ELISA detection of *Apple chlorotic leafspot virus* (ACLSV) and *Apple mosaic virus* (ApMV) in comparison to RT-PCR detection and the determination of genetic variation of these virus species in South Africa

by

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Abstract

Apple chlorotic leaf spot virus (ACLSV) and *Apple mosaic virus* (ApMV) are responsible for reduced yield in the South African deciduous fruit industry. These two diseases are regulated by the South African Deciduous Fruit Plant Certification Scheme whereby no trees infected with these viruses are permitted for plantings. Currently the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) is prescribed as the test method for routine detection of these viruses in plant material.

In the first part of this study, detection limits of DAS-ELISA and reverse transcriptase polymerase chain reaction (RT-PCR) for ApMV and ACLSV were compared. The RT-PCR was found to be 70.5 and 71 fold more sensitive than DAS-ELISA in the detection of ACLSV and ApMV, respectively. No ApMV isolates were detected by DAS-ELISA in pears, but ten isolates were detected by RT-PCR. This is of major concern as ApMV tests are not prescribed by the South African Deciduous Fruit Certification Scheme for pears, as it was not considered a host of ApMV and no ApMV symptoms have been observed.

In the second part of this study, the genetic variation of ApMV and ACLSV isolates from South Africa was investigated. Extracted RNA was used for RT-PCR of the coat protein genes which were then sequenced. Phylogenetic trees were constructed using these sequences as well as reference sequences from GenBank. Phylogenetic analysis revealed that South African isolates of ACLSV were similar to isolates from the rest of the world, grouping into 3 of 4 possible clades, and that the majority of isolates are not restricted to a particular fruit group. This indicates that cross-infection between pome- and stonefruit is possible. Certain isolates of ApMV were detected by RT-PCR, but not detected by DAS-ELISA in apples and peach. It is also concluded that ACLSV was imported from various regions of the world, since similarity with a number of different overseas isolates was found. Phylogenetic analysis of the coat protein gene sequences of ApMV isolates indicated that two major groups occur in South Africa. Phylogenetic analysis also revealed that South African isolates in individual clades are not restricted to a single fruit group, which indicates the risk of cross-infection.

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Opsomming

Apple chlorotic leaf spot virus (ACLSV) en *Apple mosaic virus* (ApMV) is verantwoordlelik vir verlaagde opbrengs in die Suid-Afrikaanse sagtevrugte industrie. Dit word gereguleer deur die Suid-Afrikaanse Sagtevrugte Plant Sertifiseringsskema waar geen aanplantings wat met hierdie virusse besmet is, toegelaat word nie. Tans word DAS-ELISA voorgeskryf as die toetsmetode waarmee roetine opsporing van hierdie viruses in plant material moet geskied.

In die eerste deel van hierdie studie, is die opsporingsdrempelgrense van DAS-ELISA en RT-PKR vir ApMV en ACLSV bepaal. Die RT-PKR tegniek was 70.5 en 71 keer meer sensitief as DAS-ELISA in die opsporing van ACLSV en ApMV onderskeidelik. Geen ApMV isolate is by pere deur DAS-ELISA opgespoor nie, terwyl 10 sulke isolate deur RT-PKR opgespoor is. Dit is ’n groot bekommernis, aangesien ApMV tans nie voorgeskryf word vir toetsing by pere deur die Suid-Afrikaanse Sagtevrugte Plant Verbeteringsskema nie, aangesien peer nie as gasheer van ApMV beskou word nie.

