Dissertation
submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by

Msc. (Genetics) Annerie Liebenberg
born in: Heidelberg, Gauteng, South Africa
Oral examination: 30 May 2013

Influence of latent apple viruses on *Malus sieboldii*-derived apple proliferation resistant rootstocks

Referees: Prof. Dr. Rüdiger Hell
PD Dr. Michael Wassenegger

CONTENTS

INDEX OF TABLES AND FIGURES

TABLES:

FIGURES:

APPENDIX A:

APPENDIX B:

APPENDIX C:

ABBREVIATIONS

µg	Microgram(s)
µl	Microliter(s)
µM	Micromolar
ACLSV	*Apple chlorotic leaf spot virus*
Amp	Ampicillin
AP	Apple Proliferation
ApLV	*Apricot latent virus*
ApMV	Apple mosaic virus
ASGV	*Apple stem grooving virus*
ASPV	*Apple stem pitting virus*
BAP	6-Benzylaminopurine
BCMV	*Bean common mosaic potyvirus*
bp	base pairs
BSA	Bovine Serum Albumin
Ca(ClO)2	Calcium Hypochlorite
Ca'. P. mali'	'*Candidatus* Phytoplasma mali'
$CaCl_2$	Calcium Chloride
CaMV	*Cauliflower mosaic virus*
CDI	Cumulative disease indices
cDNA	Complimentary Deoxynucleic Acid
cm	centimeter
CMV	*Cucumber mosaic virus*
CP	Coat protein
ct	Cycle threshold
cv	Cultivar
CTAB	Cetyltrimethylammoniumbromid
CTLV	*Citrus tatter leaf virus*
CVA	*Cherry virus A*
DAMP	Danger-associated molecular patterns
ddH2O	Double distilled water
dN	Nonsynonymous
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleoside triphosphate(s)
dS	Synonymous
dsRNA	Double Stranded Ribonucleic Acid
DTT	1,4-Dithiothreitol
EDTA	Ethylene Diamine Tetra-acetic Acid di-sodium Salt
ELISA	Enzyme-linked Immunosorbent Assay
ET	Ethylene
EtBr	Ethidium Bromide
ETS	Effector-triggered susceptibility
g/L	Gram per liter
GA_3	Gibberellic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GRSPaV	*Grapevine rupesteris stem pitting-associated virus*
h	hour
Hel	NTB-binding helicase
HOAc	Acetic Acid
HR	Hypersensitive response
IBA	Indole-3-butyric acid
IC-PCR	Immuno Capture Polymerase Chain Reaction
IC-RT-PCR	Immuno Capture Reverse Transcription Polymerase Chain Reaction
JA	Jasmonic acid
K_2HPO4	Dipotassium phosphate
kb	Kilobase
KCl	Potassium Chloride
kDa	KiloDalton
KOAc	Potassium Acetate
KOH	Potassium Hydroxide
L	Liter
LB	Luria Bertani broth
M	Molar
$MgCl_2$	Magnesium Chloride
mg/L,	Milligrams per liter
min	Minutes
ml	Milliliter
MLO	Mycoplasma Like Organism
mM	Millimolar
MP	Movement Protein
mpi	Months Post Inoculation
Mr	Molecular weight
MS medium	Murashige-Skoog Medium
Mt	Methyltransferase
na	Not Applicable
NaCl	Sodium Chloride
NaOAC	Sodium Acetate
NaOH	Sodium Hydroxide
NB-LRR	Nucleotide Binding Site Leucine Reach Repeat
ng	Nanograms
nm	Nanometer(s)
nt	Nucleotides
OD_{600}	Optical density value at 600 nm
ORF	Open Reading Frame
P:C:I	Phenol Chloroform Isoamylalcohol
PAMP	Pathogen-associated molecular patterns
PCD	Programmed Cell Death
PCMV	*Peach chlorotic mottle virus*
PCR	Polymerase Chain Reaction
P-pro	Papain-like protease
PTI	pattern triggered immunity
PVP	Polyvinylpyrrolidone

PVX	*Potato virus X*
QL medium	Quoirin-Lepoivre medium
qPCR	Quantitative Polymerase Chain Reaction
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
RdRp	RNA-dependent RNA polymerase
RE	Restriction enzyme
RFLP	Restriction Fragment Length Polymorphism
R-gene	Resistance gene
RNA	Ribonucleic Acid
rpm	Revolutions per minutes
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcription - Polymerase Chain Reaction
SA	Salicylic acid
SAR	systemic acquired resistance
SBE	Starch Binding Protein
SDS	Sodium Dodecyl Sulphate
Sec	Seconds
SSR	simple sequence repeat
ssRNA	Single Stranded Ribonucleic Acid
TCP's,	TEOSINTE BRANCHED, CYCLOIDEA, PROLIFERATING CELL FACTORS
T-DNA	Transfer DNA
TGB	Triple Gene Block
Tris-HCl	Tris Hydrochloride
tRNA	Transfer DNA
U	Units
v/v	Volume per volume
V1	Variable area 1
V2	Variable area 2
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

SUMMARY

Apple proliferation (AP) disease caused by '*Candidatus* Phytoplasma mali' is one of the most economical important diseases in the apple industry, causing tasteless undersized fruits. A project to produce AP-resistant rootstocks by crossing the natural resistant wild type *M. sieboldii* with commercial *M. domestica* apple rootstock varieties, observed severe decline and death in the progeny associated with latent apple viruses, namely Apple stem grooving virus (ASGV) and Apple stem pitting virus (ASPV). This study aimed to explain this phenomenon by gaining new information on the causative agent(s) as well as to see if there is any interaction between the different apple viruses or between virus and phytoplasma, and to study the effect of these pathogens on *M. sieboldii, M. domestica* and the hybrid progeny. The genome of the ASGV-AC strain, which was associated with the decline, was determined and variability and evolutionary studies were performed, revealing significant differences in the selection pressure of two variable areas in the genome. An *in vitro* culture system was established and ASGV, ASPV and '*Ca.* P. mali' were successfully graft-transmitted to the different *Malus* genotypes as single infection or as virus-virus and virus-phytoplasma co-infection. The transmission rate was severely affected by the *Malus* genotype, with each genotype acting differently to the different virus, virus-virus and virus-phytoplasma combinations. A hypersensitive reaction was observed on *M. sieboldii* and some of the *M. sieboldii*-derived hybrid progeny depending on the inocula. Some hybrids showed severe resistance reactions while others recovered from the initial reaction and allowed the systemic spread of the virus(es). Quantitative Real-Time PCR was established for ASGV and ASPV and used to determine the mean virus titer per plant cell. ASGV titer was significantly lower compared to ASPV in *M. domestica*. The ASGV titer was lower in *M. domestica* compared to *M. sieboldii* and the hybrid progeny confirming the tolerant nature of *M. domestica* towards ASGV. An antagonistic interaction was observed between ASGV and ASPV, with the ASGV titer decreasing by half when co-infected with ASPV, while the ASPV titer is unchanged. An antagonistic interaction was also observed between ASGV and '*Ca.* P. mali'. While the '*Ca.* P. mali' titer was significantly increased, the ASGV titer was decreased compared to the titer values obtained from single infections. This is the first study performed on the possible interaction between phytoplasma and virus.

ZUSAMMENFASSUNG

Apfeltriebsucht (AT), verursacht durch ´*Candidatus* Phytoplasma mali', gehört zu den wirtschaftlich wichtigsten Krankheiten im Apfelanbau und verursacht geschmacklose und kleinwüchsige Früchte. Ein Projekt zur Entwicklung von AT-resistenten Unterlagen, basierend auf der Kreuzung von natürlicherweise resistentem *M. sieboldii* mit kommerziellen *M. domestica* Apfelunterlagen, zeigte in der Nachkommenschaft starke Absterbeerscheinungen, wenn die Pflanzen mit latenten Apfelviren wie Apple stem grooving virus (ASGV) und Apple stem pitting virus (ASPV) infiziert waren. Das Ziel dieser Arbeit war, dieses Phänomen durch Identifizierung der verantwortlichen Pathogene zu erklären sowie die Wechselwirkungen zwischen den Viren und zwischen Viren und Phytoplasmen in den verschiedenen *Malus*-Arten zu untersuchen. Das Genom des Absterbeeerscheinungen-auslösenden Stammes ASGV-AC wurde sequenziert. Analysen der genetischen Variabilität und der evolutionären Entwicklung zeigten signifikante Unterschiede im Selektionsdruck auf zwei variable Regionen im Virusgenom. Ein *in vitro*-Kultur-System wurde etabliert, und ASGV, ASPV und '*Ca*. P. mali' wurden erfolgreich durch Pfropfung auf verschiedene *Malus*-Genotypen als Einzelinfektion oder als Virus-Virus und Virus-Phytoplasma-Mischinfektion übertragen. Die Übertragungsrate wurde stark vom *Malus*-Genotyp beeinflusst, jeder Genotyp reagierte unterschiedlich auf die verschiedenen Kombinationen von Virus, Virus-Virus und Virus-Phytoplasma. Je nach Inokulum wurde eine hypersensitive Reaktion bei *M. sieboldii* und einigen *M. sieboldii* abgeleiteten Hybriden beobachtet. Einige Hybride zeigten eine starke Resistenzreaktion, während sich andere von der anfänglichen Reaktion erholten und den Viren die systematische Ausbreitung ermöglichten. Eine quantitative Real-Time PCR wurde für ASGV und ASPV etabliert und dazu verwendet, um den mittleren Virustiter pro Pflanzenzelle zu bestimmen. Der ASGV-Titer war in *M. domestica* signifikant niedriger im Vergleich zu ASPV. Der ASGV-Titer war auch geringer in *M. domestica* im Vergleich zu *M. sieboldii* und den abgeleiteten Hybriden, was die Toleranz von *M. domestica* gegenüber ASGV bestätigt. Eine antagonistische Wechselwirkung zwischen ASGV und ASPV wurde beobachtet; in Mischinfektion mit ASPV ist der ASGV-Titer um die Hälfte reduziert, während der ASPV-Titer unverändert bleibt. Eine antagonistische Wechselwirkung wurde auch zwischen ASGV und '*Ca*. P. mali' festgestellt. Während der '*Ca.* P. mali'-Titer signifikant erhöht war, erniedrigte sich der ASGV-Titer im Vergleich zu den bei Einzelinfektion beobachteten Werten. Dies ist die erste Studie, die zu den möglichen Wechselwirkungen zwischen Phytoplasmen und Viren durchgeführt wurde.

1 INTRODUCTION

1.1 GENERAL INTRODUCTION AND PROJECT AIMS

Apple is one of the most important fruit crops in Europe. In the 28 European Union countries approximately 10.4 million tons, covering an area of 550 000 hectares was produced in 2010 (FAO stat). Germany is the 4th biggest producer of apples in the EU, behind Italy, Poland and France, producing 835 000 tons of apples, covering an area of 31820 hectares (2010). Apple proliferation disease was first described in the early 1950's and rapidly spread all over Europe to reach epidemic proportions in the year 2000. Germany and Italy was hit the hardest with 25 and 100 million euro lost due to an apple proliferation outbreak (Straus, 2009). Apple proliferation is caused by '*Candidatus* Phytoplasma mali', a wall-less, phloem-restricted, plant pathogenic gram-positive eubacteria belonging to the class *Mollicutes* (Weisburg *et al.*, 1989; Bové and Garnier, 1998). The disease causes undersized tasteless fruits, rendering them unmarketable.

In 2003 the Instituto Agrario di San Michele in Trentino and two German institutes, RLP Agroscience GmBH, Alplanta, Institute for Plant Research and the Biologische Bundesanstalt (Julius-Kühn Institut) Dossenheim started a collaboration to find a solution to fight the disease. Natural resistance to the disease was found in the wild apple type, *Malus sieboldii* but not in the commercial *M. domestica* apple trees. In the 1950s-1960s *M. sieboldii* was used to produce rootstocks but they were too vigorous for the modern apple cultivation, nevertheless the resistance in these rootstocks was proven. *M. sieboldii* was crossed with standard apple rootstock M9 since 2001 to combine resistance with other good rootstock traits. The resistance screening of the progeny took place in field trials and in the summer 2006 unexpected decline and death was observed in the potential resistant progeny. Upon further testing 3 latent viruses were identified associated with the decline: *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and *Apple chlorotic leaf spot virus* (ACLSV). Previous studies showed that *M. sieboldii* is acting hypersensitive towards these viruses. Although Apple proliferation resistant rootstocks were developed, the intolerance of the rootstocks to these four latent viruses is problematic.

To study this phenomenon, we have to consider all the influencing factors (fig.1).

- The environment: If temperature, nutrition or season plays a role in the symptom expression.
- '*Ca.* P. mali': Different strains could have different virulence factors influencing the symptom expression of the plant.
- Latent viruses: Which latent virus or combination of viruses is responsible for the decline observed and is there a difference in the virulence of isolates of the same virus?
- Apple tree: Is the *Malus* genotypes either the rootstock or the scion cultivar acting tolerant or hypersensitive to these pathogens or combinations of pathogens?

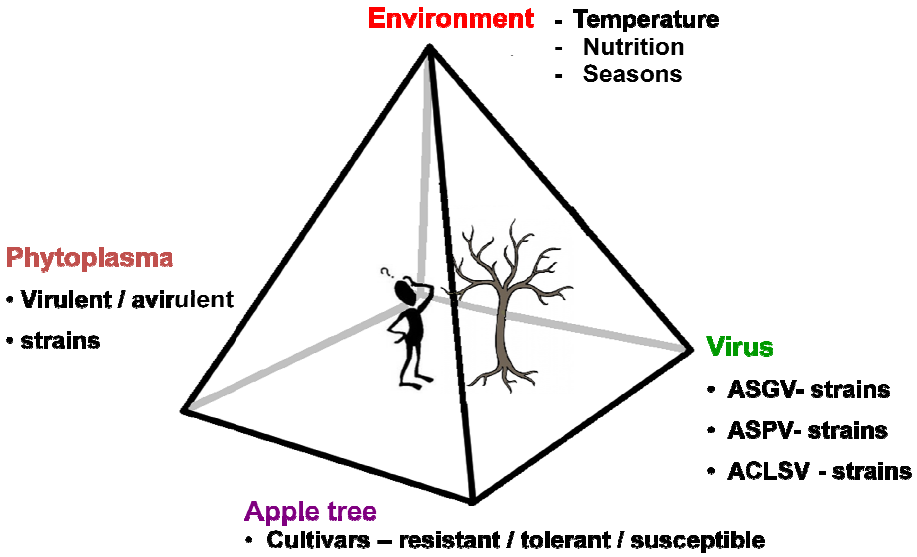

Figure 1: The factors influencing the virus-virus and virus-phytoplasma interactions

The objective of this study was to analyze virus-virus and virus-phytoplasma interactions in *Malus* genotypes and the effect thereof on the potential apple proliferation resistant rootstocks. To achieve this more information was needed on the viruses responsible for this decline observed in the field trials and a system was needed to study these interactions under controlled conditions.
Aims:

- To do variability and evolutionary studies on ASGV to select areas of importance in virus virulence for fragment swapping in the cDNA clone.

- To construct full length infectious cDNA clones of ASGV and ASPV to do fragment swopping of virulent and less virulent virus isolates, as well as easy access to a genomic stable isolate of each virus that could be used for transmission.
- To establish an *in vitro* culture system where graft transmissions of viruses and phytoplasma can be performed.
- To establish homogenous culture lines of *Malus domestica*, *M. sieboldii* and *M. sieboldii* derived hybrid rootstocks with different combinations of ASGV, ASPV and '*Ca.* P. mali' infections for virus-virus and virus-phytoplasma interaction studies.
- To reproduce symptoms observed in the field trials on the *in vitro* system and study which pathogen or combinations of pathogens are responsible for the decline observed in the field trials.
- To determine the virus and '*Ca.* P. mali' concentrations per plant cell through Real-Time PCR analysis and how it is influenced by the addition of a second pathogen.
- To analyze the effect of these different combinations of pathogens on tolerant, resistant and potential resistant hybrid genotypes.

1.2 LITERATURE REVIEW

1.2.1 Flexiviridae

The Flexiviridae family falls under the order *Tymovirales* and was recently divided into 3 new families: *Alphaflexiviridae*, *Betaflexiviridae* and *Gammaflexiviridae* (Martelli *et al.,* 2007, ICTV virus Taxonomy report, 2011). *Betaflexiviridae* includes the seven genera *Capillovirus*, *Carlavirus*, *Citrivirus*, *Foveavirus*, *Tepovirus*, *Trichovirus*, *Vitivirus* and a group of unassigned virus species within the family *Flexiviridae*. The genus *Capillovirus* includes two species, *Apple stem grooving virus* (ASGV, type member) and *Cherry virus A* (CVA). The genus *Foveavirus* includes the species *Apple stem pitting virus* (ASPV, type member), *Apricot latent virus* (ApLV), *Grapevine rupesteris stem pitting-associated virus* (GRSPaV) and *Peach chlorotic mottle virus* (PCMV).

1.2.2 Apple stem grooving Capillovirus

1.2.2.1 Morphology

The ASGV particles are non enveloped flexuous filaments with a length of 620 nm (apple strain), 650 nm (citrus strain) and 680 nm (*Actinidia* strain) (Lister *et al.,* 1965; Inouye *et al.,* 1979; Clover *et al.,* 2003), and a width of 12 nm. The filaments have a rope like structure with a cross-banding striation appearance and an obvious basic helix (fig. 2; Lister and Bar-Joseph, 1981).

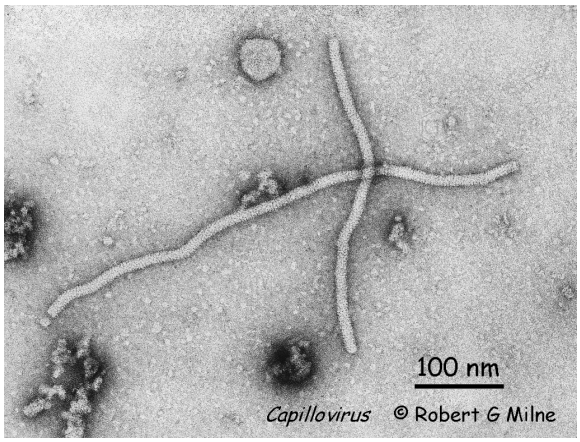

Figure 2: Electronmicrograph of *Apple stem grooving virus* particles
(R.G. Milne, http://ictvdb.bio-mirror.cn/WIntkey/Images/em_capil.htm)

1.2.2.2 Genome and Genomic Organization

ASGV has a single stranded positive sense monopartite genome of approximately 6,495 nucleotides excluding a poly-A tail. The genomic RNA has two overlapping open reading frames (ORF). ORF1 (bases 37 to 6341) encodes a 241 to 242 kDa pseudo-polyprotein (2105 amino acids) containing several domains (methyltransferase, papain-like protease, nucleotide triphosphate-binding helicase, RNA-dependent RNA polymerase) characteristic of the replicase region that is conserved along most Flexiviridae and the coat protein (CP) in the C-terminal region (Yoshikawa *et al.,* 1992; Jelkmann, 1995).

ORF2 (bases 4788-5447) encodes a 36 kDa protein containing motives for the movement protein (MP) and serine proteases (Yoshikawa *et al.,* 1992; 1993). A highly variable region is located between the conserved replicase and CP regions in ORF1, where codons 1585 to 1868 of ORF1 overlap with ORF2. This variable region has no functional motifs but could be involved in the pathogenicity of the virus (Hirata *et al.,* 2010). No ASGV isolates containing a stop codon in this region have been reported (Magome *et al.,* 1997; 1999).

The organization of the polyprotein of ASGV is unusual for members of the *Flexiviridae*, which normally encode for the CP on a separate ORF (Martelli *et al.,* 2007). Hirata *et al*. (2010) showed by mutational analysis that the expression of the full length ORF1 is not necessary for systemic infection and that infection only required the area between the N-terminus and the N-terminal of the variable region. It was also suggested that the coat protein together with the variable region is important for replication and pathogenicity and the more of the variable region is expressed the higher is the symptom expression. The replication protein encoded for by the ORF1 is expressed in two forms of different sizes: one full length polyprotein including the replicase and a second shorter replicase protein (90kDA) produced from the original ORF1 by translation frame shifting triggered by a putative stem-loop structure downstream of the conserved replicase domains (Hirata *et al.,* 2010). Two additional 3' co-terminal viral RNA species, in addition to the genomic RNA, were also detected, with sizes suggesting that the CP and the MP genes were expressed through sub-genomic RNAs (Hirata *et al.,* 2003; Tatineni *et al.,* 2009a).

1.2.2.3 Molecular Evolution

The capilloviruses are the only genus from the *Betaflexiviridae* family that has overlapping ORFs. Although capilloviruses and trichoviruses are closely related they are classified into two different genera because of their different genome organization. The study of Hirata *et al.* (2010) points to some evolutionary considerations for ASGV, as the genome organization of the truncated mutant closely resembles that of the trichoviruses (Type species, ACLSV) with the Replicase, MP and CP each encoded for by an individual ORF and could therefore have the same ancestor.

1.2.2.4 Overlapping Open Reading Frames

The two most important features of RNA viruses are their small genomes and high mutation rates. These two features have been linked by Holmes (2003), arguing that a lack of genomic space means that RNA viruses will be subjected to important evolutionary constraints because specific sequences are required to encode multiple and often conflicting functions.

One frequent attribute of small genomes is gene overlapping, where the same nucleotide sequence simultaneously encodes for two or more proteins in different ORFs as in the case of ASGV. This overlapping gene arrangement is essential to squeeze a maximum amount of information into a limited size genome (Normark *et al.*, 1983; Gibbs and Keese, 1995; Krakauer, 2000) and increase the amount of protein diversity expressed by a single nucleotide sequence (Holmes, 2009). This overlapping can be caused by several mechanisms, including RNA splicing (Pavesi, 2007), the use of non AUG start codons (Baril and Barkier-Gingras, 2005) and overprinting, where the original sequence undergoes a modification causing the elimination of a stop codon, thus printing a novel coding region over the original ORF (Keese and Gibbs, 1992; Belshaw *et al.*, 2007). Recently, Chirico *et al.* (2010) have found that the physical constraint exerted by the viral capsid size on the genome length has led to gene overlap as a mechanism for producing more proteins from the same genome length.

Overlapping ORFs influence a number of other evolutionary aspects aside from increasing the amount of genetic information stored in a limited size genome. First, synonymous nucleotide substitutions in one ORF are likely to be nonsynonymous in the second one, which complexifies evolution analyses (Hein and Støvlbæk 1995). Second, every nucleotide site within the overlap is

expected to have a major impact on the fitness and evolutionary flexibility of the virus and ORF overlaps can be considered as extreme forms of pleiotrophy. This is exemplified by the lower evolution rates observed in overlapping regions compared to non-overlapping regions in Hepatitis B virus (Zhou and Holmes, 2007) and by increased rates of deleterious mutations in these regions (Belshaw *et al.,* 2007). Third, because of the reduced urgency to create protein diversity, viruses with larger genomes tend to show less gene overlap than viruses with smaller genomes (Belshaw *et al.,* 2007).

1.2.2.5 Molecular Diversity

Apple stem grooving virus isolates from several hosts have been fully sequenced (Yoshikawa *et al*., 1992; Ohira *et al.,* 1995; Shim *et al*., 2004; Tatineni *et al*., 2009b; Terauchi *et al*., 1997; Zhao *et al*., 2012). ASGV isolates from citrus and lily (Citrus tatter leaf virus, CTLV) were first considered to be a separate virus but were later included as a strain of ASGV (Yoshikawa *et al.,* 1993; 1996; Ohira *et al.,* 1995; Magome *et al.,* 1997).

No full length sequences are available from Europe and most sequences stem from Asia and the USA. No sequence correlation was found between isolates from the same host/species (Magome *et al*., 1997; Zhao *et al*., 2012). Two areas were found to be highly conserved among ASGV isolates, the MP and the CP with amino acid identities of 92% and 94% respectively (Magome *et al*., 1997; Zhao *et al*., 2012). ASGV is highly heterogeneous and a mixture of sequence variants has been observed in single trees (Yoshikawa *et al*., 1996; Magome *et al*., 1997; LiXin *et al*., 2005). ASGV variants are distributed unevenly within an individual tree; with variants found in different leafs from different branches (Magome *et al*., 1999).

1.2.2.6 Host range and Transmission

Apple stem grooving virus has been detected in a number of important horticultural and ornamental crops, with the most important being apple, Japanese pear, European pear, cherry, citrus, kiwi and lilies. More than 40 species in 17 plant families are susceptible to infection (natural or experimental) by ASGV (*Aizoaceae, Amaranthaceae, Apocynaceae, Caryophyllaceae, Chenopodiaceae, Compositae, Convolvulaceae, Cucurbitaceae, Graminae, Labiatae, Leguminosae, Liliaceae, Pedaliaceae, Rosaceae, Rutaceae, Scrophulariaceae, Solanaceae*) (Lister *et al*., 1965 ; Semancik and Weathers, 1965; Waterworth and Gilmer, 1969; Inouye *et al*.,

1979). Diagnostic host species include *Malus sylvestris*, *M. sieboldii* (Necrosis at the graft union), *Chenopodium quinoa* (necrotic local lesions, chlorotic rings), *Nicotiana glutinosa* (yellow mosaic) and *Phaseolus vulgaris* (chlorotic local lesions) (ICTVdb). There is no known insect vector associated with ASGV transmission. Virus particles occur singly or in aggregates in the cytoplasm of mesophyll and phloem parenchyma cells (Ohki *et al*., 1989) and are transmitted through grafting and mechanical inoculation. Seed transmission has been observed in *Chenopodium quinoa* (Van der Meer, 1976; Inouye *et al*., 1979).

1.2.2.7 Disease and Geographical Distribution

Apple stem grooving virus has been reported from Australia, Bulgaria, China, Egypt, India, Italy, Japan, Korea, Netherlands, New Zeeland, Portugal, Slovenia, South Africa, USA, and probably occurs wherever apple trees are cultivated. ASGV was first reported in the 1960's on *M. sylvestris* cv. Virginia crab from the USA (Lister *et al.*1965; De Sequeira, 1967; Waterworth and Gilmer, 1969) and in Citrus from a Meyer lemon tree in California (Wallace and Drake, 1962). More recently studies where done on the incidence levels of the virus in the pomefruit growing districts of Australia and found to be 69.9% (173 trees tested; Rodoni and Constable, 2006). Youssef *et al*. (2010) found an incidence of 17% (420 trees tested) in 9 different orchards in Egypt. Polak and Zieglerova (2001) and Kundu (2003) found a 50% and 44% (420 trees tested) incidence of ASGV in field grown apple cultivars of Czech Republic, while in Jordan, Syria and Lebanon a lower incidence of 4.6% (1565 tree tested) 2.3% (754 trees tested) and 2.5% (888 trees tested) respectively, were observed (Al Jebr *et al*., 2005; Nassar *et al*., 2012; Salem *et al*., 2005). No information about the incidence of ASGV is available for Germany.

Apple stem grooving virus is latent on most commercial apple varieties and symptoms (fig. 3, necrosis, graft incompatibility, top working disease) are only observed when an infected cultivar is grafted on the sensitive cultivars *M. sieboldii* or *M. Pumila* cv. Virginia crab (Lister, 1970; Yanase, 1974; 1983). In *Citrus reticulata* trees, the infection has economic impacts by reducing the yields of mandarins by up to 25% (Takahara *et al.*, 1988). More recently James (2001) observed that ASGV-free trees appeared slightly more vigorous that those of ASGV infected trees. Similarly, Birişik and Baloğlu (2010) observed a slight decrease in trunk length in apple trees infected with ASGV.

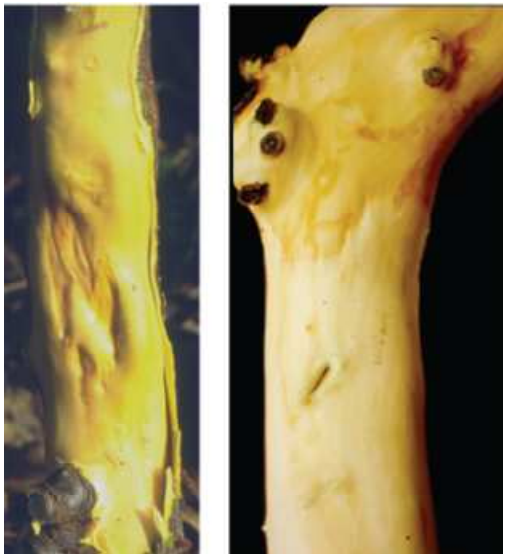

Figure 3: Symptoms caused by *Apple stem grooving virus* and *Apple stem pitting virus*. a) Stem pitting induced on Virginia Crab by *Apple stem grooving virus*; b) mild stem pitting induced on *Malus platicarpa* by *Apple stem pitting virus*. (L Giunchedi, Martelli *et al*., 2007)

1.2.2.8 Detection

Apple stem grooving virus infections could be detected by the inoculation of diagnostic plant species for symptom observation (section 1.2.2.6) or through molecular techniques (ELISA, RT-PCR and RT-qPCR). For ELISA detection, special care has to be taken on the season and part of the plant used, due to low virus titers (Fuchs *et al*., 1988; Kundu *et al*., 2003). Several RT-PCR protocols were developed for the detection of ASGV targeting either the coat protein (Kinard *et al*., 1996; James, 1998; Kirby *et al*., 2001; Kummert *et al*., 2001; Menzel *et al*., 2002; 2003) or the RNA polymerase (Marinho *et al*., 1998; Foissac *et al*., 2005). James (1998) Kirby *et al*. (2001), Kundu *et al*. (2003), Menzel *et al*. (2003) and Hassan *et al*. (2006) found RT-PCR techniques to be more reliable assays for ASGV detection than wood indexing and/or ELISA. Some RT-PCR protocols were adapted to incorporate an immune-capture-step (IC-RT-PCR; Crossley *et al*., 1998; James, 1999; Kirby *et al*., 2001) or followed by an immunoenzymatic step (RT-PCR-ELISA; Kummert *et al*., 2001; Menzel *et al*., 2003). Multiplex RT-PCR methods for the simultaneous detection of ASGV and other apple viruses were also developed (James, 1999; Ito *et al*., 2002; Menzel *et al*., 2003; Hassan *et al*., 2006 Massart *et al*., 2009). Massart *et al*. (2009) did an interlaboratory comparison of detection primers used in RT-PCR for the detection of ASGV, ASPV, ACLSV and *Apple mosaic virus* (ApMV) and found the primers to be between 94-96% sensitive for the detection of ACLSV, ASGV and ApMV, while the sensitivity was 68%

for ASPV. LiXin *et al*. (2006) reported the use a Taq-man probe based real-time fluorescent RT-PCR for ASGV detection and Gadiou and Kundu (2012) developed a SYBR Green based one step RT-qPCR assay for the detection of ASGV and found GAPDH and the ribosomal protein S19 to be the most reliable reference genes. Simplifications of the sample preparation were proposed by several authors using diluted crude extracts (Marinho *et al*., 1998; Kummert *et al*., 2001) or simpler RNA extraction procedures (James, 1999; Kundu, 2003; MacKenzie *et al*., 1997).

1.2.3 Apple stem pitting Foveavirus

1.2.3.1 Morphology

The ASPV particles are non enveloped flexuous filaments with no distinguishable helix. The particles are 800 nm in length and 12-15 nm in diameter and readily form end to end aggregates (Koganezawa and Yanase, 1990).

1.2.3.2 Genome and Genomic Organization

ASPV has a 9.3Kb (excluding the poly A tail) single stranded positive sense RNA genome consisting of 5 ORF's. ORF1 encodes for a 247kDa protein containing several domains (Methyltransferase, papain-like protease, NTB-binding helicase and RNA-dependent RNA polymerase (RdRp)), ORF2 encodes for a 25kDa Triple gene block (TGB) protein 1, ORF3 for a 13kDa TGB protein 2, ORF4 for a 7kDa TGB protein 3 and ORF5 encodes for a 42-44kDa coat protein (fig. 4; Jelkmann, 1994).

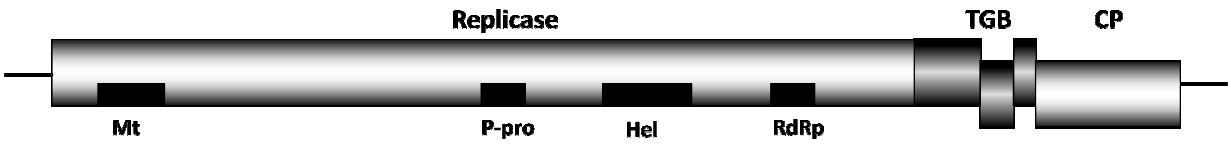

Figure 4: Genomic organization of *Apple stem pitting virus*. Methyltransferase (Mt), papain-like protease (P-pro), NTB-binding helicase (Hel), RNA-dependent RNA polymerase (RdRp), Triple Gene Block (TGB) and Coat protein (CP).

The 25kDa protein has ATPase, RNA-binding and RNA-helicase activities and is believed to increase the size exclusion limit of the plasmodesmata (Morozov and Solovyev, 2003; Martelli *et al*., 2007). Although the exact mechanism by which movement occurs is unknown, it is known

that the TGB proteins are involved in intracellular transport and membrane binding functions (Martelli *et al*., 2007; Kundu and Yoshikawa, 2008).

1.2.3.3 Molecular Diversity

Six ASPV isolates from pear and apple have been fully sequenced from Germany, China, Japan and India. (Jelkmann, 1994; Liu *et al.*, 2011; NCBI GenBank). Nucleotide sequence analysis shows identities of between 76-92%. Several studies have been done on the molecular diversity of the CP gene. Gadiou *et al*. (2010) found nucleotide sequence identities of between 80.1-81.9% (802bp N-terminus CP fragment) and Liu *et al*. (2011) 68-73% amino acid sequence identity of the full length CP. No geographic correlation between isolates was found by both research groups. Wu *et al*. (2010) divided the ASPV isolates in three groups according to CP length, and found that the three available sequences from Germany have an additional 18 aa at the 3'region of the CP. Genetic variability studies were also done on ORF1, Rodoni and Constable (2008) found high variability in the 3' region of ORF1, while Kundu (2006) found an area of high variability between the methyltransferase and the papain-like protease domains, the latter finding is consistent with findings from German-Retana *et al*. (1997) done on ACLSV. Yoshikawa *et al*. (2001) found genome heterogeneity from single tree isolates. This diverse co-existing population in a single host is caused by the lack of proofreading activity associated with RNA-dependent RNA polymerase.

1.2.3.4 Host Range and Transmission

Apple stem pitting virus is transmitted by mechanical inoculation and grafting, no insect vector has been identified to transmit the virus (Yanase *et al*., 1989). The natural host range of ASPV is restricted to pomefruits including apple, pear, quince, hawthorn and rowan (Martelli and Jelkmann, 1998; Sutic *et al*, 1999; Kundu and Yoshikawa, 2008).The following plant species from 7 plant families are susceptible to ASPV under experimental conditions: *Celosia cristata*, *Chenopodium murale*, *Chenopodium quinoa*, *Crataegus*, *Cucumis sativus*, *Gomphrena globosa*, *Malus platycarpa*, *M. sieboldii*, *M. sieboldii* var. *arborescens*, *M. sylvestris*, *Nicotiana occidentalis* (cv. 37B), *Nicotiana occidentalis* ssp. *obliqua*, *Nicotiana glutinosa*, *Nicotiana rustica*, *Physalis floridana*, *Pyronia veitchii*, *Pyrus communis*, *Sesamum indicum*, *Sorbus mitchelli*, *Tetragonia tetragonioides Celosia argentea*, *Ocimum basilicum* and *Lactuca sativa* (Yanase, 1974; Stouffer, 1989; Koganezawa and Yanase, 1990; Zhao *et al*., 2012). Diagnostic

host species include *Nicotiana occidentalis* 37B (vein yellowing and leaf necrosis), *N. occidentalis* ssp. *obliqua* (necrotic local lesions), *M. sylvestris* cv. Virginia Crab (stem pitting), *M. sieboldii* MO65 (dieback, inner bark necrosis and necrotic leaf spot) and *Pyrus communis* cv. Nouveau Poiteau (necrotic leaf spot) (Stouffer, 1989; Koganezawa and Yanase, 1990).

1.2.3.5 Disease and Geographical Distribution

Since 1954 when ASGV was first reported in *M. sylvesteris* in the USA, the virus has been detected all over the world, including Australia, Western and Eastern Europe, Asia, and South Africa. Recently studies on the incidence of the virus shows high infection rates in apple and pear orchards. In Greece 91.8% infected apple and 51% infected pear trees were found in 19 fruit growing regions (Mathioudakis *et al*., 2010); in Australia an incidence of 87.9% were found of the 173 trees tested (Rodoni and Constable, 2008); In Czech Republic 27.8-48.75% of the 15 apple orchards tested positive for ASPV (Kundu, 2002; 2003); between 33% and 36,7% of apple and cherry trees are infected with ASPV in India (Dhir *et al*., 2010; Dhir *et al*., 2011); in Lebanon an incidence of 13% was recorded on 888 apple and pear trees tested (Nassar *et al*., 2012); and Youssef *et al*. (2010) found an incidence of 13% in 420 samples collected from 9 orchards tested in Egypt. Mixed infections of ASPV and ASGV are common on apple and pear (Kundu, 2002; Gadiou *et al*., 2010). Recent studies recorded an incidence of mixed infection of 36.84% in Turkey, 27.5% in Czech Republic and 4% in Egypt (Kundu, 2002; Caglayan *et al*., 2006; Youssef *et al*., 2010).

Although ASPV as with ASGV is considered to be latent on most commercial apple varieties several cases have been reported of symptoms on apple associated with ASPV infections like green crinkle and star crack symptoms (Desvignes *et al.,* 1999), stem pitting symptoms of Virginia crab, epinasty and decline of Spy 277 (Stouffer, 1989) and top working disease when an infected rootstock is grafted on the susceptible rootstocks *M sieboldii* and *M. prunifolia* (Jelkmann, 1997). Symptoms on pear indicator cultivars include vein yellows, decline and stem pitting (Leone *et al.*, 1998).

1.2.3.6 Detection

Apple stem pitting virus infections could be detected by the inoculation of diagnostic plant species for symptom observation (section 1.2.3.4) or through molecular techniques (ELISA, RT-

PCR and RT-qPCR). Problems with virus purification made the production of reliable antiserum for immunological detection difficult (Jelkmann, 1994; Yoshikawa *et al.*, 2001). Komorowska and Malinowski (2009) produced polyclonal antiserum through the expression of the CP of 5 different ASPV isolates in E. coli, although the antiserum was successful in detecting the virus in IC-RT-PCR, it was not satisfactory in ELISA. A sensitive and reliable RT-PCR method has been developed for the detection of ASPV in infected plants (Jelkmann and Keim-Konrad, 1997; Kummert *et al*., 1998; Malinowski *et al*., 1998; Nemchinov *et al*., 1998; Schwarz and Jelkmann, 1998; Kundu, 2002). Methods combining immunocapture with RT-PCR were also developed. A highly sensitive immunocapture IC-RT-PCR was tested on a selection of ASPV isolates from apple and pear from different countries in Europe (Schwarz and Jelkmann, 1998) while Menzel *et al*. (2003) developed a multiplex RT-PCR ELISA detection method for the detection of ASGV, ASPV, ACLSV and ApMV. Salmon *et al*. (2002) successfully developed a Real-Time PCR with a fluorogenic hydrolysis probe for the detection of ASPV.

1.2.4 '*Candidatus* Phytoplasma mali'

1.2.4.1 Apple Proliferation Disease Distribution and Incidence

Apple proliferation disease was first described by Rui *et al*. (1950) in Italy and is caused by '*Ca. P*. mali' (Bonnet *et al*., 1990; Seemüller and Schneider, 2004). Apple proliferation disease / '*Ca. P*. mali' is mostly restricted to Europe and has been reported from Albania (Myrta *et al*., 2003), Austria (Németh, 1986; Danet *et al.*, 2011), Belgium (Németh, 1986; Olivier *et al*., 2010), Bulgaria (Németh, 1986; Etropolska and Laginova, 2012), Czech Republic (Bertaccini *et al*., 1997; Fialova *et al*., 2003; Franova *et al*., 2011), France (Jarausch *et al*., 1994a; Danet *et al*., 2011), Germany (Lorenz *et al*., 1995; Seemüller *et al*., 1998a; Danet *et al*., 2011), Greece (Németh, 1986; Rumbou *et al*., 2007; 2010; 2011), Hungary (Del Serrone *et al*., 1998; Paltrinieri *et al*., 2010), Italy (Firrao *et al*., 1993; Osler *et al*., 2001; Danet *et al*., 2011), Norway (Németh, 1986; Blystad *et al*., 2011), Poland (Cieslinska and Morgas, 2011), Romania (Németh,1986; Danet *et al*., 2011), Slovenia (Osler *et al*., 2001; Mehle *et al*., 2007; 2011), Serbia (Duduk *et al*., 2008; Paltrinieri *et al*., 2010) and Spain (Avinent and Llácer, 1995; Lavina *et al*., 2011). Recently, it has also been reported from Turkey (Canik and Ertunc, 2007; Sertkaya *et al*., 2008) and Tunisia (Ben Khalifa and Fakhfakh, 2011). In Finland '*Ca*. P. mali' was detected in the insect vector *Cacopsylla picta* but no report is available on the incidence in apple (Lemmetty *et al*., 2011).

Northern Italy (6.4% in 2003) and Southern Germany (up to 40% incidence) are most affected by the disease (Bliefernicht and Krczal, 1995; Springhetti *et al*., 2002; Dolzani and Branz, 2005). From the 10.3 million apple trees in Trentino, northern Italy, around 800 000 were infected with AP in 2003. In 2003 approximately 500 hectares of infected apple trees and in 2006 more than half a million infected trees had to be uprooted to control the disease (Dolzani and Branz, 2005; Baric *et al*., 2010a; 2012). High incidence was also recorded in Greece in the Pelion Mountain region where of up to 90% of apple trees were infected and yield losses of between 20-100% were recorded (Rumbou *et al*., 2011).

1.2.4.2 Disease and Symptomology

The disease causes a wide range of symptoms depending on the stage of the disease, '*Ca.* P. mali' strain, season and resistance of the host to the disease (Seemüller *et al*., 1984; Seemüller and Schneider, 2007). Trees may show symptoms every year, or they may recover for some years and later again become affected (Carraro *et al*., 2004; Seemüller and Harris, 2010). Earliest symptoms include early appearance of leafs on infected trees, unusual growth of terminal buds in autumn and late production of flowers in summer and autumn (Seemüller *et al*., 1984; Kartte and Seemüller, 1988). More unique symptoms include witches broom (the production of secondary shoots due to the lack apical dominance in affected shoots), and enlarged stipules on basal leaves (fig. 5; Kartte and Seemüller, 1988). Symptoms of economic value include a lack of vigor, undersized fruits, poorly flavored fruit and yield decreases (Kunze, 1989; Rumbou *et al*., 2007; 2011).

Phytoplasmas reside and multiply in the phloem sieve tubes, to which they were introduced by phloem-feeding insects. From there they spread systematically throughout the plant by passing through the phloem sieve plate spores (Weintraub and Beanland, 2006). Studies on infected plants have shown an inhibition of phloem transport which in turn leads to the accumulation of carbohydrates in mature leaves and a reduction in young leaves and roots (Catlin *et al*., 1975; Braun and Sinclair, 1978; Kartte and Seemüller, 1991a; Lepka *et al*., 1999; Guthrie *et al*., 2001; Maust *et al*., 2003). The phloem dysfunction could also lead to plant hormone imbalances and altered secondary metabolisms. The exact mechanism involved in symptom expression is still poorly understood (McCoy, 1979; Leon *et al.,* 1996; Lepka *et al.,* 1999; Tan and Whitlow, 2001; Maust *et al.,* 2003; Choi *et al.,* 2004).

Figure 5: Symptoms caused by *'Ca.* P. mali' on *M. domestica* a) and b) Typical witches broom symptoms (W. Jarausch; c) Undersized fruits (A. Fried); d) Enlarged stipules on basal leaves (U. Harzer). (http://www.apfeltriebsucht.info/symptome.html)

Because phytoplasmas live in the functional sieve tubes, they are almost completely eliminated from the canopy in winter and migrate to the roots. In early spring when the new phloem is formed, the phytoplasmas recolonize the aerial parts of the tree. In later years the phytoplasmas recolonize partially or not at all and no or mild symptom expression could be seen. The roots however stay colonized throughout the life of the tree. Recently, several potential virulence factors that could affect the pathogenicity have been identified (Hogenhout *et al*., 2008; Marcone, 2010). One such factor is the presence of the hflB gene that encodes for an ATP-dependent membrane-associated Zn^{2+} protease associated with protein degradation. It acts against membrane proteins like SecY (Ito and Akiyama, 2005) and is thus directly involved in membrane transport (Bonas, 1994). In most bacteria, the *hfl*B gene is present as a single copy whereas in phytoplasmas up to 24 copies are present (Arashida *et al.*, 2008; Bai *et al.*, 2006). In '*Ca.* P. mali' strain AT twelve ORFs assigned to as *hfl*B are present (Schneider and Seemüller, 2009).

1.2.4.3 Genome and Genomic Organization

'Ca. P. mali' is from the class *Mollicutes*, family *Acholeplasmataceae* and Genus '*Candidatus* Phytoplasma. The genus could then be further divided into groups and sub-groups by RFLP analysis of the 16S rRNA genes. *'Ca.* P. mali' falls in the group 16SrX (subgroup A) together with '*Ca.* P. pyri' (Pear decline, Subgroup C) and '*Ca.* P. prunorum' (European stone fruit yellows, Subgroup F) (Lee *et al*., 1998; Seemüller *et al*., 1998b; Martini *et al*., 2007; Wei *et al*., 2008).

