approaches to contain virus diseases in woody crop plants. In: First Symposium on Horticulture in Europe, 17-20 February, Vienna, Austria; Abstract book, 25.
- Laimer, M., Maghuly, F., Khan, M.A., **Borroto-Fernandez, E.G.**, Katinger, H. (2007) Stability of transgene expression in model fruit tree species over nine years. In: International Conference of Plant Transformation Technologies, 4-7 February, Vienna, Austria; Abstract book, 83.

- Laimer, M., Maghuly, F., Leopold, S., **Borroto-Fernandez, E.G.**, Khan, M.A. (2007) Analysis of transgenic grapevine (*Vitis vinifera*) including *Grapevine fanleaf Virus* Coat Protein (GFLV CP) gene. In: International Conference of Plant Transformation Technologies, 4-7 February, Vienna, Austria; Abstract book, 83.
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- Blanco, M., Castillo, R., **Borroto-Fernandez, E.G.**, Nieves, N., Borroto, C.G., Segura, M. (1999) Diferenciación de embriones somáticos en suspensiones celulares de caña de azúcar: I. Expresión de patrones de proteínas durante el proceso. In: BioVeg I International Workshop of Plant Biotechnology. Ciego de Avila, Cuba.
- Escalona, M., Lorenzo, J.C., González, J., Fundora, Z., Guerra, O., González, R., **Borroto-Fernandez, E.G.**, Herrera, L., Espinosa, P., Batista, L., Borroto, C.G. (1995) Agrobacterium-mediated transformation and regeneration of Sour orange (*Citrus aurantium* L.) from stem segments. In: of the Third International Workshop. Citrus Tristeza Virus and brown citrus aphid in the Caribbean basin: Management strategies. Lee, R., Rocha-Pena, M., et al., eds. University of Florida, IFAS, USDA. Abstract book, 96-98.
- **Borroto-Fernandez, E.G.**, Cintra, M., González, J., Borroto, C.G., Oramas, P. (1997) A report of Closteroviruslike Particle Associated with Pineapple Plants (*Ananas comosus* `Smooth Cayenne¨) Affected with Mealybug Wilt of Pineapple, at Ciego de Avila, in Cuba. In: 3rd International Workshop of Vegetal Sanity. Havana, Cuba.