In die tweede gedeelte van hierdie studie, is die genetiese variasie van Suid-Afrikaanse ApMV en ACLSV isolate ondersoek. RNA wat geïsoleer is, is vir RT-PKR van die mantelproteïen geen gebruik en die nukleotied volgorde is vervolgens bepaal. Filogenetiese bome is getrek vanaf hierdie Suid-Afrikaanse nukleotied volgordes asook verwysings isolate in GenBank. Die filogenetiese analises het getoon dat Suid-Afrikaanse ACLSV isolate in 3 van 4 moontlike klades groepeer en dat die meerderheid van Suid-Afrikaanse ACLSV isolate nie beperk is tot bepaalde vruggroepe nie. Dit dui daarop dat kruis-infeksie tussen kern- en steenvrugte ’n moontlikheid is. Sekere isolate in opgespoor in appel en perske, maar dit is nie opgespoor deur DAS-ELISA nie. Uit die resultate kon ook afgelei word dat ACLSV vanaf verskeie lande ingevoer is, aangesien sterk ooreenkomste met ’n aantal verskillende buitelandse isolate gevind is. Filogenetiese analises van die mantelproteïen volgorde van ApMV isolate het aangetoon dat twee hoof groepe in Suid Afrika voorkom. Uit die ontledings word afgelei dat ApMV isolate binne individuele klades ook nie beperk is tot ’n bepaalde vruggroep nie wat die moontlike risiko van kruis-besmetting aandui.

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Abbreviations

AL	Albania
ALM	almond
ACLSV	*Apple chlorotic leafspot virus*
AMP	*Alfalfa mosaic virus*
AP	apple
APCLSV	*Apricot pseudo clorotic leafspot virus*
ApMV	*Apple mosaic virus*
APR	apricot
ASGV	*Apple stem grooving virus*
ASPV	*Apple stem pitting virus*
BE	Belgium
bp	basepairs
BU	Bulgaria
CA	Canada
cDNA	complementary DNA
CH	China
CI	consistency index
CMV	*Cucumber mosaic virus*
CMVL	*Cherry mottle leaf virus*
CP	coat protein
CY	cherry
CR	Czech Republic
DAS-ELISA	double antibody sandwich enzyme-linked immunosorbent assay
DNA	deoxyribonucleic acid
ds	double stranded
D-IC-RT-PCR	duplex immuno-capture reverse transcriptase polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
FR	France
GINV	*Grapevine berry inner necrosis virus*
GER	Germany
HO	hops
HU	Hungary
IC-PCR	immuno-capture polymerase chain reaction
IT	Italy
JO	Jordan
KO	Korea
LA	Latvia
MP	movement protein

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mRNA	messenger RNA
NE	nectarine
NTR	non-translated region
ORF	open reading frame
PA	pear
PAUP	phylogenetic analysis using parsimony
PcMV	*Peach mosaic virus*
PD	*Prunus domestica*
PE	peach
PDV	*Prune dwarf virus*
PL	plum
PNRSV	*Prunus necrotic ringspot virus*
PO	pomefruit
PR	prune
RSA	South Africa
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
PR	Prune
RI	retention index
RNA	ribonucleic acid
S	substrate
SAPO	South African Plant Improvement Organization
SP	Spain
sp.	*species*
sRNA	small ribonucleic acid
ss	single stranded
TBR	tree bisection and reconnection
TSV	*Tobacco streak ilarvirus*
TSWV	*Tomato spotted wilt virus*
TU	Turkey
UK	Ukraine
UN	Unknown
USA	United States of America

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Table of Contents

CHAPTER 1: Introduction, Objectives and Literature Review 1

1.1 Introduction 1

1.2 Objectives of this study 2

1.3 Study rationale 2

1.4 A general background to viruses 3

1.5 *Apple chlorotic leaf spot virus* (ACLSV) 5

1.5.1 Nomenclature 5

1.5.2 Host Range and symptom expression 5

1.5.3 Transmission 7

1.5.4 Geographical distribution 7

1.5.5 Economic Importance 7

1.5.6 Genome 8

1.6 *Apple mosaic virus* (ApMV) 9

1.6.1 Nomenclature 9

1.6.2 Host range 11

1.6.3 Transmission 11

1.6.4 Symptom expression 11

1.6.5 Genome 12

1.7 Detection of viral infection in plants 14

1.7.1 Detection methods 14

1.7.2 Detection of ACLSV 16

1.7.3 Detection of ApMV 17

CHAPTER 2: Comparative detection of *Apple chlorotic leafspot virus* (ACLSV) and *Apple mosaic virus* (ApMV) by DAS-ELISA and RT-PCR in South Africa 19