Table 1: General features of the four fully sequenced '*Candidatus* Phytoplasma' chromosomes

Characteristic	**'*Ca.* P. mali' (AT)**	**'*Ca.* P. australiense'**	**'*Ca.* P. asteris' (Asteris yellows witches broom)**	**'*Ca.* P. asteris' (Onion Yellows)**
Chromosome size (Kb)	601 943	879 324	706 569	860 631
Chromosome organization	Linear	Circular	Circular	Circular
GC content	21.4	27	26.9	27.7
Protein coding regions%	78.9	74	73.7	73
Coding sequences	536	839	708	793
Genes encoding proteins	497	684	671	754
Average ORF size	955	778	779	785
Genes with assigned functions	338 (68%)	414 (60.5%)	450 (67%)	446 (59%)
No of rRNA operons	2	2	2	2
tRNA genes	32	35	31	32
Extrachromosomal DNA's	0	1	4	2
Multicopy genes	89	191	202	250

(Kube *et al*., 2008; Tran-Nguyen *et al*., 2008; Hogenhout and Music, 2010)

'*Ca.* P mali' isolate AT has been fully sequenced (Kube *et al*., 2008). Only three other genome sequences from the same genus are currently available; '*Ca.* P australiense (subgroup 16SrXII-B) (Tran-Nguyen *et al*., 2008) and two strains of '*Ca.* P asteris' (group 16SrI) (table 1; Bai *et al*., 2006; Oshima *et al*., 2004). The sequence analysis of the AT strain revealed a linear chromosome organization of 601 943 kb, making it one of the smallest bacterial genomes identified and the smallest '*Candidatus* Phytoplasma' genome recorded, with two rRNA operons, 32 tRNA genes and 497 predicted ORF's. Sixty eight percent of the protein-coding sequences have been assigned

to functions. Only 89 multi-copy genes were identified, much less than in '*Ca.* P. asteris where >250 were identified (Kube *et al*., 2008).

1.2.4.4 Molecular Diversity

Considerable variability in virulence and genomic traits for strains of '*Ca.* P. mali' has been reported by Seemüller & Schneider (2007) and Schneider and Seemüller (2009). Based on symptomology, the phytoplasma strains were defined as being avirulent to mildly, moderately, or highly virulent. Initially, '*Ca.* P. mali' strains were grouped into 3 closely related subtypes, AT-1, AT-2 and AP using PCR-RFLP detection of a nonribosomal protein (Jarausch *et al*., 2000a) in samples collected from France, Germany, Spain, Switzerland, Austria, Romania and Italy. No correlation was found between the subtypes and geographical origin. In southwest Germany, the AP strain was found to be most prevalent (75% of samples tested) (Jarausch *et al*., 2004). Strain discrimination of '*Ca.* P. mali' based on single stranded conformation polymorphism (SSCP) of the *hf*lB gene was performed by Schneider and Seemüller (2009) and they found 20 different profiles of the 44 samples tested (samples collected from Germany and Italy). The higher resolution allowed for the identification multiple distinct strains in single apple trees and revealed possible interaction among them and indicated that it could affect virulence by strain interference (Seemüller *et al*., 2010).

Seemüller and Schneider (2007) determined the concentration of '*Ca.* P. mali' in avirulent to mildly, moderately, and highly virulent or severe categories by quantitative PCR (qPCR) and found no statistical difference in the concentration indicating that the severity of the disease is not linked to the concentration of '*Ca.* P. mali' but to the virulence of the strain. Bisognin *et al*. (2008a) found a significant difference in the concentration of two '*Ca.* P. mali' strains PM4 (AP subtype) and PM6 (AT-2 subtype) on *in vitro* cultivated apple.

1.2.4.5 Vectors and Transmission

The psyllids *Cacopsylla picta* and *Cacopsylla melanoneura* have been identified as the main vectors and means of transmission of '*Ca.* P. mali' (fig. 6; Tedeschi *et al*., 2002; 2003; Jarausch *et al*., 2003). *C. picta* and *C. melanoneura* is geographically limited to Europe and the Palaearctic region, respectively (Jarausch and Jarausch, 2010). Previous studies showed that both psyllid species were present in most studied regions, but their significance as vectors depended on

region, overwintering, natural transmission, host and host prevalence. (Carraro *et al*., 2001; Jarausch *et al*., 2003; Malagnini *et al*., 2010; Baric *et al*., 2011; Lemmetty *et al*., 2011). A higher incidence of *C. melanoneura* to *C. picta* was found in orchards, but a higher percentage of *C. picta* was infected with '*Ca*. P. mali', compared to *C. melanoneura* (Jarausch *et al*., 2007; Baric *et al*., 2010b; Lemmetty *et al*., 2011).

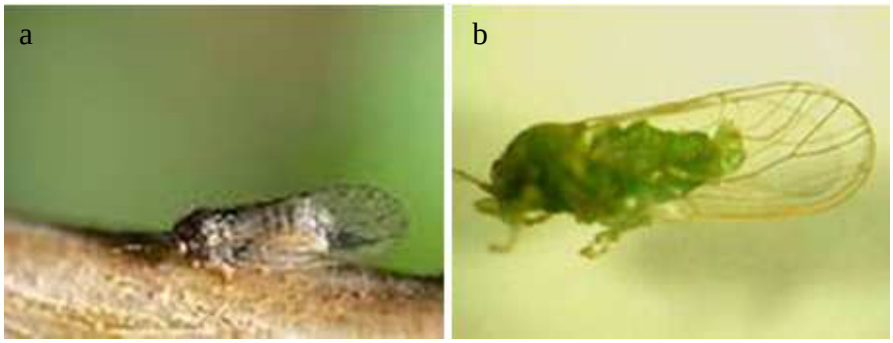

Figure 6: Psyllid vectors of '*Ca.* P. mali'. a) Young *Cacopsylla picta*; b) Overwintered *Cacopsylla melanoneura.*

C. picta is monophagous on *Malus* spp. and completes one generation per year. Adults overwinter on conifers and at the end of the winter migrate to apple trees for oviposition. The new insects feed on the apple trees until beginning of July when they leave the apple trees as adults for the overwintering period on coniferous plants (Mattedi *et al*., 2008; Tedeschi *et al*., 2009; Jarausch and Jarausch, 2010). *Cacopsylla melanoneura* is oligophagous on Rosaceae such as *Crataegus*, *Malus* and *Pyrus* spp. The life cycle is similar to that of *C. picta* but the overwintering adults appear earlier in the year and the new generation abandons the host plant earlier than *C. picta* to migrate to the overwintering plants (Mattedi *et al.*, 2008; Jarausch and Jarausch, 2010).

The ability of the leafhopper *Fieberiella florii* (Stal) to transmit '*Ca.* P. mali' was recently confirmed by Tedeschi and Alma (2006). Although it is not considered to be a major vector, its polyphagous feeding habit could further extend the host range of '*Ca.* P. mali' (Tedeschi and Alma, 2006). Transmission through grafting (Jarausch *et al*., 1999), root fusions or root grafts in medium to old aged orchards (Bliefernicht and Krczal, 1995; Baric *et al*., 2008; Ciccotti *et al*., 2008a), as well as through dodder (*Cuscuta europea*) bridges have been reported (Marwitz *et al*., 1974; Heintz, 1986).

Table 2: **List of reported plant hosts of '*Ca.* P. mali'**

Latin Name	Common Name	Reference
M. domestica	Apple	Rui *et al*., 1950; Kartte and Seemüller, 1991b; Kube *et al*., 2008; See also 1.2.4.1
M. prunifolia	Plumleaf crabapple	Seemüller and Schneider, 2004
M. pumila	Crabapple	Jarausch *et al*., 1999
Pyrus communis	Pear	Del Serrone *et al*., 1998
Catharanthus roseus*	Madagascar Periwinkle	Schneider and Seemüller, 2009
Convolvulus arvensis	Bindweed	Schneider *et al*., 1997
Corylus avellena	Hazel	Marcone *et al*., 1996
Prunus domestica	Plum	Ben Khalifa and Fakhfakh, 2011
Prunus salicina	Japanese plum	Lee *et al*., 1995
Prunus persica	Nectarine/Peach	Paltrinieri, 2001; Cieslinska and Morgas, 2011
Ribes rubrum	Redcurrant	Navratil *et al*., 2007
Vitis vinifera	Grapevine	Matus *et al*., 2008
Crataegus monogyna	Hawthorn	Tedeschi *et al*., 2009
Dahlia* x *cultorum	Dahlia	Kaminska and Silwa, 2008a
***Lilium* spp. L**	Lily	Kaminska and Silwa, 2008b
Prunus avium	Cherry	Mehle *et al*., 2007
Prunus armeniaca	Apricot	Mehle *et al*., 2007
Prunus domestica	Plum	Mehle *et al*., 2007
Nicotiana occidentalis*	Native tobacco	Berg *et al*., 1999; Seemüller and Schneider, 2007; Kube *et al*., 2008;
Nicotiana tabacum*	Cultivated tobacco	Berg *et al*., 1999; Seemüller and Schneider, 2007; Kube *et al*., 2008

*****Infected via European dodder (*Cuscuta europaea*) bridges.

1.2.4.6 Host Plants

'*Ca.* P. mali' has been reported from several hosts throughout Europe (table 2) with the major host being *M. domestica.* Kartte and Seemüller (1991b) successfully graft inoculated Apple Proliferation agent (mycoplasma like organism, MLO) on 58 ornamental and wild *Malus* species and subspecies including, *M. baccata, M. coronaria, M. domestica, M. floribunda, M. fusca, M. gloriosa, M. ionensis, M. purpurea, M. pumila, M. prunifolia, M. robusta, M. sargentii, M. sieboldii* and *M. silvesteris,* as well as on 40 *Malus* hybrids.

1.2.4.7 Detection

Before the application of molecular techniques, detection of phytoplasmas in diseased plants was difficult and relied on the observation of symptoms. Phytoplasma strains were initially differentiated and identified by their biological properties, such as the similarity of the symptoms they caused in infected plants, plant hosts, and insect vector ranges, and were often inconclusive (Bertaccini and Duduk, 2009). Recent methods for strain differentiation are described in section 1.2.4.1. Serological diagnostic techniques for the detection of phytoplasma began to emerge in the 1980s with ELISA based methods. Antisera were produced to detect '*Ca.* P. mali' and are currently commercially available and use for successful detection of '*Ca.* P. mali' (Chen *et al*., 1989; Brzin *et al*, 2003; Canik and Ertunc, 2007). Antibodies to partial sequences of immunodominant membrane proteins of '*Ca.* P. mali' have been prepared (Berg *et al.,* 1999) and used successfully in ELISA.

In the 1990s, PCR coupled with RFLP analysis allowed accurate identification of different strains and species of phytoplasma. Due to the close genetic relatedness of the apple proliferation group of phytoplasmas, specific identification often requires the digestion of the amplicons with various endonucleases and subsequent RFLP analysis (Deng and Hiruki, 1991; Ahrens and Seemüller, 1992; Lee *et al*., 1995; Schneider *et al*., 1995; Smart *et al*., 1996; Gundersen and Lee, 1996; Kison *et al*., 1997; Gibb *et al*., 1999; Jarausch *et al*., 2000a; Heinrich *et al*., 2001). Firrao *et al*. (1994) developed a 'rapid' PCR protocol for the apple proliferation organism without the need for a restriction digestion or hybridization step. Rajan and Clark (1995) used polyclonal antibodies to capture '*Ca.* P. mali' and then amplified it with universal PCR primers (IC-PCR).

In recent years several Real-Time PCR based detection methods have been devolved due to its high sensitivity (no need for restriction digests) and the direct reading of its results. Jarausch *et al*. (2004) and Galetto *et al*. (2005) developed a quantitative real-time PCR for '*Ca.* P. mali' in plants and insects from a nitroreductase gene sequence. Torres *et al*. (2005) developed a real-time PCR that will detect '*Ca.* P. mali', '*Ca.* P. prunorum', and '*Ca.* P. pyri'. Baric and Dalla-Via (2004) developed a real-time PCR for the detection of '*Ca.* P. mali' in apple plant material and included host gene from apple as an internal PCR control. Baric *et al*. (2006) compared the Baric and Dalla Via (2004) Real-Time PCR method with four conventional PCR assays and found the

Real time PCR method to have highest sensitivity and specificity and to be not susceptible to PCR inhibition.

1.2.4.8 Control of Apple Proliferation Disease

There are different strategies to help control the spread of the disease. Using '*Ca.* P. mali' free propagation material and the removal of infected plants could help reduce the spread of the pathogen. The biggest problem is re-infection through the insect vector (Weintraub and Wilson, 2010). Although the use of insecticides might help controlling vector populations and reduce intra crop transmission, conventional insecticides are inefficient because the pathogen transmission occurs faster than the insecticide can act and there is a constant influx of new vectors from surrounding habitats (Wally *et al*., 2004). The overwintering of the psyllids on conifers is also problematic especially when the oviposition coincides with the blossom period of apples, where insecticides cannot be used (Jarausch and Jarausch, 2010). Another problem is the formation of root bridges or root fusions where the phytoplasma could be transmitted without the help of a vector from infected to healthy trees (Bliefernicht and Krczal, 1995; Baric *et al*., 2008; Ciccotti *et al*., 2008a). For these reasons the most promising approach to control the disease seems to be the use of resistant plant material.

'*Ca.* P. mali' relies on intact sieve tubes for movement through the infected tree and is usually eliminated in the stem during winter due to the degeneration of the phloem. The '*Ca.* P. mali' overwinters in the roots of the infected trees where the sieve tubes are intact throughout the year (Schaper and Seemüller, 1984; Seemüller *et al*., 1984; Carraro *et al*., 2004). It is known that severity of symptom expression is related to the presence/absence of the phytoplasma in the stem and aerial part of the plant (Seemüller *et al*., 1984). This led to the conclusion that the grafting of susceptible commercial apple cultivars on apple proliferation resistant rootstocks should be efficient in the control of the pathogen, by stopping/reducing the recolonization of the pathogen to the stem of the plant (Kartte and Seemüller, 1991b; Seemüller *et al*., 2008).

1.2.4.9 Apple Proliferation Resistant Rootstocks

Studies done on established and commercial rootstocks based on *M. domestica* showed no satisfactory resistance to Apple Proliferation. Natural resistance was discovered in wild apomictic *M. sieboldii* (Kartte and Seemüller, 1988; Seemüller *et al*., 1992; Bisognin *et al*., 2008b). Crosses

of *M. sieboldii* with *M. domestica* were carried out in the 1950's and 1960's to obtain aptomictic rootstocks for easy propagation of seeds, better anchorage and higher resistance to fungal and bacterial diseases, but turned out to be too vigorous for modern apple culture. (Schmidt, 1964; 1988). Studies on these hybrids showed that some genotypes showed resistance to Apple proliferation disease and remained symptomless or recovered easily, and had no or very low phytoplasma titers (Kartte and Seemüller, 1991b; Seemüller *et al*., 1992; Bisognin *et al*., 2008b; Seemüller *et al*., 2008). Based on these studies a breading program started in 2001 by three institutes: Instituto Agrario di San Michele in Trentino, Italy, RLP Agroscience GmBH, AlPlanta, Institute for Plant Research, Neustadt an der Weinstrasse, Germany and the Institute for Plant Protection in Fruit Crops, Biologische Bundesanstalt, Dossenheim, Germany, with the aim to produce Apple Proliferation resistant rootstocks with an agronomic/commercial value.

M. sieboldii (4n), two *M. sieboldii* F1 hybrids (3n, genotypes 4551 and 4608) and six F2 hybrids that were obtained by backcrossing of F1 hybrids with M 9 (4n, genotypes H0901, H0909) or by open pollination of F1 hybrids (4n, genotypes C1907, D2118, D2212, H0801) were used as seed parents (Seemüller *et al*., 2008; Seemüller and Harris, 2010). These crosses were obtained by M9 pollination, but in a few cases with the pollination of other *M. domestica* genotypes (Seemüller *et al*., 2008; Bisognin *et al*., 2009). Graft inoculated offspring were assessed for 2 years in nurseries where the cumulative disease indices (CDI) were compared to the average CDI of all progenies. Offspring with a low CDI were than transplanted for further analysis under commercial growing conditions. Two thirds of the offspring off the crosses 4608 x M9 and D2212 x M9 showed CDI values of 1.1 and 1.3 respectively showing mild or no symptoms 1 year post inoculation, much lower than the average 4.1. These plants performed well under commercial conditions as well. The number of resistant offspring from other crosses ranged from 8-42% (Seemüller *et al*., 2008; Seemüller and Harris, 2010). Crosses/ hybrids were considered resistant when low CDI values, a high percentage of non- or little-affected trees, low incidence of the small fruit symptom, and no or little effect on vigor were recorded (Seemüller *et al*., 2008).

The process of breeding Apple Proliferation resistant rootstocks was complicated by the polyploidy of apomictic parental, making the selection process of interesting traits more difficult. Bisognin *et al*., 2009 used simple sequence repeat (SSR) analysis to determine mode of reproduction (sexually derived or apomictic), genomic constitution and ploidy level. The

offspring were divided into three groups: mother-like seedlings, which displayed the same set of alleles as the female or seed plant, Hybrid I, which displayed the whole marker profile of the apomictic seed parent with an additional derived allele at each locus from the non-apomictic paternal plant and Hybrid II or true hybrid, which displayed half of the specific alleles of both the parents. Fifty five percent of the analyzed seedlings were mother-like and showed the same level of ploidy as the female parent compared to 24% and 13% for Hybrids I and II (Bisognin *et al*., 2009). The low number of true or full recombinant offspring hinders the selection process for genotypes showing the desired properties. At present there is no data on the genetic base of resistance and no molecular markers for assisted selection are available.

A lower phytoplasma concentration in *M. sieboldii* and *M. sieboldii* hybrids were also recorded, and could be an indication of resistance (Seemüller *et al*., 1984). Bisognin *et al*. (2008b) used quantitative real time PCR to determine the phytoplasma titer in susceptible and resistant apple plants. Susceptible *M. domestica* rootstocks M9 and M11 showed a titer of 1 x 10^9 cells/g root phloem compared to 2 x 10^5- 3x 10^6 cells/g root phloem for apomictic resistant rootstocks.

No difference in resistance was found between hybrid I and mother-like plants, possibly because both groups contain the full set of alleles from the female plant accept for the 4068 offspring where the Hybrid I offspring were less affected compared to the mother-like offspring (Bisognin *et al*., 2008b; 2009). However, the resistance in the Hybrid II group was significantly lower (Bisognin *et al*., 2008b; 2009). Great differences in resistance to Apple Proliferation among and between progenies were observed. Progenies of *M. sieboldii* and the *M. sieboldii* hybrids D2212, 4608 and 4551 showed high levels of resistance, whereas *M. sargentii* (D1111 and C1828) and *M. domestica* (M9 and M11) derived hybrids showed higher susceptibility (Bisognin *et al*., 2008b). The '*Ca.* P. mali' titer in the canopy or aerial parts of trees grafted on resistant rootstocks was usually lower than in grafted susceptible rootstocks. The '*Ca.* P. mali' titer was found to be 100-5000 times lower in *M. sieboldii* and *M. sargentii* hybrids compared to *M. domestica* hybrids (Bisognin *et al*., 2008b). This indicates that phytoplasma titer alone could not be used as resistance screening tool and symptomology should be included in the screening process. The screening procedure could also affect the level of resistance. Seemüller *et al*. (2008) found smaller differences in resistance between susceptible and resistant plants when naturally infected

compared to experimental inoculation. These plants developed symptoms much later than those being graft inoculated and could be due to the different starting phytoplasma titer.

Bisognin *et al*. (2008a) also found significant differences in phytoplasma titer when the rootstocks were graft inoculated with different '*Ca.* P. mali' strains in the *in vitro* grafting trials. Higher titer values were recorded for the '*Ca.* P. mali' strain PM4 (43 987 ± 2747 copies/ng DNA) compared to the '*Ca.* P. mali' strain PM6 (23 297 ± 2123 copies/ng DNA). Differences in strain multiplication were also observed dependent on the genotype inoculated, where PM4 multiplied to a significantly higher concentration in D2212 and Golden Delicious (*M. domestica* cultivar) whereas PM6 multiplied to a higher concentration in H0909. Differences in concentration were also observed in the time post inoculation. For both strains the highest concentration was observed 6-8 months post inoculation. The PM4 strain was considered to be more pathogenic because of the higher titer values in inoculated plants, the difficulty to transmit and the negative effect on the cellular connection of the phloem tissue at the graft union (Bisognin *et al*., 2008a).

1.2.5 Sensitivity of *M. sieboldii* and *M. sieboldii* Derived Rootstocks to Latent Apple Viruses

Seemüller *et al*., 2008 observed poor development and death of some *M. sieboldii,* M. sargentii and hybrids thereof, during the nursery and field trials for resistance screening (fig.7). Most affected were the genotypes 4551, 20186, C1828 and C0725, whereas D2212 showed a lower percentage of death and stunting (table 3). 4608 seedlings were the only apomictic offspring that were not affected, similar to the *M. domestica* rootstocks M9 and M11. The presence of three latent apple viruses was observed in the affected trees, ASPV, ASGV and ACLSV. The negative effect of these latent viruses was previously observed by Schmidt (1972; 1988).

Ciccotti *et al*. (2011) studied the effect of these three latent viruses on *ex vitro M. sieboldii* hybrids. The apple plants were inoculated by chip budding with ASPV, ASGV and ACLSV, respectively, as well as cumulatively to analyze the effect of super-infection. More than 50% of the single infections failed, and hailed no usable information on which of these latent viruses are responsible for the symptom expression. The multiple virus infections on the *M. sieboldii* derived

hybrids caused a series of symptoms ranging from symptomless to severe. In contrast to the study done by Seemüller *et al*. (2008), 4551 showed slightly too moderate symptoms and D2212 showed no symptoms to the multiple virus infection (Ciccotti *et al*., 2011).

Figure 7: Poor development and death of some *M. sieboldii*, *M. sargentii* and hybrids thereof, during field trials for resistance screening (A. Fried and M. Petruschke)

1.2.6 *In vitro* Grafting

In vitro grafting or micro-grafting was initially established to obtain pathogen free material (Murashige *et al*., 1972; Alskieff and Villermur, 1978) but in 1999, Jarausch *et al*. (1999) recognized the value of the technique and developed an *in vitro* grafting system to transmit the unculturable '*Ca.* P. mali' to healthy rootstocks. '*Ca.* P. mali' was successfully transmitted to different *Malus* genotypes (Jarausch *et al*., 1999; 2000b; Bisognin *et al*., 2007; 2008a). This opened new doors for resistance screening and analysis of phytoplasma-host interactions.

The *in vitro* system has several advantages over the traditional nursery and field screening trials making it ideal for pre-selection of resistant rootstocks (Jarausch *et al*., 1999; Cicotti *et al*., 2008; Bisognin *et al*., 2008a). The high transmission rate makes it possible to obtain a high number of

infected plants of different genotypes. The system also allows for reproducibility, standardization and eliminates any environmental influences as well as the influence of exogenous pathogens. The limited space required and time efficiency for an *in vitro* grafting system leads to the ability to test a large number of hybrids simultaneously. It is also possible to reproduce symptoms observed in field trials *in vitro*. The symptom expression is also considered to be more severe and symptom expression is earlier observed compared to field trials, making it easier to select for genotypes exhibiting high levels of resistance (Jarausch *et al*., 1999). The possibility to produce *ex vitro* plants for further analysis in nurseries and field trials makes this system ideal for the pre-selection process of Apple Proliferation screening. The specific details of the *in vitro* screening protocol are further described in the Materials and Methods section.

Table 3: **Sensitivity of trees on some rootstocks to latent apple viruses**

Rootstock	Parentage	Mortality and stunting in nurseries (%)*	Mortality and stunting in orchards (%)
M. sieboldii		93	7
M. sargentii		87	13
4551	(*M domestica* cv. Laxton's superb x *M. sieboldii*)	100	0
D2212	(*M domestica* cv. Laxton's superb x *M. sieboldii*, open pollinated)	67	7
3432	((*M. sieboldii* x *M. domestica*) x M. sargentii)	73	7
20186	(*M. purpurea* cv. Eleyi x *M. sieboldii*)	80	20
C1828	(*M. sargentii* x *M. pumila*)	47	53
C0725	(*M. sargentii* x *M. pumila*)	100	0
4608	(*M. purpurea* cv. Eleyi x *M. sieboldii*)	0	0
M9	(*M. domestica*)	0	0

*15 starting trees were examined. Remaining percentage showed no stunting and/or mortality (Seemüller *et al*., 2008)

1.2.7 Plant Defense Mechanisms and Interaction with Pathogens

Pathogens pose a diverse and constant threat to plants, but surprisingly disease is an exception rather than the rule. Most pathogens are host specific and have a limited range of plants it could infect and these host plants then have defense mechanisms (passive and active) that stop or hamper the infection or propagation of the pathogen (Guest and Brown, 1997). For the pathogen to gain access to nutrients or host factors required for replication, the pathogen must first breach the natural barriers of the plant. These barriers could be physical (cell wall) or chemical (pH) and constitutes the passive or basal line of defense.

The active or induced line of defense is activated upon pathogen infection and could be divided into a rapid defense response or local response, including the hypersensitive response (HR) and a delayed response or systemic response, including systemic acquired resistance (SAR) (Guest and Brown, 1997). The activation of the induced line of defense relies on the plants ability to recognize factors or molecules that are foreign to the plant and associated with the pathogen. These pathogen-associated molecular patterns (PAMP's) could be flagelline, the bacterial elongation factor EF-Tu or chitin which upon recognition activate pattern triggered immunity (PTI). In the case of viruses, no viral PAMP have so far been identified but the plant is able to recognize endogenous elicitors released from the plants by the action of pathogens. These elicitors are considered to be danger-associated molecular patterns (DAMP's) which upon recognition also activate PTI (Zvereva and Pooggin, 2012). Another way (main) for the plant to sense viruses is through the recognition of viral RNA activating the antiviral response RNA silencing (Pallas and Garcia, 2011). Pathogens have developed counter strategies to suppress these plant responses through effectors (microbes) or suppressors (viruses) on which the plants react with another line of defense. If the plant is resistant or susceptible to a pathogen depends on the following factors: the response of the plant towards the pathogen and the ability of the pathogen to launch a counter attack (Guest and Brown, 1997; Zvereva and Pooggin, 2012).

This interaction between host and microbe was described by Jones and Dangl (2006) through the 'Zig-zag' model and adapted to include viruses by Zvereva and Pooggin (2012) (Fig. 8). In phase 1, plants detect pathogen-associated molecular patterns (PAMPs) and host danger-associated molecular patterns (DAMPs) via pattern-recognition receptors (PRRs) to induce pattern-triggered immunity (PTI) and in the case of viruses, plants additionally detect viral double-stranded RNA (dsRNA) to trigger RNA silencing. In phase 2, successful viral and non-viral pathogens deliver effectors/suppressors that interfere with both PTI and silencing, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector or suppressor is recognized directly or indirectly by a nucleotide binding site leucine reach repeat (NB-LRR) protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive response (HR) and programmed cell death (PCD). In phase 4, pathogen isolates are selected that have lost or modified the specifically recognized effector/suppressor, and perhaps gained a new effector that can help the pathogen to suppress ETI. A new plant NB-LRR allele is then evolved and selected, that can recognize the newly acquired effector, resulting again

in ETI. The final outcome of the plant-pathogen interaction depends on the sum total of ([PTI − ETS] + ETI) for microbes and [PTI + Silencing – ETS + ETI] for viruses (Jones and Dangl, 2006; Zvereva and Pooggin, 2012).

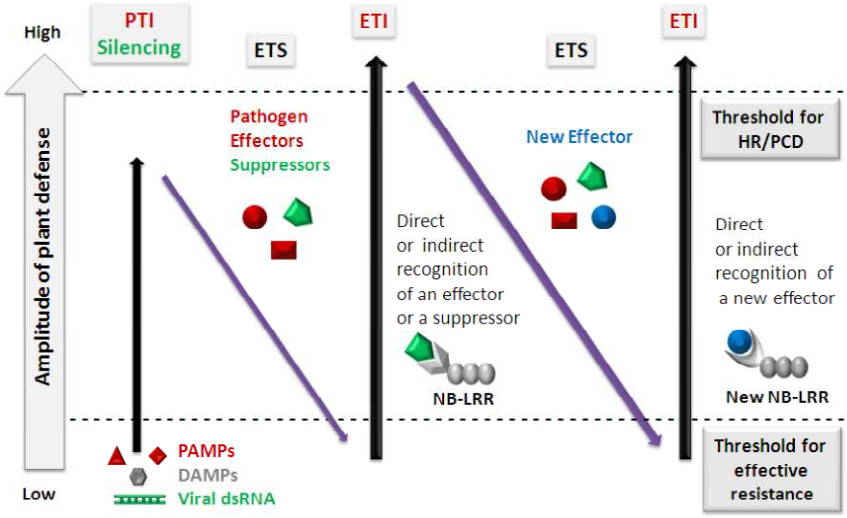

Figure 8: **Zig-zag model for evolution of innate immunity- and silencing-based plant defense against viral and non-viral pathogens** (This scheme was adapted and extended from Jones and Dangl (2006) by, Zvereva and Pooggin (2012)).

Plant hormones also play an essential role in the regulation of signal transduction pathways associated with the plant defense response. Two of the most important hormones associated with the plants response to pathogens and plant growth and development regulation are salicylic acid (SA) and jasmonic acid (JA). The SA dependant defense pathway is associated with the hypersensitive response, SAR, and resistance gene (*R*-gene) mediated resistance (Hayat and Ahmad, 2007; Loake and Grant, 2007), while the JA dependant defense pathway is involved in the activation of wound inducible genes, and SAR (Turner *et al*., 2002; Delker, 2006). These responses could be costly and could decrease the fitness of the plant, especially when more than one defense response is launched (Walters and Heil, 2007). To minimize the cost, plants allow cross-talk among these phytohormone dependant defense pathways, influencing the plants ability to adapt to the different biotic and abiotic stresses as well as the plants ability to grow and develop, especially in the case of multiple stressors and/or pathogens (Kazan and Manners, 2008). This interaction between the JA and SA dependant defense pathways allows the plant to fine-tune its response to the multiple invaders it encounters to balance the plants fitness (growth and development) and the response to the pathogen (Pieterse and Dicke, 2007).

1.2.8 Infectious Clones of Plant Viruses

A full-length viral infectious clone could be used as a tool to study viral gene expression, mechanisms of plant replication, movement and mechanisms involved in the infection cycle. They also provide further insights on the functions of different viral proteins or the mechanisms of interaction between viruses and their host plant(s) or vector(s) (Youssef *et al*., 2011). Over the years, the construction of infectious clones is routinely the first step in reverse genetic studies of RNA plant viruses, but not without limitations and difficulties. The assembly of the virus is often a long and tedious process and the infectivity is influenced by the cloning strategy and the cDNA synthesis (Boyer and Haenni, 1994; Donaldson *et al*., 2008). The viral RNA genomes are generally first reverse transcribed and PCR amplified into cDNA. The cDNA is then cloned into the bacterial vector. The viral RNA genome could be amplified in one step or in fragments that is then assembled in the bacterial plasmid. Some complications could arise in the prokaryotic plasmid due to toxicity of the viral insert and may lead to instability and random rearrangements and mutations in the viral genome (Yamshchikov *et al*., 2001). The insertion of short introns into the toxic viral genome or the use of bacterial strains that lack components of the pathway that catalyze the rearrangement and deletions of non-standard or foreign secondary and tertiary structures could help to overcome this problem (Yamshchikov *et al*., 2001).

Infectious clones of plant RNA viruses can be divided into two types:

- Infectious RNA - The cloning of a viral genome under control of a bacteriophage (T7, T3 or SP6) RNA polymerase promoter from which *in vitro* RNA transcripts can be generated (Campbell, 2000; Ryabov, 2008),
- Infectious cDNA - the cloning of a viral genome under control of a CaMV 35S promoter from which infectious viral RNAs can be produced *in vivo* from cDNA containing vectors, delivered to the plant via several different methods (Dagless *et al*., 1997; Vives *et al*., 2008).

The CaMV 35S promoter has the advantage that the RNA transcripts are synthesized inside the living plant cell, eliminating the costly *in vitro* transcription step required for the infectious RNA strategy (Boyer and Haenni, 1994).

There are several ways by which infectious clones can be introduced into a plant. Mechanical inoculation is where the leaf exterior is damaged with an abrasive material like carborundum or celite, and allows the direct introduction of nucleic acid into the injured cells (Ding *et al*., 2006; Ascencio-Ibanez and Settlage, 2007). Agro-infiltration utilizes the natural ability of agrobacterium to infect plants and launch transfer DNA (T-DNA) into the cell nucleus (Ziemienowicz *et al*., 2000). The T-DNA is substituted with the cDNA clone of the virus and so delivered directly into the nucleus where it will be transcribed and transported to the cytoplasm (Grimsley *et al*., 1987; Leiser *et al*., 1992). In the biolistic approach, gold or tungsten microcarriers coated with the desired nucleic acids are shot directly into plant tissues with the assistance of a gene gun (Turnage *et al*., 2002).

Ohiro *et al*., (1995) successfully constructed a full length cDNA clone of an ASGV isolate (CTLV-lily) by assembling the virus in a pITCL vector containing a T7 RNA promoter. This clone was then used in a study by Hirata *et al*., (2003; 2010) to study the expression strategies of the ASGV ORFs by generating truncated mutants trough the insertion of termination codons into the variable region located between the replicase and the coat protein coding regions. These mutants were capable of systemic infection, although their pathogenicity was attenuated.

2 MATERIAL AND METHODS

2.1 TECHNICAL EQUIPMENT

Equipment	Model	Company
Autoclave	HST 4x5x6	Zirbus Technology GmbH, Germany
Incubator	EB 18	Jouan, USA
Plant growth Chamber	Kamrath Kälte	Kamrath GmbH & Co., Germany
Incubation Shaker	Orbital incubator	Gallenkamp, UK
Agarose Gel Electrophoreses Chamber	Mini 440.000	Harnischmacher Polymehr, Germany
Agarose Gel Electrophoreses Power Supply	Microcomputer electrophoresis power supply E865	Consort, Belgium
Gel Documentation system	Dunkelhaube DH-30/32 Biostep argus x1	Biostep GmbH, Germany
Speedvac	RC1010	Jouan, USA
Micro Centrifuge	Mikro 200	Hettich Lab Technology, Germany
Micro Centrifuge (cooling)	Mikro 200R	Hettich Lab Technology, Germany
Bench Centrifuge	Rotanta 460R	Hettich Lab Technology, Germany
Plate centrifuge	Axygen C1000-AXY	Axygen Scientific Inc., USA
Clean Bench	DNA/RNA UV-cleaner UVT-B-AR	Biosan, UK
Sterile Bench Lamina flow	KH 170 Basic	Kojair Tech OY, Finland
Gradient Cycler	Mastercycler EP gradient	Eppendorf, Germany
Thermal Cycler	Gene Amp PCR System 9700	Applied Biosystems GmBH, Germany
Real-Time Thermal Cycler	Chromo4 Real-Time PCR Detector, BioRad DNA Engine,	BioRad, Germany
Real-Time Thermal Cycler	Chromo4 Real-Time PCR Detector, PTC-200, Peltier Thermal Cycler	MJ Research, Canada
pH-Meter	pH level 1	VWR International GmbH, Germany
Pipette	P10, P20, P200, P1000	Gilson Inc., USA
Spectrophotometer	NanoDrop ND-1000	Thermo Fisher Scientific Inc, USA
Heating block	Digi-Block JR	Sigma Aldrich, Germany
UV-Table	TFX-20.M	Vilbert Lourmat, Germany
Microscope	SMZ-143 series	Motic GmBH, Germany
Vortex	MS1 Minishaker	IKA-Werke GmbH 6 Co

2.2 CHEMICALS AND CONSUMEABLES

2.2.1 Chemicals

Chemical	Company
Agarose for DNA electrophoresis	Serva, Germany
Acetosyringone	Sigma Aldrich, Germany
Ampicillin	Duchefa Biochemie, The Netherlands
Bacto Tryptone	BD Bioscience, Germany
Bacto yeast extract	BD Bioscience, Germany
BAP	Duchefa Biochemie, The Netherlands
Bentonite	Sigma Aldrich, Germany
Bovine Serum Albumin (BSA)	Roth, Germany
Ca(ClO)2	Roth, Germany
$CaCl_2$	Sigma Aldrich, Germany
Chloroform	Roth, Germany
Celite	Sigma Aldrich, Germany
CTAB	Roth, Germany
EDTA	Roth, Germany
EtBr	Sigma Aldrich, Germany
Ethanol	Roth, Germany
GA_3	Duchefa Biochemie, The Netherlands
Glucose	Sigma Aldrich, Germany
Glycerol	Sigma Aldrich, Germany
HOAc	Sigma Aldrich, Germany
IBA	Sigma Aldrich, Germany
Isoamyl Alcohol	Roth, Germany
Isopropanol	Roth, Germany
KCl	Sigma Aldrich, Germany
K_2HPO4	Sigma Aldrich, Germany
KOAc	Sigma Aldrich, Germany
KOH	Sigma Aldrich, Germany
Micro Agar	Duchefa Biochemie, The Netherlands
MS Medium	Duchefa Biochemie, The Netherlands
NaCl	Sigma Aldrich, Germany
NaOAC	Sigma Aldrich, Germany
NaOH	Sigma Aldrich, Germany
Nicotine	Sigma Aldrich, Germany
PVP40	Sigma Aldrich, Germany
QL Medium	Duchefa Biochemie, The Netherlands
SDS	Sigma Aldrich, Germany
Sucrose	Duchefa Biochemie, The Netherlands
Thiamine	Sigma Aldrich, Germany
Tris-HCl	Sigma Aldrich, Germany
X-gal	Duchefa Biochemie, The Netherlands

2.2.2 DNA Gel Standards

Product	Company
GeneRuler™ DNA Ladder Mix	Fermentas, Germany
GeneRuler™ 100 bp DNA Ladder	Fermentas, Germany
1 kb Ladder	Invitrogen, Germany

2.2.3 Enzymes and Kits

Product	Company
5 Prime Taq DNA Polymerase kit	5Prime, Germany
D-Genos RNA extraction kit	Ivagen, France
NucleoSpin Gel and PCR cleanup Kit	Macherey Nagel, Germany
QIAprep spin miniprep kit	Qiagen, Germany
Restriction Enzymes	Fermentas, Germany
Superscript III Platinum SYBR Green One-Step RT-qPCR kit	Invitrogen, Germany
Superscript III One step RT-PCR system with Platinum Taq	Invitrogen, Germany
T4 DNA Ligase	Fermentas, Germany

2.3 BIOLOGICAL MATERIAL

2.3.1 Chemical Competent Bacterial Strains

E coli strains	Relevant Properties
INVα F (Invitrogen)	F´ endA1 recA1 hsdR17 (rk-, mk+) supE44 thi-1 gyrA96 relA1 80lacZ M15 lacZYA-argF
JM110 (Stratagene)	rpsL (Str^r) thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB) [F´ traD36 proAB $lacI^qZ\Delta M15$]
SURE cells (Stratagene)	e14-(McrA-) Δ(mcrCB-hsdSMR-mrr)171 endA1 gyrA96 thi-1 supE44 relA1 lac recB recJ sbcC umuC::Tn5 (Kanr) uvrC F´ proAB lacIqZΔM15 Tn10 (Tetr)]. (Genes listed signify mutant alleles. Genes on the F´ episome, however, are wild-type unless indicated otherwise)

2.3.2 Plant Material

Micro-propagated *M. sieboldii* derived genotypes, derived from a plant collection present at the Julius Kühn-Institute (JKI) Dossenheim (Germany) were established and maintained by RLP Agroscience, AlPlanta Institute for Plant Research (table 4).

Table 4: **Parentage and ploidy of *Malus* rootstocks and cultivars**

Name	Parental	Parental	Ploidy
Rubinette*	*M. domestica* cv. Golden Delicious	*M. domestica* cv. Cox orange pippin	2n
Golden Delicious*	*M. domestica* cv. Grimmes golden	*M. domestica* cv. Golden reinette	2n
M9	*M. domestica* cv. Jaune de metz	*M. domestica* cv. Paradise	2n
M. sieboldii*	-	-	4n
Supporter 1	M9	*M. baccata* var himalaica	2n
Laxton superb	*M. domestica* cv. Cox orange pippin	*M. domestica* cv. Wyken pippin	2n
4551	Laxton's superb	*M. sieboldii*	3n
4556	Laxton's superb	*M. sieboldii*	3n
4608	M. *purpurea* cv Eleyi	*M. sieboldii*	3n
H0909	4556	M9	4n
C1907	4608	open polynated	4n
D45*	C1907	Supporter 1	3n
O45	4551	M9	4n
CC38*	C1907	M9	3n
CAT5*	Laxton's superb	*M. sieboldii*	3n
B89*	4608	M9	4n
B63*	4608	M9	4n
B323*	4608	M9	4n
W355*	HO909	M9	3n
Royal Gala*	*M. domestica* cv. Golden Delicious	*M. domestica* cv. Kidds orange red	2n

*Genotypes used in this study. Other genotypes were included for a better understanding of the parental of the genotypes used in this study.

2.3.3 Virus Isolates and Phytoplasma Strains

2.3.3.1 Apple Stem Grooving Virus

The ASGV-AC isolate was obtained from infected *M. domestica* from Neustadt an der Weinstrasse, Germany, and maintained *in vitro* on *M. domestica* plantlets. Additional ASGV isolates (table 12) from infected *M. domestica* material were obtained from Australia (Dr. Fiona Constable, Department of Plant Science, Waite Campus, University of Adelaide, Glen Osmond, South Australia, 5064), Austria (Dr. Margit Laimer, Plant Biotechnology, Unit, Institute of Applied Microbiology, University of agricultural sciences, Nussdorffer lande 11, 1190 Vienna), Canada (Dr. Mike Bernardy, Pacific Agri-Food Research Centre, Agriculture & Agri-Food

Canada, Summerland, British Columbia V0H 1Z0), France (Dr. Pascal Gentit, Centre Technique Inter professionnel des Fruits et Legumes (CTIFL), 22 rue Bergere, 75009 Paris) and Germany (Michael Petruschke, Landwirtschaftliches Technologiezentrum Augustenberg-Außenstelle Stuttgart, Rötestrasse 16a, 70197 Stuttgart). The ASGV isolates AC and Stutt were established and maintained *in vitro* by Alplanta, Institute for Plant Research. Canadian and French ASGV isolates were graft-inoculated at Alplanta to *ex vitro* plants of M. *domestica* cv. Royal Gala.

2.3.3.2 Apple Stem Pitting Virus

ASPV isolates were obtained from infected *M. domestica* from Dossenheim and Stuttgart, Germany, and established *in vitro* on *M. domestica* plantlets by AlPlanta, Institute for Plant Research. The isolate ASPV-LFP (Stuttgart) was the main isolate used in the graft inoculation studies.

2.3.3.3 *'Candidatus* Phytoplasma mali'

Three different strains of '*Ca.* P. mali' were used in this study: PM4 (Subtype AP, Neustadt an der Weinstrasse, Germany) (Jarausch *et al*., 2000a; Bisognin *et al*., 2008a; Schneider and Seemüller, 2009), PM6 (Subtype AT2, San Michele Institute for Agriculture, Trentino, Italy) (Jarausch *et al*., 2000a; Bisognin *et al*., 2008a; Schneider and Seemüller, 2009) and PM5 (Subtype AP, Meckenheim, Germany) (Schneider and Seemüller, 2009). All three strains were maintained *in vitro* on *M. domestica* plantlets.

2.4 METHODS

2.4.1 *In vitro* Culture Establishment and Tissue Culture

Actively growing shoots from *Malus* trees were cut in segments containing one or two buds. The segments were incubated in ddH_2O overnight, washed with commercial detergent and incubated for 15 min in a 1% sterilium solution. The segments were surface sterilized for 1-2 min in 70% (v/v) ethanol and 5 min in 2.5% (v/v) freshly prepared Ca(ClO)2. The segments were washed two times with 0,254% (v/v) $CaCl_2$ and three times with ddH_2O under sterile conditions (Jarausch *et al*., 1994b; Ciccotti *et al*., 2008b). The sterilized nodal explants were placed in 25 x 150 mm glass tubes containing 10 ml modified MS medium (Murashige and Skoog, 1962) modified as described by Van der Salm *et al*. (1994) with micro-and macro-elements and vitamins; 1 mg/L

thiamine, 30 g/L sucrose, 8 g/L micro agar. Two different media were used for micro-propagated plantlets depending on the genotype. MLO6 medium (Modified MS as described above, with the addition of 0,05 mg/L auxine IBA, 0,1 mg/L gibberelline GA_3 and 1 mg/ cytokine BAP)(Jarausch *et al*., 1999; Ciccotti *et al*., 2008b) was used for genotypes, *M. sieboldii*, D45, O45, CAT5, CC38, W355 and *M. domestica* cultivars, Rubinette, Royal gala and Golden Delicious. QL6 medium (Quoirin Lepoivre medium, with micro and macro elements, 1 mg/L thiamine, 30g/L sucrose, 8 g/L micro agar 0,05 mg/L, auxine IBA, 0,1 mg/L gibberelline GA_3 and 1 mg/ cytokine BAP) was used for genotypes B323, B89, and B63. Micro-propagated plantlets were kept on respective media and placed in an usual growth chamber at 23/18 °C ± 1°C day/night and 16h photoperiod under cool-white fluorescent lights (60 µE m-2 s-1). Plantlets were subcultured every 6 weeks (Jarausch *et al*., 1996; Ciccotti *et al*., 2008b).

2.4.2 *In vitro* Grafting

Micro-propagated apple plantlets infected with '*Ca.* P. mali' strains PM4, PM5 or PM6 were used as inoculums for graft transmission to different *M. domestica* and *M. sieboldii* and *M. sieboldii* derived hybrids. Micro-propagated apple plantlets containing ASPV, or ASGV (isolates AC or stutt) were also used for graft transmission to different *Malus* genotypes. In addition to the single infections, the inoculum plantlets were also used to establish cultures containing different combinations of pathogens (virus-virus and virus-phytoplasma).