2.1 Abstract 19

2.2 Introduction 19

2.3 Materials and Methods 20

2.3.1 Sample collection for ACLSV detection 20

2.3.2 Sample collection for ApMV detection 21

2.3.3 Sample collection for ApMV detection in pears 22

2.3.4 Sample preparation for DAS-ELISA and RT-PCR 22

2.3.5 Plant sample homogenization for DAS-ELISA 22

2.3.6 Plant sample homogenization for RT-PCR 23

2.3.7 Total RNA extractions for RT-PCR 23

2.3.8 Dilution series prepared for comparative ACLSV detection by DAS-ELISA and RT-PCR 23

2.3.9 Dilution series prepared for comparative ApMV detection by DAS-ELISA and RT-PCR 24

2.3.10 DAS-ELISA procedure 24

2.3.11 RT-PCR procedure for ACLSV detection 24

2.3.12 RT-PCR procedure for ApMV detection 25

2.3.13 Electrophoretic analysis of RT-PCR-amplified products 25

2.3.14 Statistical analysis of DAS-ELISA and RT-PCR comparative results 26

2.3.15 Detection of ApMV in pears using DAS-ELISA and RT-PCR 27

2.4 Results 27

2.4.1 RT-PCR and DAS-ELISA detection of ACLSV 27

2.4.1.1 RT-PCR detection of ACLSV 27

2.4.1.2 DAS-ELISA detection of ACLSV 28

2.4.1.3 Diagnostic assay comparison: DAS- ELISA and RT-PCR detection of ACLSV 28

2.4.1.4 Statistical analyses: DAS- ELISA and RT-PCR detection of ACLSV 32

2.4.2 RT-PCR and DAS-ELISA detection of ApMV 32

2.4.2.1 RT-PCR detection of ApMV 32

2.4.2.2 DAS-ELISA detection of ApMV 33

2.4.2.3 Diagnostic assay comparison: DAS- ELISA and RT-PCR detection of ApMV 33

2.4.2.4 Statistical analyses: DAS- ELISA and RT-PCR detection of ApMV 39

2.4.3 Detection of ApMV in pears 39

2.5 Discussion 39

Stellenbosch University http://scholar.sun.ac.za

CHAPTER 3: Determination of genetic variation of *Apple chlorotic leafspot virus* (ACLSV) in South Africa 44
3.1 Abstract 44
3.2 Introduction 44
3.3 Materials and Methods 45
3.3.1 Sample collection and DAS-ELISA detection 45
3.3.2 RNA isolation 45
3.3.3 RT-PCR amplification of CP gene 45
3.3.4 Electrophoretic analysis of RT-PCR amplification products 46
3.3.5 Purification of RT-PCR amplification products 46
3.3.6 Sequencing of RT-PCR amplification products 46
3.3.7 Nucleotide sequence analysis and alignment 47
3.3.8 Phylogenetic analysis 47
3.4 Results 49
3.4.1 DAS-ELISA screening 49
3.4.2 Electrophoretic analysis of RT-PCR-amplified products of ACLSV 51
3.4.3 Nucleotide sequence analysis and alignment 51
3.4.4 Phylogenetic analysis of ACLSV CP gene sequences 51
3.5 Discussion 54
CHAPTER 4: Determination of genetic variation of *Apple mosaic virus* (ApMV) in South Africa 59
4.1 Abstract 59
4.2 Introduction 59
4.3 Materials and Methods 60
4.3.1 Sample collection 60
4.3.2 Sample preparation for RT-PCR 61
4.3.3 Electrophoretic analysis of RT-PCR-amplified products 61
4.3.4 Sequencing of RT-PCR amplification products 62
4.3.5 Nucleotide sequence analysis and alignment 62
4.3.6 Phylogenetic analysis 62

Stellenbosch University http://scholar.sun.ac.za

4.4 Results 63

4.4.1 DAS- ELISA and electrophoretic analysis of RT-PCR-amplified products of ApMV 63

4.4.2 Nucleotide sequence analysis and alignment 65

4.4.3 Phylogenetic analysis of ApMV CP gene sequences 66

4.5 Discussion 69

CHAPTER 5: Conclusions and future perspectives 72

REFERENCES 74

ADDENDUM A 86

ADDENDUM B 104

ADDENDUM C 107

ADDENDUM D 125

CHAPTER 1

INTRODUCTION, OBJECTIVES AND LITERATURE REVIEW

1.1 Introduction

South Africa produces pomefruit (apples and pears) and stonefruit (peaches, plums, prunes, apricots and nectarines) to the value of approximately R7.32 billion annually. It amounts to a world market share of between 0.7% (peaches/nectarines) and 1.6% (pears). South Africa is one of the top 10 exporters of apples (eighth) and pears (fourth) in the world. South Africa is also rated as the third and eighth highest country with regard to pear and apple production efficiency worldwide respectively.

Kotze (2011) reported that a total of 52 116 hectares (ha) were planted to pomefruit (apples and pears) and stonefruit (peaches, plums, prunes, apricots and nectarines) in South Africa in 2011. Pomefruit to the value of R5.96 billion were sold during 2011, whilst stonefruit to the value of R1.36 billion were marketed (Kotze, 2011). Apples and pears yield around 55 and 45 ton/ha respectively, whilst stonefruit yield between 20 and 25 ton/ha. Production of fruit takes place mainly in the Western Cape province of South Africa where an estimated 31 446 ha of a total of 33 481 ha of pomefruit and 14 988 ha of a total of 18 635 ha of stonefruit are cultivated (Kotze, 2011).

Establishment costs of pome- and stonefruit orchards are high ranging between R187 394 and R192 060 per ha for pomefruit and between R114 935 and R155 899 for stonefruit depending on the type of fruit planted. Due to the large financial layout, producers prefer to maintain orchards for many years. In line with this, 52% and 59% of apple and pear orchards respectively are older than 15 years. For stonefruit, 21% to 46% of orchards are older than 15 years depending on the particular fruit group (Kotze, 2011).

Viruses tend to shorten the lifespan of trees and cause a decrease in yield (Desvignes & Boyé, 1989) with a consequent substantial loss of income. Two important viruses that affect pome- and stonefruit are *Apple chlorotic leaf spot virus* (ACLSV) and *Apple mosaic virus* (ApMV). ACLSV decreases the growth, mean fruit weight and yield of trees (Cosba et al., 1986). Severe strains of ACLSV can cause severe fruit necrosis (German-Retana et al., 1997) and mottling and pitting on leaves and fruits (Desvignes & Boyé, 1989) rendering them unmarketable. ACLSV can also cause symptoms of pseudopox or false plum pox on stonefruit (Jelkman & Kunze, 1995) which resembles a South African quarantine virus, *Plum pox virus*, which also renders the fruit unmarketable. ApMV infection results in a reduction of production and tree decline (Desvignes, 1999) and significant yield losses have occurred in some regions on sensitive varieties (Nemeth, 1986; Desvignes, 1999). Since the viruses are transmitted by infected plant material and new orchards are established by using buds from existing orchards, these viruses can be transmitted indefinitely.

In South Africa both ACLSV and ApMV are tested under the South African Deciduous Fruit Plant Certification Scheme. The laboratory which tests most of the pome- and stonefruit material under certification in South Africa is the South African Plant Improvement Organization (SAPO) Trust laboratory (Stellenbosch). The laboratory tests plant material for ApMV and ACLSV, as well as *Apple stem grooving virus* (ASGV), *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) using double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). These tests are carried out annually to five-yearly depending on the classification of the trees. Trees classified as nucleus and foundation trees are tested annually for all of these viruses. Trees classified as mother block trees are tested annually for PDV and PNRSV, due to their pollen transmissible nature. The mother block trees are tested once every five years for ACLSV and ApMV and once during the lifetime of the block for ASGV using DAS-ELISA (these frequencies are currently under revision). The DAS-ELISA tests are conducted during the spring months from the end of September to the middle of December. Leaf samples are collected from most pome- and stonefruit growing regions, especially the Western Cape and North West Provinces. The samples are stored in cooler boxes and delivered to the laboratory within a week after collection. If a sample from the nucleus block tests positive, the applicable plants are removed from the nucleus block. If the variety is of importance, a thermal therapy process is followed to eliminate the virus from the plants and the plants are only re-introduced into the nucleus block once the elimination process was successful. When samples from the foundation blocks are found to be infected, the originating plants are destroyed and new plants are established from the uninfected nucleus block. If a sample from a mother block tests positive for one of the viruses, the block is discarded from the certification scheme and no further plant material is issued from the block. New mother blocks are established from the uninfected foundation blocks.