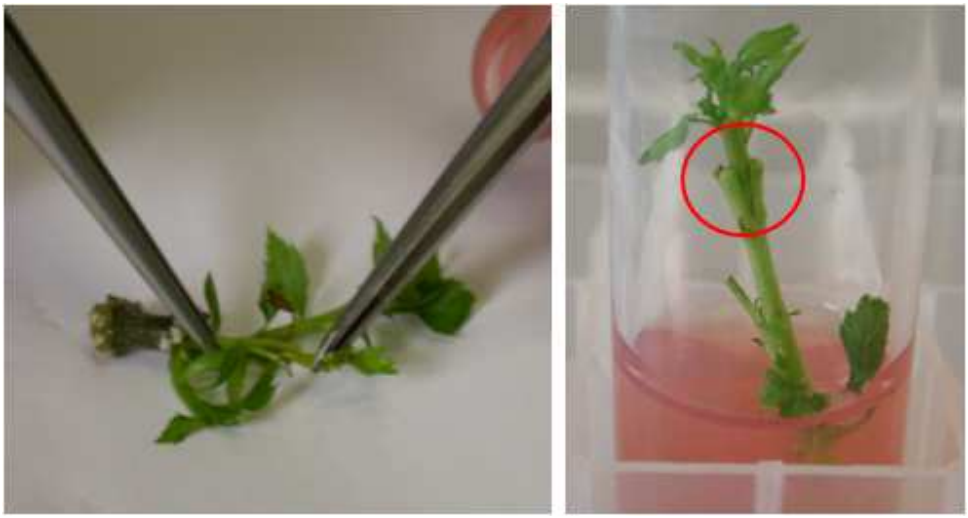

Figure 9: ***In vitro* System a) Subculture of micro-propagated *Malus* plantlets under sterile conditions; b) Compatible graft union between rootstock and inoculums tip**

The *in vitro* grafting technique described by Jarausch *et al*., 1999 was used for all pathogen transmissions (fig. 9a). Healthy micro-propagated shoots, of an adequate size (1-2 cm), which served as the rootstocks, were subcultured and a small cut (2mm) were made at the top of the shoot and auxiliary shoots removed. Shoot tips infected with the desired pathogen were prepared as scion by cutting a wedge at the base. The tip was then inserted into the cut of the rootstock to form a compatible union or graft (fig. 9b) and placed on fresh medium and maintained under the conditions described above.

For each grafting experiment a minimum of ten repetitions were performed. After 4 weeks the tip was separated from the shoot and the strength of the graft union tested by pulling the tip out of the rootstock. Graft tips that had to be removed using force were considered to be strong grafts. The transmission of '*Ca.* P. mali' from an infected tip to a healthy rootstock requires a good phloem connection. Grafts with weak graft unions were not considered for further analysis. Graft tips were stored at -20°C or -80°C depending if the graft tip was infected with '*Ca.* P. mali' or ASGV and ASPV. The rootstocks were sub-cultured and transferred to fresh media for another 6 weeks after which they were sampled and tested for the desired pathogen through polymerase chain reaction. Graft tips of negative rootstocks which displayed a strong graft union were tested for the presence of the pathogen. Graft inoculations with negative tips were not further analyzed or included in transmission rate analysis. As control, healthy grafts were performed using the same genotypes and conditions as the pathogen grafts. All grafts were also analyzed for graft union necrosis associated with graft incompatibility as well as premature death.

2.4.3 Growth and Symptom Expression Analysis

Different experiments were designed to analyze the effect of one factor on the growth and symptom expression of the micro-propagated *Malus* plantlets, for instance the effect of different pathogen combinations on the same genotype or the effect of one pathogen on different genotypes (table 5). In all of these experiments the micro-propagated plants were sub-cultured to a size of 1cm on the same date, one shoot per glass tube and kept under the same conditions for 4 weeks. The plantlets were then monitored for three symptoms: size, (an indication of stunting) the amount of shoots, (an indication of proliferation) and leaf size. Plantlets were also sampled and tested with PCR/RT-PCR dependant on the pathogen, to confirm the infection. The data obtained were then statistically analyzed as described in section 2.4.15.

Table 5: **Experiments designed to analyze the effect of '*Ca*. P. mali', ASGV and ASPV or combinations thereof on growth and symptom expression of different *Malus* genotypes**

Experiment	*Malus* genotype	Pathogen
The effect of different ASGV isolates on Royal Gala	*M. domestica* cv Royal Gala	Virus and '*Ca.* P. mali' free ASGV-AC ASGV-CO2 ASGV-CO4-1 ASGV-CO4-2 ASGV-CO6 ASGV-CO7 ASGV-stutt ASGV-W157
The effect of ASGV and/or ASPV on Rubinette	*M. domestica* cv Rubinette	Virus and '*Ca.* P. mali' free ASGV-AC ASGV-stutt ASGV-AC + ASPV-LFP ASGV-stutt + ASPV-LFP
The effect of ASGV-AC on the symptom expression of different '*Ca.* P. mali' strains on Rubinette	*M. domestica* cv Rubinette	Virus and '*Ca.* P. mali' free PM4 + ASGV-AC PM5 PM5 + ASGV-AC PM6 PM6 + ASGV-AC
The effect of an additional pathogen on the of symptom expression of PM6 infected Rubinette	*M. domestica* cv Rubinette	Virus and '*Ca.* P. mali' free PM6 PM6 + ASGV-AC PM6 + ASGV-stutt PM6 + ASPV-LFP
The effect of PM4 + ASGV-AC on susceptible and tolerant *Malus* genotypes	*M. domestica* cv Rubinette B323 D45	PM4 + ASGV-AC
The effect of ASGV-AC on susceptible and tolerant *Malus* genotypes	*M. domestica* cv Rubinette CC38 *M. sieboldii*	ASGV-AC Virus and '*Ca.* P. mali' free

2.4.4 Nucleic Acid Extraction

Total RNAs were isolated of infected apple plant material using the D-Genos RNA extraction kit (Ivagen, France).This procedure were only used when larger fragments needed to be amplified. Total nucleic acid were extracted using a modified CTAB extraction method (Jarausch *et al*., 2011) Approximately 0.1-0.5 g of Micro-propagated *Malus* material were homogenized in Bioreba ELISA bags with 4 ml of TexDir extraction buffer (2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 100 mM Tris-HCl (pH 8.0), and 2% (w/v) PVP). Two milliliters of the homogenate were transferred to a 2 ml tube and centrifuged for 4 min at 2000 rpm. 1 ml of the supernatant were transferred to a 2 ml safelock eppendorf tube and incubated at 60°C for 30- 60 min. One milliliter of chloroform were added, inverted for 1 min and centrifuged for 5 min at 13 000rpm. The supernatant were transferred to a new 1.5 ml tube, precipitated with isopropanol (10min) and centrifuged at 4°C for 15 min at 13000rpm. The pellet was washed with 70% ethanol, dried and eluted in 100 ml ddH_2O. The total nucleic acid extraction method was used for all virus and '*Ca.* P. mali' detections as well as quantitative real time PCR reactions.

2.4.5 Polymerase Chain Reaction

2.4.5.1 Detection

For the detection of ASGV and ASPV Superscript III One step RT-PCR system with Platinum Taq were used, the protocol was adapted for a 10 µl final volume reaction.

RT-PCR mix	**RT-PCR reaction parameters**	
5 µl of 2X Reaction mix (containing 0.4 mM of each dNTP, 3.2 mM $MgSO_4$)	30 min at 50°C 2 min at 94°C	
0.5 µl of 100 µM Sense primer	15 sec at 94°C	} X 40
0.5 µl of 100 µM Anti-sense primer	20 sec at X°C (table 6)	
0.1 µl of Superscript II RT/Platinum Taq mix	30 sec at 72°C	
1 µl Template (Total nucleic acid)	7 min at 72°C	
2.9 µl ddH2O	∞ at 15°C	

For the detection of '*Ca.* P. mali', 5 Prime Taq DNA Polymerase kit (5Prime) was used and the protocol adapted for 20µl final volume reactions.

PCR mix	PCR reaction parameters
2 µl of 10X Taq Buffer advanced	5 min at 95°C
0.2 µl of 100µM Sense primer	15 sec at 95°C
0.2 µl of 100µM Anti-sense primer	20 sec at X°C (table 6) } X 35
0.5 µl of dNTPs (5 mM each)	60 sec at 72°C
0,5 µl of Taq DNA polymerase (5 u/µl)	7 min at 72°C
2 µl of DNA	∞ at 15°C
15.6 µl ddH2O	

2.4.5.2 Gradient PCR

The optimal annealing temperature for the primers used in section 2.5.3 and 2.5.4 were optimized using gradient RT-PCR. The thermal gradient cycler Mastercycler EPgradient (Eppendorf) was used to produce a temperature gradient at different positions on the thermoblock. The most optimal annealing temperature where then selected for further testing.

Table 6: **Primers used for the detection of ASGV, ASPV and '*Ca.* P. mali'**

Primer name	Sequence (5'-3')	Position (nt)*	Annealing (°C)	Reference
ASPV 4F	GAGTCTGATTATGAGGCATTTGATGC	5952-5977	55	Massart *et al.*, 2009
ASPV 4R	GCTTCCCTCCCATTGAGATCATAC	6202-6179		
ASGV 5F	CCTGAATTGAAAACCTTTGCTGC	6018-6040	55	Massart *et al.*, 2009
ASGV 5R	CACGACTCCTAACCCTCCAGTTCC	6361-6338		
U4	GAAGTCGAGTTGCAGACTTC	265446-265427 451668-451687	50	Ahrens & Seemüller, 1992
U5	CGGCAATGGAGGAAACT	264505-264521 450746-450762		Lorenz *et al.*, 1995
AP3	GAAACATGTCCTATTGGTGG	364271-364291	57	Jarausch *et al.*, 1994a
AP4	CCAATGTGTGAAATCTGTAG	364130-364149		

*Position was based on the reference genome sequences available on ncbi. NC_011047 ('*Ca.* P. mali'), NC_003462 (ASPV), NC_01749 (ASGV)

2.4.5.3 Amplification of the *Apple stem grooving virus* Genome

For the amplification of the various ASGV fragments Superscript III One step RT-PCR system with Platinum Taq were used, the protocol was adapted for a 20 µl final volume reaction. The complete genome of ASGV was amplified using the primers described in table 7. All primers incorporated a Restriction enzyme (RE) recognition site for the assembling of a full length cDNA

clone (section 2.4.8). All primers designed were based on the AC isolate (JX080201, Liebenberg *et al*., 2012).

RT-PCR mix	RT-PCR reaction parameters
10 µl of 2X Reaction mix (containing 0.4 mM of each dNTP, 3.2 mM $MgSO_4$)	30 min at 50°C 2 min at 94°C
2 µl of 10µM Sense primer 2 µl of 10µM Anti-sense primer 0.25 µl of Superscript II RT/Platinum Taq mix	30sec at 94°C 30 sec at X°C (table 7) 30-90 sec at 72°C (60sec per Kb extension) } X 40
X µl Autoclaved distilled water 2-4 µl RNA (0.2-0.5 µg)	7 min at 72°C ∞ at 15°C

Table 7: **Primers used in the amplification of the full length ASGV-AC genome, incorporating restriction enzyme recognition sites for assembling in pCass2 vector.**

Primer name	Sequence (5'-3')	Annealing (°C)	Cloning step
AG370-Afl-as	GC**TTTAAA**CTGAGTGCAT**CTTAAG**AA	50	5' end (1)
AG1-Dra-s2	GC**TTTAAA**TTTAACAGCGCTTAATTTCC		
RNA2-OdT	GGTACCGAATTC**GAGCTC**TTTTTTTTTTTTTTTTT	56	3' end (2)
AG6350-Xhos	GTC**GAGCTC**GCAATCTGGAA**CTCGAG**GGTTAGAAGTCG		
AG6350-Xhoas	CGACTTCTAACC**CTCGAG**TTCCAGA	53	3
AG5870-E52s2	GTC**GGTACC**ACCCAGTTTC**CGGCCG**TTGGATTTGATAC		
AG5870-E52as	CAA**CGGCCG**GAAACTGGGTCTTGTC	55	4
AG4800-BHIs	TC**GGTACC**TGAAGGGATT**GGATCC**GAATGGCAATCG		
AG4800-BHIas	CATTC**GGATCC**AATCCCTTCACC	50	5
AG3850-Bgl-s	TC**GGTACC**AACCACTCAGAA**AGATCT**GACATTG		
AG2800-Hpa-s	TC**GGTACC**CTCGATCTAAT**GGTTAAC**ACCATCAAACAC	55	6
AG3850-Bgl-as	CAATGTC**AGATCT**TTCTGAG		
AG1968-Stu-s	TC**GGTACC**AGATGAAGATAA**AGGCCT**GTCG	50	7
AG2800-Hpa-as	GATGGT**GTTAAC**CATTAGATCG		
AG1968-Stu-as	CGAC**AGGCCT**TTATCTTCATC	52	8
AG1280-Bal-s	TC**GGTACC**TGTTGAATGACC**TGGCCA**CT		
AG370-Afl-s	TTTT**CTTAAG**ATGCACTCAG	50	9
AG1280-Bal-as	TTAG**TGGCCA**GGTCATTCAAC		

2.4.5.4 Apple stem grooving virus Variable Areas

The two variable regions (V1 and V2) of ASGV were amplified from ASGV isolates receive from Germany, France, Austria, Canada and Australia (table 8), using the protocol described at section 2.4.5.1. Two sets of primers were designed for the amplification of each fragment. The design and development of all new primers used in this study was performed with the online web service of primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) using the standard

parameters. Primers were manufactured by Sigma and Invitrogen, Germany. Different annealing temperatures were used to obtain the desired fragment, depending on the isolate amplified (46-50 °C for V1 and 49-56° C for V2).

Table 8: Degenerate primers designed for the amplification of the two ASGV variable areas.

Primer name	Sequence (5'-3')	Variable region
A-V1-1142s	TGAGRGAYGAYTTTGAYGTSTCAGT	1
A-V1-2049as	CATAYTTYCTRTTAGRACTGCCAT	1
A-V2-4662s	TACACAATYYTGAAAGAAGA	2
A-V2-5866as	ACAGCRGGRAACTGGGTCTT	2
a-V1-1180s	GGRATGAGRGAYGACTTTGA	1
a-V1-2100as	ACTTCYTCAATCATYTCATG	1
a-V2-4650s	CTACAGRTTAGGTGAGAGGC	2
a-V2-5800as	CAYAGAAGAAGGTAAAGCTC	2

2.4.5.5 Quantitative Real-Time PCR

The primers developed for the real-time PCR analysis were first optimized using the gradient RT-PCR conditions as described in section 2.4.5.2. The data were collected using the Opticon Monitor 3.1 software (Bio-Rad Laboratories). Samples were tested in duplicate, and the reaction was repeated in a second run, to produce 4 data sets per sample tested. The data generated were exported to Microsoft Excel for data editing and analysis. Statistical analysis was performed using the biostatistics program R (R development core team, 2.9.0) as described in section 2.4.15.

2.4.5.6 Preparation of Dilution Series

A dilution series were prepared from plasmid DNA containing the corresponding clone insert. A stock with a concentration of 10^9 molecules/µl was prepared for each plasmid based on their initial concentration and size. Subsequently a ten-fold dilution series was prepared (10^8-10^1). A dilution series were prepared for each pathogen tested as well as for the internal control. The same stock solution was used for all RT-qPCR reactions to produce comparable data.

2.4.5.7 Quantitative Real-Time RT-PCR for ASGV and ASPV

Quantitative real-time RT-PCR was used to determine the ASGV copy number per plant cell. A variety of ASGV and or ASPV infected *M. domestica*, *M. sieboldii* and *M. sieboldii* derived genotypes were analyzed to determine the effect on the virus tier per plant cell. The effect of an additional pathogen (ASPV or '*Ca.* P. mali' strains) on the ASGV titer per plant cell was analyzed as well as the virulence of different ASGV isolates. All quantitative real-time RT-PCR reactions were performed using the Superscript III Platinum SYBR Green One-Step RT-qPCR kit.

RT-qPCR mix	RT-qPCR reaction parameters
10 µl of 2X SYBR Green Reaction mix (containing SYBR green I, dNTP's, $MgSO_4$, and stabilizers) 0.5 µl of 100 µM Sense primer 0.5 µl of 100 µM Anti-sense primer µl of Superscript II RT/Platinum Taq mix X µl Autoclaved distilled water 1 µl total nucleic acid	1. 10 min at 50°C 2. 2 min at 95°C 3. 15 sec at 95°C 4. 30 sec at 60°C 5. Plate read 6. Go to line 3 for 39 more times 7. 4 min at 72°C 8. Melting curve from 50°C to 95°C (read every 1°C, hold 10sec) 9. ∞ at 15°C

Table 9: **Primers used for Real-time PCR quantification**

Primer name	Binding	Primer sequence 5'-3'
ASGV_uni-1685-F	ASGV	GAGTCAAATCCGACGAAAGC
ASGV_AC-1876-R	ASGV	GAACATCAATGCCGAGGT
qASPVrev	ASPV	GGGAGCCCCAACATTGCCACC
qASPVfor	ASPV	CTTACAGTGACGCGCCGCCA
SBE1-F	Starch Binding Protein	GGCACATGTTGGAATGAGTAGC
SBE1-R	Starch Binding Protein	GTTCCAGATCTACTGCTGACGGC

2.4.5.8 Quantitative Real-Time PCR for '*Ca.* P. mali'

Quantitative Real-Time RT-PCR was used to determine the '*Ca.* P. mali' copy number per plant cell. A variety of infected *M. domestica*, *M. sieboldii* and *M. sieboldii* derived genotypes were analyzed to determine the copy number per plant cell, an indication of resistance to '*Ca.* P. mali'. The effect of an additional pathogen (ASPV or ASGV) on the copy number per plant cell as well as the virulence of different '*Ca.* P. mali' strains was also analyzed. All quantitative real-time

PCR reactions were performed using the 5Prime DNA polymerase kit together with the SYBR green as detection method. The primers AP3 and AP4 were used for all '*Ca.* P. mali' Real-Time qPCR's (table 6).

qPCR mix	qPCR reaction parameters
2 µl of 10X Taq Buffer advanced	1. 2 min at 95°C
0.2 µl of 100µM Sense primer	2. 15 sec at 95°C
0.2 µl of 100µM Anti-sense primer	3. 30 sec at 57°C
0.5 µl of dNTPs (5mM each)	4. 30 sec at 72°C
0,5 µl of Taq DNA polymerase (5u/µl)	5. Plate read
1 µl SYBR Green (BioRad)	6. Go to line 2 for 39 more times
2 µl of DNA	7. 4 min at 72°C
X µl Autoclaved distilled water	8. Melting curve from 50°C to 90°C
	9. (read every 1°C, hold 10sec)
	10. ∞ at 15°C

2.4.5.9 Quantitative Real-Time PCR for SBE1 Gene

The Starch Binding Protein gene (SBE) was used as internal control for standardization of the pathogen copy number per plant cell. The real-time PCR reactions were performed as described in section 2.4.5.8.

Reaction parameters
1 min at 95°C
15 sec at 95°C
45 sec at 60°C
Plate read
Go to line 2 for 29 more times
4 min at 72°C
Melting curve from 50°C to 90°C (read every 1°C, hold 10sec)
∞ at 15°C

2.4.6 Agarose Gel Electrophoresis

The DNA fragments were separated by agarose gel electrophoresis, according to Sambrook *et al.* (1989). DNA fragment separation was performed on 1-2% (w/v) agarose gel, depending on the fragment size, in 1 x TAE(40 mM Tris, 0.114% (v/v) HOAc, 1 mM EDTA pH 8) at 100 V for

30-60 min. 0.8 µg/ml ethidium bromide was added to the agarose gel for ultra violet visualization. 1/3 volume DNA-loading buffer (50% (v/v) glycerin, 0.25% (w/v) bromophenol blue) was added to the samples prior to application to the gel.

2.4.7 TA Cloning of Amplified Products

The pTPCR vector (Appendix A, Wassenegger *et al*., 1994) was used for all basic TA cloning reactions. The vector is based on a standard PUC vector system and prepared by digesting 10 µg with 5 u of Xcml for 3 hours at 37°C. The digested pTPCR was purified using the Phelon:Chlorofrom:Isoamyalcohol method (2.4.8) and the pellet dissolved in 50 µl of ddH_2O and alliquated to 50 ng of pTPCR per tube. The ligation reaction was performed using 50ng of pTPCR vector, with, 1:1, 3:1or 5:1 molar ratio PCR product together with the T4 DNA ligase kit, according to the manufacturer's specifications. If unspecific products were present after PCR amplification the desired fragment were cut from the agarose gel and purified using the NucleoSpin Gel and PCR cleanup kit (Macherey-Nagel) according to the manufacturer's specifications before ligation.

2.4.8 Assembling of cDNA clones

The amplified products of ASGV-AC were first cloned in the TA cloning vector pTPCR before subcloned into pCass2 (Appendix A, Shi *et al*., 1997) consisting of a partially duplicated 35S promoter and 35S terminator. It contains also a resistance gene against ampicillin and a LacZ gene expressing β-galactosidase. The fragments and vector were linearized using the appropriate restriction enzymes (2 h, 37°C), and the enzyme activity heat inactivated (according to the enzyme's specifications). The linearized vector were dephosphorylated by adding 5 µl of 5 u/µl Alkaline phosphatase, 5 µl 10x Alkaline phosphatase Buffer to 40ul of linearized DNA (1 h, 37°C). Dephosphorilation decrease the vector background in cloning by removing the 5´ phosphoryl termini required by ligases, thus preventing self-ligation.

The digested, dephosphorylated plasmid DNA/vector, were purified using the Phenol Chloroform Isoamylalcohol (P:C:I) purification protocol for the removal of all enzymes and smaller undesired DNA products that could influence the ligation negatively (Sambrook *et al*., 1989). The digested vector was diluted with autoclaved distilled water to a volume of 100 µl, an equal

volume of P:C:I (25:24:1) was added, inverted and centrifuged (3 min, 13000 rpm). The supernatant was collected and precipitated (2-4 h, -20°C) with 1/10 of the volume 3 M NaOAC (pH 5.2) and 2.5 times the volume 99.6% ethanol. The precipitated was pelleted (15 min, 13000, 4°C) and washed (5 min, 13000 rpm, 4°C) with 70% (v/v) ethanol. The pellet was dried and dissolved in 15µl autoclaved double distilled water. The products were step by step ligated into pCass2 (fig. 16) using the T4 DNA ligase kit. The amount of insert added to the ligation was calculated using the following formula (for a 3:1 Molar ratio ligation):

Amount of inserts = insert size/vector size x 3(molar ratio of the insert/ molar ratio of the vector) x amount of vector

For agro infiltration the full length ASGV and ASPV cDNA is ligated into the binary vector pPZP200 (Hajdukiewicz *et al.*, 1994) containing a bom site, which permits the mobilization of the plasmid DNA from *E. coli* to Agrobacterium, an origin of replication functional in both *E. coli* and Agrobacterium and a resistance gene against spectinomycin.

2.4.9 Preparation of Chemically Competent *E. coli* cells

Chemically competent *E. coli* cells were prepared according to the method described by Sambrook *et al*., 1989. A single E. coli colony was incubated overnight (37°C, 155 rpm) in 10 ml Luria Bertani broth (LB, 0.5% (w/v), yeast extract, 1% (w/v)tryptone, 1% (w/v) NaCl, pH 7, NaOH) and used as inoculum for 1000 ml LB media. The culture was incubated (37°C, 225 rpm) until an optical density (OD_{600}) value of approximately 0.6 was reached. The culture was centrifuged (5000 rpm, 5 min, 4°C), the pellet resuspended in 250 ml of ice cold $CaCl_2$ and incubated on ice for 15 min. The suspension was centrifuged (5000 rpm; 5 min; 4°C) and resuspended 25 ml of ice cold $CaCl_2$ containing 15% glycerol. The suspension was aliquated in pre-cooled 1.5 ml tubes and flash frozen in liquid nitrogen before storage at -80°C.

2.4.10 Transformation into Competent *E. coli* cells

Transformations were performed according to the protocol specified by Sambrook *et al*. (1989). One hundred microliters of the chemical competent cells was defrosted on ice, added to the ligation reaction (section 2.4.7), gently mixed and incubated on ice for 10 min. The cells were

heat shocked (45 sec, 42°C heatblock) and directly incubated on ice for another 5 min. Four hundred microliters of SOC (20 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 0.5 g/L NaCl, 2.5 ml 1M KCl, pH 7.0, 1 M glucose) were added to the transformation reaction and incubated (60 min, 155 rpm, 37°C). One too two hundred microliters were pelleted on LB agar plates containing 100 µg/ml Ampicillin (Amp) and 40 µg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), for blue-white selection. The plates were incubated at 37°C overnight.

2.4.11 Colony PCR

Colonies were preselected for the correct insert by performing colony PCR, especially in the cases where blue-white selection was not possible. The 5 Prime Taq DNA Polymerase kit and primers described in section 2.4.5.1 were used in the colony PCR reactions together with insert and/or vector specific primers (table 10).

Table 10: **Vector specific Primers used for colony PCR**

Primer name	Sequence	Vector
pCass Ev	AAAGCAAGTGGATTGATGTGATATC	pCass2 (universal)
35Tas-pCass	ATGCTCAACACATGAGCG	pCass2 (universal)
U-FWD	GTA AAA CGA CGG CCA GT	Universal PUC (pT-PCR)
Puc-UR	AGC GGA TAA CAA TTT CAC ACA GG	Universal PUC (pT-PCR)

2.4.12 Plasmid Miniprep

The QIAprep spin miniprep kit (Qiagen) was used according to the manufacturer's specifications. When large quantities of plasmid DNA were purified, a modified version of the plasmid alkaline lysis mini-prep method was used (Sambrook *et al*., 1989). Three to five milliliters of overnight culture was centrifuged (13 000 rpm, 1 min) and the supernatant discarded. The pellet was resuspended in 100 µl of solution 1 (25 mM Tris-HCl pH8, 50 mM Glucose, 10 mM EDTA) and incubated for 5 min at room temperature. Two hundred microliters of solution 2 (100 mM NaOH, 1% (w/v) SDS) was added, inverted 4 times and incubated on ice for 5 min. Thereafter 150 µ of ice cold solution 3 (3 M KOAc, 5 M HOAc, pH 4.8) was added, inverted 4 times incubated for 5 min on ice and centrifuged (13 000, 10 min, 4°C). The supernatant was recovered, transferred to a new 1.5 ml tube and precipitated with 2.5 volumes of ice cold Isopropanol. The solution was incubated (2 min, room temperature) and centrifuged (13 000 rpm, 5 min, 4°C). The supernatant was discarded and the pellet washed with 70% (v/v) ethanol (13 000 rpm, 5 min, 4°C). The pellet

was dried and resuspended in 20-50 µl of autoclaved distilled water. DNA concentrations were determined using the Nanodrop ND-1000 (Thermo Fisher) spectrophotometer according to the manufacturer's instructions. Plasmid DNA was screened for containing the correct insert size using Restriction Enzyme analysis according to the manufacturer's specifications.

2.4.13 Sequencing

Sequencing reactions were performed by 4Base lab GmbH, Reutlingen, Germany and GATC Biotech AG, European Custom Sequencing Centre, Cologne, Germany. Plasmid DNA samples, PCR products and primers were prepared according to the company's instructions.

2.4.14 Sequence and Phylogenetic Analysis

BioEdit (Ver. 7.0.4) (Hall, 1999) and DNAStar (Ver. 4.0) were used to perform sequence editing and compilation. Sequence comparisons were performed using the BLAST algorithm (Altschul *et al*., 1990) against the GenBank database of the NCBI (www.ncbi.nlm.nih.gov). Sequence alignments were performed using the ClustalW (ver. 1.4) algorithm embedded in the BioEdit software. The maximum likelihood method implemented in PhyML version 3.0 (Guindon and Gascuel, 2003) and the neighbor joining method implemented in MEGA version 4 software (Tamura *et al*., 2007) were used to infer the tree topologies. Branch support was calculates with 100 bootstrap replicates. Bootstrap percentages of 75% were considered as well supported, between 75 and 50% as moderately supported and values below 50% as weakly supported. Bootstrap percentages below 50% are not indicated on the phylograms.

2.4.15 Statistical Analysis

All statistical analysis was performed using the biostatistics program R (R development core team, 2.9.0). The specific analysis of these empiric data obtained during this study was based on the initial discrimination between normal or nonparametrical distribution of values by the Saphiro-Wilk normality test. The calculations were adapted to our particular data sets (Jarausch *et al*., 2011). Data obtained for the phytoplasma and virus concentration as well as data obtained from the growth and symptomology analysis was tested for parametrical distribution through the Shapiro-Wilk normality test, the homogeneity of the variances was calculated by using the Bartlett test of homogeneity and the Kruskal-Wallis rank sum test was adopted to determine

differences among means. If the Kruskal- Wallis rank sum test showed differences among means, a Duncan test (confidence level = 95%) was adopted to rank these differences.

2.4.16 Selection Intensity Analysis of the ASGV Genome

Sequences produced of the full length ASGV-AC isolate as well as from the two ASGV variable regions were sent for selection pressure analysis due to the unavailability of the programs in the laboratory where I conducted my research (Liebenberg *et al*., 2012). The analysis was performed by B. Moury (INRA, UR407 Pathologie Vegetale, Domaine St Maurice, BP94, 84140 Montfavet, France) and N. Sabath (Institute of Evolutionary Biology and Environmental Studies, University of Zurich, 8057 Zurich, Switzerland; The Swiss Institute of Bioinformatics, Basel, Switzerland) using the RDP version 2 software for Recombination analysis (Martin *et al*., 2005) and the SNAP program (Korber, 2000) and the Simultaneous estimation of dN/dS in overlapping genes method (Sabath *et al*., 2008) for synonymous(dS) and nonsynonymous (dN) distribution. The dN/dS rate was analyzed using the Nei-Gajobori method using the MEGA version 4.0 (Nei and Gojobori, 1986; Tamura *et al*., 2007).

2.4.17 Seed Germination

C. quinoa, *N. glutinosa* and *N. occidentalis* seeds were sterilized with 70% (v/v) Ethanol, and washed with dd$H_2$0. 7% (v/v) Ca(ClO)2 were added and incubated for 20 min, washed with dd$H_2$0 (5 times) and placed on MS-medium. The *N. occidentalis* seeds were additionally treated with GA3 for seed germination activation before placed on MS-medium. The seeds were incubated (21°C, 14 h light and 10 h dark) for 2-3 weeks. The plantlets were then transferred to soil and grown for another 3-4 weeks in the greenhouse.

2.4.18 Mechanical Infiltration of the cDNA Clone

pCass2 plasmid DNA containing the full length ASGV and ASPV clones were diluted in bentonite buffer (0.03 M K_2HPO_4, 0.05 M Glycine (pH 9.2, KOH), 1% (w/v) Bentonite, 1% (w/v) Celite) or Nicotine buffer (2% (v/v) Nicotine, 1% (w/v) celite) and mechanically infiltrated on *C. quinoa*, *N. glutinosa* and *N. occidentalis* greenhouse plants by leaf rubbing. Plants were grown under normal greenhouse conditions and observed for symptom expression.

2.4.19 Agro-Infiltration

Agrobacterium tumefaciens chemical competent cells were prepared according to the protocol specified by Sambrook *et al*. (1989). A single *E. coli* colony was incubated overnight (28°C, 160rpm) in 50 ml LB medium until an optical density (OD_{600}) value of approximately 0.3 was reached. The culture was incubated on ice (15 min), centrifuged (5000 rpm, 5 min, 4°C) and the pellet resuspended in 10 ml of ice cold 100 mM $MgCl_2$. The suspension was incubated (60 min) on ice, centrifuged (5000 rpm; 5 min; 4°C) and resuspended 2ml of ice cold 20 mM $CaCl_2$ (2 h on ice). Five hundred microliters of Glycerol was added and flash frozen in 50 µl aliquots in liquid nitrogen. The aliquots were stored at -80°C.

For the transformation of the cDNA clone into agrobacterium the full length virus genome was first cloned into the binary pPZP200 vector. Plasmid DNA was transformed into the agrobacterium competent cells by adding 1 µg of plasmid DNA to the 50 µl competent agrobacterium aliquot (defrosted on ice) and gently mixed. The mixture was flash frozen in liquid nitrogen, incubated (5 min, 37°C) and 200 µl of SOC added (1 h, 28°C, 160 rpm). The bacterial culture were transferred to LB-agar plates (spectinomycin) and incubated at 28°C for 36-48 h.

For the infiltration procedure, 5 ml of LB (spectinomycin) was inoculated with a single colony of the transformed agrobacterium and incubated (overnight, 28°C, 200 rpm). A large LB media suspension was then inoculated with the overnight culture and grown at 28°C to an OD_{600} of ± 1.0. The cells were harvested by centrifugation at 5000 rpm for 2 min and resuspended in 1 ml of infiltration buffer (10 mM $MgCl_2$ and 100 µM acetosyringone) (Bhaskar *et al*., 2009). The bacterial suspension was taken in a syringe and infiltrated through the abaxial surface of the leaf. Before infiltration, a small incision was made at the site of infiltration using a sterile scalpel to enhance the efficiency of infiltration. Plants were grown under normal greenhouse conditions and observed for symptom expression.

3 RESULTS

3.1 MOLECULAR EVOLUTION OF THE GENOMIC RNA OF APPLE STEM GROOVING VIRUS

3.1.1 Genome characteristics of the German isolate AC of ASGV

The ASGV-AC isolate was extracted from infected micropropagated *M. domestica*, amplified through RT-PCR and sequenced. The complete nucleotide sequence of ASGV-AC was 6496 nucleotides (nt) long, excluding the poly (A) tail (JX080201, Liebenberg *et al*., 2012). Computer analysis revealed one large open reading frame (ORF) (nt 37–6354), encoding a 2,105 amino acid (aa) polypeptide containing methyltransferase-like, papain-like protease, helicase-like, and RdRp-like domains, and a tricho-like coat protein (CP) (237 aa) located at the carboxy-terminal end of the polyprotein. This ORF was preceded by a 36 nucleotide-long non-coding region at the 5' end of the genome, and followed by a 142 nucleotide-long non-coding region at the 3' end.

Table 11: Sequence comparison between ASGV isolates. Full length sequences of ASGV isolates available in Genbank ncbi were compared to the ASGV-AC isolate with the pairwise Blast program available on ncbi.

Accession number	Country	Host	Genome (nt)	ORF1(aa)	ORF2 (aa)	CP (aa)
HE978837	India	*M. domestica*	97%	95%	98%	99%
D14995	Japan	*M. domestica*	83%	88%	96%	98%
FJ355920	Taiwan	*Citrus sinensis*	82%	88%	97%	97%
JQ765412	China	Citrus	82%	88%	97%	95%
JX416228	Taiwan	*Citrus reticulata*	82%	88%	97%	95%
AY646511	Taiwan	Kumquat	82%	88%	97%	96%
JN701424	China	*Pyrus pyrifolia*	82%	87%	97%	98%
EU553489	USA	Meyer lemon	82%	87%	96%	96%
D16681	Japan	Lily	82%	87%	96%	99%
AB004063	Japan	Lily	82%	87%	96%	98%
JQ308181	China	*M. domestica*	82%	87%	95%	99%
AY596172	South Korea	*Pyrus pyrifolia*	81%	87%	94%	96%

nt: nucleotide, aa: amino acid

A second smaller ORF was found nested in ORF1 (nt 4788 to 5750), encoding a 320 amino acid polypeptide, with similarities to the 30-kDa superfamily of cell-to-cell movement proteins (fig.

10; Melcher, 2000). When compared to other isolates of ASGV, the ASGV-AC isolate showed between 82 and 97% nucleotide identity for the complete genome. Amino acid comparison revealed 87 to 95% identity for ORF1, 94-98% for ORF2 and between 95 and 99% for the coat protein (table 11).

3.1.2 Analyses of variable genome regions of ASGV isolates

Two regions of high variability were detected in the genome (fig. 10), variable region 1 (V1, amino acids 532–570) and variable region 2 (V2, amino acids 1,583–1,868). Interestingly, variable region 2 which corresponds to the overlapping region between ORF1 and ORF2, showed a high level of variability in ORF1, but was conserved in ORF2 (fig. 12). This means that the nucleotide changes which induced amino acid substitutions in ORF1 were in most cases silent in ORF2, putting a constraint on the evolutionary fitness of the virus, by restraining the amount of advantageous mutations allowed in this area. In order to analyze more thoroughly the genetic variability in the V1 and V2 regions, additional isolates from Germany and other countries were collected (table 12) and their variable genome regions cloned and sequenced. For a few of the plant samples however, only one of the variable regions could be amplified (table 12), even with varying annealing temperatures and additional primer combinations the amplification was unsuccessful. This could be due to insufficient complementarities between the primers and the viral RNAs in this variable region.

In the V1 region, most of the isolates from Germany, France, Canada and India showed less variability when compared to the ASGV-AC isolated, with the exception of 3 isolates (fig. 12, Fr3, CO4b, CO2), whereas the isolates from Asia, (China, Taiwan, Japan, South Korea) and the isolate form the USA, showed much more variability when compared to the ASGV-AC isolate and with each other. Similar results were obtained with the phylogenetic analyses of the V1 region, the ASGV-AC isolate grouped within one clade of closely related isolates containing European and Canadian isolates (fig. 12). Sequences from Asia, the USA and Australia on the other hand showed much more variability between themselves and with other isolates, and were distributed in several clades, in which clustered also one French (Fr3) and two Canadian isolates (CO4b and CO2).

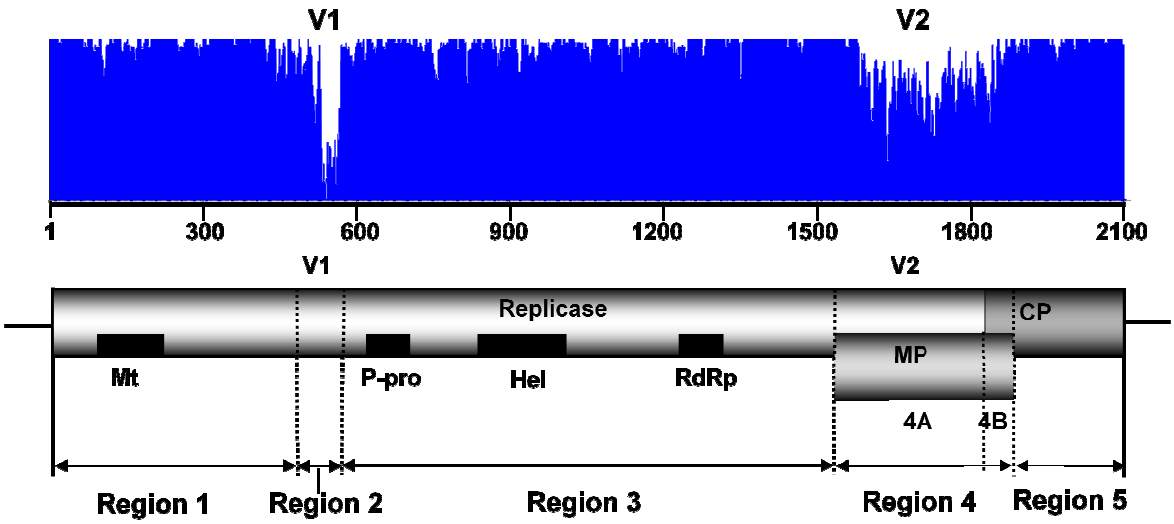

Figure 10: **Similarity plot of ASGV-AC with amino acid sequences of other ASGV isolates available on NCBI**. The two most variable regions V1 and V2 are indicated with dotted lines. Open boxes represent the open reading frames (ORF), ORF1 encoding the replicase and coat protein (CP), and ORF2 encoding the movement protein (MP). Black boxes represent the protein domains: Mt: methyltransferase, P-pro: papain-like protease, Hel: nucleotide triphosphate-binding helicase, RdRp: RNA-dependent RNA polymerase. The five indicated regions were analyzed separately for selection intensity (Table 2)

In the variable region 1 the sequenced isolates from Europe (Germany, Austria and France) as well as from India show less variability compared to the isolates from Asia (China, Taiwan, Japan, South Korea) and Australia, with the exception of the isolates CO6, Fr3 and CO2, which show higher variability (fig. 11 and 12). This could most likely be explained by vegetative propagation and exchange of infected material between countries. Phylogenetic analyses of the V2 regions from the different isolates revealed a similar tree topology as the one observed with V1 (fig.14).

Table 12: **Origin of the ASGV isolate**

Isolate	Country	Host	V1	V2
AC ASGV	Germany	*M. domestica*	JX080201	JX080201
Fr3 ASGV	France	*M. domestica*	JX080217	JX080225(a) JX080226(c)
Fr5 ASGV	France	*M. domestica*	JX080209	ns
Fr7 ASGV	France	*M. domestica*	JX080210	JX080227
Fr8 ASGV	France	*M. domestica*	JX080211	ns
CO2 ASGV	Canada	*M. domestica*	JX080215	ns
CO3 ASGV	Canada	*M. domestica*	JX080205	ns
CO4 ASGV	Canada	*M. domestica*	JX080204 (a) JX080218 (b)	JX080222
CO5 ASGV	Canada	*M. domestica*	JX080206	ns
CO6 ASGV	Canada	*M. domestica*	JX080207	JX080223
CO7 ASGV	Canada	*M. domestica*	JX080208	JX080224
GO7 ASGV	Canada	*M. domestica*	JX080212	JX080228
W157 ASGV	Germany	*M. domestica*	JX080213	JX080232
W199 ASGV	Germany	*M. domestica*	JX080214	JX080233
Stutt1 ASGV	Germany	*M. domestica*	JX080202	JX080229
Stutt2 ASGV	Germany	*M. domestica*	JX080203	JX080230
AUS ASGV	Australia	*M. domestica*	ns	JX080219
VC40 ASGV	Austria	*M. domestica*	ns	JX080231
LCd-NA-1-CTLV*	Taiwan	*Citrus sinensis*	FJ355920	FJ355920
LI-23-ASGV	Japan	Lily	AB004063	AB004063
CTLV*	USA	Meyer lemon	EU553489	EU553489
PBNLSV*	South Korea	*Pyrus pyrifolia*	AY596172	AY596172
Kumquat-1-CTLV*	Taiwan	Kumquat	AY646511	AY646511
Lily-CTLV*	Japan	Lily	D16681	D16681
ASGV	Japan	*M. domestica*	D14995	D14995
ASGV-CHN[a]	China	*M. domestica*	JQ308181	JQ308181
ASGV-HH[a]	China	*Pyrus pyrifolia*	JN701424	JN701424
CTLV-Pk[a]*	Taiwan	*Citrus reticulata*	JX416228	JX416228
CTLV-Chatang[a]	China	*Citrus*	JQ765412	JQ765412
ASGVp12[a]	India	*M. domestica*	HE978837	HE978837

* Citrus tatter leaf virus (CTLV) and pear black necrotic leaf spot virus (PBNLSV) are two recognized isolates of ASGV. [a] These isolates were not available on ncbi when the phylogenetic and statistical analysis were performed and were only used for basic sequence comparisons.
V1: Variable region 1, V2: Variable region 2. ns: not sequenced

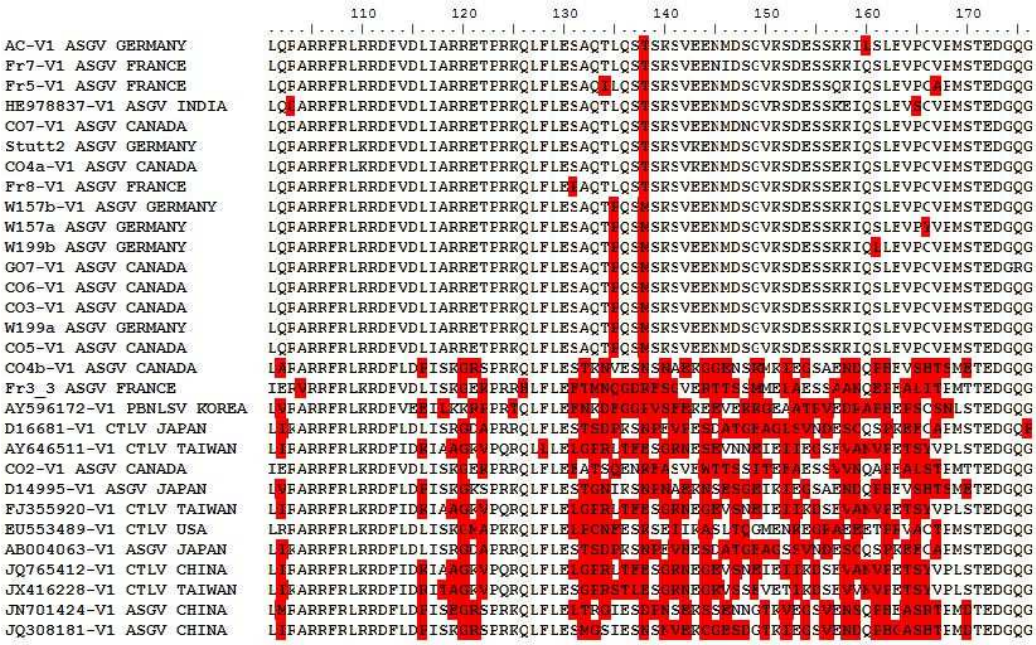

Figure 11: **Sequence alignment corresponding to V1 region. The variable amino acids are indicated in red.**

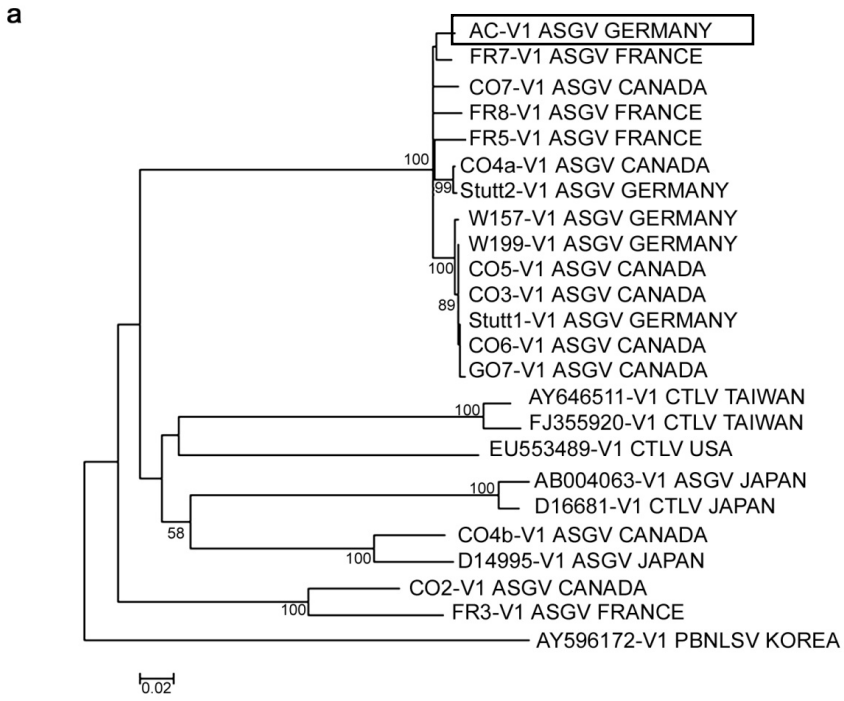

Figure 12: **Neighbor joining phylogenetic trees of the nucleotide sequences of regions and V1 of ASGV.** The German AC isolate sequenced in indicated by the box. Bootstrap percentages above 50% are shown. The scale bar indicates branch lengths in substitutions per nucleotide

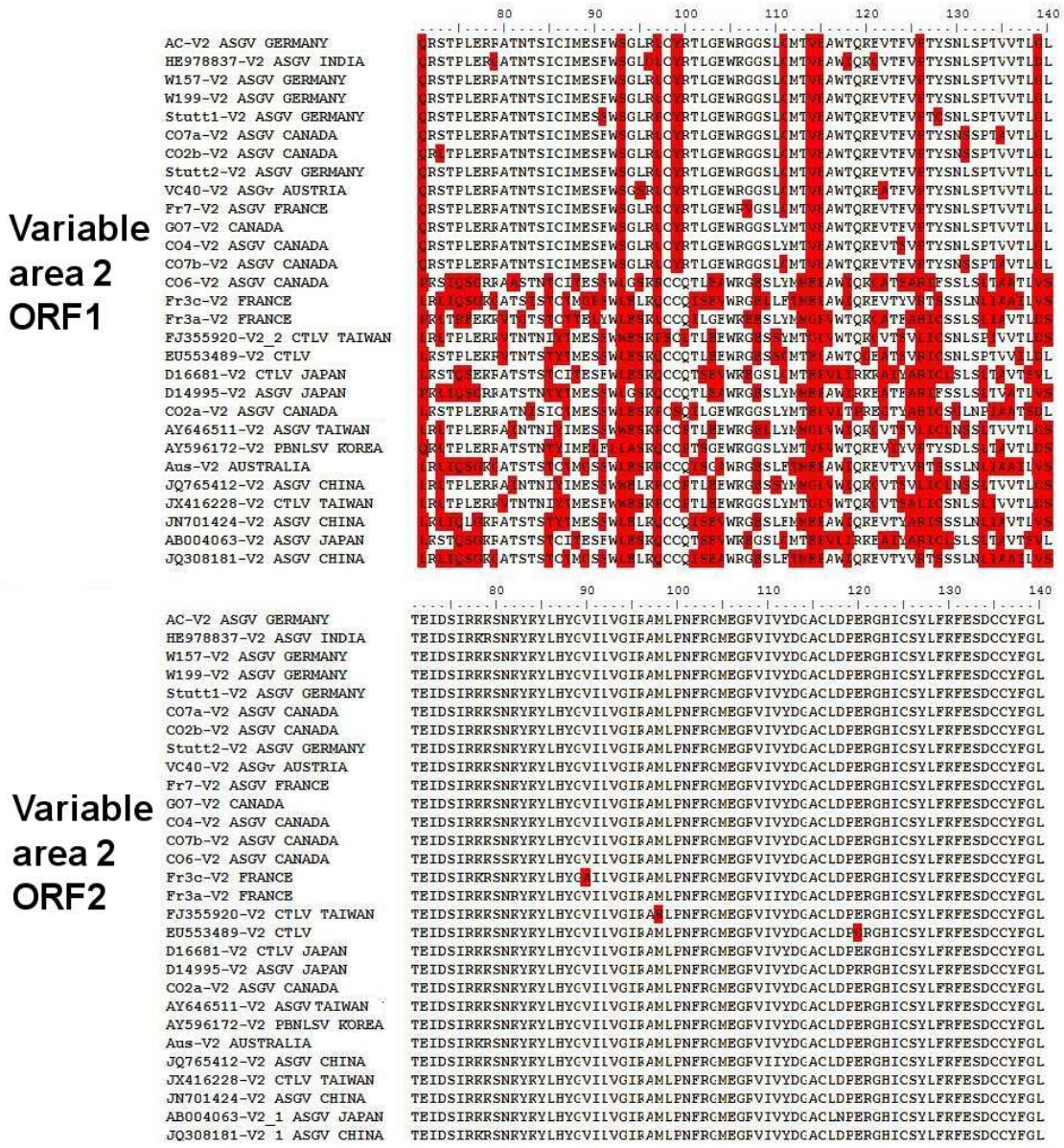

Figure 13: **Sequence alignment corresponding to V2 region.** The variable amino acids are indicated in red. Variability found in ORF1 is silent in ORF2.