1.2 Objectives of this study

In recent years concerns have arisen as to whether the DAS-ELISA can detect all isolates of these viruses and whether genetic variants occur which are not detected by current testing. As the financial implications of a lack of detection of ACLSV and ApMV are severe, the first objective of this study was to compare DAS-ELISA detection to reverse transcriptase polymerase chain reaction (RT-PCR) detection of ApMV and ACLSV and determine whether some isolates are not detected by DAS-ELISA. The second objective was to determine the genetic variation within ApMV and ACLSV in South Africa in pome- and stonefruit with a view to determine how many genetic variants occur in South Africa and whether all the strains are detected by DAS-ELISA. To this end, sequences of the coat protein genes of each of the viruses were determined and subjected to phylogenetic analysis. This would allow the development of management strategies for the control of these viruses in South African pome- and stone fruit.

1.3 Study rationale

The research presented in this thesis therefore focusses on South African isolates of ApMV and ACLSV. A brief description of the host range, transmission, geographical distribution, genome and detection techniques of ApMV and ACLSV is given in Chapter 1 of this thesis. In Chapter 2 a

comparison of the sensitivity of DAS-ELISA with RT-PCR for the detection of ACLSV and ApMV is described and the outcomes of this research are discussed. In Chapter 3 genetic variation of ACLSV species in South Africa was determined by means of coat protein gene sequencing. Phylogenetic analysis of the coat protein genes was used to identify the grouping of South African isolates relative to those from other areas of the world. In Chapter 4 genetic variation of ApMV species in South Africa was determined by means of coat protein gene sequencing. The molecular variability was again determined by means of phylogenetic analysis. The conclusion and future perspectives of this study are presented in Chapter 5. It should be noted that Chapters 2, 3 and 4 are written in publication format in order to facilitate their subsequent publication. However, to avoid duplication, a single reference list is given at the end of the thesis. This is followed by addenda of the percentage nucleotide sequence similarities of the CP genes of ACLSV and ApMV isolates and the ACLSV and ApMV sequences generated in this study.

1.4 A general background to viruses

A virus was defined by Matthews (1981) as “a set of one or more nucleic acid template molecules, normally encased in a protective coat, or coats of protein or lipoprotein, which is able to organise its own replication only within suitable host cells. Within such cells virus production is (a) dependent on the host’s protein synthesising machinery, (b) organised from pools of the required materials rather than by binary fission, and (c) located at sites which are not separated from the host cell contents by a lipoprotein, bilayer membrane” (Walkey, 1991).

Viruses are diverse entities that use animals, plants, bacteria, fungi, vertebrates or invertebrates as hosts (Figure 1). Viruses can have different impacts on their host species.