Phylogenetic analysis of the V2 regions from the different isolates revealed a similar tree topology as the one observed with V1 and were consistent between the two reconstruction methods used (maximum likelihood and neighbor joining). In these trees, internal branches were associated with low bootstrap values while branches at intermediate levels were particularly long and had high bootstrap values (fig. 14). When considering the phylogenetic clustering of the ASGV isolates in relation to their hosts, the three citrus isolates clustered together, as did the two

lily isolates. The single pear isolate on the other hand was linked to a separated branch in the tree. Apple isolates of ASGV were grouped into 4 major clusters. However, this apparent larger diversity could also be due to the higher number of sequences from this host species.

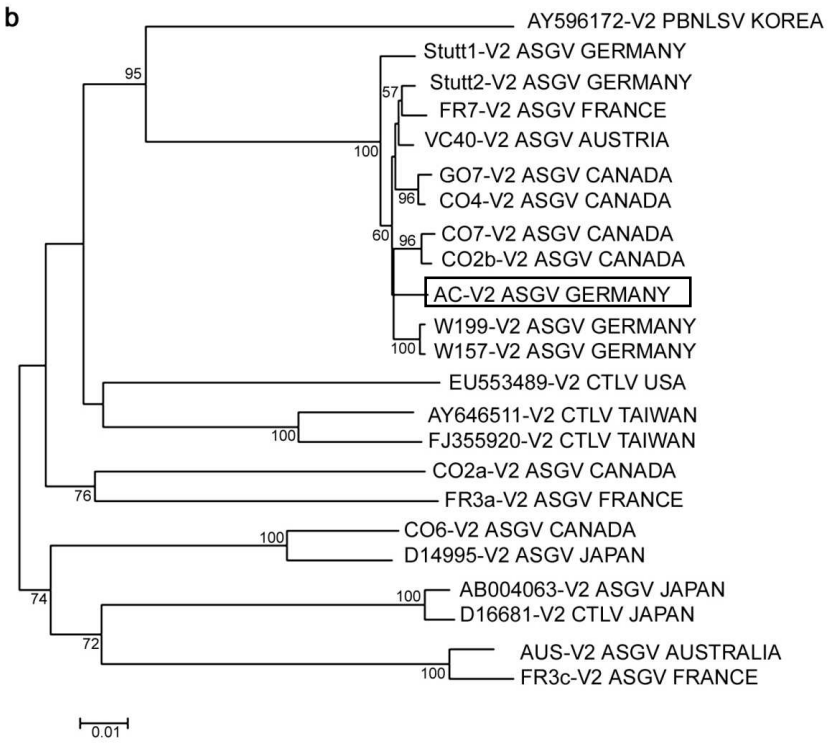

Figure 14: **Neighbor joining phylogenetic trees of the nucleotide sequences of regions and V2 of ASGV.** The German AC isolate sequenced in indicated by the box. Bootstrap percentages above 50% are shown. The scale bar indicates branch lengths in substitutions per nucleotide.

3.1.3 Selection Intensity and Evolutionary Patterns along the ASGV Genome

To analyze the selection intensity and the evolutionary constraints or advantages in the ASGV genome and especially in the 2 variable regions the synonymous/nonsynonymous (dN/dS) mutation rate was analyzed. Nonsynonymous mutations cause a change in the translated amino acid while synonymous mutations do not change the translated amino acid. The comparison between the number of nonsynonymous mutations to the number of synonymous mutations can suggest whether, on the molecular level, natural selection is acting to promote the fixation of advantageous mutations (positive selection) or to remove deleterious mutations (negative or purifying selection). When positive selection dominates the dN/dS ratio is greater than 1 and the

diversity at the amino acid level is favored, likely due to the advantage in fitness provided by the mutation. When purifying selection dominates the dN/dS ration is less than 1 and the deleterious changes are selected against.

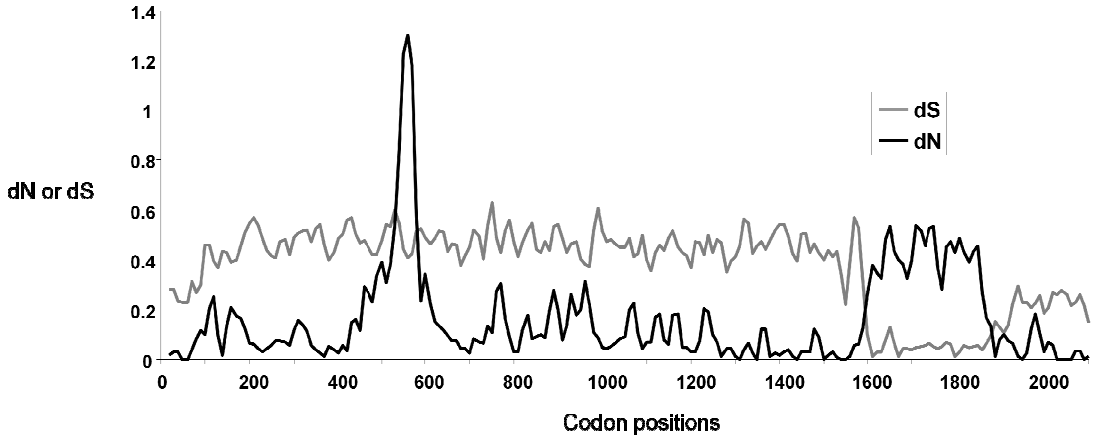

Figure 15: **Sliding-window analysis of rates of synonymous (dS, in gray) and nonsynonymous (dN, in black) substitutions along ASGV ORF1.** The x axis represents the codon positions and the y axis represents the average rates of synonymous or nonsynonymous mutations estimated with a 20 codon window size and a 10 codon step size

A sliding-window analysis of dN and dS along ORF1 using the eight full genome sequences of ASGV showed that negative selection predominated (dN<dS) except in the two regions of higher diversity V1 and V2 identified previously, where dN was higher than dS (fig. 15). Except in these two regions, dN/dS were roughly constant along ORF1. Compared to the background of ORF1, dN increased in region V1 while dS remained constant. By contrast, in the variable region V2, dN increased compared to the background but dS showed a sharp decrease (Fig. 15). This was confirmed by more thorough comparisons of dN and dS in these different regions.

For regions 2 and 4 (fig. 10), no recombination signal was detected with any of the seven methods implemented into RDP2 at the $P = 0.05$ threshold. For regions 3 and 5, weak ($0.001<P<0.01$) recombination signals were detected by three of the seven RDP2 methods only. For region 1, one sequence (accession number AY646511) was detected as recombinant by a majority of the methods (four of seven; $0.001<P<0.01$), but the recombinant region was very short (about 100 nucleotide long). As a consequence, no putative recombination events introduced biases in the positive selection analyses of regions 2 and 4 and only slightly affected

the estimates of dN and dS (regions 1, 3 and 5), which were verified by analyzing datasets where the putative recombinants were withdrawn.

Table 13: **Mean number of nonsynonymous and synonymous nucleotide substitutions per site, with the corresponding standard errors between parentheses, in different regions of ORF1 and in ORF2 of ASGV** (see Fig. 10)

Gene region	Number of sequences	Nucleotide positions [a]	dN [b]	dS [b]	dN/dS
ORF1 region 1	8	36 -1238	0.032 (0.004)	0.649 (0.020)	0.049
ORF1 region 2 (V1)	8	1239 - 1994	**0.165 (0.014)**	0.779 (0.019)	0.212
ORF1 region 2 (V1)	23	1239 - 1994	0.117 (0.010)	0.559 (0.016)	0.209
ORF1 region 2 (V1 sub-region)	8	1629 - 1748	**0.520 (0.027)**	0.718 (0.029)	0.724
ORF1 region 2 (V1 sub-region)	23	1629 - 1748	0.391 (0.021)	0.610 (0.025)	0.641
ORF1 region 3	8	1995 - 4787	0.039 (0.003)	0.729 (0.012)	0.053
ORF1 region 4 (V2)	8	4788 - 5747	**0.158 (0.006)**	**0.078 (0.012)**	2.026
ORF1 region 5	8	5748 - 6350	**0.017 (0.004)**	**0.349 (0.028)**	0.049
ORF2 region 4 (V2)	8	4787 - 5746	0.025 (0.003)	0.521 (0.021)	0.048

[a] According to GenBank accession number D14995
[b] dN or dS values indicated by in bold are significantly different from the background of ORF1 corresponding to regions 1 and 3 using Student t tests with Bonferroni correction for multiple tests

As shown in table 13, the ratio dN/dS between the mean number of nonsynonymous substitutions per site and that of synonymous substitutions was close to 0.05 and remarkably constant in ORF2 and in ORF1 (except in regions V1 and V2 of ORF1). This confirms that ORF1 and ORF2 are overall evolving under strong negative selection. A different feature was observed in regions V1 and V2 of ORF1. In region V1, dN was significantly higher than in the surrounding parts of ORF1 (except V2) but dS was not significantly different (table 13). This was particularly obvious for the region spanning nucleotides 1629 to 1748, for which a dN/dS ratio from 0.64 to 0.72 was estimated. This value, close to unity, suggests that some codon positions in this region might undergo positive selection. In region V2, both dN and dS varied significantly from the background of ORF1 (table 13) and a dN/dS ratio of 2.02 was estimated. Again, this could be

indicative of positive selection. It can also be noticed that in the non-overlapping region of the CP-coding region (region 5 in Table 13), both dS and dN were significantly lower than in the background of ORF1, while dN/dS remained unchanged.

Table 14: **Mean number of nonsynonymous and synonymous substitutions per site and their ratio with the corresponding standard errors between parentheses, in the V2 variable region estimated from the 22 available sequences**

Gene region and ORF	Length (nt)	dN	dS	dN/dS-1 [a]	dN/dS-2 [b]
ORF1, overlapping region 4A (MP/replicase)	852	0.148 (0.005)	0.050 (0.008)	2.960	0.95 (0.26)
ORF2, overlapping region 4A (MP/replicase)	852	0.015 (0.002)	0.501 (0.019)	0.030	0.019 (0.022)
ORF1, overlapping region 4B (MP/CP)	111	0.010 (0.005)	0.146 (0.042)	0.068	0.18 (0.12)
ORF2, overlapping region 4B (MP/CP)	108	0.055 (0.014)	0.018 (0.010)	3.056	0.76 (0.65)

a Estimated with MEGA that does not consider overlapping between ORFs
b Joint estimates in the two overlapping ORFs were obtained with Sabath *et al*.'s method (2008)

Consequently, the method of Sabath *et al*. (2008) for joint estimation of the selection intensity in both ORFs was used. Since ORF2 overlaps with regions of ORF1 which encode two different proteins (the replicase and the CP) of different functions, the estimations were performed separately for region A where the MP gene overlaps with the replicase gene and for region B where the MP gene overlaps with the CP gene (table 14). In overlapping region A, the MP gene ORF (ORF2) appears highly constrained with dN/dS = 0.019, while ORF1 (replicase) shows a neutral pattern of evolution (dN/dS = 0.95 ± 0.26). Opposite results were obtained for overlapping region B. The MP gene ORF (ORF2) appears poorly constrained with dN/dS=0.76 while ORF1 (CP) is highly constrained (dN/dS = 0.18). However, the dN/dS estimate for region B of ORF2 is rather imprecise because of its small length.

Further analyses were performed to confirm or refute previous hypotheses about positive selection. For region V1, analysis of the alignment of the 23 available sequences with PAML did not allow the detection of any codon position under positive selection. The best-fit model M3 in PAML indicated that about 9% of codon positions in this genomic region evolved close to neutrality (dN/dS = 1). In region V2, the use of the dN/dS ratio to estimate selection intensity and

to detect positive selection is problematic since the basic assumption is that synonymous substitutions are neutral is violated because of overlapping ORFs.

3.2 FULL LENGTH cDNA CLONE

3.2.1 Constructing of Full Length cDNA Clones

To construct the full length cDNA clone of ASGV-AC the virus was extracted from infected *M. domestica*, *in vitro* plantlets, and amplified in 9 fragments. Primers were used that incorporated a restriction enzyme recognition site at the 3' and 5' of each fragments. The incorporation of the restriction enzyme sites only produced silent changes, and there was no change in the amino acid sequence. Each restriction enzyme recognition site was unique and not present in the virus or vector sequence. After amplification the fragments were cloned into the TA cloning vector (pT-PCR) sequenced and digested with the appropriate enzyme for sub-cloning into the pCass2 vector (fig. 16). This method was performed for each fragment until the full length ASGV-AC cDNA was assembled in pCass2 (fig. 16). All transformations were performed in the E. coli strain JM110. Some difficulties arose with the ligation of the last fragment (fragment 9) in pCass2. The ligation of fragment 9 (containing the methyltranferase domain) caused rearrangement of the virus cDNA or did not ligate at all. To try and counter this rearrangement event, we changed the formation of assembly. The virus cDNA genome was first ligated (assembled) into two separate pCass2 vectors, after which the two parts were ligated to assemble the full lenght clone, but again it resulted in rearrangement events. The competent *E. coli* strain, SURE (Stop Unwanted Rearrangement Events) lacks components of the pathways that catalyze the rearrangement and deletion of nonstandard secondary and tertiary structures. Cloning of the last fragment was performed in SURE cells and the rearrangement event was overcome and the full length ASGV-AC cDNA was successfully assembled in pCass2.

A similar experience was made with the assembly of full length ASPV cDNA clone. The assembly of all fragments, except the last was performed by at AlPlanta Institute for Plant Research (Barth, 2010). The ligation of the last fragment also resulted in the rearrangement of the ASPV cDNA. After the successful assembling of the ASGV cDNA clone using the SURE cells, I corrected and successfully assembled the ASPV cDNA clone.

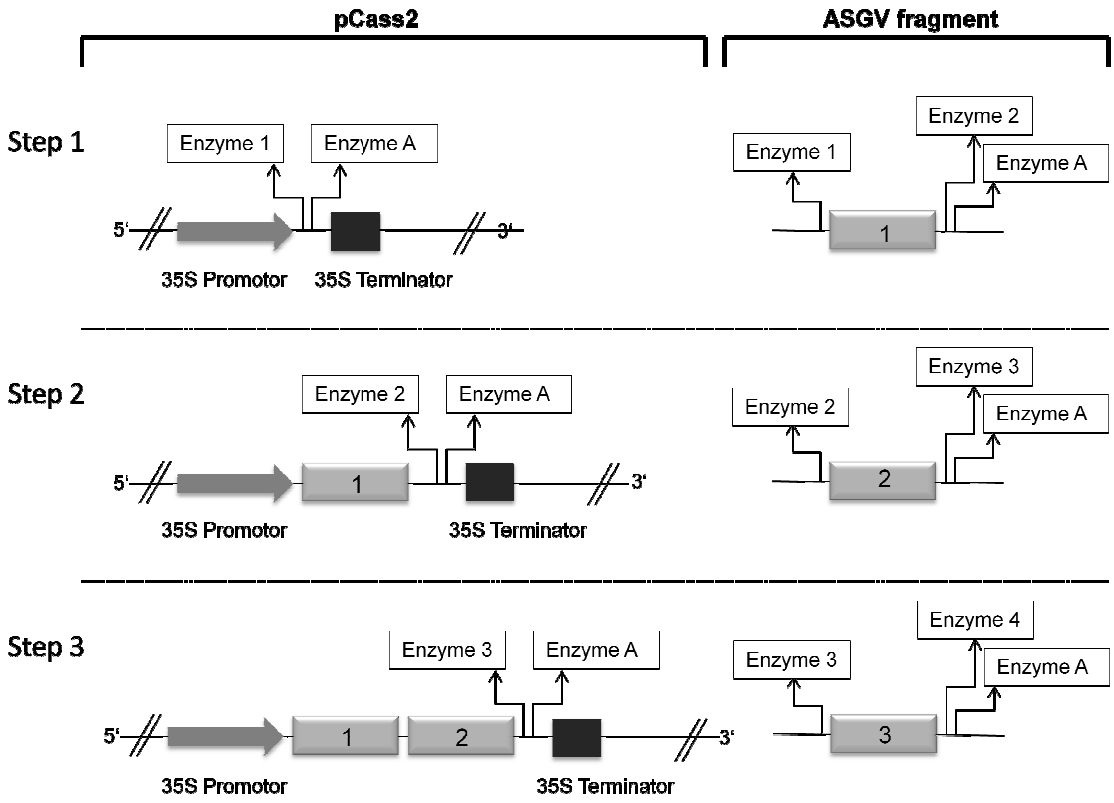

Figure 16: **Schematic presentation of the fragment assembling strategy. Step 1:** The pCass2 vector and the first fragment were digested with enzyme 1 and A, and ligated. The restriction enzyme recognition site for Enzyme 2 was incorporated in fragment 1.**Step 2:** The pCass2 vector and fragment 2 were digested with Enzyme 2 and A, and ligated. The restriction enzyme recognition site for Enzyme 3 was incorporated in fragment 2. **Step 3:** The pCass2 vector and fragment 3 were digested with Enzyme 3 and A, and ligated. The restriction enzyme recognition site for Enzyme 4 was incorporated in fragment 3. The strategy was continued until all 9 fragments were assembled in pCass2.

3.2.2 Testing for Infectivity

The full length cDNA clones (pCass2) were tested for infectivity by mechanical inoculation (5 µg of cDNA clone per plant) *of N. occidentalis*, *N. glutinosa* and *C. quinoa*, diagnostic host species of ASGV and/ or ASPV. Positive controls were performed by mechanically inoculating the diagnostic hosts with virus infected *Malus* sap. Negative controls were performed using virus-free *Malus* sap. No symptom expression was observed in plants infected with the cDNA clones and all plants tested negative with RT-PCR as well as ELISA. Positive controls tested positive for ASGV and ASPV respectively and showed stunting, leaf deformation and yellowing (fig. 17).

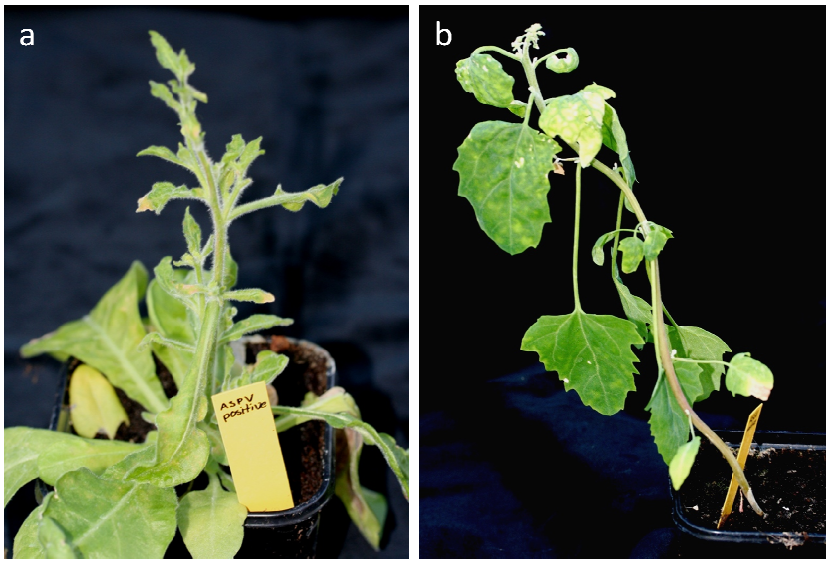

Figure 17: **Symptoms expressed after mechanical inoculation of ASGV-AC and ASPV-LFP infected sap. a**) *N. occidentalis* showing leaf deformation, stunting and yellowing after inoculated with ASPV-LFP infected *Malus* sap. b) *C. quinoa* showing chlorotic spots and leaf deformation after inoculated with ASGV-AC infected *Malus* sap.

As no infectivity was observed with mechanical inoculation, the ASGV and ASPV full length cDNA was cloned into the pPXP200 binary vector for agro-infiltration of *N. occidentalis* and *C. quinoa*. None of the plants showed any symptoms and tested negative for the respective viruses. Both cDNA clones were sequenced and no unwanted mutations, deletions or insertions in the viral cDNA were observed.

3.3 ESTABLISHMENT OF AN IN VITRO CULTURE SYSTEM FOR STUDING VIRUS-VIRUS AND VIRUS-PHYTOPLASMA INTERACTION ON DIFFERENT MALUS GENOTYPES

3.3.1 Pathogen-free and Virus and/or Phytoplasma infected *Malus* Genotypes

Most of the pathogen-free *Malus* genotypes used in this study were already established micro-propagated plantlets maintained at AlPlanta, Institute for Plant Research (table 4). New virus-and-phytoplasma-free hybrids were received during the study and their culture was initiated. Each culture line was established from a single tree, and not from different seedlings. This is important because the recombination event for each seedling from the same parents could be different and it is important to have a homogenous culture line that could be maintained over years for comparable data. *M. domestica* cultures infected with ASGV-AC, '*Ca*. P. mali' strains

PM4+ASGV-AC, PM5 and PM6 were already established by AlPlanta, Institute for Plant Research. After successful transmission through *in vitro* grafting of the different pathogens to the different *Malus* genotypes, the plantlets were tested for the presence of the appropriate pathogen every 6 weeks, to confirm the infection. After 6 months these cultures were considered as homogenously infected, replicated and used as inoculum or for further tests.

In this study, 1180 grafts were performed from which 771 (including healthy controls) were considered to be successful by testing the graft strength, and the infectivity of the inoculum (when appropriate). Ten different *Malus* genotypes were tested and graft inoculated with 12 combinations of ASGV, ASPV, and '*Ca*. P. mali' (Appendix C1). Two different ASGV isolates and 3 different '*Ca*. P. mali' strains were used in this study. At the end of the study 98 combinations were tested. Some of the *Malus* genotypes were only received later in the study and not all pathogen combinations could be graft-inoculated, due to the time constraint of the study. Of the 625 successful grafts 309 (49%) tested positive of a period of 6 months for the appropriate pathogen.

The percentage of hypersensitivity (3-8%) and transmission rate (40-65%) was not influenced by the season and we could conclude that it is dependent on the *Malus* genotype and the inoculums used (Appenedix 7B).

3.3.2 Successful Transmission of ASGV and ASPV through *in vitro* Grafting

Micro-propagated *M. domestica* infected with ASGV-AC and ASPV-LFP were the main isolates used in this study. ASGV-AC and ASPV-LFP were associated with the decline and death observed in the field trials of the potential Apple Proliferation resistant rootstocks and to reproduce the symptoms observed in the field trials in the *in vitro* system, the same virus isolates were used. Only grafts with a strong graft union were considered as successful and used for further analysis. The inoculum graft tips were tested for the desired pathogen when the rootstock tested negative for the transmission. Grafts whose tips tested negative were also not considered successful and were not included in the data.

ASGV-AC was successfully transmitted to different *Malus* genotypes. The transmission rate was dependent on the rootstock genotype, with the hybrid genotypes W355, B89 and *M. sieboldii*

with the highest transmission rate of 83, 69 and 50% respectively. It was not possible to transmit ASGV-AC to the hybrid genotype CAT5 (table 15). For comparison another German isolate from Stuttgart (ASGV-stutt) was also transmitted to different *Malus* genotypes (table 16). The transmission rate for ASGV-stutt was the highest in the hybrid genotype B89 with 89% and a 50% transmission rate for hybrid genotypes D45, B323, W355 and *M. domestica* cv. Rubinette. No transmission to the hybrid genotype CC38 and a very low transmission rate to CAT5 (8%) was observed. A higher overall transmission rate was observed for ASGV-stutt compared to ASGV-AC.

Table 15: **Transmission and survival rate of the different *Malus* genotypes graft-inoculated with the ASGV-AC isolate.**

Rootstock	Tip	Inoculum	Amount of successful grafts	ASGV positive (%)	3 mpi	6 mpi
CAT5	Rubinette	ASGV-AC	6	0%	nt	nt
Golden Del	Rubinette	ASGV-AC	30	10%	100%	100%
O45	Rubinette	ASGV-AC	9	11%	100%	100%
D45	Rubinette	ASGV-AC	11	18%	100%	100%
B323	Rubinette	ASGV-AC	10	30%	100%	100%
Rubinette	Rubinette	ASGV-AC	6	33%	100%	100%
CC38	Rubinette	ASGV-AC	12	42%	100%	80%
M. sieboldii	Rubinette	ASGV-AC	10	50%	80%	80%
B89	Rubinette	ASGV-AC	16	69%	100%	36%
W355	Rubinette	ASGV-AC	6	83%	83%	17%

The survival of the newly established cultures were also recorded, the survival rate 3 and 6 months post transmission/inoculation (mpi) were between 80-100% for most genotypes infected with ASGV-AC (table 15). The two genotypes that showed the highest ASGV-AC transmission rate showed the lowest survival rate 6 mpi: B89 (36%) and W355 (17%). The survival rate for the plantlets infected with the ASGV-stutt isolate showed a survival rate of between 25-100% 3 mpi, with only 25% for *M. sieboldii* and D45 (table16). After 6 months the survival rate of most cultures was lower than after 3 mpi. Only two cultures had a 100% survival rate, CAT5 and W355. None of the ASGV-stutt infected *M. sieboldii* survived 6mpi.

Table 16: **Transmission and survival rate of the different *Malus* genotypes graft-inoculated with the ASGV-stutt isolate.**

Rootstock	Tip	Inoculum	Amount of successful grafts	ASGV positive (%)	3 mpi	6 mpi
CC38	Rubinette	ASGV-stutt	4	0%	na	na
CAT5	Rubinette	ASGV-stutt	12	8%	100%	100%
O45	Golden Del	ASGV-stutt	16	19%	100%	33%
Golden Del	Rubinette	ASGV-stutt	24	25%	66%	50%
M. sieboldii	Golden Del	ASGV-stutt	16	25%	25%	0%
B323	Rubinette	ASGV-stutt	8	50%	100%	75%
D45	Rubinette	ASGV-stutt	10	50%	25%	25%
Rubinette	Golden Del	ASGV-stutt	6	50%	66%	66%
W355	Rubinette	ASGV-stutt	4	50%	100%	100%
B89	Rubinette	ASGV-stutt	9	89%	100%	77%

ASPV-LFP was also transmitted to different *Malus* genotypes and the transmission and survival rates were recorded (table 17). The transmission rate was between 14-100% depending on the genotype. The highest transmission rate was observed in *M. sieboldii* (100%) and O45 (80%), with an 80 and 40% survival rate respectively, 6 mpi. The transmission rate was the lowest in genotype D45 (17%) and the *M. domestica* cv. Golden Delicious (GD) (14%). The transmission rate was much higher for ASPV-LFP compared to ASGV in genotypes *M. sieboldii*, CAT5 and O45. The survival rate of the different genotypes infected with ASPV-LFP was between 40-100% 3 mpi and declined 6 mpi. Golden delicious had a very low transmission and survival rate, although the inoculum culture used for the transmissions was an established Golden delicious genotype infected with ASPV-LFP.

In most of the potential Apple Proliferation resistant rootstocks that showed decline and death in the field trials, more than one latent virus was observed. The multiple transmissions were grouped into two pathways; co-transmission and super-transmission. Co-transmission is when two or more pathogens were simultaneously transmitted to the micro-propagated *Malus* cultivar. In super-transmission different pathogens were transmitted to the micro-propagated *Malus* cultivar at different times. The effect of the second pathogen in co-transmission and super-transmission trials on the transmission could indicate how these pathogens affect the defense system of the plant.

Table 17: **Transmission and survival rate of the different *Malus* genotypes graft-inoculated with the ASPV-LFP isolate.**

Rootstock	Tip	Inoculum	Amount of successful grafts	ASPV positive (%)	3 mpi	6 mpi
B323	Golden Del	ASPV-LFP	10	70%	100%	85%
B89	Golden Del	ASPV-LFP	10	60%	100%	50%
CAT5	Golden Del	ASPV-LFP	9	67%	67%	33%
CC38	Golden Del	ASPV-LFP	10	50%	40%	20%
D45	Golden Del	ASPV-LFP	6	17%	100%	100%
Golden Del	Golden Del	ASPV-LFP	7	14%	100%	0%
M. sieboldii	Golden Del	ASPV-LFP	5	100%	100%	80%
O45	Golden Del	ASPV-LFP	5	80%	50%	50%
Rubinette	Golden Del	ASPV-LFP	10	60%	83%	83%
W355	Golden Del	ASPV-LFP	7	71%	100%	40%

First *M. domestica* cv. Rubinette cultures, infected with one pathogen, were super-infected by graft-inoculation with ASGV-AC, ASGV-stutt, ASPV-LFP or '*Ca.* P. mali' (table 18). These double infected lines were then used as inoculum to co-transmit both pathogens to the different genotypes. '*Ca.* P. mali' strain PM6 had a 100% transmission rate to Rubinette infected with ASGV-AC compared to a transmission rate of only 20% when it was transmitted to virus-free Rubinette. The ASGV-stutt transmission rate was enhanced from 50 to 90% when super-transmitted to Rubinette ASPV-LFP. The transmission rate of ASPV-LFP to Rubinette was also enhanced (from 60 to 100%) when super-transmitted to Rubinette ASGV-AC, while ASGV-stutt and PM6 had no effect. In all cases a 100% survival rate was observed 6mpi.

In the ASGV-AC + ASPV-LFP co-transmission trials CC38 and GD showed a 100 and 80% transmission rate respectively for both viruses, while CAT5 could not be infected. *M. sieboldii* showed a 50% transmission rate for both viruses, the same as for ASGV-AC alone, with half the plants surviving 6 mpi (table 19). For the combination ASGV-stutt + ASPV-LFP the co-transmission rate was lower than with the combination ASGV-AC + ASPV-LFP and single virus transmissions were more prevalent (table 22). In *M. sieboldii* only ASPV-LFP was transmitted.

Table 18: Transmission and survival rate of the different *Malus* genotypes graft-inoculated with ASGV-AC + ASPV-LFP

			ASGV			ASPV			ASGV & ASPV		
Rootstock	**Inoculum**	**Successful grafts**	**%**	**3 mpi**	**6 mpi**	**%**	**3 mpi**	**6 mpi**	**%**	**3 mpi**	**6 mpi**
CAT5	ASGV-AC + ASPV	5	0%	na	na	0%	na	na	0%	na	na
CC38	ASGV-AC + ASPV	6	100%	83%	0%	100%	83%	0%	100%	83%	0%
Golden Del	ASGV-AC + ASPV	5	80%	100%	100%	80%	100%	100%	80%	100%	100%
M. sieboldii	ASGV-AC + ASPV	4	50%	50%	50%	50%	50%	50%	50%	50%	50%
O45	ASGV-AC + ASPV	6	17%	100%	0%	83%	83%	83%	17%	100%	0%

Table 19: **Transmission and survival rate of the different *Malus* genotypes graft-inoculated with ASGV-stutt + ASPV-LFP**

			ASGV			ASPV			ASGV & ASPV		
Rootstock	**Inoculum**	**Successful grafts**	**%**	**3 mpi**	**6 mpi**	**%**	**3 mpi**	**6 mpi**	**%**	**3 mpi**	**6 mpi**
M. sieboldii	ASGV-stutt + ASPV	1	0%	na	na	100%	100%	100%	0%	na	na
CAT5	ASGV-stutt + ASPV	7	43%	67%	67%	71%	80%	60%	29%	100%	100%
CC38	ASGV-stutt + ASPV	6	33%	100%	100%	33%	50%	50%	0%	na	na
D45	ASGV-stutt + ASPV	2	100%	0%	0%	50%	0%	0%	50%	0%	0%
W355	ASGV-stutt + ASPV	4	50%	100%	100%	75%	100%	67%	50%	100%	100%

Table 20: Transmission and survival rate of the different *Malus* genotypes graft-inoculated with ASGV-AC and the 'Ca. P. mali' strain PM4

			ASGV			'Ca. P. mali' (PM4)			ASGV & PM4		
Rootstock	**Inoculum**	**Successful grafts**	**%**	**3 mpi**	**6 mpi**	**%**	**3 mpi**	**6 mpi**	**%**	**3 mpi**	**6 mpi**
B323	PM4 + ASGV-AC	8	100%	50%	50%	38%	100%	100%	38%	100%	100%
B89	PM4 + ASGV-AC	8	75%	100%	67%	38%	100%	67%	38%	100%	67%
CAT5	PM4 + ASGV-AC	5	0%	na	na	0%	na	na	0%	na	na
CC38	PM4 + ASGV-AC	8	25%	100%	100%	25%	100%	100%	25%	100%	100%
D45	PM4 + ASGV-AC	2	50%	100%	100%	50%	100%	100%	50%	100%	100%
Golden Del	PM4 + ASGV-AC	13	31%	100%	75%	54%	71%	57%	23%	100%	67%
M. sieboldii	PM4 + ASGV-AC	7	0%	na	na	14%	100%	0%	0%	na	na
O45	PM4 + ASGV-AC	5	0%	na	na	20%	100%	0%	0%	na	na
W355	PM4 + ASGV-AC	3	67%	100%	100%	0%	na	na	0%	na	na

Genotype CC38 also showed no co-transmission and only ASGV-stutt or ASPV-LFP was transmitted. There was also a difference in transmission rate of ASGV alone depending on the isolate in certain genotypes. The transmission rate of ASGV-AC to *M. sieboldii* alone (50%) or when in combination (50%) with ASPV-LFP was much higher than the transmission rate of ASGV-stutt alone (25%) or in combination (0%) with ASPV-LFP.

Table 21: Super-transmissions performed on *M. domestica* cv. Rubinette

Rootstock Status	Inoculum	Number of successful grafts	Positive for Inoculum Pathogen (%)	3 mpi	6 mpi
ASGV-AC	PM6	7	100%	100%	100%
PM6	ASGV-stutt	10	90%	100%	100%
PM6	ASPV-LFP	6	50%	100%	100%
ASGV-AC	ASPV-LFP	7	100%	100%	100%
ASGV-stutt	ASPV-LFP	12	75%	100%	100%

The effect '*Ca.* P. mali' strain PM4 on the co-transmission rate of ASGV-AC and the survival rate of the genotypes were also analyzed (table 21). Both pathogens could be co-transmitted to the genotypes Golden delicious (23%), CC38, (25%), B89 (38%), B323 (38%) and D45 (50%). No transmission was observed to the genotype CAT5. While in the genotypes *M. sieboldii* (14%) and O45 (20%) a low transmission rate of PM4 alone was observed, but these plants did not survive 6 mpi. ASGV-AC could be transmitted to *M. sieboldii* alone or in combination with ASPV-LFP but no transmission was observed in combination with '*Ca.* P. mali' strain PM4. Grafting experiments to transmit the '*Ca.* P. mali' strains PM5 and PM6 to *M. sieboldii* were successful but all transmissions with the addition of ASGV-AC or ASPV-LFP were unsuccessful.

Interestingly, even with the addition of ASPV-LFP or '*Ca.* P. mali' strain PM4 in the co-transmission trails, some of the hybrid genotypes acted similar as they did when infected with ASGV-AC or ASGV-stutt alone, for example: the hybrid genotype CAT5 could not be infected with ASGV-AC regardless whether it was graft-inoculated alone or in combination with ASPV-LFP or '*Ca.* P. mali' strain PM4. Similar results were observed in the hybrid genotypes O45 (low or no transmission rate of ASGV-AC alone or in combination with ASPV-LFP or '*Ca.* P. mali' strain PM4). Here it is important to note that both genotypes CAT5 and O45 have a similar genetic background: the entire genome 4551 (see table 3). In contrast, high transmission rates of

ASGV-AC alone or in combination with ASPV-LFP or '*Ca.* P. mali' strain PM4 strain were observed for the genotype CC38 while transmission of ASGV-stutt was always low. This could indicate that the hypersensitivity of the hybrid genotypes towards ASGV was not influenced by a second pathogen. The opposite observation was made in the ASGV tolerant *M. domestica* cv. Golden Delicious, where the addition of a second pathogen in the co-transmission trials enhanced the transmission rate for ASGV-AC as well as for ASPV-LFP.

3.3.3 Stable Virus Culture Lines

The variable area 1 of ASGV-AC was sequenced 3 times over a period of 3 years (2008, 2009, 2010) and the ASPV-LFP coat protein 2 times over a period of 2 years (2010, 2012). Sequence comparisons for both the ASGV variable area 1 and the ASPV coat protein show an identity of 100% for all sequences obtained respectively. These results provide prove that a define isolate could be stably maintained in *in vitro Malus* plantlets.

3.4 HYPERSENSITIVITY ANALYSIS OF THE MALUS GENOTYPES INFECTED WITH ASGV, ASPV AND/OR 'CA. P. MALI'

All the *in vitro* grafting experiments were analyzed for graft union necrosis, an indication of hypersensitivity to ASGV and ASPV (fig. 18). Necrosis and or death of the graft tip alone were not considered, because it could be due to an insufficient connection at the graft union. A weak graft connection could hamper the flow of nutrients to the graft tip and cause necrosis and death. ASGV, ASPV and '*Ca.* P. mali'-free grafts were performed as control for all graft transmission experiments (table 22). The 'healthy' grafts showed no necrotic reaction except for *M. sieboldii* that had an 8% incidence. Some of the grafts showed graft union necrosis but died before it could be tested for the appropriate pathogen; in these cases the data were not included as successful graftings as the transmission of the pathogen to the rootstock could not be confirmed, but the necrosis and rapid death of the rootstocks were also considered as a hypersensitive reaction. No hypersensitive reaction was observed in the *M. domestica* cv. Golden Delicious and Rubinette as well as the *M. sieboldii* derived hybrids B323 and B89 (table 22).

Table 22: **Percentage of necrotic reaction at the graft union of the different *Malus* genotypes to the different pathogen combinations**

	Healthy	ASGV-AC	ASGV-stutt	ASPV-LFP	ASGV-AC + ASPV-LFP	ASGV-stutt + ASPV-LFP	ASGV-AC + PM4
Golden Del	0%	0%	0%	0%	0%	-	0%
Rubinette	0%	0%	0%	0%	-	-	-
B323	0%	0%	0%	10%	-	-	0%
B89	0%	0%	0%	0%	-	-	0%
W355	0%	17% (14%)	0%	0%	-	0%	0%
D45	0%	0% (21%)	0%	17% (33%)	-	0%	0%
O45	0%	0%	19% (15%)	0%	17%	-	20% (17%)
CC38	0%	0%	0%	0%	0% (14%)	33%	0%
CAT5	0%	0% (25%)	25%	0%	0%	0%	0% (38%)
M. sieboldii	8%	0% (9%)	40% (6%)	0% (29%)	0%	0%	43% (13%)

Values in brackets are the percentage of rootstocks that showed necrosis and sudden death.

M. sieboldii micro-propagated plants showed a high percentage of hypersensitivity when grafted with ASGV, ASPV, and ASGV-AC + '*Ca*. P. mali' strain PM4. Local or graft union necrosis was observed associated with both latent viruses. The necrotic reaction was divided into two groups. Plants that showed necrosis and tested positive, and plantlets that showed necrosis and sudden death (indicated between brackets on table 22). Only 9% of the *M. sieboldii* plantlets infected with ASGV-AC and 29% of the ASPV-LFP infected plantlets showed necrosis and plant death, while most of the infected *M. sieboldii* infected plantlets showed no necrotic reaction and survived 6mpi and could be maintained. None of the plantlets infected with ASGV-AC or ASPV-LFP that showed necrosis survived. ASGV-stutt induced an initial necrotic reaction after which the plantlets recovered from the initial symptoms, were homogenously infected and could be maintained. In the case of ASGV-AC + '*Ca*. P. mali' strain PM4, 43% of the plants showed graft union necrosis preventing further virus propagation.

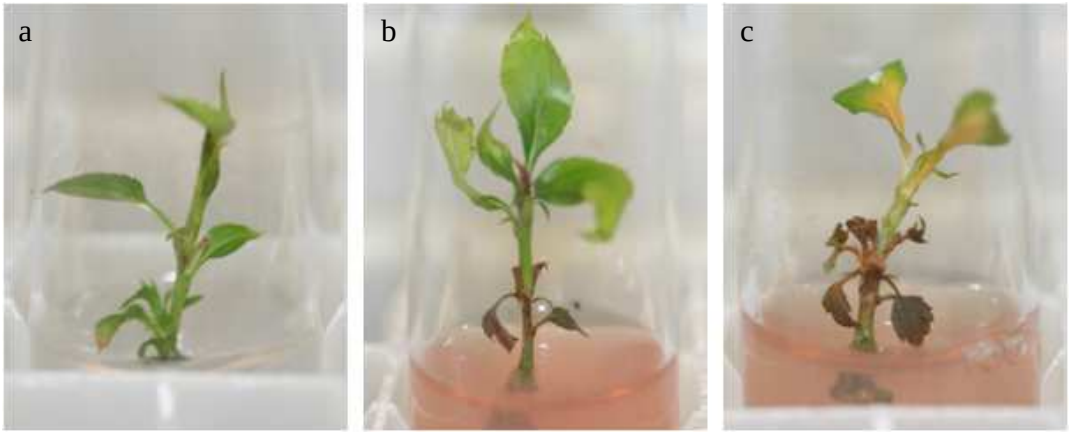

Figure 18: **Graft union analysis one month post graft transmission.** a) Healthy graft union showing no graft union necrosis. b) Graft union necrosis c) severe graft union necrosis.

The hybrid genotypes W355, D45, O45, CC38 and CAT5 showed hypersensitivity of between 0-38% depending on the inoculum. W355 only showed necrosis associated with ASGV-AC. CC38 reacted hypersensitive only in association with ASPV-LFP, whereas CAT5 reacted hypersensitive in the presence of ASGV but only if ASPV-LFP is absent. O45 and CAT5 were the only hybrids that reacted hypersensitive to ASGV-AC + '*Ca*. P. mali' strain PM4

3.5 GROWTH AND SYMPTOM ANALYSIS

After homogenous culture lines were established with different combinations of ASGV, ASPV and '*Ca.* P. mali', the effect thereof on the growth and symptom expression, were analyzed. All the plantlets were subcultured under to approximately 1 cm in size and grown for 6 weeks under the same conditions (section 2.4.3). The growth and symptom expression was then recorded by measuring the leave size, the height and the amount of shoots which is indicative for the vigor of the plantlets. In all experiments a pathogen free culture was included as control. All the data obtained were statistically analyzed. Different combinations of pathogens on the same genotype were analyzed to determine the effect on the genotype. In other cases the same pathogen was analyzed on different genotypes to see how the different genotypes respond to the same pathogen (table 5). Data obtained from the biostatistics program R for all growth analyses is summarized in Appendix B.

Figure 19: ***M. domestica* cv. Rubinette plantlets infected with different pathogens subcultured to 1 cm.**

3.5.1 ASGV Isolates on *M. domestica* cv. Royal Gala

M. domestica cv. Royal Gala infected with 5 different Canadian isolates were established through culture initiation from previous graft inoculated ex-vitro plants and three German isolates were transmitted to healthy Royal Gala plantlets through *in vitro* grafting. After all the infected Royal Gala plantlets were established and homogenously infected, they were subjected to analysis. The experiment was repeated three times (10 cultures per isolate times 3) and the data pooled for statistical analysis. Statistical differences were observed in all three aspects analyzed.

Table 23: **Symptom expression data and statistical difference of Royal Gala infected with different ASGV isolates.**

Pathogen (s)	Amount of shoots		Shoot height		Leaf size	
	Mean	Statistical difference	Mean	Statistical difference	Mean	Statistical difference
Virus-free	2.44	c	1.22	d	0.50	de
ASGV-AC	3.44	bc	1.50	cd	0.73	c
ASGV-CO2	2.70	c	2.13	bc	0.55	d
ASGV-CO4-1	4.75	a	1.55	cd	0.50	de
ASGV-CO4-2	2.47	c	2.53	b	0.97	b
ASGV-CO6	3.6	bc	1.24	d	1.57	a
ASGV-CO7	4.15	ab	1.63	c	0.64	cd
ASGV-stutt	3.00	bc	1.20	d	0.81	bc
ASGV-W157	4.00	ab	4.00	a	0.30	e

a-e Each letter represents a statistical different mean value

The Kruskal-Wallis chi-square test showed statistical differences in the mean amount of shoots per culture, grouping the isolates in 3 statistically different categories (table 23). The virus-free, CO2 and CO4-2 isolates showed the lowest amount of shoots (2.4-2.7 shoots per culture) while the W157, CO4-1, and CO7 isolates showing the highest amount of shoots per culture (4.0-4.75). Statistical difference was also observed in the mean shoot height, grouping the isolates in 4 statistical groups. The average height for the isolates was between 1.2 and 2.5 cm for most cultures except for CO6, which grew to a mean size of 4 cm. The mean leaf size of the ASGV infected Royal Gala was between 0.3 (W157) and 1.6 cm (CO6) and grouped the isolates in 5 statistical different categories. For most of the cultures a mean of 0.5-0.8 cm were recorded, similar to that of the virus-free control. No extreme values were recorded for ASGV-AC and ASGV-stutt infected cultures.

3.5.2 The Effect of a Second Latent Virus on the *M. domestica* cv. Rubinette

The same parameters (shoot proliferation, height and leaf size) were also analysed for *M. domestica* cv. Rubinette infected with ASGV-AC or ASGV-stutt with or without ASPV-LFP. This was done to analyse the effect of the ASGV on symptom expression if a second virus is present. Although a slighly higher mean amount of shoots was observed with the addition of ASPV, it was not statistical significant. Statistical differences were observed when the shoot height was analysed (table 24). In both cases the addition of ASPV-LFP slightly increased the height of the cultures infected with ASGV alone. The leaf size was more variable between the

different combinations ranging from an avarage size of 0.54 to 0.85 cm. The leaf size was slightly bigger in Rubinette ASGV-stutt + ASPV-LFP compared to ASGV-stutt alone, while the leaf size was slightly reduced in ASGV-AC + ASPV-LFP compared to ASGV-AC alone.

Table 24: **Symptom expression data and statistical difference of Rubinette infected with ASGV alone compared to ASGV co-infected with ASPV-LFP**

Pathogen (s)	Amount of shoots		Shoot height		Leaf size	
	Mean	Statistical difference	Mean	Statistical difference	Mean	Statistical difference
Pathogen free	2.96	na	2.37	bc	0.68	b
ASGV-AC	2.82	na	2.12	bc	0.82	a
ASGV-stutt	2.79	na	2.04	c	0.54	c
ASGV-AC + ASPV-LFP	3.05	na	3.05	a	0.70	b
ASGV-stutt + ASPV-LFP	2.92	na	2.54	b	0.85	a

a-c Each letter represents a statistical different mean value. Na, not applicable, no statistical difference observed.

3.5.3 The Effect of ASGV on the Reaction of *M. domestica* cv. Rubinette Infected with '*Ca.* P. mali'

It is important to know if the addition of ASGV-AC is effecting the symptom expression of Rubinette when '*Ca.* P. mali' is present, and could be an indication of the plant response to the addition of a second pathogen (table 25). *M. domestica* cv. Rubinette infected with '*Ca*. P. mali' strain PM5 showed a slight enhancement of shoot proliferation when co-infected with ASGV-AC. No change in shoot height or leaf size was observed in *M. domestica* cv. Rubinette co-infected with '*Ca.* P. mali' and ASGV-AC compared to *M. domestica* cv. Rubinette infected with '*Ca.* P. mali' alone. Statistical difference was however observed when '*Ca.* P. mali' strains were compared. With PM6 showing less stunted growth and bigger leaf sizes when compared to the strains PM4 and PM5. The data indicate that the presence of ASGV-AC has little effect on the symptom expression of the plantlets when infected with '*Ca.* P. mali'.

Unfortunately '*Ca.* P. mali' strain PM4 without ASGV-AC was only recently established and not included in this study, but no differences were observed when PM4 was compared with PM4 + ASGV-AC cultures (data not shown). In all three measurements a statistical difference was observed when the '*Ca.* P. mali' infected cultures were compared to the virus and phytoplasma free control (fig. 20).

Table 25: **Symptom expression data and statistical difference of Rubinette infected with different '*Ca.* P. mali' strains alone compared to co-infections with ASGV-AC**

Pathogen (s)	Amount of shoots		Shoot height		Leaf size	
	Mean	Statistical difference	Mean	Statistical difference	Mean	Statistical difference
Pathogen free	2.96	b	2.37	a	0.85	a
ASGV-AC + PM4	4.07	a	1.07	c	0.25	c
PM5	3.06	b	1.12	c	0.23	c
ASGV-AC + PM5	4.26	a	1.05	c	0.25	c
PM6	4.31	a	1.49	b	0.41	b
ASGV-AC + PM6	4.23	a	1.47	b	0.40	b

a-c Each letter represents a statistical different mean value.

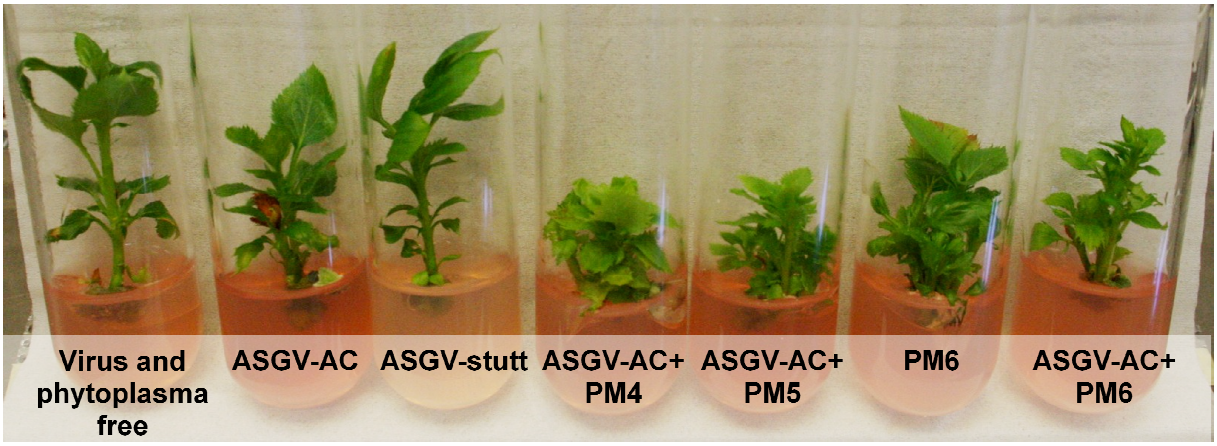

Figure 20: Symptom expression of *M. domestica* cv. Rubinette with different combinations of ASGV and '*Ca.* P. mali' strains

3.5.4 The effect of ASGV-stutt, ASGV-AC and ASPV-LFP on the Reaction of *M. domestica* cv. Rubinette infected with '*Ca.* P. mali' strain PM6

After the conclusion that the ASGV-AC isolate had no effect on the severity of the symptoms caused by '*Ca.* P. mali' infection, the effect of another ASGV isolate (stutt) and ASPV-LFP on the symptom expression was analyzed. The '*Ca.* P. mali' strain PM6 without the addition of a virus was compared to PM6 co-infected with ASGV-AC, ASGV-stutt or ASPV-LFP. No statistical difference was observed in all three variables leading to the conclusion that the symptom expression of '*Ca.* P. mali' is not enhanced or reduced by the addition of ASGV or ASPV (Appendix 4B).

3.5.5 The effect of ASGV-AC on *M. sieboldii*, *M. domestica* cv. Rubinette and the Hybrid Culture CC38.

To analyze the effect of ASGV-AC on a tolerant and sensitive genotype and a potential apple proliferation resistant hybrid genotype, the symptom expression was monitored for ASGV-AC infected Rubinette, *M. sieboldii* and CC38 (table 26). A virus-free control of each genotype was also included. No statistical difference was found in the amount of shoots per genotype. For the shoot height analysis *M. sieboldii* and CC38 showed some decrease in shoot height for the cultures infected with ASGV-AC compared to the virus-free cultures. In the *M. domestica* cv. Rubinette trial, the ASGV-AC infected culture had a higher shoot height compared to the virus-free culture. No statistical difference between the ASGV-AC infected and virus-free Rubinette and *M. sieboldii* cultures were observed in the leaf size examination. Statistical difference was observed for the CC38 culture, with the virus-free culture showing bigger leafs when compared to the ASGV-AC-infected culture.

Table 26: **Symptom expression data and statistical difference of different *Malus* genotypes infected with ASGV-AC compared to virus-free *Malus* genotypes.**

Genotype/Pathogen (s)	Amount of shoots		Shoot height		Leaf size	
	Mean	Statistical difference	Mean	Statistical difference	Mean	Statistical difference
CC38	3.00	na	1.56	cd	0.61	b
CC38/ASGV-AC	2.75	na	1.17	d	0.38	c
M. sieboldii	2.15	na	2.00	bc	0.38	c
M. sieboldii/ASGV-AC	2.90	na	1.43	d	0.42	c
Rubinette	3.10	na	2.35	ab	0.78	a
Rubinette AC	2.95	na	2.73	a	0.79	a

a-d Each letter represents a statistical different mean value. Na, not applicable, no statistical difference observed.

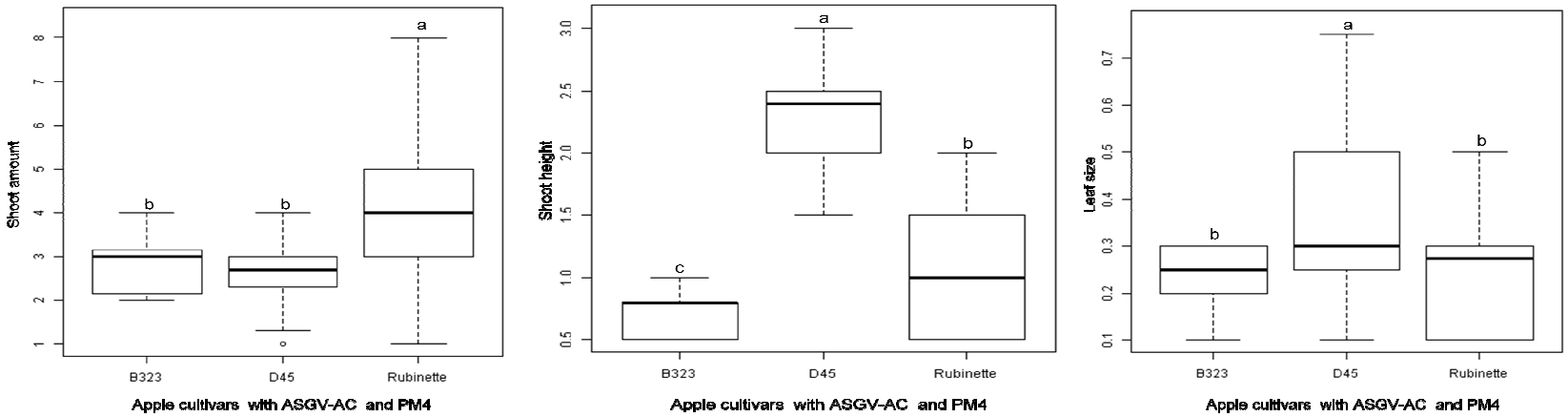

Figure 21: **Boxplot analysis of the symptom expression analysis of the micro-propagated *Malus* genotypes co-infected with PM4 and ASGV-AC**

3.5.6 The effect of '*Ca.* P. mali' strain PM4 + ASGV-AC on *M. domestica* cv. Rubinette and the Hybrid Cultures D45 and B323

How do the different hybrid genotypes react when co infected with ASGV-AC and '*Ca.* P. mali' strain PM4? The hybrid genotypes D45 and B323 co- infected with ASGV-AC and '*Ca.* P. mali' strain PM4 were analyzed (table 27). Rubinette was included as ASGV-AC tolerant control. Unfortunately after three years of grafting experiments no transmission of '*Ca.* P. mali' strain PM4 and ASGV-AC to *M. sieboldii* was possible and the ASGV-AC sensitive control could not be included in this symptom expression comparison.

Table 27: **Symptom expression data and statistical difference of different *Malus* genotypes co-infected with '*Ca.* P. mali' strain PM4 and ASGV-AC**

Genotype	Amount of shoots		Shoot height		Leaf size	
	Mean	Statistical difference	Mean	Statistical difference	Mean	Statistical difference
Rubinette	3.99	a	1.07	b	0.25	b
B323	2.88	b	0.74	c	0.23	b
D45	2.73	b	2.26	a	0.36	a

a-c Each letter represents a statistical different mean value.

Rubinette showed a higher amount of shoots per culture compared to the hybrids B323 and D45 (fig. 21). The hybrid culture D45 had the least stunted growth, and biggest leaf size compared to the *M. domestica* cv. Rubinette and B323. Furthermore the amount of shoots (2.2) and the height of the shoots (2.5 cm) were similar to the virus-and-phytoplasma free culture of D45, only the leaf size was smaller (0.64 cm). This could be an indication of possible resistance to '*Ca.* P. mali'.

3.6 ESTABLISHING OF QUANTITATIVE REAL-TIME

The real-time analysis was performed using SYBR Green I as detection method. The SYBR Green I molecule binds specifically double-strand DNA. In this state, after excitation from a 492 nm light-source, it emits a fluorescence signal with a 513 nm wavelength. The emitted signal is proportional to the quantity of SYBR Green I® bound and therefore to the DNA quantity and length. The continuous fluorescence detection of the amplified products allows the calculation of the quantity of the template initially present in the sample. There is a direct relationship between the initial amount of template present in the reaction and the cycles required before a significant

increase in the fluorescence signal is detected. In other words, the higher the initial copy number in the sample the fewer cycles are necessary to detect a signal.

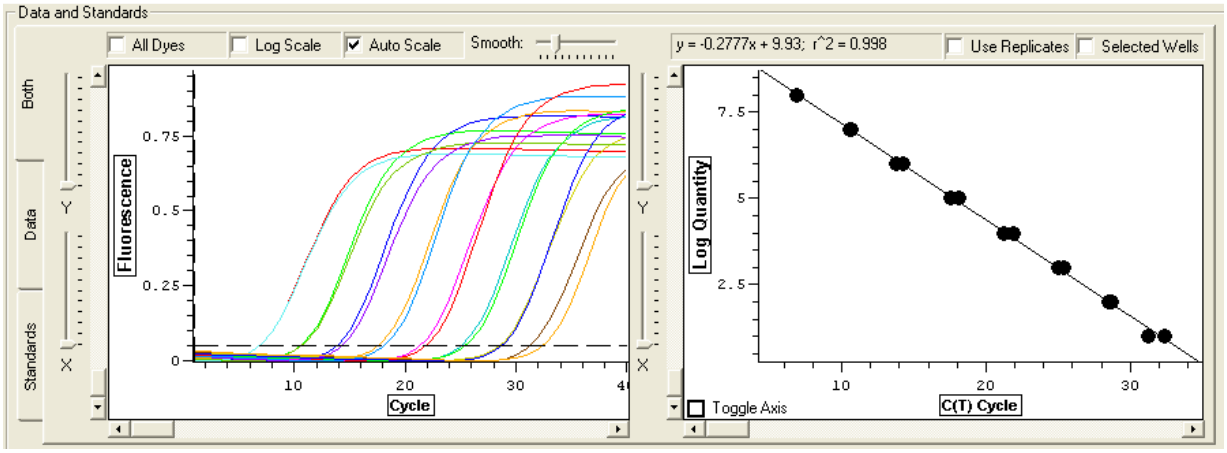

Figure 22: **Fluorescence and standard curve of the standard dilution series used in '*Ca.* P. mali' qPCR reactions**

The cycle threshold (ct) or quantification cycle (Cq) line was manually adjusted to the best fit of linearity in the $\log_{10}$ curves of all samples, excluding any background noise of samples and non template control. Negative template controls (NTC) were also included in all qPCR and RT-qPCRs. For each run a dilution series of the fragment to be amplified was included as quantification standard and the quantity (known copy number) was plotted against the cycle were the fluorescence exceeds the cycle threshold (fig. 22). The quantity of template of the unknown samples was then calculated automatically by applying to the standard curve. The R-square (R^2) value indicates how well the fit of the standard curve describes the variation in the data. The value of R-square can vary between 0 and 1, with values closer to 1 signifying a good fit. All runs showed an R-square value of between 0.994 and 0.999, indicating that 99% of the variance in the data could be explained by the standard curve. The dilution series were run in duplicate and outlying points were deselected. The primers for each reaction was carefully designed and optimized to exclude primer dimer formation. The presence of primer dimers could enhance the signal observed and could lead to false data.

The quantity of the initial template data was then adapted to an efficiency of 100%. Each sample was run in duplicate and each run repeated to analyze the intra-assay variation and variation between runs. The four values obtained for each sample were used to calculate the mean copy

number of the sample. In the case where one value showed significant variation compared to the other 3 values obtained for the same sample, the value was eliminated and the mean value was only calculated from the remaining 3 values. This was performed to exclude samples with pipetting errors. The absolute quantification with standard curve was used for all qPCR and RT-qPCR reactions including the SBE1 qPCR. The mean virus or '*Ca*'. P. mali' copy number per sample was normalized against (divided by) the mean SBE1 copy number for the same sample. The normalization calculation was adapted to the ploidy of each *Malus* genotype. The normalized values were then subjected to statistical analysis and statistical comparisons using the biostatistics program R.

3.6.1 Normalization with the Starch Binding Protein Gene1

For the comparison of virus and '*Ca.* P. mali' titers in the *in vitro* plantlets, a reference is required for the normalizing of the Real-Time PCR /RT-PCR. As reference a single copy gene from the apple genome was selected, the starch binding protein gene 1 (SBE1). A fragment of the SBE1 gene of all *M. domestica*, *M. sieboldii* and hybrids used in this study was amplified and sequenced and all 21 sequences obtained showed a 100% nt identity at the primer binding site. Because the different hybrids and *Malus* cultivars have different polyploidy levels, the number of SBE1 alleles per *Malus* genotype was different. Table 4, shows the ploidy level of each *Malus* genotype. The ploidy number was then used to standardize the data analysis.

To analyze the effect of ASGV-AC on the SBE1 gene copy number, the SBE1 copy number of *M. sieboldii* and *M. domestica* cv. Rubinette was determined. For extracts from each genotype were tested in duplicate and the run repeated. The addition of ASGV-AC had no influence on the SBE1 copy number of *M. sieboldii* or Rubinette and the data were normally distributed. (Shapiro-Wilk normality test, W = 0.9335, p-value = 0.5154). *M. sieboldii* (tetraploid) had 1.965 times more SBE1copies per gram fresh weight than the *M. domestica* cv. Rubinette (diploid), confirming our assumption that the ploidy level of a genotype is an indication of the amount of SBE1 alleles present.

The effect of the addition of '*Ca.* P. mali' on the SBE1 copy number was analyzed by comparing the copy number of *M. domestica* cv Rubinette infected with ASGV-AC (93 extracts) to *M. domestica* cv Rubinette co-infected with ASGV-AC and '*Ca.* P. mali' strain PM4 (110 extracts).

The data were not normally distributed (Shapiro-Wilk normality test, W = 0.9787, p-value = 8.184×10^{-6}), and statistical difference between the mean copy numbers was observed (p-value = 1.622×10^{-3}). Rubinette co-infected with '*Ca.* P. mali' strain PM4 and ASGV-AC (3×10^6 copies) showed a higher copy number per gram of fresh weight that Rubinette infected with ASGV-AC alone (2×10^6). This could be due to more compact cells in '*Ca.* P. mali' symptomatic plants having a higher amount of cells per gram of fresh weight. This result also confirms the choice of using a normalizing gene rather than the traditional copies per fresh weight analysis.

The effect of different '*Ca.* P. mali' strains co-infected with ASGV-AC on the amount of SBE1 copies per gram fresh weight were analyzed to see if there is any changes in the SBE1 copy number. The data were normally distributed (p = 0.08668) and no statistical difference were found between the copy numbers of Rubinette infected with PM4, PM5 or PM6 co-infected with ASGV-AC, indicating that the '*Ca.* P. mali' strain has no effect on the SBE1 copy number of '*Ca.* P. mali'-infected Rubinette.

To be certain that there is no inhibition in qPCR analysis, a dilution series (100 ng/µl, 10 ng/µl, 1 ng/µl) of Rubinette and *M. sieboldii* total nucleic acids was tested. The 4 dilution series' were made from 4 different Rubinette and 4 different *M. sieboldii* cultures and tested in duplicate. For each series step the SBE1 copies were decreasing with 1×10^1, confirming that there is no inhibition in the qPCR reaction.

3.6.2 ASGV Quantitative Real-Time RT-PCR

Primers were designed to amplify a 210bp fragment in the Variable region 1. The primers were designed using the nucleotide sequences obtained from the Variable region study described in section 3.1.2. The RT-qPCR reactions were first optimized on RNA extracts (DGenos) before it was used on the CTAB total nucleic acid extracts. No difference in amplification efficiency was observed between the two extraction methods. The same extractions were also used to test for SBE1 gene as well as for '*Ca.* P. mali' (when applicable). Virus and phytoplasma free controls were included for each run. All data obtained from the biostatistics R program for are summarized in table 31.

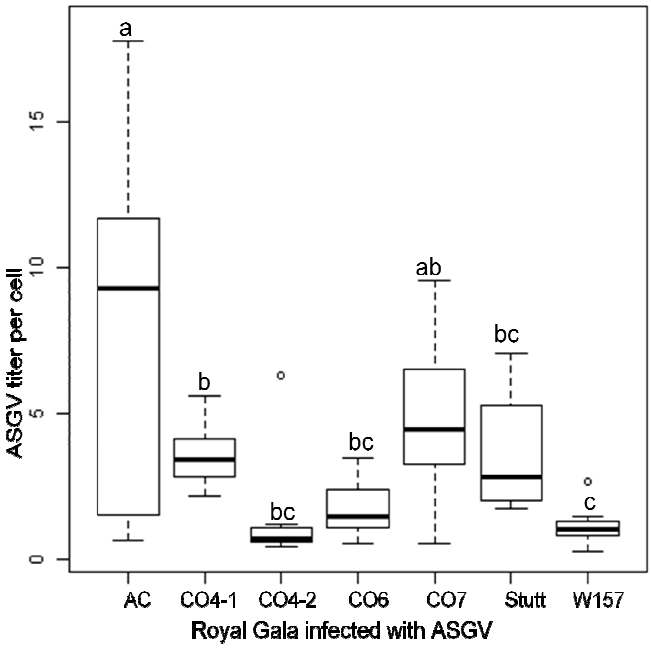

Figure 23: **Boxplot analysis of the ASGV titer per plant cell of Royal Gala infected with different ASGV isolates from Germany and Canada**

3.6.2.1 Analysis of the replication efficiency of different ASGV Isolates on *M. domestica* cv.Royal Gala

The virus titer from established micro-propagated Royal Gala plantlets infected with different ASGV isolates were determined to analyze the replication efficiency of the different isolates and compare it with the symptom expression analysis results. Statistical difference in the mean ASGV copy number per cell from the different isolates was observed (table 28). The titer varied greatly among isolates with ASGV-AC having the highest titer mean of 7.92 viruses per cell and ASGV-W157 the lowest with 1.16 copies per cell (table 28). ASGV-AC also showed much more variability in the titer per cell within the Royal Gala culture compare to the other ASGV isolates. ASGV-AC showed no extreme symptom expression values and the shoot length, shoot height and leaf size was similar to the ASGV-AC free culture. The W157 isolate showed the lowest virus titer but in the symptom expression analysis extreme values were recorded. Similar results were also observed in Rubinette; the ASGV-AC isolate had a mean copy number per cell of 6.94 while ASGV-stutt had a copy number per cell of 2.76.

Table 28: **Statistical difference determined in the ASGV titer of Royal Gala infected with different ASGV isolates.**

Pathogen (s)	Mean	Statistical difference
ASGV-AC	7.92	a
ASGV-CO4-1	3.56	b
ASGV-CO4-2	1.52	bc
ASGV-CO6	1.73	bc
ASGV-CO7	4.95	ab
ASGV-stutt	3.60	bc
ASGV-W157	1.16	c

a-c Each letter represents a statistical different mean value

3.6.2.2 The Influence of a Second Pathogen on the ASGV-AC Replication Efficiency

To analyze if there is an effect of a second pathogen on the replication efficiency of ASGV-AC, the titer of Rubinette ASGV-AC cultures co-infected with ASPV-LFP or the '*Ca.* P. mali' strains PM4, PM5 and PM6 were determined (fig. 24a, table 29). The replication efficiency of ASGV-AC was reduced when co-infected with ASPV-LFP and/or '*Ca.* P. mali'. Although a slight decrease in the ASGV-AC titer was observed when the titers of Rubinette ASGV-AC + PM6 were compared to Rubinette ASGV-AC titer alone, there was no statistical difference in the mean titer values (fig. 24a).

Table 29: **Statistical difference determined in the ASGV-AC titer in the presence of a second pathogen**

Rubinette infected with	ASGV-AC virus titer	Statistical difference
ASGV-AC	6.94	a
ASGV-AC + ASPV	3.66	b
ASGV-AC + ASPV + '*Ca.* P. mali' strain PM6	2.34	b
ASGV-AC + '*Ca.* P. mali' strain PM4	3.97	b
ASGV-AC + '*Ca.* P. mali' strain PM5	2.89	b
ASGV-AC + '*Ca.* P. mali' strain PM6	6.46	a

a-b Each letter represents a statistical different mean value.

In all other combinations the titer decreased by more than half of the titer of the ASGV virus alone. The '*Ca.* P. mali' strain PM6 is considered to be less virulent when compared to the PM4 and PM5 strains which could explain why the titer was not reduced. In the case where PM6 is co-

infected with ASGV-AC and ASPV-LFP the ASGV titer is significantly reduced, possibly because of completion for the plant enzymes and cellular machinery needed for replication.

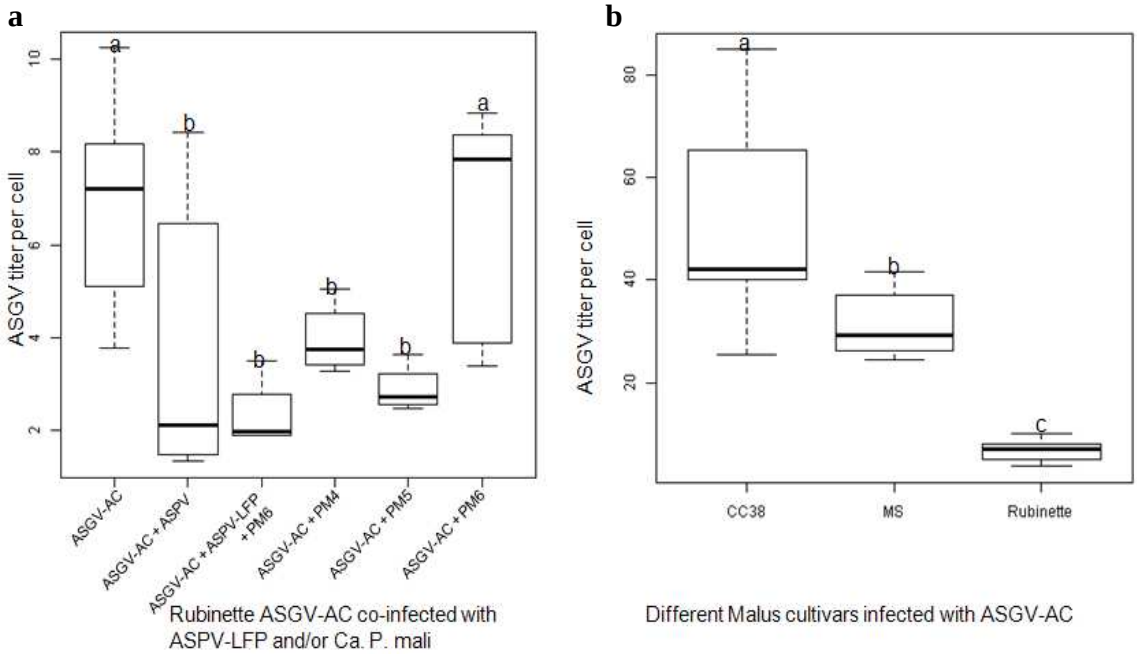

Figure 24: **Boxplot analysis of the ASGV-AC titer per plant cell** a) The influence of a second pathogen on the ASGV titer per plant cell b) ASGV-AC titer per plant cell in different *Malus* genotypes

3.6.2.3 The Replication Efficiency of ASGV-AC in Different Malus Genotypes

The replication efficiency of the virus could indicate why certain *Malus* genotypes react sensitive or tolerant to the ASGV virus. The ASGV-AC titer per cell was significantly lower in *M. domestica* cv. Rubinette (6.94) compared to *M. sieboldii* (31.94) and the hybrid culture CC38 (51.57, fig. 24b). Previous results also show a lower ASGV-AC titer in the *M. domestica* cv. Royal Gala (7.92). The high replication efficiency of ASGV-AC in the *M. sieboldii* and CC38 genotypes could be an indication of why these genotypes react sensitive to ASGV infection.

3.6.2.4 The Replication Efficiency of ASGV-AC in Different *Malus* Genotypes co-infected with '*Ca.* P. mali' strain PM4

The hybrid genotypes B323, B63, D45 co-infected with ASGV-AC and '*Ca.* P. mali' strain PM4 were subjected to ASGV-AC RT-qPCR for the titer determination, to study if the replication efficiency in the hybrids is similar or higher than those of the ASGV tolerant *M. domestica* cv.

Rubinette. The ASGV-AC titer in all three hybrid genotypes was significantly higher compared to Rubinette (table 30).

Table 30: **Statistical difference determined in the ASGV-AC titer in different *Malus* genotypes co-infected with '*Ca.* P. mali'**

***Malus* genotype infected with ASGV-AC + PM4**	**ASGV-AC virus titer**	**Statistical difference**
Rubinette	3.97	b
B63	25.38	ab
B323	45.82	a
D45	48.85	a

a-b Each letter represents a statistical different mean value

Table 31: **Results of the R-test per experimental data set for the ASGV-titer per plant cell.**

Experiment	**Shapiro-Wilk normality test**	**Bartlett test of homogeneity of variances**	**Kruskal-Wallis chi-squared test**	**Duncan test to compare means**
ASGV titer per plant cell of Royal Gala infected with different ASGV isolates	W = 0.7937 p-value = 5.18×10^{-7} Not normally distributed	K-square = 39.2223 df = 6 p-value = 6.474×10^{-7} Variance not homogenous	chi-squared = 21.5544 df = 6 p-value = 0.001476 Differences in mean values	Confidence level 0.95 Variation Coefficient: 83.99%
The effect of a second pathogen on the ASGV-AC copies per Rubinette plant cell	W = 0.9172 p-value = 0.01187 Not normally distributed	K-square = 16.1468 df = 5 p-value = 0.006437 Variance not homogenous	chi-squared = 18.0949 df = 5 p-value = 0.00283 Differences in mean values	Confidence level 0.95 Variation Coefficient: 42.08%
ASGV titer per plant cell of different *Malus* cultivars infected with ASGV	W = 0.8414 p-value = 0.003032 Not normally distributed	K-square = 28.2604 df = 2 p-value = 7.3×10^{-7} Variance not homogenous	chi-square = 15.7741 df = 2 p-value = 0.003761 Differences in mean values	Confidence Level 0.95 Variation Coefficient: 47.64%
ASGV titer per plant cell of different *Malus* cultivars infected with ASGV and PM4	W = 0.869 p-value = 0.005039 Not normally distributed	K-square = 36.9786 df = 3 p-value = 4.65×10^{-8} Variance not homogenous	chi-square = 14.006 df = 3 p-value = 0.002896 Differences in mean values	Confidence Level 0.95 Variation Coefficient: 70.57%

3.6.3 ASPV Quantitative Real-Time RT-PCR

To analyze if the addition of ASGV-AC or ASGV-stutt influence the replication efficiency or fitness of ASPV-LFP the mean titer per plant cell was determined in *M. domestica* cv. Rubinette infected with ASPV-LFP, ASPV-LFP + ASGV-AC and ASPV-LFP + ASGV-stutt. No statistical difference was observed between the mean titer per plant cell of the single ASPV infected plantlets compared to the Rubinette plantlets co-infected with either of the ASGV isolates. The titer of ASPV-LFP was however significantly higher when compared to the ASGV titer (7 copies per plant cell) in single infected *M. domestica* cv. Rubinette.

Table 32: **Statistical difference determined in the ASPV-LFP titer in *M. domestica* cv. Rubinette co-infected with ASGV**

Rubinette infected with ASPV and/or ASGV	ASPV-LFP virus titer	Statistical difference
ASPV-LFP	48.5	na
ASPV-LFP + ASGV-AC	33.46	na
ASPV-LFP + ASGV-stutt	25.7	na

Table 33: **Results of the R-test per experimental data set for the ASPV-titer per plant cell.**

Experiment	Shapiro-Wilk normality test	Bartlett test of homogeneity of variances	Kruskal-Wallis chi-squared test	Duncan test to compare means
ASPV titer per plant cell of Rubinette co-infected with different ASGV isolates	W = 0.7633 p-value = 0.01145 Not normally distributed	K-square = 5.7139 df = 2 p-value = 0.05744 Variance is homogenous	chi-squared = 0.6944 df = 2 p-value = 0.7066 No differences mean values	Confidence level 0.95 Variation Coefficient: 51.06%

3.6.4 *'Ca.* P. mali' Quantitative Real-Time PCR

3.6.4.1 The Effect of ASGV-AC on the *'Ca.* P. mali' Replication Efficiency in *M. domestica* cv. Rubinette

The addition of a *'Ca.* P. mali' is reducing the replication efficiency of ASGV-AC, but how is ASGV-AC changing the replication efficiency of *'Ca.* P. mali'? The same extractions used for the ASGV-AC titer determination were used for this study. Rubinette infected with *'Ca.* P. mali' strain PM4 was only established at the end of the project and was not included in this study. No statistical difference was observed between the mean *'Ca.* P. mali' titer per plant cell in the co-

infections PM4 + ASGV-AC and PM5 + ASGV-AC (table 34, fig. 25a). Statistical differences in the '*Ca.* P. mali' titer per cell were observed for PM6 +ASGV-AC when compared to PM4 and PM5 co-infected with ASGV-AC, with a titer per Rubinette plant cell of 18.21 compared to 34.35 and 34.18 respectively. For both PM5 and PM6 the titer per Rubinette plant cell was enhanced when co-infected with ASGV-AC. All obtained from the biostatistics R program for are summarized in table 36.

Table 34: **Statistical analysis of the effect of ASGV-AC on the titer per plant cell of *'Ca.* P. mali'**

Rubinette infected with ASGV-AC and *'Ca.* P. mali' strain	***'Ca.* P. mali' titer**	**Statistical difference**
PM4 + ASGV-AC	34.35	a
PM5	16.90	bc
PM5 + ASGV-AC	34.18	a
PM6	10.59	c
PM6 + ASGV-AC	18.21	b

a-c Each letter represents a statistical different mean value

3.6.4.2 The effect of Latent Apple Viruses on the Replication Efficiency of the *'Ca.* P. mali' strain PM6 in *M. domestica* cv. Rubinette

The effect of ASPV-LFP and a second ASGV isolate (stutt) were analyzed to see if they also enhance the '*Ca.* P. mali' replication efficiency as in the case of ASGV-AC. The '*Ca.* P. mali' titer per Rubinette plant cell were slightly increased in the Rubinette co-infected with PM6 and ASGV-stutt compared to Rubinette infected with PM6 alone (table 35, fig. 25b). A slight decrease was recorded in Rubinette co-infected with PM6 and ASPV-LFP but with the addition of a 3 pathogen (ASGV-AC + ASPV-LFP + PM6) a more significant decreased in '*Ca.* P. mali' titer was recorded.

Table 35: Statistical analysis of the effect of a latent virus on the titer per plant cell of *'Ca.* P. mali' strain PM6

Rubinette infected with *'Ca.* P. mali' strain PM6 + a latent apple virus	*'Ca.* P. mali' titer	Statistical difference
PM6	10.59	bc
PM6 + ASGV-AC	18.21	a
PM6 + ASGV-stutt	13.73	b
PM6 + ASPV-LFP	9.53	c
PM6 + ASGV-AC +ASPV-LFP	6.06	d

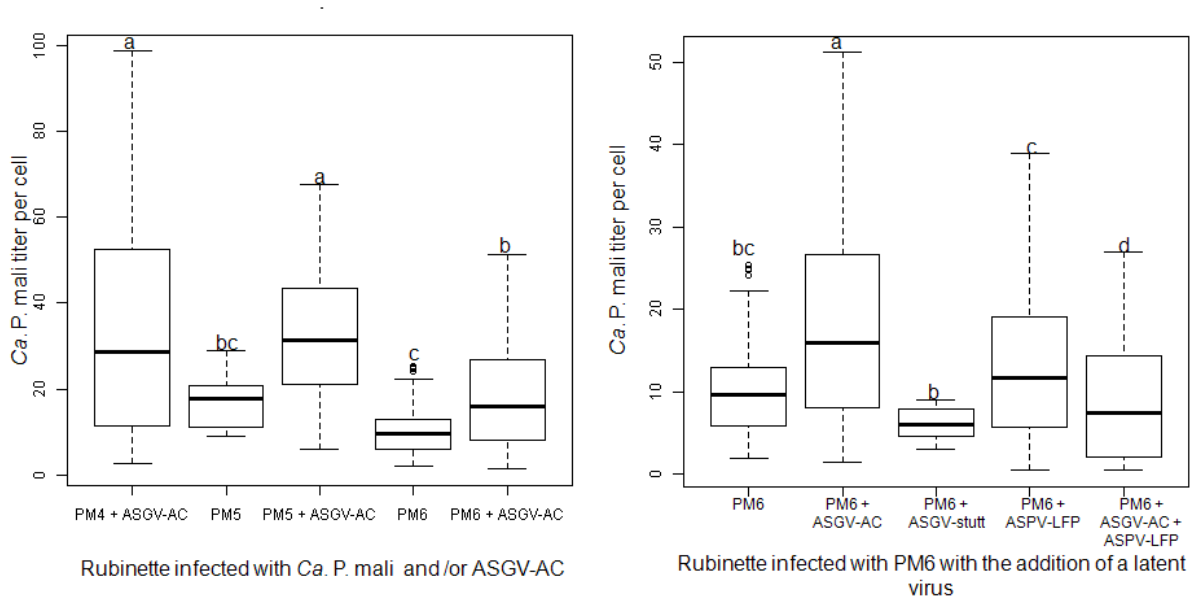

Figure 25: **Boxplot analysis of the *'Ca.* P. mali' titer.** a) The effect of ASGV-AC on the titer per plant cell of different of '*Ca.* P. mali' strains. b) The effect of a latent apple virus on the titer per plant cell of '*Ca.* P. mali' strain PM6.

3.6.4.3 The Replication Efficiency of *'Ca.* P. mali' strain PM4 in different *Malus* Genotypes co-infected with ASGV-AC

The '*Ca.* P. mali' titer per cell was determined in the *M. domestica* cv. Rubinette and the hybrid genotypes B323, D45 and B63 to analyze the replication efficiency of '*Ca.* P. mali' in comparison to the ASGV-AC replication efficiency as well as the symptom expression of these cultures. No statistical difference was found in the '*Ca.* P. titer between the genotypes Rubinette, B323 and B63 and a titer per plant cell of between 34.35 and 45.46 was recorded (fig. 26). A significantly lower '*Ca.* P. mali' titer was recorded in the hybrid genotype D45 (12.91). The growth analysis also showed less stunting in the D45 genotype compared to the B323 and B63

hybrids. There could be a link between '*Ca.* P. mali' strain PM4 titer values and symptom expression. Hybrids with a lower '*Ca*. P. mali' titer showed fewer symptoms.

Table 36: Results of the R-test per experimental data set for '*Ca.* P. mali' titer analysis.

Experiment	Shapiro-Wilk normality test	Bartlett test of homogeneity of variances	Kruskal-Wallis chi-squared test	Duncan test to compare means
Rubinette infected with different '*Ca.* P. mali' strains with or without the addition of a second pathogen	W = 0.8473 p-value = 8.754×10^{-15} Not normally distributed	K-square =119.9884 df = 4 p-value < 2.2×10^{-16} Variance not homogenous	chi-squared = 76.4741 df = 4 p-value = 9.718×10^{-16} Differences in mean values	Confidence level 0.95 Variation coefficient = 70.17%
The effect of latent apple viruses on Rubinette infected with '*Ca. P.* mali' strain PM6	W = 0.8965 p-value = 2.478×10^{-11} Not normally distributed	K-square =44.0832 df = 4 p-value = 6.166×10^{-9} Variance not homogenous	chi-squared = 24.4298 df = 4 p-value 6.549×10^{-5} Differences in mean values	Confidence level 0.95 Variation coefficient = 72.62%
***Malus* cultivars infected with PM4 and ASGV-AC**	W = 0.8981 p-value = 2.566×10^{-5} Not normally distributed	K-square = 19.0451 df = 3 p-value = 0,0002676 Variance not homogenous	Chi-square = 12.5324 df = 3 p-value = 0,005765 Differences in mean values	Confidence level 0.95 Variation coefficient = 71.68%

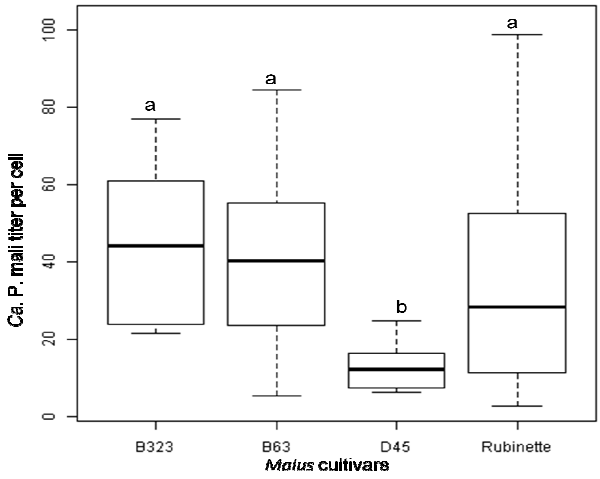

Figure 26: **'*Ca.* P. mali' titer of different *Malus* cultivars infected with PM4 and ASGV-AC**

4 DISCUSSION

The apple industry is one of Germany's most important fruit industries, and is considered as the most important fruit tree in Germany in 2012. Over two thirds of the area under fruit trees (70%) in Germany is used for apple production (Statistisches Bundesamt, Pressemitteilung 2012, 325/12, www.destatis.de). In 2001 Germany lost 25 million Euro due to an outbreak of Apple proliferation disease, causing the fruits to be tasteless and undersized (Strauss, 2009). The fast spreading and economic impact of this disease started a boom in phytoplasma research, especially in the production of Apple proliferation resistant rootstocks. In 2006, this process was complicated by three latent apple viruses, ASGV, ASPV and ACLSV causing the potential resistant apple rootstocks to decline and die. This opened the following questions: What do we know about these viruses? What is the best system to study these viruses? Which of these viruses were responsible for the decline? Is there any interaction between the latent viruses or between virus and '*Ca.* P mali'? How do the hybrid rootstocks react upon these infections? In the next section I will discuss the results I obtained in this study in an attempt to answer these open questions.

4.1 WHAT DO WE KNOW ABOUT THE LATENT VIRUSES

Hypersensitive reactions of some *M. sieboldii* derived hybrids were also observed in the *in vitro* culture system at AlPlanta, Institute for Plant Research. After the detection of the three latent viruses in the declining *Malus* trees, tests were performed at AlPlanta, Institute for Plant Research, and the presence of ASGV and ASPV was detected in the '*Ca*. P. mali' inoculum plantlets. This led to the focus of ASGV and ASPV as causative agents.

Because not much is known about ASGV, especially not for the German isolates, we started our study on the genomic variability and molecular evolution of ASGV. Variability studies and molecular characterization of ASPV were performed by a collogue Jessica Barth (Bachelor study at Fachhochshule Bingen) and will not be discussed here. The complete genome of ASGV-AC, the isolate associated with the decline observed in previous studies, was sequenced. This is the first report of the full length sequence of ASGV from Germany and Europe and showed a nucleotide identity of between 82-97% compared to full length sequences on Genbank ncbi. The German isolate ASGV-AC is closest to an Indian isolate. The genomic organization of ASGV-

AC was similar to those of previously described isolates of ASGV (Yoshikawa *et al*., 1992; Ohira *et al*., 1995; Shim *et al*., 2004; Tatineni *et al*., 2009b). There is a high degree of conservation among the different isolates, except for two variable areas, variable region 1 (V1, amino acids 532–570) and variable region 2 (V2, amino acids 1,583–1,868) (Tatineni *et al*., 2009b). These areas were further compared to isolates obtained from France and Canada as well as sequences obtained from Genbank ncbi and used for selection pressure analysis. Additionally, no stop codon was found in any of the variable sequences of ORF1, confirming previous results by Magome *et al*. (1997) and Tatineni *et al*. (2009b) where no stop codons were found in any of the isolates analyzed.

The initial results from the classical dN/dS estimates obtained from the PAML software showed that the Variable region 1 and 2 have a high amino acid diverisity compared to the rest of the genome. These results suggested that at least some of the codon positions in these regions could be subjected to positive selection and involved in adaptation of the virus. The analysis of positive selection in region V2 was more difficult because it consists of overlapping ORFs. “Classical” dN/dS analyses are not suited to analyze selection in overlapping ORFs since they assume that synonymous substitutions are neutral, which is not true in the case of overlapping ORFs because synonymous substitutions in one ORF are frequently nonsynonymous (and consequently frequently not neutral) in the other ORF. In that case, a joint estimation of dN/dS in both ORFs is necessary. Using the dN/dS joint estimation in overlapping ORFs developed by Sabath *et al*. (2008) showed that evolution of region V2 was very close to neutrality for the replicase OR. As expected, this estimate contrasted greatly with the “classical” estimate from PAML which predicted positive selection.

Taxonomically, capilloviruses are closest to tricho- and citriviruses in the family Beta-Flexiviridae. However, only capilloviruses encode both the replicase and coat protein in a single ORF (Hirata *et al*., 2010). The introduction of a stop codon in the region V2 of the replicase gene would split the replicase from the CP, as for the tricho- and citriviruses (fig. 27). Phylogenetic analysis based on genome sequences shows extensive homology between capillovirus and trichovirus, while the viruses in both genera were historically classified into independent taxa primarily because of the difference in their genome organizations (Coffin and Coutts, 1993).

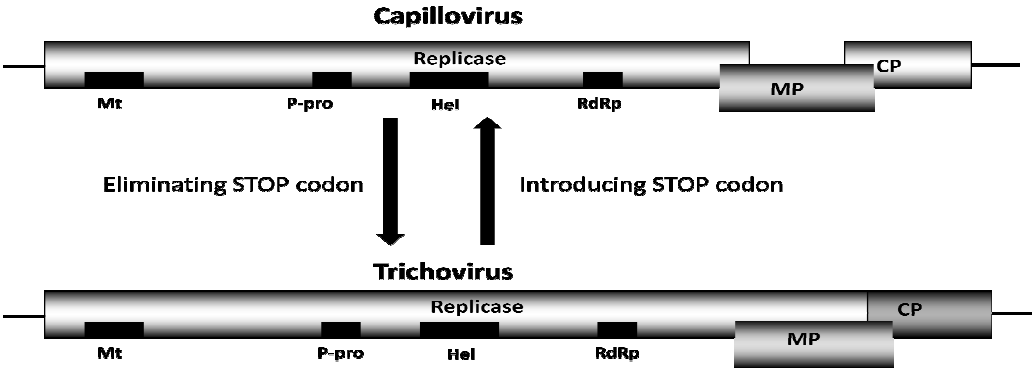

Figure 27: **The introduction of a stop codon in the region V2 of the replicase gene of cappiloviruses would split the replicase from the coat protein. This would cause the formation of a third ORF and a similar genome organization as for the tricho- and citriviruses.**

In a recent study, Hirata *et al*. (2010) used an ASGV infectious clone to introduce artificial stop codons in the V2 region of ORF1 at the end of the replicase gene, which lead to truncated polyproteins lacking the CP fragment. Four of five constructs were infectious and capable of systemic movement. The only non-infectious construct was mutated at the beginning of region V2 (at position 4795 as compared to position 4825 or beyond in the case of the other four) of the ASGV genome. This demonstrates that the V2 region of the replicase gene downstream of position 4825 is not essential for the replicase activity and systemic movement, confirming the low evolutionary constraint in this region, as attested to by the dN/dS analysis. Our analysis, together with the data of Hirata *et al*. (2010), fuels the hypothesis of the "compaction theory" (Keese and Gibbs 1992; Belshaw *et al*., 2007), where the elimination of a stop codon and the overprinting of a novel coding region over the ancestral ORF, increases the amount of genetic data that can be stored in viruses with small genomes.

Evolution analyses of regions V1 and V2 of ORF1 suggest that they correspond to regions with relaxed evolutionary constraints but do not show significant positive selection. They could consequently act as bridges between important functional domains, *i.e*. between methyltransferase and protease domains of the replicase for region V1 and between the replicase and the CP for region V2 (fig. 10). The only constraint in regions V1 and V2 is the absence of stop codons that would produce truncated forms of the replicase, lacking important functional domains. As the CP gene is suspected to be produced from a sub-genomic RNA (Hirata *et al*.,

2003; Tatineni *et al*., 2009a), our results suggest that a CP linked to the C terminal extremity of the replicase could be functionally important for the virus, which would explain the absence of stop codons in region V2.

A clear difference was observed between the selection pressure on regions A and B of ORF2 (fig. 10, table 14). Surprisingly, region B of ORF2, which overlaps with the functionally important CP, was much less constrained (and not significantly different from neutral evolution; table 14) than region A, which overlaps with the low constrained part of the replicase. This suggests that region B of ORF2 might have a more recent origin than region A and could be a new extension of ORF2 with lower selection pressure.

4.2 ASSEMBLING AND TESTING FOR INFECTIVITY OF ASGV AND ASPV cDNA CLONES

Although an ASGV-AC and ASPV-LFP full length clones were successfully assembled no infectivity was established. The attempt to construct the infectious clones were performed for two reasons: first we wanted genomic stable ASGV-AC and ASPV-LFP virus isolates that could be transmitted to *Malus* genotypes for interaction and symptom expression studies. The most variable areas ASGV-AC (V1 and V2) and ASPV-LFP (CP) was sequenced three times over a period of three years and no change in the nucleotide sequence of both ASGV-AC and ASPV-LFP was observed, and indicates that we were able to established and maintain genomic stable ASGV-AC and ASPV-LFP isolates. These isolates are easily accessible and were successfully used in interaction and symptom expression studies.

Secondly, we wanted to perform gene or fragment swopping between virulent and avirulent/less virulent virus isolates to determine areas associated with virus virulence. Hirata *et al*. (2010) suggested that the two variable areas of ASGV could be involved in pathogenicity or virulence of ASGV. Unfortunately, the variability studies done on ASGV-AC and other ASGV isolates from France, Germany, Austria and Canada showed low variability when compared with each other and only high variability was observed when compared to ASGV sequences from Asia, making it hard to select a virulent isolate based on sequence variability. ASGV-AC showed the highest

mean titer per plant cell compared to the other ASGV isolates tested, an indication that the German (AC) strain has a high propagation efficiency and virulence.

Unfortunately due to the time constraints of this study, the full length clones were not further tested for infectivity, and the focus of my studies therefore shifted to experimental procedures that could deliver answers without the use of the cDNA clones.

4.3 THE USE OF *IN VITRO* GRAFTING TO TRANSMIT APPLE VIRUSES AND TO ESTABLISH HOMOGENOUS VIRUS INFECTED *MALUS* PLANTLETS

In vitro graft inoculation has been successfully exploited for the transmission of '*Ca*. P. mali' to micro-propagated *Malus* (Jarausch *et al*., 1999; Bisognin *et al*., 2008a) as well as for the transmission Plum pox virus (PPV) to micro-propagated *Prunus* (Lansac *et al*., 1998; Lichtenegger *et al*., 2010). Although the micro-propagation of ASGV and ASPV infected *Malus* cultures have been reported (Knapp *et al*., 1998; James, 2010), this is the first report of transmission of ASGV and ASPV to *Malus* genotypes through *in vitro* grafting. ASGV and ASPV were successfully transmitted and maintained in the biologically important *M. domestica*, *M. sieboldii* and *M. sieboldii* derived hybrid genotypes. The homogeneity if each infection was analyzed through extensive RT-PCR testing for the appropriate pathogen. The homogenous establishment and maintenance of different latent virus isolates alone or in combination on micro-propagated *Malus* plants, under controlled and comparable conditions, opens a new way of research for virus and virus-host interaction studies. This technique is less time consuming and requires less space compared to the traditional field and greenhouse trails (Lansac *et al*., 1998; Jarausch *et al*., 1999). Virus-'*Ca.* P. mali' combinations were also successfully established and the reproduction of symptoms was successful with different *Malus* genotypes acting similar to those observed in the field trials. The *in vitro* system gives you the advantage to study the virus and virus-virus or virus-phytoplasma interactions on the economic important natural host and allows you easy access to a genomic stable replicating virus. This is the first report of virus interactions studied in an *in vitro* woody plant system.

In the co-transmission trials the hybrid genotypes reacted differently upon different inoculums, some acted similar to the tolerant *M. domestica* genotypes and others reacted similar to the incompatible *M. sieboldii* genotype (CAT5 and O45), while most of the hybrid genotypes acted somewhere between the two (CC38, D45, W335). ASGV-AC could not be transmitted to CAT5 alone or co-transmitted with ASPV-LFP or '*Ca*. P. mali' strain PM4. CAT5 (*M. domestica* cv. Laxton superb x *M. sieboldii*) and O45 (4551 (*M. domestica* cv. Laxton superb x *M. sieboldii*) x M9) have both the full maternal (*M. sieboldii*) genome and acted similar to *M. sieboldii*.

4.4 THE MALUS GENOTYPES REACT DIFFERENTLY TO THE VIRUSES ALONE, VIRUS-VIRUS AND VIRUS-PHYTOPLASMA COMBINATIONS

Lichtenegger *et al.* (2010) used the *in vitro* grafting as a tool for the investigation of hypersensitivity of *P. domestica* to PPV and corresponding observations to *ex vitro* grafting plants were made. In this study the hypersensitive response observed on the *Malus* field trials were reproduced in the established *in vitro* culture system. The response/ symptom expression of the *Malus* genotypes upon infection were divided into two groups for this study, incompatible (extreme resistance and hypersensitive) and compatible (tolerant and sensitive) (fig. 28). Tolerance is a manifestation of resistance in which plants show mild or no symptoms as a function of infection, and in most cases a lower virus titer (Bruening, 2006), while sensitivity allows the plant to be infected, showing symptom expression and a moderate virus titer. Extreme resistance is when a necrotic reaction occurs when a plant defense response is activated upon infection, initiated by the plant resistance genes (*R* genes) and infected cells are killed off to prevent further infection (no transmission) (Palukaitis and Carr, 2008; Pallas and Garcia, 2011). In the hypersensitive response, the necrotic reaction is activated but the virus escapes this initial response, allowing the virus to spread before the necrotic response is activated, killing all infected cells. This response might prove to be lethal for the plant (Palukaitis and Carr, 2008; Pallas and Garcia, 2011). Another hypersensitive response is also possible; the viral factor could activate a pathological response by inducing a resistance mechanism (necrotic reaction) but then suppresses it, allowing the virus to propagate through the plant without activating further necrosis or cell death (Pallas and Garcia, 2011).

Previous studies on the screening of *M. sieboldii* derived rootstocks referred to resistance as having a low pathogen concentration and no symptom expression, while tolerant cultivars were described as showing no hypersensitivity, high pathogen titers and no symptom expression and sensitive cultivars were those which exhibited high pathogens titers and severe symptoms (Jarausch *et al*., 1999; Bisognin *et al*., 2008; Ciccotti *et al*., 2008; Seemüller *et al*., 2007; 2008) In the discussion I will classify the *Malus* reactions towards the different pathogens according to the above mentioned method (fig 28).

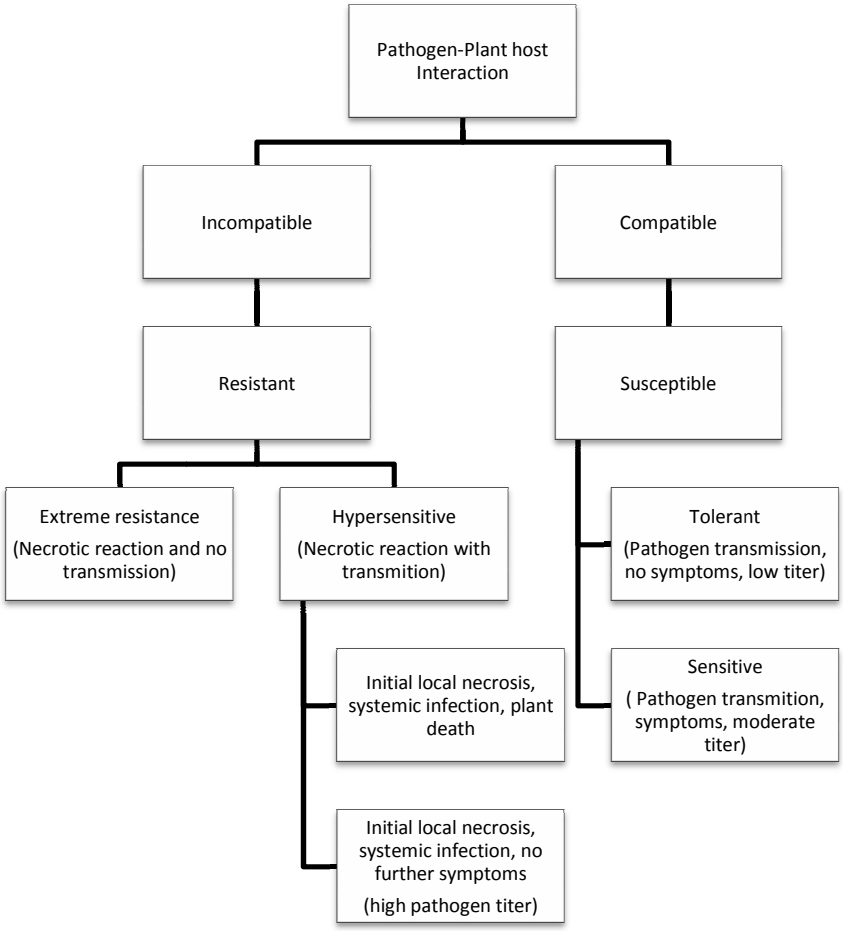

Figure 28: **Schematic illustration of the plants' reaction against pathogens**

The *M. domestica* cv. Rubinette and Golden Delicious could be infected with ASGV, ASPV and '*Ca.* P mali' and combinations thereof without showing any necrosis, cell or plant death and are considered being tolerant to the two latent viruses. The hybrid genotypes B323, B89 and B63 acted similar than the tolerant *M. domestica* cultures and showed no virus induced necrotic

reactions. Interestingly, all three hybrid genotypes stem from seedlings of the same parentage (4608 (*M. purpurea* x *M. sieboldii*) x M9 (*M. domestica* x *M. domestica*) and obtained only half of the *M. sieboldii* genome.

M. sieboldii confers tolerance to '*Ca*. P. mali' and hypersensitivity to ASGV and ASPV to some of the *M. sieboldii* hybrids, indicating that not all four alleles carry these traits or that these traits could be recessive (Palukaitis and Carr, 2008), with hybrids containing half the *M. sieboldii* genome showing less or no hypersensitivity towards the viruses and sensitivity (symptoms) towards '*Ca*. P. mali'. Another possibility is that these traits are allele dosage dependent. One example is the dosage-dependant *I* gene allele of *Pahselus vulgaris* (bean). Collmer *et al.* (2000) observed variation in the defense response of different bean cultivars towards *Bean common mosaic potyvirus* (BCMV), with plants showing extreme resistance, hypersensitivity, or systemic necroisis upon BCMV infection. These reactions were dependent on the amount of *I* gene alleles in the different cultivars. Allele dosage dependence of resistance towards *Barley yellow dwarf virus* was also observed in *Triticum* (Ayala *et al*., 2001).

In most of the hypersensitive reactions observed in this study, an initial or local necrotic reaction was observed after which the virus replicated and spread causing necrosis and death of the plantlet or had no further effect at all. In the latter, the plantlets were homogenously infected and could be maintained at least 6 mpi. Extreme resistance was observed in CAT5, where an initial necrotic reaction was observed with no further transmission.

M. sieboldii micro-propagated plants showed a high number of hypersensitivity when grafted with ASGV, ASPV, and ASGV-AC + '*Ca*. P. mali' strain PM4 compared to other *Malus* genotypes. All three levels of incompatibility were observed in *M. sieboldii*. ASGV-AC and ASPV alone caused necrosis and plant death in some plantlets. While ASGV-stutt only activated the initial or local necrosis after which the plantlets recovered and the virus infected plantlets could be maintained. *M. sieboldii* co-transmitted with ASGV-AC and '*Ca*. P. mali' strain PM4 caused extreme resistance, and no transmission was observed. The difference in reaction of *M. sieboldii* to the infection of ASGV-AC and ASPV-LFP alone could be due to the virus titer of the inoculum tip, with higher virus titers activating a more severe response (Stoimenova *et al*., 2005).

Unfortunately the tips were only tested for the presence of the virus and no titer determination was performed.

The CAT5 hybrid genotype showed the highest percentage of incompatibility of all the hybrid genotypes tested and acted similar to *M. sieboldii* with the only exception that no hypersensitivity was observed in association with ASPV-LFP. CAT5 could not be infected with ASGV-AC or ASGV-AC co-infected with ASPV-LFP or '*Ca*. P. mali' strain PM4 but could be infected with ASGV-stutt and/or ASPV-LFP. The hybrid genotypes CC38, W355, D45 and O45 showed different levels of hypersensitivity dependant on the inoculums. Both ASGV and ASPV were able to activate the hypersensitive reaction observed in the field trials, with the hybrid genotypes reacting differently to the viruses alone or in combination with each other or with '*Ca. P*. mali'.

Figure 29: **CC38 micro-propagated plants infected with ASGV-AC and ASPV-LFP dying 6 mpi.**

The ASGV titer was significantly lower in the tolerant *M. domestica* cv. Rubinette compared to the virus titer in the recovered *M. sieboldii* plantlets. A similar response was also found in *N. benthamiana* plants infected with *Tomato ringspot nepovirus* (ToRSV) that activates an initial necrotic response after which the plants recover but still shows high virus titers (Jovel *et al*., 2007). The recovery phenomenon of some *Malus* genotypes after the initial necrotic reaction could be due to the virus suppressing the plant response. The p30 like MP of ACLSV (*Betaflexiviridae*) has recently been found to be involved in suppressing systemic RNA silencing but not local silencing in *N. benthamiana* (Yaegashi *et al*., 2007). ASGV also contains a p30 like MP and although no such study has been done on ASGV, this could explain the recovery of *M.*

sieboldii and *M. sieboldii* derived hybrid genotypes after the initial or local necrotic reaction induced by ASGV. Similar observations were also made with the TGB protein 1 of *Potato virus* X (PVX, Genus: *Potexvirus*, Family: *Alphaflexiviridae*). The TGB1 protein has been found to be recognized by the *R* gene encoded proteins of plants and activates the plants hypersensitive response (Malcuit *et al*., 1999). The same protein has also been shown to suppress the activity or production of the mobile silencing signal in *N. Benthamiana* (Voinnet *et al*., 2000; Martelli *et al*., 2007). This could explain the recovery observed of the *M. sieboldii* hybrids after the initial necrotic reaction initiated by ASPV.

4.5 IS THE SYMPTOM EXPRESSION OF 'CA. P. MALI' ENHANCED BY THE ADDITION OF THE LATENT VIRUSES OR IS IT DEPENDANT ON THE 'CA. P. MALI' STRAIN

Although ASGV and ASPV is considered to be latent in commercial apple varieties like *M. domestica*, some vigor reductions have been reported in *Malus* associated with ASGV. The effect of the different ASGV isolates on the vigor of the Royal Gala were analyzed and showed some contradicting results. James (2001) observed a decrease in the vigor of *M. domestica* trees infected with ASGV compared to virus-free trees and we expected similar results (decrease in shoot height, leaf size and amount of shoots for the infected plantlets). But the opposite result was observed with most cultures showing a higher shoot height, leaf size and amount of shoots when compared to the virus-free culture. Unfortunately it is difficult to compare our results with those of James (2001) because different ASGV isolates and different *M. domestica* cultivars were used and the study was performed under different conditions (*in vitro* vs. field). Birisk *et al*. (2010) looked at the effect of the ASGV on different commercial cultivars and reported a decrease in trunk diameter of infected trees compared to uninfected trees, but found no statistical difference when the amount of shoots and height of the trees were analyzed, indicating that the measurement of the trunk diameter could be a better indicator for vigor measurement. Unfortunately it was impossible to measure the trunk diameter of our cultures because of their small sizes.

The effect of the addition of ASPV-LFP on the symptom expression of ASGV infected Rubinette was analyzed. Only slight enhancement of the shoot length was observed when the ASGV

infected Rubinette cultures were compared to Rubinette infected with only ASGV-AC or ASGV-stutt. The symptom expression of Rubinette infected with different '*Ca.* P. mali' strains was not affected with the addition of ASGV-AC, ASGV-stutt or ASPV-LFP. The symptom expression was however more extreme in the '*Ca.* P. mali' strains PM4 and PM5 infected Rubinette compared to the PM6 infected Rubinette. This could be an indication of virulence of these '*Ca.* P. mali' strains. The effect of ASGV-AC on the sensitive *M. sieboldii* and *M. sieboldii* derived hybrid genotype CC38 revealed a slight reduction in the shoot height of both CC38 and *M. sieboldii* when compared to the virus-free cultures, while in Rubinette the shoot height was increased, confirming the results obtained in the Royal Gala ASGV trials. The effect of PM4 + ASGV-AC was analyzed on the symptom expression of the *M. sieboldii* hybrid genotypes B323 and D45 and compared to the '*Ca.* P. mali' susceptible cultivar Rubinette. D45 showed the least severe symptom expression when compared with Rubinette and B323, with less shoot proliferation and stunting. D45 is the only hybrid genotype with Supporter 1 (*M. baccata* cv. Himalaica) as parent and *M. baccata* together with *M. sieboldii* could enhance the tolerance towards '*Ca.* P. mali'.

4.6 VIRUS-VIRUS AND VIRUS-PHYTOPLASMA SYNERGISTIC OR ANTAGONISTIC INTERACTION?

To analyze if there is an interaction between the ASGV and ASPV or between the viruses and '*Ca.* P. mali' the titer values of *Malus* single infections were first determined through RT-qPCR. Although the Minimum Information for Publication of Quantitative Real-Time PCR experiments (MIQE) is mainly optimized for relative quantification of gene expression studies, it was used as guideline to establish the absolute qPCR and RT-qPCR protocols used in this study (Bustin *et al.*, 2009). The experimental design is fully explained in this study, including data on sample preparations, optimization and design of primers, target information, qPCR protocol information and programs used for data analysis. The reactions were validated by intra-assay comparisons as well as individual run comparisons. Sample efficiencies and calibration curve values (r^2) were comparable between runs and non template controls were included for each assay.

A SYBRGreen based RT-qPCR method for ASGV and ASPV were established and the titers were normalized using the SBE1 gene. At the start of this study, the SBE1 gene was the only

one-copy/ reference gene studied in *Malus* (Han *et al*., 2007). In 2012, Gadiou and Kundu also developed a RT-qPCR technique for the quantification of ASGV and found the GAPDH and S19 *Malus* genes as the best reference genes, but the SBE1 gene was not analyzed in the study. For the quantification of '*Ca*. P. mali' the method described by Jarausch *et al*. (2004) were used, with the adaptation of using SBE1 as normalizer to determine the copy number per plant cell. Previous studies performed on the quantification of '*Ca.* P. mali' were performed on the copy number per gram of fresh weight or per copy number per host plant DNA (Jarausch *et al*., 2004; Bisognin *et al*., 2008a). No difference in the relative changes of the '*Ca.* P. mali' copy numbers were observed when these two techniques (copy number per cell vs. copy number per gram fresh weight) were compared. For the quantification of the virus titers in virus infected *Malus* compared to co-infections with '*Ca*. P. mali', this method was insufficient, because the titer per gram fresh weight was influenced by the symptom expression of '*Ca*. P. mali' (a higher amount of cells per gram fresh weight in '*Ca*. P. mali' + ASGV infected Rubinette cells, compared to the amount of cells per gram fresh weight of ASGV or ASPV infected Rubinette plantlets). This result was confirmed by the number of copies per gram fresh weight of the SBE1 gene in '*Ca.* P. mali' infected compared to the amount of copies in ASGV infected plantlets. Baric *et al*. (2004; 2006) used a *Malus* chloroplast gene coding for tRNA leucine as internal PCR control, but this gene was not used as reference in titer quantification. This is the first report for the quantification of '*Ca*. P. mali' and ASPV quantification using a *Malus* gene as reference.

The mean ASGV-AC titer per plant cell in the tolerant genotype Rubinette was 4 times lower compared to *M. sieboldii* and 7 times lower compared to CC38. Interestingly, no hypersensitivity was observed in CC38 towards ASGV-AC alone, and only hypersensitive reactions (and delayed death) was observed when ASPV-LFP were co-transmitted, while in *M. sieboldii* the plantlets showed initial necrosis and recovered or extreme resistance (necrosis and no transmission). One hypothesis for this result is that in the tolerant Rubinette, a low or delayed plant defense response is triggered, while in *M. sieboldii* a severe defense response is triggered, activating the virus to suppress the response (fig 8, Zvereva and Pooggin, 2012).

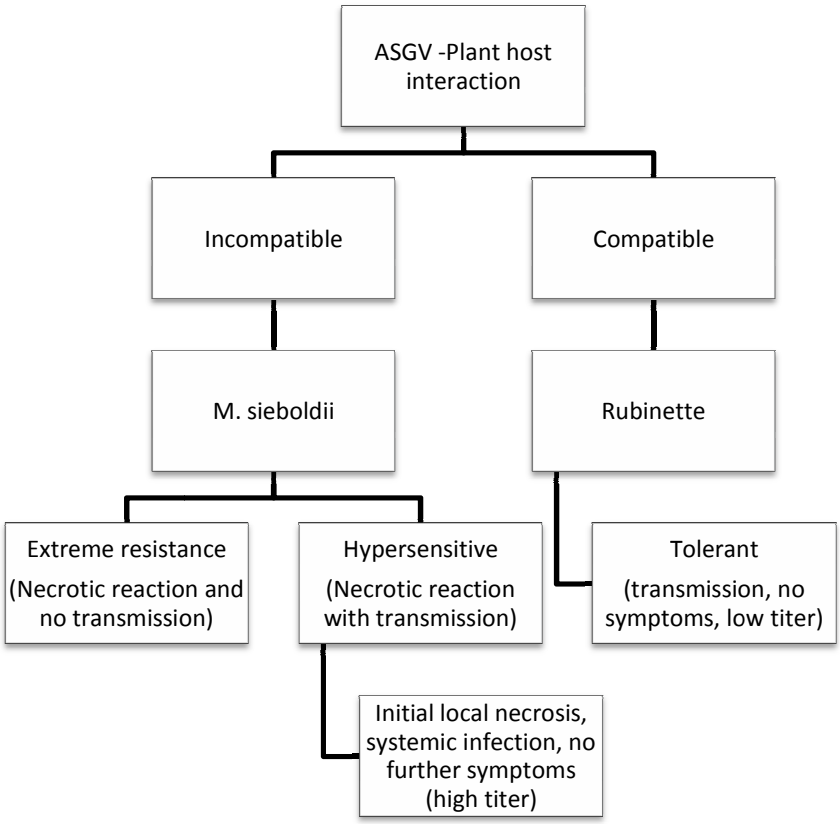

Figure 30: **Schematic illustration of the *Malus* defense reaction against ASGV in this study.**

An antagonistic interaction between ASGV-AC and ASPV-LFP in the tolerant *M. domestica* cultivar Rubinette was observed. The ASGV-AC titer was reduced by half when co-infected with ASPV-LFP, while the ASPV-LFP titer was unchanged. The ASPV-LFP titer per plant cell was 10 times higher compared to the ASGV titer in the co-infected Rubinette. Because no previous studies were performed on the interaction of these two viruses we can only hypothesize: 1) Both viruses are single stranded positive sense viruses from the family *Betaflexiviridae* and it could be possible that both viruses use the same host factors to form replication complexes and that ASPV is outcompeting ASGV (Clarke *et al*., 1994; Hurst and Lindquist, 2000). 2) The TGB could constitute more efficient movement of ASPV and enhance its fitness, compared to the p30 like MP of ASGV. 3) Another possibility is the ability of ASPV to suppress the plant response more adequately compared to ASGV (fig. 31).

Differences in the titer of '*Ca.* P. mali' strains, PM4, PM5 and PM6 were observed. PM4 and PM5 titers per cell were double as high as for PM6 in both single infections and co-infected with

ASGV-AC. A previous study performed by Bisognin *et al*. (2008b) on the concentration of different '*Ca.* P. mali' strains in the *M. domestica* cultivars Rubinette and Golden delicious, showed significant higher PM4 titers compared to the titer obtained for PM6. These results were confirmed by this study. These results also confirm the results obtained from our symptom expression study the symptoms observed in the PM6 infected Rubinette were less severe compared to the symptoms observed in PM4 and PM5 infected Rubinette.

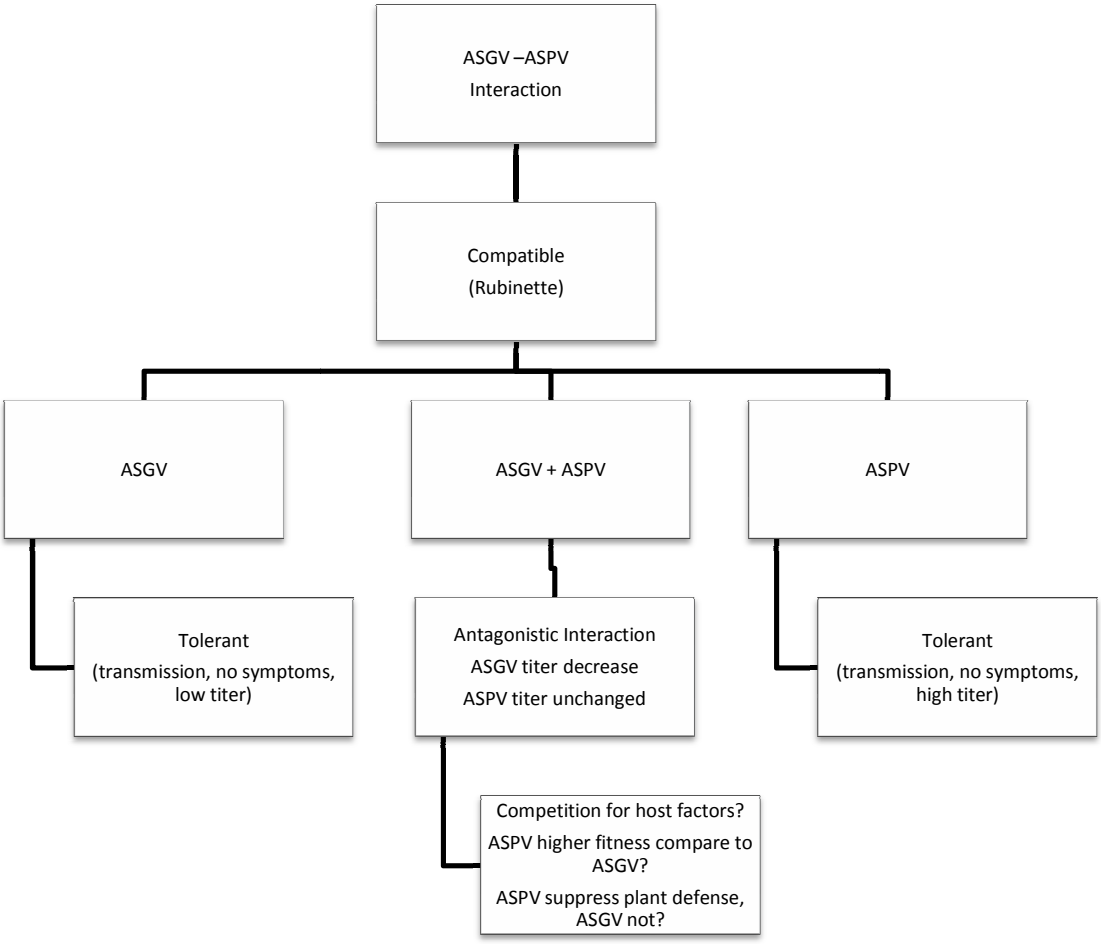

Figure 31: **Schematic illustration of the *Malus* defense reaction against ASGV observed in this study.**

An antagonistic interaction between ASGV-AC and '*Ca.* P. mali' strain PM5 (PM4) was observed in Rubinette. The ASGV-AC titer per cell was reduced by half (similar to the effect observed when co-infected with ASPV), while the '*Ca.* P. mali' titer per plant cell was increased (doubled). Co-infections with the '*Ca.* P. mali' strain PM6 and ASGV-AC revealed an increase

in PM6 titer (doubled) but no statistical difference in the ASGV titer compared to the titer observed in Rubinette infected with ASGV-AC alone, was observed. No statistical difference was observed in the titer of PM6 when co-infected with ASPV-LFP or ASGV-stutt. A decrease in PM6 titer was observed when PM6 was co-infected with ASGV-AC and ASPV-LFP. Although the titer of '*Ca.* P. mali' was increased by the presence of ASGV-AC, no influence on the symptom expression was recorded.

The '*Ca.* P. mali' strain PM4 and ASGV-AC concentration was also determined in the hybrid genotypes co-infected with both pathogens. The hybrid cultivar D45 had a significantly lower PM4 titer compared to B323, B63 and Rubinette. While all the hybrid genotypes B63 and B323 had significantly higher ASGV-AC titers compared to Rubinette. D45 had a moderate ASGV-AC titer and is the only hybrid genotype that showed some degree of tolerance (low '*Ca.* P. mali' titer and moderate ASGV-AC titer) towards the co-infection of ASGV-AC and PM4, confirming the results obtained in the symptom expression analysis.

These results open the question if there is two independent or related plant defense mechanisms activated against the two different pathogens and if there is cross talk involved where one response is favored or influence the severity of the second response? And if so, how are these different responses regulated in the different *Malus* genotypes? No previous study has been done on the interaction between phytoplasmas and viruses, and not much is known about the *Malus* or any plant defense response/s triggered by '*Ca.* P. mali', ASGV, or ASPV. This makes it extremely difficult to construct a scientific hypothesis without further experiments on the *Malus* defense response. To explain this remarkable observation I tried to find a plant defense model that could fit to my data, and could be the basis for further research.

The Salicylic acid (SA) and Jasmonic acid (JA) plant defense responses have been extensively studied in the last years and revealed that there is some degree of cross talk or interaction between the two pathways (Mur *et al*., 2006; Pieterse *et al*., 2009; Thaler *et al*., 2012). Multiple defenses to multiple pathogens can be costly for plants and reduce plant's fitness. The cross talk or interaction between these two independent response pathways provides the plant the regulatory potential to tailor its defense to the most severe or dangerous pathogen (Pieterse *et al*., 2009; Thaler *et al*., 2012). It is also known that both viruses and phytoplasmas are able to activate the

SA response pathway (Murphy *et al*., 1999; Ahmad and Eveillard, 2011; Hren *et al*., 2009), affecting the replication and systemic movement of viruses (Murphy *et al*., 1999), and could induce resistance towards phytoplasmas (Sánchez-Rojo *et al*., 2011). In the following paragraphs I will use this model to construct a possible hypothesis to explain the results observed in this study.

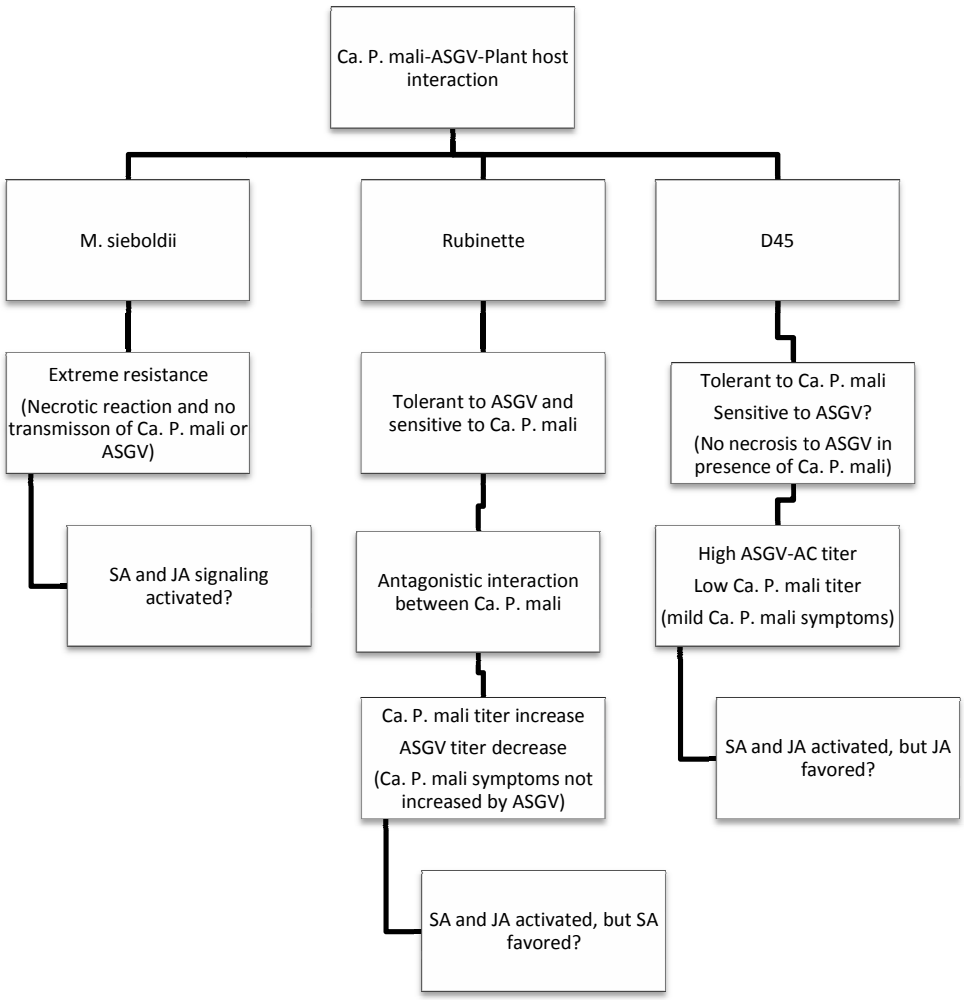

Figure 32: **Schematic illustration of the *Malus* defense reaction against the co-infection of '*Ca.* P. mali' strain PM4 and ASGV-AC observed in this study.**

The hypersensitive reaction or necrotic reaction associated with virus infection is normally associated with Salicylic acid plant response, while the systemic, non-necrotizing virus infections do not normally trigger the biosynthesis of Salicylic acid or the induction of SA response genes (Alvarez, 2000; Palukaitis and Carr, 2008). However a study done on *Cucumber mosaic virus*

(CMV) showed a mild induction of SA biosynthesis only after systemic movement of the virus and was not activated before (Ji and Ding, 2001, Whitham *et al*, 2003). If this is true for ASGV-AC in the tolerant cultivar Rubinette, it could explain the low virus titer. A recent study on the response of tomato to the stolbur phytoplasma was performed by Ahmad and Eveillard (2011) and revealed that different strains of the same phytoplasma could activate different plant defense responses. The milder isolate, causing less severe symptoms activated the SA, ET and JA plant defense responses, while in the more severe strain (causing severe symptom expression) only the SA and ET was activated while the JA was significantly suppressed. A possible explanation for the increase in '*Ca.* P. mali' titer in association with ASGV-AC could be that both the virus and '*Ca.* P. mali' induce salicylic acid response and that these responses are milder in single infections, compared to the response activated in the co-infection of Rubinette. The higher level of salicylic response causes the inhibition or suppression of ASGV, and hence the lower virus titer. Because there is mostly an antagonistic relationship between the two independent response pathways JA and SA, it could be possible that the JA is suppressed to a level that the '*Ca.* P. mali' could multiply and spread to a higher concentration than in single infected plants. Phytoplasmas also produce effectors that have been found to be involved in signaling pathways alterations and modulating the production of phytohormones that regulate plant defense responses (Sugio *et al*., 2011). One example is the effector SAP11 produced by the '*Ca.* P. Asteris' (AY-WB) that is involved in the destabilization of plant TCP's, which in turn reduces the Lox2 expression and impairs the JA synthesis, which enables the leafhopper nymphs to develop and so guaranty the further transmission of the phytoplasma (Sugio *et al*., 2010). But this specific effector was not found in the sequenced '*Ca*. P. mali' strain AT (Sugio *et al*., 2010). So far none of the 13 effectors produced by '*Ca.* P. mali' strain AT have been studied, but we cannot exclude that '*Ca.* P. mali' strain PM4 does not have an effector with a similar function. The presence of such an effector could explain the high titer of '*Ca.* P. mali' strain PM4 in Rubinette compared to the more tolerant hybrid genotype D45. If the '*Ca.* P. mali' strain PM4 is able to suppress the JA response to a certain extend in the sensitive cultivar Rubinette, but not in the possible tolerant hybrid genotype D45, and the JA is favored over the SA response it could cause a low '*Ca.* P. mali' titer, and a high ASGV-AC titer. In the co-infection of ASGV-AC and '*Ca.* P. mali' (PM4) it could be possible that the suppression of JA is double strong, first by the suppression by the SA response and secondly by the effectors produced by '*Ca.* P. mali' (PM4), resulting in a higher '*Ca.* P. mali' (PM4) titer.

If this hypothesis is correct, why is there no effect of the '*Ca.* P. mali' strain PM6 on the ASGV-AC titer, compared to the decrease in titer observed in the presence of the more virulent '*Ca.* P. mali' strain PM4? A lower PM6 titer as well as less severe symptom expression was observed in the single infections of Rubinette compared to the more virulent strain PM5. Ahmad and Eveillard (2011) found that with the milder stolbur phytoplasma strain, both the SA and JA response pathways were activated. It is possible that the '*Ca*. P. mali' strain PM6 is not able to suppress the JA response, hence the lower titers observed.

In the ASGV-AC hypersensitive cultivar *M. sieboldii*, extreme resistance was observed when ASGV-AC and PM4 was used as inoculum. A low percentage of *M. sieboldii* plantlets acted hypersensitive to ASGV-AC alone but with the addition of '*Ca*. P. mali' strain PM4, 56% of the plants showed necrosis and the transmission of both pathogens was not possible. We know that the hypersensitive reaction (necrosis) is associated with salicylic acid plant response (Alvarez, 2000; Palukaitis and Carr, 2008) and we can assume that the SA is also activated in *M. sieboldii* upon infection with ASGV (at least in those plants that showed necrosis). If '*Ca.* P. mali' also activates the SA response, this super activation could be responsible for the extreme resistance response observed in *M. sieboldii* when co-infected with ASGV-AC and PM4.

5 SHORT SUMMARY OF THE FINDINGS AND FUTURE PROSPECTS

- The ASGV isolate (AC) associated with the decline observed in previous trials was successfully sequenced and showed two areas of high variability (V1 and V2).
- Selection pressure analysis of these variable areas showed significant positive selection compared to the rest of the genome which predominantly showed negative selection pressure.
- However, the V2 area includes overlapping ORFs, making the estimate biased. Joint estimates of the selection intensity in the different ORFs indicated that this region of ORF1 was in fact evolving close to neutrality. Suggesting that the elimination of a stop codon caused the overprinting of a novel coding region over the ancestral ORF.
- An *in vitro* culture system was established were ASGV and ASPV could be successfully transmitted through *in vitro* grafting and the infected cultures could be maintained and propagated *in vitro*.
- The establishing of the *in vitro* system allowed for studies on the interaction of ASGV and ASPV as well as for the interaction studies of '*Ca*. P. mali' and ASGV by eliminating all environmental factors that could influence these interactions.
- Symptoms observed in field trials were reproduced, with different degrees of hypersensitivity observed in the potential AP resistant *M. sieboldii*-derived hybrid genotypes.
- ASPV and ASGV are the causative agents for the decline observed. The hypersensitivity observed was different for each *M. sieboldii*-derived hybrid genotypes. With some acting hypersensitive upon ASGV infections, while other reacted hypersensitive towards ASPV or a combination of both viruses.
- No enhancement of '*Ca*. P. mali' symptom expression was observed with the addition of ASGV in *M. domestica* cultivars.
- The highest percentage of hypersensitivity was observed in the co-infection trials of '*Ca*. P. mali' strain PM4 and ASGV-AC, an important finding for the screening of potential '*Ca*. P. mali' and ASGV tolerant *Malus* genotypes.

- A quantitative Real-Time PCR and RT-PCR using the *Malus* normalizing gene SBE1 was established for ASPV, ASGV, and '*Ca*. P. mali' and successfully used for titer determinations per plant cell.
- An antagonistic interaction was observed between ASGV and ASPV, with the ASGV titer being reduced by half, and the ASPV titer staying unchanged in co-infections.
- An antagonistic interaction was also observed between ASGV-AC and '*Ca.* P. mali' strain PM4. With the ASGV titer per plant being significantly reduced and the '*Ca*. P. mali' titer being significantly increased in co-infections.
- This result also suggests that care should be taken in host-phytoplasma interaction studies, because the addition of a second pathogen could influence these results.

This is the first study done on the ASGV-ASPV and ASGV-'*Ca.* P. mali' interaction, and possibly the first interaction study performed on viruses and phytoplasma. Now that it has been established that an interaction between these biologically distant pathogens exist and a in vitro system was established to easily study these interactions, further studies can be done to determine how this interaction is possible between a phloem restricted pathogen and a cytoplasm restricted virus. The analysis of gene expression involved in the salicylic acid and jasmonic acid defense pathways could be a start to understand and explain this indirect interaction between virus and phytoplasma. Ahmad and Eveillard (2011) studied the expression of the pathogen related PR1, PR2 and PR5 genes involved in the salicylic acid defense pathway and the PIN2, GluB and LoxD genes involved in the jasmonic acid defense pathway upon infection of stolbur phytoplasmas in tomato. Whitham *et al.* (2003) studied the expression of genes involved in plant defense upon infection of diverse RNA viruses and found that the PR1, PR5 and PAD4 genes involved in the salicylic acid defense pathway were up-regulated in response to *Oilseed rape tobamovirus* (ORMV) and *Cucumber mosaic virus* (CMV). These genes could be of interest in studying the pathways activated by '*Ca.* P. mali' and ASGV. Functional studies on the 13 effectors produced by the '*Ca*. P. mali strain' AT as well as for the PM4 and PM6 strains needs to be performed to determine if any of the effectors are involved in suppressing the plant defense.

6 REFERENCES

Ascencio-Ibanez, J. Settlage, S. (2007). DNA abrasion onto plants is an effective method for geminivirus infection and virus-induced gene silencing. Journal of Virological Methods 142: 198-203.

Ahmad, J.N., Eveillard, S. (2011). Study of the expression of defense related protein genes in stolbur C and stolbur PO phytoplasma-infected tomato. Bulletin of Insectology 64 Supplement: S159-S160.

Ahrens, U., Seemüller, E. (1992). Detection of DNA of plant pathogenic mycoplasma like organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82: 828-832.

Ayala, L., Henry, M., Gonzalez-de-Leon, D., van Ginkel, M., Mujeeb-Kazi, A., Keller, B., Khairallah, M. (2001). A diagnostic molecular marker allowing the study of *Th. intermedium*-derived resistance to BYDV in bread wheat segregating populations. Theoretical and Applied Genetics 102: 942-949.

Al Jeb, K., Ismaeil, F., Mando, M.J., Al-Saadoun, E., and Al-Chaabi, S. (2005). First record of pome fruit viruses in Syria. Journal of Plant Pathology 87: 243.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

Alvarez, M.E. (2000). Salicylic acid in the machinery of hypersensitive cell death and disease resistance. Plant Molecular Biology 44: 429-442.

Arashida, R., Kakizawa, S., Hoshi, A., Ishii, Y., Jung, H.J., Kagiwada, S., Yamaji, Y., Oshima, K., Namba, S. (2008). Heterogenic dynamics of the structures of multiple gene clusters in two pathogenetically different lines originating from the same phytoplasma. DNA and Cell Biology 27: 209-217.

Alskieff, J., Villermur, P. (1978). Greffage *in vitro* d'apex sur des plantules decapite´es de pommier (*Malus pumila* Mill.). Compte Rendu de l'Acade´mie des Sciences, Se´rie D 287, 1115-1118.

Avinent, L., Llácer, G. (1995). Detection of phytoplasmas in fruit trees by polymerase chain reaction (PCR) in Spain. Acta Horticulturae 386: 480-483.

Bai, X., Zhang, J., Ewing, A., Miller, SA., Jancso Radek, A., Shevchenko, D.V., Tsukerman, K., Walunas, T., Lapidus, A., Campbell, J.W., Hogenhout, S.A. (2006). Living with genome instability: the adaptation of phytoplasmas to diverse environments of their insect and plant hosts. Journal of Bacteriology 188: 3682-3696.

Baric, S., Dalla Via, J. (2004). A new approach to apple proliferation detection: a highly sensitive realtime PCR assay. Journal of Microbiological Methods 57: 135-145.

Baric, S., Kerschbamer, C., and Dalla Via, J. (2006). TaqMan real-time PCR versus four conventional PCR assays for detection of apple proliferation phytoplasma. Plant Molecular Biology Reporter 24: 169-184.

Baric, S., Kerschbamer, C., Vigl, J., Dalla Via, J. (2008). Translocation of apple proliferation phytoplasma via natural root grafts - a case study. European Journal of Plant Pathology 121: 207-211.

Baric, S., Kerschbamer, C., Berger, J., Cainelli, C., Dalla Via, J., Österreicher, J. (2010a). Ausbreitung der Apfeltriebsucht in Südtirol in zwei Wellen. Obstbau Weinbau 47: 70-73.

Baric, S., Öttl, S., Dalla Via, J. (2010b). Infection rates of natural psyllid populations with 'Candidatus Phytoplasma mali' in South Tyrol (Northern Italy). Julius Kühn Archiv 427: 189-192.

Baric, S., Berger, J., Cainelli, C., Kerschbamer, C., Letschka, T., Dalla Via, J. (2011). Seasonal colonisation of apple trees by 'Candidatus Phytoplasma mali'' revealed by a new quantitative TaqMan real-time PCR approach. European Journal of Plant Pathology 129: 455-467.

Baric, S. (2012). Molecular Tools Applied to the Advancement of Fruit Growing in South Tyrol: a Review. Erwerbs-Obstbau 54: 125-135.

Baril, M., Barkier-Gingras, L. (2005). Translation of the F protein of Hepatitis C virus is initiated at a non-AUG codon in a reading frame relative to the polyprotein. Nucleic Acid Research 33: 1474-1486.

Barth, J. (2011). Molekulare Charakterisierung des *Apple stem pitting virus* (ASPV). Bachelorthesis, Fachochschule Bingen, University of Applied Sciences in collaboration with AlPlanta, Institute for Plant Research.

Bhaskar, P.B., Venkateshwaran, M., Wu, L., Ane´, J.M., Jiang, J. (2009). Agrobacterium-Mediated Transient Gene Expression and Silencing: A Rapid Tool for Functional Gene Assay in Potato. PloS ONE 4(6): e5812.

Belshaw, R., Pybus, O.G., Rambaut, A. (2007). The evolution of genome compression in RNA viruses. Genome Research 17:1496-1504.

Ben Khalifa, M., Fakhfakh, H. (2011b). First report of *'Candidatus'* Phytoplasma prunorum infecting almonds in Tunisia. Phytoparasitica 39(4): 411-414.

Berg, M., Davies, D.L., Clark, M.F., Vetten, H.J., Maier, G., Marcone, C., Seemüller, E. (1999). Isolation of the gene encoding an immunodominant membrane protein of the apple proliferation phytoplasma, and expression and characterization of the gene product. Microbiology 145(8): 1937-1943.

Bertaccini, A., Vibio, M., Janeckova, M., Franova-Honetslegrova, J. (1997). Molecular detection of phytoplasmas in apple with rubbery wood symptoms in the Czech republic. Proceedings of the 17th Symposium on virus and virus like diseases of temperate fruit crops, pp. 102-103.

Bertaccini, A., Duduk, B. (2009). Phytoplasma and phytoplasma diseases: a review of recent research. Phytopathologia Mediterranea 48: 355-378.

Birişik, N., Baloğlu, S. (2010). Determination of the effects of *Apple stem grooving virus* on some commercial apple cultivars. Proceedings of the 21st international conference on virus and other graft transmissible diseases of fruit crops. Julius-Kühn- Archiv 427: 240-244.

Bisognin, C., Ciccotti; A., Salvadori; A., Moser; M., Grando, M.S., Jarausch, W. (2007). Effect of different strains of 'Candidatus Phytoplasma mali' in susceptible and resistant *Malus* genotypes inoculated by *in vitro* grafting. Bulletin of Insectology 60 (2): 205-206.

Bisognin, C., Ciccotti; A., Salvadori; A., Moser; M., Grando, M.S., Jarausch, W. (2008a). *In vitro* screening for resistance to apple proliferation in *Malus* species. Plant Pathology 57(6): 1163-1171.

Bisognin, C., Schneider, B., Salm, H., Grando, M.S., Jarausch, W., Moll, E. Seemüller, E. (2008b). Apple proliferation resistance in apomictic rootstocks and its relationship to phytoplasma concentration and simple sequence repeat genotypes. Phytopathology 98, 153-158.

Bisognin, C., Seemüller, E., Citterio, S., Velasco, R., Grando, M.S., Jarausch, W. (2009). Use of SSR markers to assess sexual vs apomictic origin and ploidy level of breeding progenies

derived from crosses of apple proliferation-resistant *Malus sieboldii* and its hybrids with *Malus* × *domestica* cultivars. Plant Breeding 128: 507-513.

Bliefernicht, K., Krczal, G. (1995). Epidemiological studies on apple proliferation disease in southern Germany. Acta Horticulturae 386: 444-447.

Blystad, D-R., Toppe, B., Brurberg, M-B., Sørum, O. (2011): New cases of apple proliferation disease in Norway. Bullet of Insectology LXIV (Supplement).

Bonas, U. (1994). *Hrp* genes of phytopathogenic bacteria. Current Topics of Microbiological Immunology 192: 79-98.

Bonnet, F., Saillard, C., Kollar, A., Seemüller, E., Bové, J.M. (1990). Detection and differentiation of the mycoplasma-like organism associated with the apple proliferation disease using cloned DNA probes. Molecular Plant-Microbe Interactions 3: 438-443.

Bové, J.M., Garnier, M. (1998). Walled and wall-less eibacteria from plants: sieve-tube-restricted plant pathogens. Plant Cell, Tissue and Organ Culture 52: 7-16.

Boyer, J-C., Haenni A-L. (1994). Infectious transcripts and cDNA clones of RNA viruses. Virology 198: 415-426.

Braun, E.J. Sinclair, W.A. (1978) Translocation in phloem necrosis-diseased American elm seedlings. Phytopathology 68: 1733-1737.

Bruening, G. (2006). Resistance to infection. In: Loebenstein, G., Carr, J.P. (eds). Natural Resistance Mechanisms of Plants to Viruses, pp 211-240. Springer, Dordrecht, the Netherlands.

Brzin, J., Ermacora, P., Osler, S., Loi, N., Ravnikar, R., Petrovic, N. (2003). Detection of apple proliferation phytoplasma by ELISA and PCR in growing and dormant apple trees. Journal of Plant Diseases and Protection 110(5): 476-483.

Bustin, S.A:, Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 55(4): 611-622.

Caglayan, K., Ulubas Serce, C., Gazel, M., Jelkmann, W. (2006). Detection of four apple viruses by ELISA and RT-PCR assays in Turkey. Turkish Journal of Agriculture and Foresty 30: 241-246.

Campbell, M.S., Pletnev, A.G. (2000). Infectious cDNA clones of langat tick-borne flavivirus that differ from their parent in peripheral neurovirulence. Virology 269: 225-237.

Canik, D., Ertung, F. (2007). Distribution and molecular characterization of apple proliferation phytoplasma in Turkey. Bulletin of Insectology 60(2): 335-336.

Catlin, P.B., Olsson, E.A. and Beutel, J.A. (1975). Reduced translocation of carbon and nitrogen from leaves with symptoms of pear curl. Journal of the American Society for Horticultural Science 100: 184-187.

Carraro, L., Osler, R., Loi, N., Ermacora, P., Refatti, E. (2001). Fruit tree phytoplasma diseases diffused in nature by psyllids. Acta Horticulturae 550, 345-350.

Carraro, L., Ermacora, P., Loi, N., Osler, R. (2004). The recovery phenomenon in apple proliferation-infected apple trees. Journal of Plant Pathology 86: 141-146.

Chen, T.A., Lei, J.D., Lin, C.P. (1989). Detection and identification of plant and insect mollicutes. In: Whitcomb, R.F., Tully, J.G. (eds). The Mycoplasmas, Academic Press Inc., San Diego, USA, Vol. V, pp. 393-424.

Chirico, N., Vianelli, A., Belshaw, R. (2010). Why genes overlap in viruses. Proceedings of the Royal Society Biological Sciences 277(1701): 3809-3817.

Choi, Y.H., Tapias, E.C., Kim, H.K., Lefeber, A.W.M., Erkelens, C., Verhoeven, J.T.J., Brzin, J., Zel, J., Verpoorte, R. (2004). Metabolic discrimination of *Catharanthus roseus* leaves infected by phytoplasma using 1H-NMR spectroscopy and multivariate data analysis. Plant Physiology 135: 2398-2410.

Clarke, D.K., Duarte, E.A., Elena, S.F., Moya, A., Domingo, E., Holland, J. (1994). The red queen reigns in the kingdom of RNA viruses. Proceedings of the National Acadamy of Science of the USA 91: 4821-4824.

Collmer, C.W., Marston, M.F., Taylor, J.C., Jahn, M. (2000). The *I* Gene of Bean: A Dosage-Dependent Allele Conferring Extreme Resistance, Hypersensitive Resistance, or Spreading Vascular Necrosis in Response to the Potyvirus *Bean common mosaic virus*. Molecular Plant-Microbe Interactions 13: 1266-1270.

Ciccotti, A.M., Bianchedi, P.L., Bragagna, P., Deromedi, M., Filippi, M., Forno, F., Mattedi, L. (2008a). Natural and experimental transmission of '*Candidatus* Phytoplasma mali' by root bridges. Acta Horticulturae 781: 459-464.

Ciccotti, A.M., Bisognin, C, Battocletti, I., Salvadori, A., Herdemertens, M., Jarausch, W. (2008b). Micropropagation of *Malus* sieboldii hybrids resistant to apple proliferation disease. Agronomy research 6: 445-458.

Ciccotti, A.M., Bisognin, C., battocletti, I., Deromedi, M., Bragagna, P., Fillipi, M. (2011). Response of apple proliferation-resistant *Malus* sieboldii hybrids to multiple infections with latent apple viruses. Bulletin of Insectology 64: S273-S274.

Cieslinska, M., Morgas, H. (2011). Detection and identification of *'Candidatus'* Phytoplasma prunorum, *'Candidatus'* Phytoplasma mali' and *'Candidatus'* Phytoplasma pyri in stone fruit trees in Poland Journal of Phytopatholy 159: 217-222.

Clover, G.R.G., Pearson, M.N., Elliot, D.R., Tang, Z., Smales, T.E., Alexander, B.J.R. (2003). Characterization of a strain of *Apple stem grooving virus* in *Actinidia chinensis* from China. Plant Pathology 52: 371-378.

Coffin, R.S., Coutts, R.H., (1993). The closteroviruses, capilloviruses and other similar viruses: a short review. Journal of General Virology 74: 1475-1483.

Crossley, S.J., Jacobi, V., Adams, A.N. (1998). IC-PCR amplification of apple stem grooving virus isolates and comparison of polymerase and coat protein gene sequences. Acta Horticulturae 272: 113-118.

Dagless, E.M., Shintaku, M.H., Nelson, R.S., Foster, G.D. (1997). A CaMV 35S promoter driven cDNA clone of tobacco mosaic virus can infect host plant tissue despite being uninfectious when manually inoculated onto leaves. Archives of Virology 142(1): 183-91.

Danet, J.L., Balakishiyeva, G., Cimerman, A., Sauvion, N., Marie-Jeanne, V., Labonne, G., Lavina, A., Batlle, A., Krizanac, I., Skoric, D., Ermacora, P., Serce, U.S., Caglayan, K., Jarausch, W., Foissac, X. (2011). Multilocus sequence analysis reveals the genetic diversity of European fruit tree phytoplasmas and supports the existence of inter-species recombination. Microbiology 157: 438-450.

Deng, S., Hiruki, C. (1991). Amplification of 16S rRNA from culturable and non-culturable mollicutes. Journal of Microbiological Methods 14: 53-61.

Delker, C., Stenzel, I., Hause, B., Miersch, O. (2006). Jasmonate biosynthesis in *Arabidopsis thaliana* - enzymes, products, regulation. Plant Biology 8: 297-306.

Del Serrone, P., La Starza, S., Krystai, L., Kolber, M., Barba, M. (1998). Occurrence of apple proliferation and pear decline phytoplasmas in diseased pear trees in Hungary. Journal of Plant Pathology 80: 53-58.

De Sequeira, A.O. (1967). Studies on a virus causing stem grooving and graft union abnormalities in Virginia crab apple. Annals of Applied Biology 60: 59-66.

Desvignes, J. C. (1999). Virus Diseases of Fruit Trees, pp.202. Ctifl, Paris.

Dhir, S., Tomar, M., Thakur, P.D., Ram, R., Hallan, V., Zaidi, A.A., (2010). Molecular evidence for *Apple stem pitting virus* infection in India. Plant Pathology 59(2): 393-393.

Dhir, S., Ram, R., Hallan, V., Zaidi, A.A. (2011). Molecular characterization of an Indian variation of Apple stem pitting virus: Evidence of recombination. Journal of Plant Pathology 93(2):471-478.

Ding, X.S., Schneider, W.L., Chaluvadi, S.R., Mian, M.A.R., Nelson, R.S. (2006). Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. Molecular Plant-Microbe Interaction 11: 1229-1239.

Dolzani, F., Branz, A. (2005). Strategie di contenimento delgi scopazzi. Terra trentina 51(3): 21-23.

Donaldson, E.F., Yount, B., Sims, A.C., Burkett, S., Pickles, R.J., Baric, R.S. (2008). Systematic Assembly of a Full-Length Clone of Human Coronavirus NL63. Journal of Virology 82: 11948-11957.

Duduk, B.; Paltrinieri, S.; Ivanović, M.; Bertaccini, A. (2008). Phytoplasmas infecting fruit trees in Serbia. Acta Horticulturae 781: 351-358.

Etropolska, A., Laginov, M. (2012). Monitoring distribution of fruit phytoplasmas in Bulgaria from 2007 until 2011. 22nd International Conference on virus and other Graft Transmissible diseases of Fruit Crops. (Abstract) pp. 22.

Fialova, R., Navratil, M., Valova, P. (2003). Phytoplasma occurrence in apple trees in Czech Republic. Plant Protection Science 39(1): 7-12.

Firrao, G., Gobbi, E., Locci, R. (1993). Use of polymerase chain reaction to produce oligonucleotide probes for mycoplasmali'ke organisms. Phytopathology 83(6): 602-607.

Firrao, G., Gobbi, E., Locci, R. (1994). Rapid diagnosis of apple proliferation mycoplasma-like organism using a polymerase chain reaction procedure. Plant Pathology 43: 669-674.

Foissac, X., Svanella-Dumas, L., Gentit, P., Dulucq, M.J., Marais, A., and Candresse, T. (2005). Polyvalent degenerate oligonucleotides reverse transcription-polymerase chain reaction: A polyvalent detection and characterization tool for trichoviruses, capilloviruses, and foveaviruses. Phytopathology 95: 617-625.

Franova, J., Ludvikova, H., Paprszein, F. (2011). Detection and characterization of phytoplasmas infecting apple tree in Czech Republic during 2010. Bullet of Insectology 64 Supplement): 51-52.

Fuchs, E., Grüntzig, M., Al Kai, B. (1988). Der serologische Nachweis mechanisch übertragbarer Viren des Kern- und Steinobstes. Nachrichtenblatt Pflanzenschutz DDR 10: 208-211.

Gadiou, S., Kundu, J.K., Paunovic, S., Garcia-Diez, P., Komorowska, B., Gospodaryk, A., Handa, A., Massart, S., Birisik, N., Takur, P.D., Polischuk, V. (2010). Genetic diversity of flexiviruses infecting pome fruit trees. Journal of Plant Pathology 92 (3): 685-691.

Gadiou, S., Kundu, J.K. (2012). Evaluation of Reference Genes for the Relative Quantification of *Apple stem grooving virus* and *Apple mosaic virus* in Apple Trees. Indian Journal of Virology 23(1): 39-41.

Galetto, L., Bosco, D., Marzachi, C. (2005). Universal and group-specific real-time PCR diagnosis of flavescence doree (16Sr-V), bois noir (16Sr-Xii) and apple proliferation (16Sr-X) phytoplasmas from fieldcollected plant hosts and insect vectors. Annals of Applied Biology 147: 191-205.

German-Retana, S., Bergey, B., Delbos, R. P., Candresse, T. Dunez, J. (1997). Complete nucleotide sequence of the genome of a severe cherry isolate of apple chlorotic leaf spot trichovirus (ACLSV). Archives of Virology 142: 833-841.

Gibb, K.S., Constable, F.E., Moran, J.R., Padovan, A.C. (1999). Phytoplasmas in Australian grapevines - detection, differentiation and associated diseases. Vitis 38(3): 107-114.

Gibbs, A., Keese, P.K. (1995). In search of the origin of viral genes. In: Gibbs A, Calisher HC, Garcia-Arenal F (eds) Molecular basis of virus evolution. Cambridge University Press, Cambridge, pp 79-90.

Grimsley, N., Hohn, T., Davies, J.W., Hohn, B. (1987). Agrobacterium-mediated delivery of infectious maize streak virus into maize plants. Nature 325: 177-179.

Guest, D., Brown, J.F. (1997). Plant Defenses Against Pathogens. In: Brown, J.F., Ogle, H.J. (Eds). Plant Pathogens and Plant Diseases, pp.263-286. Rockvale Publications, Armidale, Australia.

Guindon, S., Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systems Biology 52: 696-704.

Gundersen, D.E., Lee, I.-M. (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea 35: 144-151.

Guthrie, J.N., Walsh, K.B., Scott, P.T. Rasmussen, T.S. (2001). The phytopathology of Australian papaya dieback: a proposed role for the phytoplasma. Physiological and Molecular Plant Pathology 58: 23-30.

Hajdukiewicz, P., Svab, Z., Mali'ga, P. (1994). The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Molecular Biology 25(6): 989-994.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

Han, Y., Gasic, K., Sun, F., Xu, M., Korban, S.S. (2007). A gene encoding starch branching enzyme I (SBEI) in apple (*Malus* x *domestica*, *Rosaceae*) and its phylogenetic relationship to Sbe genes from other angiosperms. Molecular Phylogenetic Evolution 43: 852-863.

Hassan, M., Myrta, A., and Polak, J. (2006). Simultaneous detection and identification of four pome fruit viruses by one-tube pentaplex RT-PCR. Journal of Virological Methods 133: 124-129.

Hayat, S., Ali, B., Ahmad, A. (2007). Salicylic Acid: Biosynthesis, Metabolism and Physiological Role. In: Hayat, S., Ahmad, A.(Eds). Plants Salicylic Acid: A Plant Hormones, pp.1-14. Springer, the Netherlands.

Hein, J., Støvlbæk, J. (1995). A maximum-likelihood approach to analyzing nonoverlapping and overlapping reading frames. Journal of Molecular Evolution 40: 181-189.

Heinrich, M, Botti, S., Caprara,L., Arthofer, W., Strommer, S., Hanzer, V., Katinger, H., Bertaccini, A., Machada, M.L.D.C. (2001). Improved detection methods for fruit tree phytoplasmas. Plant Molecular Biology Reporter 19: 169-179.

Heintz, W. (1986). *Cuscuta odorata* – an effective vector for mycoplasma-like organisms (MLO). Nachrichtenblatt des Deutschen Pflanzenschutzdienstes 38: 138-141.

Hirata, H., Lu, X., Yamaji, Y., Kagiwada, S., Ugaki, M., Namba, S. (2003). A single silent substitution in the genome of Apple stem grooving virus causes symptom attenuation. Journal of General Virology 84: 2579-2583.

Hirata, H., Yamaji, H., Komatsu, H., Kagiwada, S., Oshima, K., Okano, Y., Takahashi, S., Ugaki, M., Namba, S. (2010). Pseudo-polyprotein translated from the full-length ORF1 of capillovirus is important for pathogenicity, but a truncated ORF1 protein without variable and CP regions is sufficient for replication. Virus Research 152: 1-9.

Holmes, E.C. (2003). Error thresholds and the constraints to RNA virus evolution. Trends in Microbiology 11: 543-546.

Holmes, E.C. (2009). The evolution and emergence of RNA viruses. In: Harvey, P.H., May, R.M. (eds.). Oxford series in Ecology and Evolution, Oxford University Press Inc., New York, pp 114-116.

Hogenhout, S.A., Music, M.S. (2010). Phytoplasma Genomics, from Sequencing to Comparative and Functional Genomics -What Have We Learnt? In: P.G. Weintraub P.G., Jones, P. (eds). Phytoplasmas: Genomes, Plant Hosts and vectors, CAB International, pp. 19-36.

Hogenhout, S.A., Oshima, K., Ammar, E.-D., Kakizawa, S., Kingdom, H.N., Namba, S. (2008). Phytoplasmas: bacteria that manipulate plants and insects. Molecular Plant Pathology 9, 403-423.

Hren, M., Nikolić, P., Rotter, A., Blejec, A., Terrier, N., Ravnikar, M., Dermastia, M., Gruden, K. (2009). 'Bois noir' phytoplasma induces significant reprogramming of the leaf transcriptome in the field grown grapevine. BMC Genomics 2009 10: 460-477.

Hurst, C.J., Linquist, H.D.A. (2000). Defining the Ecology of Viruses. In: Hurst, C.J. (eds). Viral Ecology, Academic press limited, London, Uk, pp. 19-32.

ICTVdB Management (2006). 00.056.0.06.001. Apple stem grooving virus. In: *ICTVdB - The Universal Virus Database*, version 4. Büchen-Osmond, C. (Ed), Columbia University, New York, USA

Inouye, N., Maeda, T., Mitsuhata, K. (1979). Citrus tatter leaf virus isolated from Lily. Annals of Phytopatholy Society of Japan 45: 712-720.

Ito, T., Ieki, H., Ozaki, K. (2002). Simultaneous detection of six citrus viroids and Apple stem Grooving virus from Citrus plants by multiplex reverse transcription polymerase chain reaction. Journal of Virological Methods 106: 235-239.

Ito, K., Akiyama, Y. (2005). Cellular functions, mechanism of action, and regulation of FTSH protease. Annual Reviews of Microbiology 59: 211-231.

James, D. (1998). Factors affecting the detection of apple stem grooving virus by PCR analysis. Acta Horticulturae 472: 119-124.

James, D. (1999). A simple and reliable protocol for the detection of apple stem grooving virus by RT-PCR and in a multiplex PCR assay. Journal of Virological Methods 83: 1-9.

James, D. (2001). Long term assessment of the effects of *in vitro* chemotherapy as a tool for apple stem grooving virus elimination. Acta Horticultura 550: 559-462.

James, D. (2010). Confirmation of the elimination of *Apple stem grooving virus* from apple trees by *in vitro* chemotherapy. 21st International Conference on Virus and other Graft Transmissible Diseases of Fruit Crop. Julius-Kühn-Archiv 427: 47-50

Jarausch, W., Saillard, C., Dosba, F., Bove J.M. (1994a). Differentiation of mycoplasmali'ke organisms (MLOs) in European fruit trees by PCR using specific primers derived from the sequence of a chromosomal fragment of the apple proliferation MLO. Applied and Environmental Microbiology 60(8): 2916-2923.

Jarausch, W., Lansac, M., Dosba, F. (1994b). Micropropagation for maintenance of mycoplasma-like organisms in infected *Prunus marianna* GF 8-1. Acta Horticulturae 359: 169-76.

Jarausch, W., Lansac, M., Dosba, F. (1996). Long-term maintenance of non-culturable apple proliferation phytoplasmas in their micro-propagated natural host plant. Plant Pathology 45: 778-786.

Jarausch, W., Lansac, C., Bliot, B., Dosba, F. (1999). Phytoplasma transmission by *in vitro* graft inoculation as a basis for a preliminary screening method for resistance in fruit trees. Plant Pathology 48: 283-287.

Jarausch ,W., Lansac, M., Portanier, C., Davies, D.L., Decroocq, V. (2000a). *In vitro* grafting: a new tool to transmit pome fruit phytoplasmas to non-natural fruit hosts. Advances in Horticultural Science 14: 29-32.

Jarausch, W., Saillard, C., Helliot, B., Garnier, M., Dosba, F. (2000b). Genetic variability of apple proliferation phytoplasmas as determined by PCR-RFLP and sequencing of a non-ribosomal fragment. Molecular and Cellular Probes 14(1): 17-24.

Jarausch, B., Schwind, N., Jarausch, W., Krczal, G. (2003). First report of *Cacopsylla picta* as a vector of apple proliferation phytoplasma in Germany. Plant Disease 87(10): 101.

Jarausch, W., Peccerella, T., Schwind, N., Jarausch, B., Krczal, G. (2004). Establishment of a quantitative real-time PCR assay for quantification of apple proliferation phytoplasmas in plants and insects. Acta Horticulturae 657: 415-420.

Jarausch, B., Fuchs, A., Schwind, N., Krcal, G. Jarausch, W. (2007). *Cacopsylla picta* as most important vector for 'Candidatus Phytoplasma mali' in Germany and neighbouring regions. Bulletin of Insectology 60: 189-190.

Jarausch, B., Jarausch, W. (2010). Psyllid vectors and their control. In: Phytoplasmas: Genomes, Plant Hosts and vectors. Weintraub, P.G., Jones, P. CAB International, pp.250-271.

Jarausch, B., Schwind, N., Fuchs, A., Jarausch, W. (2011). Characteristics of the Spread of Apple Proliferationby Its Vector *Cacopsylla picta*. Phytopathology 101(12): 1471-1480.

Jelkmann, W. (1994). Nucleotide sequences of apple stem pitting virus and of the coat protein gene of a similar virus from pear associated with vein yellows disease and their relationship with potex- and carlaviruses. Journal of General Virology 75: 1535-1542.

Jelkmann, W. (1995). Cherry virus A: cDNA cloning of dsRNA, nucleotide sequence analysis and serology reveal a new plant capillovirus in sweet cherry Journal of General Virology 76: 2015-2024.

Jelkmann, W. (1997). Apple stem pitting virus. In: Vesely, D. Monette, P. (eds). Filamentous Viruses of Woody Plants, Research Signpost, Trivandrum- 695008, India, pp. 133-141.

Jelkmann, W., Keim-Konrad, R. (1997). An immuno-capture polymerase chain reaction and plate-trapped ELISA for the detection of apple stem pitting virus. Journal of Phytopathology 145: 499-504.

Ji, L.H., Ding, S.W. (2001). The suppressor of transgene RNA silencing encoded by *Cucumber mosaic virus* interferes with salicylic acid-mediated virus resistance. Molecular Plant Microbe Interaction 14: 715-724.

Jones, J.D.G., Dangl, J.L. (2006). The plant immune system. Nature 444: 323-329.

Jovel, J., Walker, M., Sanfaçon, H. (2007). Recovery of *Nicotiana benthamiana* Plants from a Necrotic Response Induced by a Nepovirus Is Associated with RNA Silencing but Not with Reduced Virus Titer. Journal of Virology 81(22): 12285-12297.

Kaminska, M., Sliwa, H. (2008a). Mixed infection of dahlia plants in Poland with apple proliferation and aster yellows phytoplasmas. Plant Pathology 57: 363.

Kaminska, M., Sliwa, H. (2008b). First report of 'Candidatus Phytoplasma mali' in oriental lilies and its association with leaf scorch in Poland. Plant Pathology 57: 363.

Kartte, S., Seemüller, E. (1988). Variable response within the genus *Malus* to the apple proliferation disease. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 95(1):25-34.

Kartte, S., Seemüller, E. (1991a). Histopathology of apple proliferation in *Malus* taxa and hybrids of different susceptibility. Journal of Phytopathology 131: 149-160.

Kartte, S., Seemüller, E. (1991b). Susceptibility of grafted *Malus* taxa and hybrids to apple proliferation disease. Journal of Phytopathology 131: 137-148.

Kazan, K., Manners, J.M. (2008). Jasmonate Signaling: Toward an Integrated View. Plant Physiology 146: 1459-1468.

Keese, P.K., Gibbs, A. (1992). Origins of genes: "big bang" or continuous creation? Proceedings of the National Acadamy of Science of the USA 89: 9489-9493.

Kinard, G.R., Scott, S.W., Barnett, O.W. (1996). Detection of apple chlorotic leafspot and apple stem grooving viruses using RT-PCR. Plant Disease 80: 616-621.

Kirby, M. J., Guise, C.M., Adams, A.N. (2001). Comparison of bioassays and laboratory assays for apple stem grooving virus. Journal of Virological Methods 93: 167-173.

Kison, H., Schneider, B., Seemüller, E. (1994). Restriction fragment length polymorphisms within the apple proliferation mycoplamsalike organism. Journal of Phytopathology 141: 395-401.

Knapp, E., Hanzer, V., Mendonca, D., da Camara Machado, A., Katinger, H., Laimer de Camara Machado, M. (1998). Improved virus detection in rosaceous fruit trees *in vitro*. Plant Cell, Tissue and Organ culture 52: 3-6.

Koganezawa, H., Yanase, H. (1990). A new type of elongated virus isolated from apple trees containing the stem pitting agent. Plant Disease 74: 610-614.

Komorowska, B., Malinowski, T. (2009). The Attempts to produce antiserum against apple stem pitting virus coat protein (ASPV-CP) obtained in prokaryotic and eukaryotic expression systems. Journal of Fruit and Ornamental Plant Research 17(2): 21-30.

Korber, B. (2000). HIV signature and sequence variation analysis. In: Allen Rodrigo G, Learn GH (eds) Computational analysis of HIV molecular sequences. Kluwer Academic Publishers, Dordrecht, pp 55-72.

Krakauer, D.C. (2000). Stability and evolution of overlapping genes. Evolution 54: 731-739.

Kube, M., Schneider, B., Kuhl, H., Dandekar, T., Heitmann, K., Migdoll, A.M., Reinhardt, R., Seemüller, E. (2008). The linear chromosome of the plant-pathogenic mycoplasma 'Candidatus Phytoplasma mali'. BMC Genomics 9: 306.

Kummert, J., Marinho, V. L. A., Rufflard, G., Colinet, D. Lepoivre, P. (1998). Sensitive detection of Apple stem grooving and Apple stem pitting viruses from infected apple trees by RT-PCR. Acta Horticulturae 472: 97-105.

Kummert, J., Vendramme, M., Lepoivre, P., Steyer, S. (2001). Development of routine RT-PCR ELOSA tests for fruit tree certification. Acta Horticulturae 550: 45-52.

Kundu, J. K. (2002). The application of RT-PCR assay for the detection of *Apple stem pitting virus* and *Apple stem grooving virus* in four apple cultivars. Plant Protection Science 38: 13-17.

Kundu, J. K. (2003). The occurrence of *Apple stem pitting virus* and *Apple stem grooving virus* within field-grown apple cultivars evaluated by RT-PCR. Plant Protection Science 39(3): 88-92.

Kundu, J.K. (2006). Detection, distribution and genetic diversities of *Apple stem pitting virus* and *Apple stem grooving virus* in the Czech Republic. Acta Horticulturae 781: 135-142.

Kundu, J.K., Svoboda, J., Polak, J. (2003). Detection of Apple Stem Grooving Virus in different tissues of apple trees throughout the year. Plant Protection Science. 39: 93-96.

Kundu, J.K., Yoshikawa, N. (2008). **Apple stem pitting virus.** In: Rao, G.P., Myrta, A., Ling, K-L. (eds). Characterization, diagnosis & management of plant viruses: horticultural crops, Chapter 1, vol 2. Studium Press, Houston, pp 1-15.

Kunze, L. (1989). Apple proliferation. In: Friedlung, P.R. (eds).Virus and viruslike diseases of pome fruits and simulating noninfectious disorders. Cooperative extension, Washington State University, Pullman, pp. 284-295.

Lansac, M., Chalak, L., Cardona, B., Sorbier, A., Bodin-Ferri, M., Dosba, F., Labonne, G., Quiot, L., Quiot, J.B. (1998). *In vitro* Inoculation of Prunus species with Plum pox potyvirus. Acta Horticulturae 472: 455-460.

Lavina, A., Sebate, J., Battle, A. (2011). 'Candidatus Phytoplasmas mali': identification of potential insect vectors in Spanish apple orchards. Bulletin of Insectology 64 (Supplement): 125-126.

Lee, I. M., Bertaccini, A. Vibio, M., Gundersen, D.E. (1995). Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. Phytopathology 85(6): 728-735.

Lee, I.M., Gundersen-Rindal, D.E., Davis, R.E., Bartoszyk, I.M. (1998). Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. International Journal of Systematic Bacteriology 48: 1153-1169.

Leiser, R.M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Lemaire, O., Guilley, H., Richards, K., Jonard, G. (1992). Agroinfection as an alternative to insects for infecting plants with beet western yellows luteovirus. Proceedings of the Natonal Acadamy of Science of the USA 89: 9136-9140.

Lemmetty, A., Tuovinen, T., Kemppainen, R. (2011). 'Candidatus Phytoplasma mali' infected *Cacopsylla picta* found in apple orchards in South-Western Finland. Bulletin of Insectology 64 (Supplement), 257-258.

Leon, R., Santamaria, J.M., Alpizar, L., Escamilla, J.A., Oropeza, C. (1996). Physiological and biochemical changes in shoots of coconut palms affected by lethal yellowing. New Phytologist 134: 227-234.

Lepka, P., Stitt, M., Moll, E. Seemüller, E. (1999). Effect of phytoplasmal infection on concentration and traslocation of carbohydrates and amino acids in periwinkle and tobacco. Physiological and Molecular Plant Pathology 55, 59-68.

Leone, G., Lindner, J. L., Jongedijk, G., van der Meer, F. A. and Schoen, C. D. (1998). Symptoms on apple and pear indicators after back-transmission from *Nicotiana occidentalis* confirm the identity of *Apple stem pitting virus* with *Pear vein yellows virus*. Acta Horticulture 472: 61-65.

Lichtenegger, L., Neumüller, M., Treutter, D. (2010). *In vitro* grafting as a tool for investigating the hypersensitivity of European plum (Prunus domestica L.) to the Plum pox virus. Acta Horticulturae 874: 243-248.

Liebenberg, A., Moury, B., Sabath, N., Hell, R., Kappis, A., Jarausch, W., Wetzel, T. (2012). Molecular Evolution of the Genomic RNA of Apple Stem Grooving Capillovirus. Journal of Molecular Evolution 75: 92-101.

Lister, R.M., Bancroft, J.B., Nadakavukaren, M.J. (1965). Some sap-transmissible viruses from apple. Phytpathology 55:859-870.

Lister, R.M. (1970). Apple stem grooving virus. CMI/AAB Descriptions of plant viruses, no. 31. Kew, UK: Commonwealth Mycological Institute.

Lister, R.M., Bar-Joseph, M. (1981). Closteroviruses. In Kurstak, E. (eds). *Handbook of Plant Virus Infections and Comparative Diagnosis*, Amsterdam, Elsevier, pp. 809-844.

Liu, N., Niu, J., Zhao, Y. (2011). Complete genomic sequence analyses of Apple stem pitting virus isolates from China. Virus Genes 44(1): 124-130.

LiXin, G., BenChun, X., HungYun, C., WeiJun, D., ShuiFang, Z. (2005). Sequence analysis of the capsid protein gene of KRL-1 isolate of Apple stem grooving virus. Journal Fruit Science 22: 576-578.

LiXin, G., BenChun, X., HungYun, C., WeiJun, D., HongJun, C., ShuiFang, Z. (2006). Detection of Apple stem grooving virus by real-time fluorescent RT-PCR one step assay. Acta Phytopathologica Sinica 36: 57-61.

Loake, G., Grant, M. (2007). Salicylic acid in plant defence - the players and protagonists. Current Opinion in Plant Biology 10: 466-472.

Lorenz, K.H., Schneider, B., Ahrens, U., Seemüller, E. (1995). Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. Phytopathology 85(7): 771-776.

MacKenzie, D.J., McLean, M.A., Mukerji, S., Green, M. (1997). Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. Plant Disease 81: 222-226.

Magome, H., Yoshikawa, N., Takahashi, T., Ito, T., Miyakawa, T. (1997). Molecular variability of the genomes of capilloviruses from apple, Japanese pear, European pear, and citrus trees. Phytopathology 87: 389-396.

Magome, H., Yoshikawa, N., Takahashi, T. (1999) Single-strand conformation polymorphism analysis of apple stem grooving capillovirus sequence variants. Phytopathology 89: 136-140.

Malagnini, V., Pedrazzoli, F., Gualandri, V., Zasso, R., Bozza, E., Flamingo, F., Pozzebon, A., Mori, N., Ioriatt, C. (2010). Detection of 'Candidatus Phytoplasma mali' in different populations of *Cacopsylla melanoneura* in Italy. Bulletin of Insectology 63 (1): 59-63.

Malcuit, I., Marano, M.R., Kavanagh, T.A., De Jong, W., Forsyth, A., Baulcombe, D.C. (1999). The 25-kDa movement protein of PVX elicits Nb-mediated hypersensitive cell death in potato. Molecular Plant Microbe Interaction 12: 536-543.

Malinowski, T., Komorowska, B., Golis, T. and Zawadzka, B. (1998). Detection of Apple stem pitting virus and pear vein yellows virus using reverse transcription polymerase chain reaction. Acta Horticulturae 472: 87-95.

Marcone, C., Ragozzino, A., Seemüller, E. (1996). Association of phytoplasmas with the decline of European hazel in southern Italy. Plant Pathology 45: 857-863.

Marcone, C. (2010). Movement of Phytoplasmas and the Development of Disease in the Plant. In: Weintraub, P.G. Jnes, P. (eds). Phytoplasmas: Genomes, Plant Hosts and vectors. CAB International, pp.114-131.

Marinho, V.L.A., Kummert, J., Rufflard, G., Colinet, D., Lepoivre, P. (1998). Detection of apple stem grooving virus in dormant apple trees with crude extracts as template for one-step RT-PCR. Plant Disease 82: 785-790.

Martelli, G. P. and Jelkmann, W. (1998). *Foveavirus*, a new plant genus. Archives of Virology 143: 1245-1249.

Martelli, G.P., Adams, M.J., Kreuse, J.F., Dolja V.V. (2007). Family Flexiviridae: a case of study in virion and genome plasticity. Annual Review of Phtopathology 45: 73-100.

Martin, D.P., Williamson, C., Posada, D. (2005). RDP2: recombination detection and analysis from sequence alignments. Bioinformatics 21: 260-262.

Martini, M., I. Lee, I.M., Bottner, K.D., Zhao, Y., Botti, S., Bertaccini, A., Harrison, N.A., Carraro, L., Marcone, C., Khan, A.J., Osler, R. (2007). Ribosomal protein gene-based

phylogeny for finer differentiation and classification of phytoplasmas. International Journal of Systematic and Evolutionary Microbiology 57: 2037-2051.

Marwitz, R., Petzold, H., Kunze, L. (1974). Investigations on the transfer of the possible causal agent of apple proliferation to an herbaceous host. Phytopathologische Zeitschrift 81: 85-91.

Massart, S., Brostaux, Y., Barbarossa, L., Batlle, A., Cesar, V., Dutrecq, O., Fonseca, F., Guillem, R., Komorowska, B., Olmos, A., Steyer, S., Wetzel, T., Kummert, J. and Jijakli, M.H. (2009). Interlaboratory evaluation of two Reverse-transcriptase Polymeric Chain Reaction-based methods for detection of four fruit tree viruses. Annals of Applied Biology, 154:133-141.

Mathioudakis, M.M., Mali'ogka, V.I., Dovas, C.I., Vasilakakis, M., Katis, N.I. (2006). First record of the *Apple stem pitting virus* (ASPV) in Quince in Greece. Journal of Plant Pathology 88: 225-225.

Mattedi, L., Forno, F., Cainelli, C., Grando, M.S., and Jarausch, W. (2008). Research on *'Candidatus'* Phytoplasma mali' transmission by insect vectors in Trentino. Acta Horticulturae 781: 369-374.

Matus, J.T., Andrea, V., Rodrigo, L., Carolina, S., Soledad, C., Patricio, A. (2008). Phytoplasma and virus detection in commercial plantings of *Vitis vinifera* cv. Merlot exhibiting premature berry dehydration. Electronic Journal of Biotechnology 11(5): 1-10.

Maust, B.E., Espadas, F., Talavera, C., Aguilar, M., Santamaria, J.M. and Oropeza, C. (2003). Changes in carbohydrate metabolism in coconut palms infected with the lethal yellowing phytoplasma. Phytopathology 93: 976-981.

McCoy, R.E. (1979). Mycoplasmas and yellows diseases. In: Whitcomb, R.F., Tully, J.G. (eds) The Mycoplasmas, Vol. III. Academic Press, New York, pp. 229-264.

Mehle, N., Brzin, J., Boden, J., Hren, M., Frank, J., Petrovic, N., Gruden, K., Dreo, T., Zezlina, I., Seljak, G., Ravnikar, M. (2007). First report of '*Candidatus*' Phytoplasma mali' in Prunus avium, P. armeniaca and P. *domestica.* Plant pathology 56(4): 721

Mehle, N., Ravnikar, M., Seljak, G., Knapie, V., Dermastia, M. (2011). The most widespread phytoplasmas, vectors and measures for disease control in Slovenia. Phytopathogenic Mollicutes 1(2): 65-76.

Melcher, U. (2000). The '30K' superfamily of viral movement proteins. Journal of General Virology 81: 257-266.

Menzel, W., Jelkmann, W., Maiss, E. (2002). Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. Journal of Virological Methods 99: 81-92.

Menzel, W., Zahn, V., Maiss, E. (2003). Multiplex RT-PCR-ELISA compared with bioassay for the detection of four apple viruses. Journal of General Methods 110: 153-157.

Morozov, S.Y., Solovyev, G.A. (2003). Triple gene block: modular design of a multifunctional machine for plant virus movement. Journal of General Virology 84: 1351-1366.

Mur, L.A.J., Kenton, P., Atzorn, R., Miersch, O., Wasternack, C. (2006). The Outcomes of Concentration- Specific Interactions between Salicylate and Jasmonate Signaling Include Synergy, Antagonism, and Oxidative Stress Leading to Cell Death. Plant Physiology 140: 249-262.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco culture. Physiologia Plantarum 15: 473-97.

Murashige, T., Bitters, W.P., Rangan, T.S., Nauer, E.M., Roistacher, C.N., Holliday, P.B. (1972). A technique of shoot apex grafting and its utilization towards recovering virus-free *Citrus* clones. Hortscience 7: 118-119.

Murphy, A.M., Chivasa, S., Singh, D.P., Carr, J.P. (1999). Salicylic acid-induced resistance to viruses and other pathogens: a parting of the ways? Review Article. Trends in Plant Science 4: 155-160.

Myrta, A., Ermacora, P., Stamo, B., Osler, R. (2003). First report of phytoplasma infections in fruit trees and grapevine in Albania. Journal of Plant Pathology 85(1): 64-64.

Nassar, E., Choueiri, E., Minoia, S., Di Serio, F., Djelouah K. (2012). First survey on pome fruit viruses in Lebanon. Proceedings of the 22st international conference on virus and other graft transmissible diseases of fruit crops Abstracts pp.123.

Navratil, M., Pribylova, J., Valova, P., Fialova, R., Savarova, D., Spak, J., Kubelkova, D., Petrzik, K., Karesova, R., Spakova, V. (2007). Detection and identification of phytoplasmas in *Ribes rubrum*. Bulletin of Insectology 60 (2): 123-124.

Nei, M., Gojobori, T. (1986) Simple method for estimating the numbers of synonymous and nonsynonymous substitutions. Molecular Biology Evolution 3: 418-426.

Nemchinov, L., Hadidi, A., Faggioli, F. (1998). PCR-detection of Apple stem pitting virus from pome fruit hosts and sequence variability among viral isolates. Acta Horticulturae 472: 67-73.

Németh, M. (1986). Virus, mycoplasma and rickettsia diseases of fruit trees. Martinus Nijhoff Publishers, Budapest, Hungary pp. 841.

Normark, S., Bergstrom, S., Edlund, T., Grundstrom, T., Jaurin, B., Lindberg, F.P., Olsson, O. (1983). Overlapping genes. Annual Review of Genetics 17: 499-525.

Ohira, K., Namba, S., Rozanov, M., Kusumi, T., Tsuchizaki, T. (1995). Complete sequence of an infectious full-length cDNA clone of citrus tatter leaf capillovirus: comparative sequence analysis of capillovirus genomes. Journal of General Virology 76: 2305-2309.

Ohki, S. T., Yoshikawa, N., Inouye, N., Inouye, T. (1989). Comparative electron microscopy of *Chenopodium quinoa* leaves infected with apple chlorotic leaf spot, apple stem grooving, or citrus tatter leaf virus. Annals of the Phytopathological Society of Japan 55: 245-249.

Olivier, T., Steyer, S., Demonty, E., Laurent, P. (2010). First report of molecular identification of '*Candidatus*' Phytoplasma mali' in apple trees in Belgium. New disease reports 21: 11.

Oshima, K., Kakizawa, S., Nishigawa, H., Jung, H.Y., Wei, W., Suzuki , S., Arashida, R., Nakata, D., Miyata, S., Ugaki, M., Namba, S. (2004) Reductive evolution suggested from the complete genome sequence of a plant-pathogenic phytoplasma. Nature Genetics 36: 27-29.

Osler, R., Ermacora, P., Loi, N., Cararro, L., Ferrini, F., Refatti, E. (2001). Control strategies of Apple proliferation, a serious disease occuring both in Slovenia and in Italy In: Milevoj, L. (ed). 5th Slovenian Conference on Plant Protection. Ljubljana University,Biotechnical Faculty, Slovenia, pp. 238-243.

Pallas, V., Garcia, J.A. (2011). How do plant viruses induce disease? Interactions and interference with host components. Review. Journal of General Virology 92: 2691-2705.

Paltrinieri, S., Martini, M., Stefani, E., Pondrelli, M., Fideghelli, C., Betaccini, A. (2001). Phytoplasma infection in peach and cherry in Italy. Acta Horticulturae 550: 365-370.

Paltrinieri, S., Duduk, B., Dal Molin, F., Mori, N., Comerlati, G., Bertaccini, A. (2010). Molecular characterization of 'Candidatus Phytoplasma mali' strains in outbreaks of apple proliferation in north eastern Italy, Hungary, and Serbia. Proceedings of the 21st international conference on virus and other graft transmissible diseases of fruit crops. Julius-Kühn-Archiv 427: 178-182.

Palukaitis, P., Carr, J.P. (2008). Invited Review: Plant Resistance Response to Viruses. Journal of Plant Pathology 90(2): 153-171.

Pavesi, A. (2007). Pattern of nucleotide substitution in the overlapping nonstructural genes of influenza A virus and implication for the genetic diversity of the H5N1 subtype. Gene 402: 28-34.

Pieterse, C.M.J., Dicke, M. (2007). Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends in Plant Science 12: 564-569.

Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S., Van Wees, S.C.M. (2009). Networking by small-molecule hormones in plant immunity. Nature Chemical Biology 5: 308-316.

Polak, J., Zieglerova, J. (2001). Distribution of Apple stem grroving virus in Czech Republic. Plant Protection Science UZPI 37: 1212-2580.

Rajan, J., Clark, M.F. (1995). Detection of apple proliferation and other MLOs by immune-capture PCR (IC-PCR). Acta Horticulturae 386: 511-514.

Rodoni, B.C., Constable F.E. (2008). The incidence and strain variation of Apple stem grooving and Apple stem pitting viruses in Australian pome fruit. Acta Horticulturae 781: 167-174.

Rui, D., Ciferri, R., Refatti, E. (1950). La virosi degli "scopazzi del melo" nel Veronese. Notiziario sulle malattie delle piante 13: 7-11.

Rumbou, A., Carraro, L., Nanos, G., Boutla, I., Rumbos, I.C. (2007). First report of 'Candidatus Phytoplasma mali' in Greece and correlation with small apple fruit disorder occurring in the orchards of the Pelion Mountain. Acta Horticulturae, 781: 505-510.

Rumbou, A., Nanos, G., Rumbos, I.C. (2010).Ten years of apple proliferation epidemics in the apple orchards of Pelion Mountain in Greece In: Bertaccini, A., Lavina, A., Torres, E. (eds). Current status and perspectives of phytoplasma disease research and management, Sitges, Spain. p. 91.

Rumbou, A., Miliordos, D., Athanasiou, C., Koutroumpas, A., Sahinglou, A., Rumbos, I.C. (2011). Investigation on the apple proliferation epidemics in the orchards of the Pelion Mountain and preliminary observations on possible phytoplasma vectors. Bulletin of Insectology 64 (Supplement): 259-260.

Ryabov, E.V. (2008). Construction of infectious cDNA clones for RNA viruses: Turnip crinkle virus. Methods of Molecular Biology 451: 491-502.

Sabath, N., Landan, G., Graur, D. (2008) A method for the simultaneous estimation of selection intensities in overlapping genes. PLoS ONE 3(12):e3996. doi:10.1371/journal.pone.0003996

Salem, N., Mansour, A., Al-Musa, A. (2005). Viruses of pome fruit trees in Jordan. Journal of Plant Pathology 87: 123-126.

Salmon, M.A., Vendrame, M., Kummert, J., Lepoivre, P. (2002). Rapid and Homogenous Detection of *Apple stem pitting* Virus by RT-PCR and a Fluorogenic 3' Minor Groove Binder-DNA Probe. European Journal of Plant Pathology 108: 755-762.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). Molecular cloning: a laboratory manual. 2nd edition. Cold Springs Harbour Laboratory Press, New York, USA.

Sánchez-Rojo, S.; López-Delgado, H., Mora-Herrera, M., Almeyda-León, H., Zavaleta-Mancera, H., Espinosa-Victoria, D. (2011). Salicylic Acid Protects Potato Plants-from Phytoplasma-associated Stress and Improves Tuber Photosynthate Assimilation. American Journal of Potato Research 88(2): 175-183.

Schaper, U., Seemüller, E. (1984). Recolonization of the stem of apple proliferation and pear decline-diseased trees by the causal organisms in spring. Journal of Plant Disease and Protection 91: 608-613.

Schmidt, H. (1964). Beiträge zur Züchtung apomiktischer Apfelunterlagen. Z. Pflanzenzüchtung 52: 27-102.

Schmidt, H. (1972). The effect of latent virus infections on the yield of maiden tress on 20 apomictic apple seedling rootstocks. Journal of horticultural Science 47: 159-163.

Schmidt, H. (1988). Criteria and procedure for evaluating apomictic rootstocks for apple. HortScience 23, 104-107.

Schneider, B., Seemüller, E., Smart, C.D., Kirkpatrick, B. (1995). Phylogenetic classification of plant pathogenic mycoplasmali'ke organisms or phytoplasmas. In: Razin S., and Tully, J.D. (eds.) Molecular and Diagnostic Procedures in Mycoplasmatology, Academic Press, San Diego,USA, pp. 369-380.

Schneider, B., Marcone, C., Kampmann, M., Ragozzino, A., Lederer, W., Cousin, M.T., and Seemüller, E. (1997). Characterization and classification of phytoplasmas from wild and

cultivated plants by RFLP and sequence analysis of ribosomal DNA. European Journal of Plant Pathology 103: 675-686.

Schneider, B., Seemüller, E. (2009). Strain differentiation of *'Candidatus'* Phytoplasma mali' by SSCP- and sequence analyses of the *HFLB* gene. Journal of Plant Pathology 91(1): 103-112.

Schwarz, K., Jelkmann, W. (1998). Detection and characterization of European *Apple stem pitting virus* sources from apple and pear by PCR and partial sequence analysis Acta Horticulturae 472: 75-85.

Seemüller, E., U. Schaper, Zimbelmann, F. (1984). Seasonal variation in the colonization patterns of mycoplasmali'ke organisms associated with apple proliferation and pear decline. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 91(4): 371-382.

Seemüller, E., Kartte, S., Kunze, L. (1992). Resistance in established and experimental apple rootstocks to apple proliferation disease. Acta Horticulturae 309: 245-251.

Seemüller, E., Kison, H., Lorenz, K.H. (1998a). On the geographic distribution and prevalence of the apple proliferation phytoplasma in low intensity orchards in Germany. Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz 105(4): 404-410.

Seemüller, E., Marcone, C., Lauer, U., Ragozzino, A., Goschl, M. (1998b). Current status of molecular classification of the phytoplasmas. Journal of Plant Pathology 80: 3-26.

Seemüller, E., Schneider, E. (2004). 'Candidatus Phytoplasma mali', 'Candidatus Phytoplasma pyri' and 'Candidatus Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. International Journal of Systematic and Evolutionary Microbiolog 54: 1217-1226.

Seemüller, E., Schneider, E. (2007). Differences in virulence and genomic features of strains of 'Candidatus Phytoplasma mali', the apple proliferation agent. Phytopathology 97(8):964-970.

Seemüller, E., Moll, E., Schneider, B. (2008). Apple proliferation resistance of *Malus* sieboldii-based rootstocks in comparison to rootstocks derived from other *Malus* species. European Journal of Plant Pathology 121: 109-119.

Seemüller, E., Kiss, E., Sule, S., Schneider, B. (2010). Multiple infection of apple trees by distinct strains of 'Candidatus Phytoplasma mali' and its pathological relevance. Phytopathology 100: 863-870.

Seemüller, E., Harris, H. (2010). Plant Resistance. In: Weintraub P.G., Jones, P. (eds). Phytoplasmas: Genomes, Plant Hosts and vectors. CAB International, pp. 147-169.

Semancik, J.S., Weathers, L.G. (1965). Partial purification of a mechanically transmissible virus associated with tatter leaf of citrus. Phytopathology 55: 1354-1358.

Sertkaya, G., Martini, M., Osler, R. (2008). First report of '*Candidatus*' phytoplasma mali' in Turkey. Journal of Plant Pathology 90: 143.

Shi, B-J., Ding, S-W., Symons, R.H. (1997). Plasmid vector for cloning infectious cDNAs from plant RNA viruses: high infectivity of cDNA clones of tomato aspermy cucumovirus Journal of General Virology 78: 1181-1185.

Shim, H., Min, Y., Hong, S., Kwon, M., Kini, D., Kim, H., Choi, Y., Lee, S., Yang, J. (2004). Nucleotide sequence of a Korean isolate of apple stem grooving virus associated with black necrotic leaf spot disease on pear (Pyrus pyrifolia). Molecular Cells 18: 192-199.

Smart, C.D., Schneider, B., Blomquist, C.L., Guerra, L.J., Harrison, N.A., Ahrens, U., Lorenz, K.H., Seemüller, E., Kirkpatrick, B.C. (1996). Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. Applied Environmental Microbioliogy 62: 2988-2993.

Springhetti, M., Janes, P.G., Dallago, G. (2002). Diffusione degli scopazzi (AP) nel melo nelle valli del noce. In: Brunelli A., Canova A. (eds). Proceedings Giornate Fitopatologiche, vol. II, Baselga di Piné, Trento, pp. 599-606.

Stoimenova, E., marinova, G., Garcia-Luque, I. (2005). Influence of virus concentration on expression of cucumber mosaic virus resistance in pepper. Biotechnology & Biotechnological Equipment 19: 36-41.

Stouffer, R.F. (1989). Apple stem pitting. In: Fridlund P.R. (ed). Virus and Virus-like Diseases of Pome Fruits and Simulating Non-infectious Disorders. Washington State University, Pullman, WA, USA, pp. 138-144.

Strauss, E. (2009). Phytoplasma Reasearch Begins to Bloom. Science 325: 388-390.

Sugio, A., Kingdom, H.N., Nicholls, V.M., Hogenhout, S.A. (2010). The phytoplasma effector protein SAP11 improves vector fitness. 18th Congress of the International Organization of Mycoplasmology Abstract: 47:82.

Sugio, A., MacLean, A.M., Kingdom, H.N., Grieve, V.M., Manimekalai, R., Hogenhout, S.A. (2011). Diverse Targets of Phytoplasma Effectors: From Plant Development to Defense Against Insects. Annual Review of Phytopathology 49: 175-95.

Sutic, D.D., Ford, R.E., Tosic, M.T. (1999). Virus diseases of fruit trees. In: Sutic D.D., Ford R.E., Tosic M.T. (eds). Handbook of Plant Virus Diseases, CRC Press, Boca Raton, FL, USA, pp. 321-432.

Takahara, T., Kawase,K., Ono, S., Iwagaki, I., Hirose, K., Yoshinaga, K. (1988). Rootstocks for Ponkan (Citrus reticulata Blanco) in relation to tatter leaf virus. Bulletin Fruit Tree Research. Station D 10: 35-45.

Tamura, K., Dudley, J., Nei, M., Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 40. Molecular Biology Evolution 24: 1596-1599.

Tan, P.Y., Whitlow, T. (2001). Physiological responses of *Catharanthus roseus* (periwinkle) to ash yellows phytoplasmal infection. New Phytologist 150: 757-769.

Tatineni, S., Afunian, M.R., Gowda, S., Hilf, M.E., Bar-Joseph, M., Dawson, W.O. (2009a). Characterization of the 5- and 3-terminal subgenomic RNAs produced by a capillovirus: evidence for a CP subgenomic RNA. Virology 385: 521-528.

Tatineni, S., Afunian, M.R., Hilf, M.E., Gowda, S., Dawson, W.O., Garnsey, S.M. (2009b). Molecular characterization of Citrus tatter leaf virus historically associated with Meyer Lemon trees: complete genome sequence and development of biologically active *in vitro* transcripts. Phytopathology 99: 423-431.

Tedeschi, R., Bosco, D., Alma, A. (2002). Population dynamics of *Cacopsylla melanoneura* (Homoptera: Psyllidae), a vector of apple proliferation phytoplasma in Northwestern Italy. Journal of Economic Entomology 95(3): 544-551.

Tedeschi, R., Visentin, C., Alma, A. Bosco, D. (2003): Epidemiology of apple proliferation (AP) in northwestern Italy: evaluation of the frequency of AP-positive psyllids in naturally infected populations of *Cacopsylla melanoneura* (Homoptera: Psyllidae). Annals of applied Biology 142: 285-290.

Tedeschi, R., Alma, A. (2006). *Fieberiella florii* (Homoptera: Auchenorrhyncha) as a vector of "Candidatus Phytoplasma mali'". Plant Disease 90(3): 284-290.

Tedeschi, R., Lauterer, P., Brusetti. L., Tota, F., Alma, A. (2009). Composition, abundance and phytoplasma infection in the hawthorne psyllid fauna of northwestern Italy. European Journal of Plant Pathology 123: 301-310.

Terauchi, H., Magome, H., Yoshikawa, N., Takahashi, T., Inouy, N. (1997). Construction of an infectious cDNA clone of the apple stem grooving capillovirus (isolate Li-23) genome containing a cauliflower mosaic virus 35S RNA promoter. Annals of Phytopatholy Society Japan 63: 432-436.

Thaler, J.S., Humphrey, P.T., Whiteman, N.K. (2012). Special Issue: Specificity of plant–enemy interactions Evolution of jasmonate and salicylate signal crosstalk. Trends in Plant Science 17(5): 260-270.

Torres, E., Bertolini, E., Cambra, M., Monton, C., and Martin, M.P. (2005). Real-time PCR for simultaneous and quantitative detection of quarantine phytoplasmas from apple proliferation (16SrX) group. Molecular and Cellular Probes 19: 334-340.

Tran-Nguyen, L.T.T., Kube, M., Schneider, B., Reinhardt, R., Gibb, K.S. (2008). Comparative genome analysis of "Candidatus Phytoplasma australiense" (subgroup tuf-Australia I; rp-A) and "*Ca*. phytoplasma asteris" strains OY-M and AY-WB. Journal of Bacteriology 190(11): 3979-3991.

Turnage, M.S., Muangsan, N., Peele, C.G., Robertso, D. (2002). Geminivirus-based vectors for gene silencing in Arabidopsis. Plant Journal 30: 107-117.

Turner, J.G., Ellis, C., Devoto, A. (2002). The Jasmonate Signal Pathway. The Plant Cell Online 14: S153-S164.

Van der Meer, F.A. (1976). Observation on apple stem grooving virus. Acta Horticultura 67: 293-304.

Van der Salm, T.P.M., Van der Toorn, C.J.G., Hänisch ten Cate, C.H., Dubois, L.A.M., De Vries, D.P., Dons, H.J.M. (1994). Importance of the iron chelate formula for micropropagation of *Rosa hybrida* L. 'Moneyway'. Plant Cell, Tissue and Organ Culture 37: 73-77.

Vives, M.C., Martin, S., Ambros, S., Renovell, A., Navarro, L., Pina, J.A., Moreno, P., Guerri, J. (2008). Development of a full-genome cDNA clone of Citrus leaf blotch virus and infection of citrus plants. Molecular Plant Pathology 9(6): 787-97.

Voinnet, O., Ledere, C., Baulcombe, D.C. (2000). A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. Cell 103: 157-67.

Walters, D., Heil, M. (2007). Costs and trade-offs associated with induced resistance. Physiological and molecular plant pathology 71: 3-17.

Waterworth, H.E., Gilmer, R.M. (1969). Dark green epinasty of Chenopodium quinoa, a symdrome induced by a virus latent in apple and pear. Phytopathology 59: 334-338.

Wallace, J.M., Drake, R.J. (1962). Tatterleaf, a previously undescribed virus effect on Citrus. Plant Disease Report. 46: 211.

Wally, O., Daayf, F., Khadhair, A.H., Adam, L., Elliott, B., Shinners-Carnelley, T., Iranpour, M., Northover, P. Keyworth, S. (2004). Incidence and molecular detection of yellows-type disease in carrots, associated with leafhoppers in southern Manitoba, Canada. Canadian Journal of Plant Pathology 26: 498-505.

Wassenegger, M., Heimes, S., Sänger, H.L. (1994). An infectious viroid RNA replicon evolved from an *in vitro*-generated non-infectious viroid deletion mutant via a complementary deletion *in vivo*. Journal of EMBO 13: 6172-6177.

Wei, W., Lee, I.-M., Davis, R.E., Suo, X., Zhao, Y. (2008). Automated RFLP pattern comparison and similarity coefficient calculation for rapid delineation of new and distinct phytoplasma 16Sr subgroup lineages. Journal of Systematic and Evolutionary Microbiology, 58(10): 2368-2377.

Weintraub, P.G., Beanland, L. (2006). Insect vectors of phytoplasmas. Annual Review of Entomology 51: 91-111.

Weintraub, P.G., Wilson, M.R. (2010). Control of Phytoplasma Diseases and Vectors In: Weintraub, P.G., Jones, P. (eds). Phytoplasmas: Genomes, Plant Hosts and vectors. CAB International, pp. 234-249.

Weisburg, W. G., Tully, J. G., Rose, D. L., Petzel, J.P:, Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T.G., Van Etten, J., Maniloffs, J., Woese, C.R: (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. Journal of Bacteriology 171: 6455-6467.

Whitham, S.A., Quan, S., Chang, H.S., Cooper, B., Estes, B., Zhu, T., Wang, X., Hou, Y.M. (2003). Diverse RNA viruses elicit the expression of common sets of genes in susceptible *Arabidopsis thaliana* plants. Plant Journal 33: 271-283.

Wu, Z.-B., Ku, H.-M., Su, C.-C., Chen, I.-Z., Jan, F.-J. (2010). Molecular and biological characterization of an isolate of Apple stem pitting virus causing pear vein disease in Taiwan. Journal of Plant Pathology 92: 721-728.

Yaegashi, H., Takahashi, T., Isogai, M., Kobori, T., Ohki, S.,Yoshikawa, N. (2007). Apple chlorotic leaf spot virus 50 kDa movement protein acts as a suppressor of systemic silencing without interfering with local silencing in *Nicotiana benthamiana*. Journal of General Virology 88: 316-24.

Yamshchikov, V., Mishin, V., Cominelli, F. (2001). A new strategy in design of (+) RNA virus infectious clones enabling their stable propagation in E. coli. Virology 281: 271-280.

Yanase, H. (1974). Studies on apple latent viruses in Japan. Bullet of Fruit Tree Research Station Japan, series C: 47-109.

Yanase, H. (1983). Back transmission of apple stem grooving virus to apple seedlings and induction of symptoms of apple topworking disease in Mitsuba Kaido (*Malus sieboldii*) and Kabano Zumi (*Malus sieboldii* var.*arborescens*) rootstocks. Acta Horticulturae 130: 117-122.

Yanase, H., Koganezawa, H., Fridlund, P.R. (1989). Correlation of pear necrotic spot with pear vein yellows and apple stem pitting, and a flexuous filamentous virus associated with them. Acta Horticulturae 235: 157-158.

Yoshikawa, N., Sasaki, E., Kato, M.,and Takahashi, T. (1992). The nucleotide sequence of apple stem grooving capilovirus genome. Virology 191: 98-105.

Yoshikawa, N., Imaizumi, M., Takahashi, T., Inouye, N. (1993). Striking similarities between the nucleotide-sequence and genome organization of citrus tatter leaf and apple stem grooving capillovirus. Journal of General Virology 74: 2743-2747

Yoshikawa, N., Sasamoto, K., Sakurada, M., Takashi, T., Yanase, H. (1996). Apple stem grooving and citrus tatter leaf capilloviruses obtained from a single shoot of Japanese pear (*Pyrus serotina*). Annals of Phytopatholy Society Japan 62: 119-124.

Yoshikawa, N., Matsuda, H., Oda, Y., Isogai, M., Takahashi, T., Ito, T., Yoshida, K. (2001). Genome heterogeneity of *Apple stem pitting virus* in apple trees. Acta Horticulturae 550: 285-290.

Youssef, S.A., Moawad, S.M., Nosseir, F.M., Shalaby, A.A. (2010). Detection and identification of *Apple stem pitting virus* and *Apple stem grooving virus* affecting apple and pear

trees in Egypt. Proceedings of the 21st international conference on virus and other graft transmissible diseases of fruit crops. Julius-Kühn-Archiv 427: 248-252.

Youssef, F., Marais, A., Faure, C., Gentit, P., Candresse, T. (2011). Strategies to facilitate the development of uncloned or cloned infectious full-length viral cDNAs: *Apple chlorotic leaf spot virus* as a case study. Virology Journal 8: 488-490.

Zhou, Y., Holmes, E.C. (2007). Bayesian estimates of the evolutionary rate and age of hepatitis B virus. Journal of Mol Evolution 65: 197-205.

Zhao, L., Hao, X., Liu, P., Wu, Y. (2012). Complete sequence of an Apple stem grooving virus (ASGV) isolate from China. Virus genes 45(3): 596-599.

Ziemienowicz, A., Tinland, B., Bryant, J., Gloeckler, V., Hohn, B. (2000). Plant enzymes but not Agrobacterium VirD2 mediate TDNA ligation *in vitro*. Molecular Cellular Biology 20: 6317-6322.

Zvereva, A.S., Pooggin, M.M. (2012). Silencing and Innate Immunity in Plant Defense Against Viral and Non-Viral Pathogens. Viruses 4: 2578-2597.

APPENDIX A

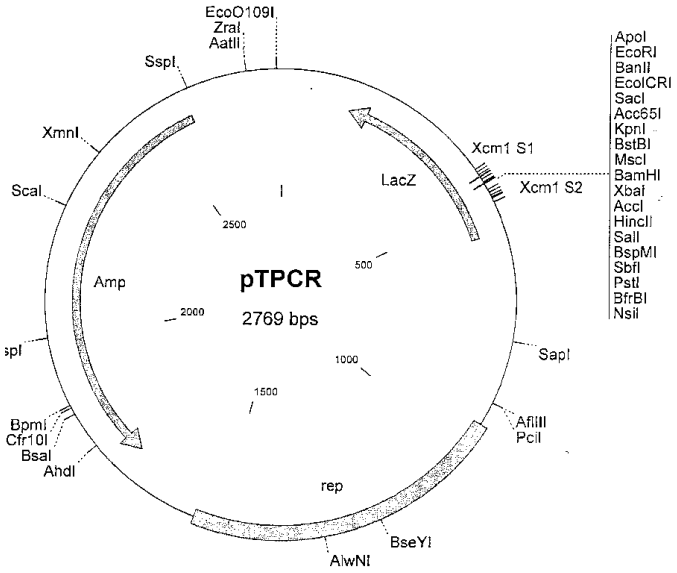

Figure A1: Molecular structure and restriction map of the PUC based TA cloning vector pTPCR

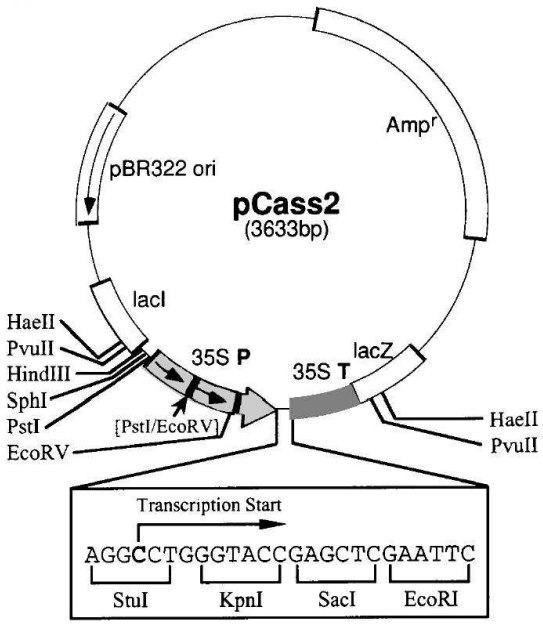

Figure A2: **Molecular structure and restriction map of the cloning vector pCass2 (Shi *et al.*, 1997)**

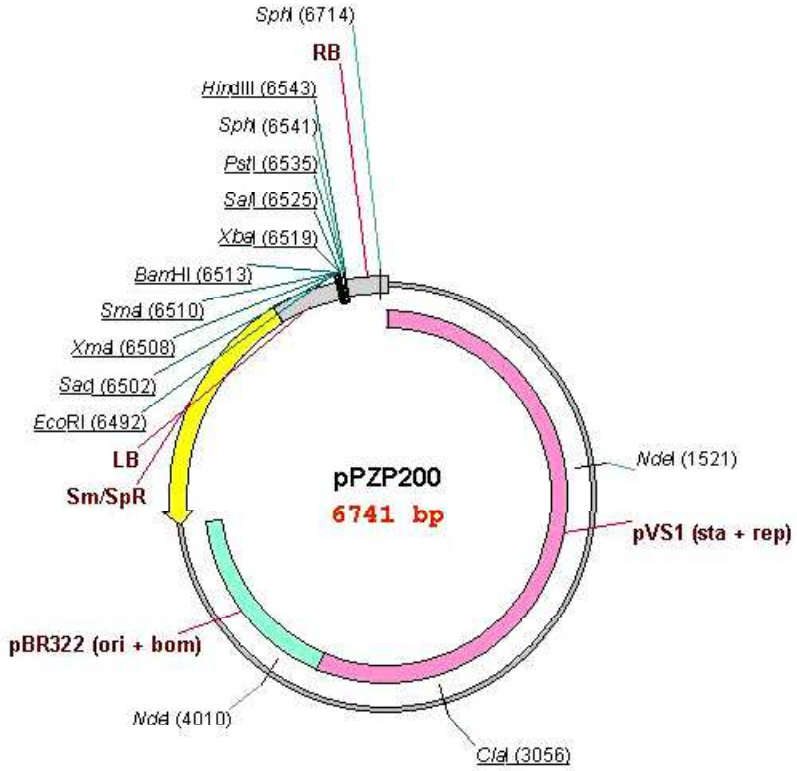

Figure A3: **Molecular structure and restriction map of the binary vector pPXP200 (Hajdukiewicz *et al*., 1994)**

APPENDIX B

Table 1B: **Results of the R-test per experimental data set for Royal Gala growth experiments infected with different ASGV strains**

Experiment	Shapiro-Wilk normality test	Bartlett test of homogeneity of variances	Kruskal-Wallis chi-squared test	Duncan test to compare means Confidence level:0.95
Amount of shoots	W = 0.9 p-value = 3.787×10^{-8} Not normally distributed	K-square = 34.1 df = 8 p-value = 3.912×10^{-5} Variance not homogenous	chi-squared = 25.3 df = 8 p-value = 0.001364 Differences in mean values	Variation Coefficient: 48.62%
Shoot height	W = 0.7159 p-value = 3.973×10^{-15} Not normally distributed	K-square = 110.0209 df = 8 p-value < 2.2×10^{-16} Variance not homogenous	chi-squared = 38.0219 df = 8 p-value = 7.459×10^{-6} Differences in mean values	Variation Coefficient: 64.54%
Leaf size	W = 0.7453 p-value = 2.213×10^{-13} Not normally distributed	K-square = inf df = 8 p-value < 2.2×10^{-16} Variance not homogenous	chi-squared = 59.8267 df = 8 p-value = 5.04×10^{-10} Differences in mean values	Variation Coefficient: 46.18%

Table 2B: **Results of the R-test per experimental data set for the symptom expression of Rubinette *in vitro* plants infected with ASGV with or without the addition of ASPV**

Experiment	Shapiro-Wilk normality test	Bartlett test of homogeneity of variances	Kruskal-Wallis chi-squared test	Duncan test to compare means Confidence level:0.95
Amount of shoots	W = 0.932 p-value = 1.444×10^{-7} Not normally distributed	K-squared = 20.842 df = 4 p-value = 0.0003403 Variance not homogenous	chi-squared = 1.013 df = 4 p-value = 0.9078 There is no differences in the mean values	Variation Coefficient: 45.81%
Shoot height	W = 0.9347 p-value = 2.221×10^{-7} Not normally distributed	K-squared = 3.784 df = 4 p-value = 0.436 Variance homogenous	chi-squared = 16.059 df = 4 p-value = 0.00294 Differences in mean values	Variation Coefficient: 40.88%
Leaf size	W = 0.852 p-value = 3.136×10^{-12} Not normally distributed	K-squared = 8.403 df = 4 p-value = 0.07786 Variance homogenous	chi-squared = 34.408 df = 4 p-value = 6.146×10^{-7} Differences in mean values	Variation Coefficient: 30.91%

Table 3B: **Results of the R-test per experimental data set for the symptom expression of Rubinette *in vitro* plants infected with '*Ca. P.* mali' strains with/without an addition of ASGV**

Experiment	Shapiro-Wilk normality test	Bartlett test of homogeneity of variances	Kruskal-Wallis chi-squared test	Duncan test to compare means Confidence level:0.95
Amount of shoots	W = 0.8988 p-value = 7.021×10^{-14} Not normally distributed	K-squared = 54.889 df = 5 p-value = 1.376×10^{-10} Variance not homogenous	chi-squared = 21.377 df = 5 p-value = 0.0006875 Differences in mean values	Variation Coefficient: 54.01%
Shoot height	W = 0.8617 p-value < 2.2×10^{-16} Not normally distributed	K-squared = 67.074 df = 5 p-value = 4.16×10^{-13} Variance not homogenous	chi-squared = 100.048 df = 5 p-value < 2.2×10^{-16} Differences in mean values	Variation Coefficient: 38.84%
Leaf size	W = 0.8746 p-value = 1.013×10^{-15} Not normally distributed	K-squared = 18.138 df = 5 p-value = 0.002779 Variance not homogenous	chi-squared = 141.270 df = 5 p-value < 2. X 10^{-16} Differences in mean values	Variation Coefficient: 41.57%

Table 4B: **Results of the R-test per experimental data set for the symptom expression of Rubinette *in vitro* plants infected with PM6 with/without an addition of ASGV-AC, ASGV-stutt or ASPV**

Experiment	Shapiro-Wilk normality test	Bartlett test of homogeneity of variances	Kruskal-Wallis chi-squared test	Duncan test to compare means Confidence level:0.95
Amount of shoots	W = 0.888 p-value = 2.26×10^{-11} Not normally distributed	K-squared = 41.943 df = 3 p-value = 4.125×10^{-9} Variance not homogenous	chi-squared = 4.012 df = 3 p-value = 0.2601 There is no differences in the mean values	Variation Coefficient: 56.63%
Shoot height	W = 0.880 p-value = 3.795×1^{-12} Not normally distributed	K-squared = 3.431 df = 3 p-value = 0.3298 Variance is homogenous	chi-squared = 1.756 df = 3 p-value = 0.6245 There is no differences in the mean values	Variation Coefficient: 34.39%
Leaf size	W = 0.888 p-value = 1.688×10^{-11} Not normally distributed	K-squared = 3.198 df = 3 p-value = 0.362 Variance is homogenous	chi-squared = 1.424 df = 3 p-value = 0.6999 There is no differences in the mean values	Variation Coefficient: 47.12%

Table 5B: **Results of the R-test per experimental data set for the symptom expression of M. sieboldii, *M. domestica cv.* Rubinette and the Hybrid Culture CC38.**

Experiment	Shapiro-Wilk normality test	Bartlett test of homogeneity of variances	Kruskal-Wallis chi-squared test	Duncan test to compare means Confidence level:0.95
Amount of shoots	W = 0.9212 p-value = 0.0001321 Not normally distributed	K-squared = Inf df = 5 p-value = $< 2.2 \times 10^{-16}$ Variance not homogenous	chi-squared = 8.3436 df = 5 p-value = 0.1383 There is no differences in the mean values	Variation Coefficient: 36.90%
Shoot height	W = 0.8967 p-value = 1.119×10^{-5} Not normally distributed	K-squared = 32.3626 df = 5 p-value = 5.035×10^{-6} Variance not homogenous	chi-squared = 30.935 df = 5 p-value = 9.646×10^{-6} Differences in mean values	Variation Coefficient: 36.01%
Leaf size	W = 0.8805 p-value = 2.563×10^{-6} Not normally distributed	K-squared = 5.0549 df = 5 p-value = 0.4092 Variance is homogenous	chi-squared = 41.382 df = 5 p-value = 7.854×10^{-8} Differences in mean values	Variation Coefficient: 28.79%

Table 6B: **Results of the R-test per experimental data set for the symptom expression of different *in vitro Malus* genotypes infected ASGV-AC and '*Ca*. P. mali' strain PM4.**

Experiment	Shapiro-Wilk normality test	Bartlett test of homogeneity of variances	Kruskal-Wallis chi-squared test	Duncan test to compare means Confidence level:0.95
Amount of shoots	W = 0.9257 p-value = 9.257×10^{-7} Not normally distributed	K-squared = 34.470 df = 2 p-value = 3.272×10^{-8} Variance not homogenous	chi-squared = 36.070 df = 2 p-value = 1.47×10^{-8} Differences in mean values	Variation Coefficient: 31.39%
Shoot height	W = 0.9063 p-value = 2.028×10^{-8} Not normally distributed	K-squared = 26.401 df = 2 p-value = 1.85×10^{-6} Variance not homogenous	chi-squared = 101.59 df = 2 p-value < 2.2×10^{-16} Differences in mean values	Variation Coefficient: 30.32%
Leaf size	W = 0.8749 p-value = 3.976×10^{-10} Not normally distributed	K-squared = 16.220 df = 2 p-value = 0.0003005 Variance not homogenous	chi-squared = 15.345 df = 2 p-value = 0.0004655 Differences in mean values	Variation Coefficient: 46.68%

Table B: 7: **Results of the R-test per experimental data set for the seasonal analysis of the transmission rate, of ASGV, ASPV, '*Ca.* P. mali, and necrotic reactions.**

Experiment	Shapiro-Wilk normality test	Bartlett test of homogeneity of variances	Kruskal-Wallis chi-squared test	Duncan test to compare means Confidence level:0.95
ASGV transmission per season	W = 0.8893 p-value = 2.76×10^{-6} Not normally distributed	K-squared = 10.7741 df = 3 p-value = 0.01303 Variance not homogenous	chi-squared = 3.6782 df = 3 p-value = 0.2984 No differences in mean values	Variation Coefficient: 85.05%
ASPV transmission per season	W = 0.9056 p-value = 0.01798 Not normally distributed	K-squared = 3.0111 df = 3 p-value = 0.3899 Variance are homogenous	chi-squared = 0.5149 df = 3 p-value = 0.9156 No differences in mean values	Variation Coefficient: 53.28%
'Ca. P. mali' transmission per season	W = 0.8693 p-value = 3.932×10^{-5} Not normally distributed	K-squared = 1.5363 df = 3 p-value = 0.6739 Variance are homogenous	chi-squared = 3.3647 df = 3 p-value = 0.3387 No differences in mean values	Variation Coefficient: 95.97%
Necrosit reaction per season	W = 0.5138 p-value = 2.2×10^{-16} Not normally distributed	K-squared = 21.8537 df = 3 p-value = 6.997×10^{-5} Variance not homogenous	chi-squared = 6.6622 df = 3 p-value = 0.08348 No differences in mean values	Variation Coefficient: 220.2%%

APPENDIX C

Table C 1: Graft-Inoculation experiments performed in this study

Rootstock	Rootstock Status	M. domestica Inoculum	Grafts performed	Successfull grafts
B323	Healthy	ASGV-AC	10	10
B323	Healthy	ASGV-stutt	10	8
B323	Healthy	ASPV-LFP	10	10
B323	Healthy	Healthy	10	10
B323	Healthy	PM4 + ASGV-AC	10	8
B323	Healthy	PM5	10	10
B323	Healthy	PM6	10	4
B89	Healthy	ASGV-AC	10	10
B89	Healthy	ASGV-AC	10	6
B89	Healthy	ASGV-stutt	10	9
B89	Healthy	ASPV-LFP	10	10
B89	Healthy	Healthy	10	10
B89	Healthy	PM4 + ASGV-AC	10	8
B89	Healthy	PM5	10	8
CAT5	Healthy	ASGV-AC	10	6
CAT5	Healthy	ASGV-AC + ASPV	10	5
CAT5	Healthy	ASGV-stutt	20	12
CAT5	Healthy	ASGV-stutt + ASPV	10	7
CAT5	Healthy	ASPV-LFP	10	9
CAT5	Healthy	Healthy	10	9
CAT5	Healthy	PM4 + ASGV-AC	10	5
CAT5	Healthy	PM5	10	8
CAT5	Healthy	PM5 + ASGV-AC	10	6
CAT5	Healthy	PM6	10	6
CAT5	Healthy	PM6 + ASGV-AC	10	8
CAT5	Healthy	PM6 + ASGV-stutt	10	7
CC38	Healthy	ASGV-AC	20	12
CC38	Healthy	ASGV-AC + ASPV	10	6
CC38	Healthy	ASGV-stutt	10	4
CC38	Healthy	ASGV-stutt + ASPV	10	6
CC38	Healthy	ASPV-LFP	10	10
CC38	Healthy	Healthy	10	5
CC38	Healthy	PM4 + ASGV-AC	10	4
CC38	Healthy	PM5	10	4
CC38	Healthy	PM6	10	2
CC38	Healthy	PM6 + ASGV-AC	10	2
CC38	Healthy	PM6 + ASGV-stutt	10	3
D45	Healthy	ASGV-AC	20	11

D45	Healthy	ASGV-stutt	10	10
D45	Healthy	ASGV-stutt + ASPV	10	2
D45	Healthy	ASPV-LFP	10	6
D45	Healthy	Healthy	10	6
D45	Healthy	PM4 + ASGV-AC	10	2
D45	Healthy	PM5	10	2
D45	Healthy	PM6	10	7
D45	Healthy	PM6 + ASGV-AC	10	3
D45	Healthy	PM6 + ASGV-stutt	10	4
Golden Delicious	ASGV-stutt	ASPV-LFP	10	9
Golden Delicious	ASGV-Stutt	PM6	10	1
Golden Delicious	Healthy	ASGV-AC	30	30
Golden Delicious	Healthy	ASGV-AC + ASPV	10	5
Golden Delicious	Healthy	ASGV-stutt	30	24
Golden Delicious	Healthy	ASPV-LFP	10	7
Golden Delicious	Healthy	Healthy	20	14
Golden Delicious	Healthy	PM4 + ASGV-AC	20	13
Golden Delicious	Healthy	PM5	10	8
Golden Delicious	Healthy	PM5 + ASGV-AC	10	7
Golden Delicious	Healthy	PM6	10	6
Golden Delicious	Healthy	PM6 + ASGV-AC	10	5
Golden Delicious	PM6	ASGV-stutt	10	3
M. sieboldii	Healthy	ASGV-AC	10	10
M. sieboldii	Healthy	ASGV-AC + ASPV	10	4
M. sieboldii	Healthy	ASGV-stutt	20	16
M. sieboldii	Healthy	ASGV-stutt + ASPV	10	1
M. sieboldii	Healthy	ASPV-LFP	10	5
M. sieboldii	Healthy	Healthy	70	62
M. sieboldii	Healthy	PM4 + ASGV-AC	10	7
M. sieboldii	Healthy	PM5	10	5
M. sieboldii	Healthy	PM5 + ASGV-AC	20	16
M. sieboldii	Healthy	PM6	10	6
M. sieboldii	Healthy	PM6 + ASGV-AC	10	3
M. sieboldii	Healthy	PM6 + ASGV-stutt	10	6
O44	Healthy	Healthy	10	9
O45	Healthy	ASGV-AC	10	9
O45	Healthy	ASGV-AC + ASPV	10	6
O45	Healthy	ASGV-stutt	20	17
O45	Healthy	ASPV-LFP	10	5
O45	Healthy	PM4 + ASGV-AC	10	5
O45	Healthy	PM5	20	12
Rubinette	ASGV-AC	ASPV-LFP	10	7
Rubinette	ASGV-AC	PM6	10	7

Rubinette	ASGV-stutt	ASPV-LFP	10	3
Rubinette	Healthy	ASGV-AC	10	6
Rubinette	Healthy	ASGV-stutt	10	6
Rubinette	Healthy	ASPV-LFP	10	10
Rubinette	Healthy	Healthy	20	15
Rubinette	Healthy	PM5	10	7
Rubinette	PM6	ASGV-stutt	10	7
Rubinette	PM6	ASPV-LFP	10	6
W355	Healthy	ASGV-AC	10	6
W355	Healthy	ASGV-stutt	10	4
W355	Healthy	ASGV-stutt + ASPV	10	4
W355	Healthy	ASPV-LFP	10	7
W355	Healthy	Healthy	10	6
W355	Healthy	PM4 + ASGV-AC	10	3
W355	Healthy	PM6	10	3
W355	Healthy	PM6	10	5
W355	Healthy	PM6 + ASGV-stutt	10	3
		Total	**1180**	**771**

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to the following people and institutions:

- Dr. Wolfgang Jarausch, I cannot convey in words how much I appreciate your and Barabara's help during my time here at Alplanta, especially at my arrival in this new and interesting country. You took me under your wings, and helped me where you could. On a more scientific note, thank you for your invaluable scientific input, your patience and your help with abstract, presentation and thesis correcting.
- Dr. Thierry Wetzel, thank for making it possible to come to Germany for this study, as well as for your help and scientific input through the study.
- Dr. Gabi Krczal, for giving me the opportunity to do my studies at this prestigious Institute.
- Alex, Michelle, Michele and Migo for your wonderful friendship and scientific support during my study. You will always have a special place in my heart.
- To everybody working at Alplanta, thank you for making me feel welcome, your friendship and providing me a pleasant and (sometimes fun) working environment.
- Prof. R. Hell for allowing me to perform my study at the University of Heidelberg.
- The ´Fonds zur Entwicklung Ländlicher Räume in Rheinland Pflaz` for the funding of the project.
- And most importantly, thank you my Skattebol, for your love, support and understanding, especially during the thesis writing times. You are my rock.

General Statement

I declare that I am the sole author of this submitted dissertation and that I did not make use of any sources or help apart from those specifically referred to. Experimental data or material collected from or produced by other persons is made easily identifiable.

I also declare that I did not apply for permission to enter the examination procedure at another institution and that the dissertation is neither presented to any other faculty, nor used in its current or any other form in another examination.

_____	_____
City, Date	Annerie Liebenberg