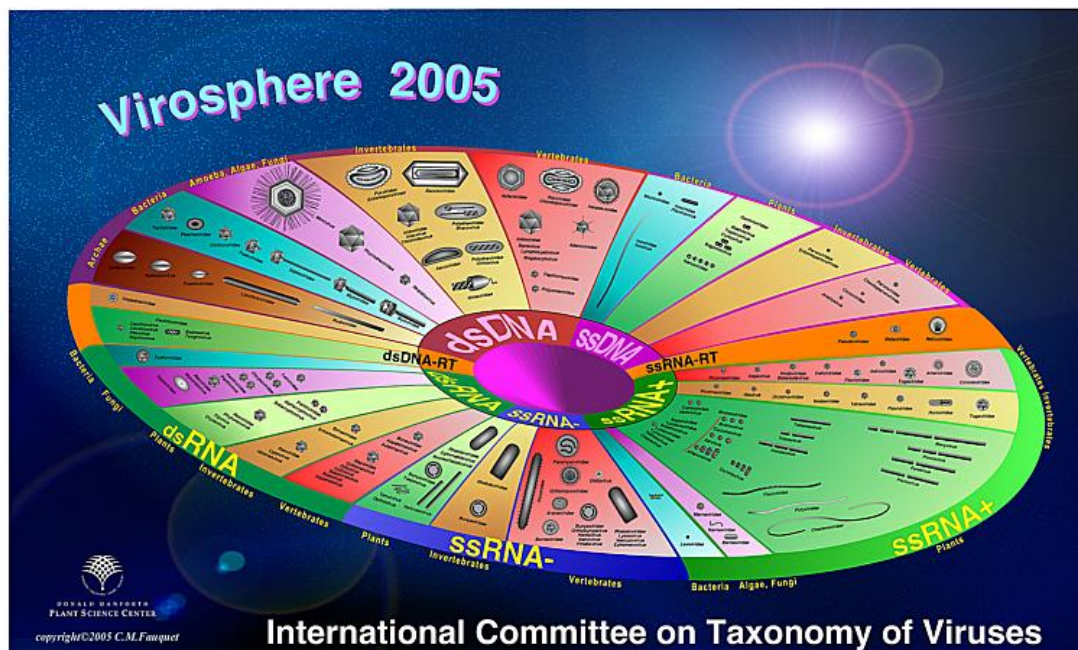

Figure 1. Viruses divided into classification groups depending on their characteristics. RNA = ribonucleic acid, DNA = deoxyribonucleic acid, ss = single stranded, ds = double stranded, - = negative sense, + = positive sense, RT = reverse transcriptase (Fauquet, 2005).

The virus may affect the hosts's health which could include killing the host, it could actively multiply without any detrimental effect on the host, or it may just even remain within the host cell without any activity (Dimmock et al., 2007).

Viruses are divided into various groups depending on their characteristics (Figure 1). The nucleic acid of a virus particle, referred to as the genome, consists of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) (Walkey, 1991). Plant viruses mostly contain RNA, but some viruses belonging to the caulimovirus and geminivirus groups, contain DNA (Walkey, 1991). Viruses have a small genome in comparison to the genomes of prokaryotic and eukaryotic cells, but genomes of various viruses vary in size from *c.* 3000 nucleotides (nts) to 1 200 000 nts (Dimmock et al., 2007). In addition, viruses can either contain negative or positive sense RNA. Some positive sense RNA viruses have RNA genes that function directly as mRNA (messenger RNA) without further modification. Negative sense RNA viruses have RNA that is a complimentary copy of mRNA and these viruses rely on the cell's or their own RNA polymerase enzyme to make mRNA (Dimmock et al., 2007). Retroviruses differ from other viruses since they contain RNA-dependent DNA polymerase that directs the synthesis of a DNA form of the viral genome after infection of a host cell. The RNA is then transcribed into DNA in the host cell. This is integrated into the host cell's genome and then undergoes the usual transcription and translational processes to express the genes carried by the virus (Vogt, 1997).

The coat protein, called the capsid, is composed of individual protein sub-units (polypeptide chains) referred to as the capsomeres (Walkey, 1991). The capsomeres of viruses can have various shapes (Figure 1) and in the case of plant viruses shapes such as bacilliform, rod-shaped or isometric occur (Walkey, 1991). The capsid allows the virus to identify the correct host cell and gain entry into the cytoplasm (Dimmock et al., 2007).

Entry of the virus to a cell is essential for virus replication. Plant cells have cell walls composed of cellulose, which are more complex to enter than animal cells. The plant cell wall tissue needs to be damaged in order for viruses to enter the plant. Thus natural virus entry occurs by mechanical damage, such as wind or passing animals or by vectors such as insects with piercing mouthparts, grazing animals or invading fungi (Dimmock et al., 2007). Once access to the plant has been gained, entry into the cytoplasm is gained by attachment via the capsid protein by uncoating (Dimmock et al., 2007).

Viruses can only multiply within living cells and make use of the cell's reproduction system to replicate. Replication, transcription of mRNA and translation into proteins occurs within the host cell by using ribosomes provided by the host cell. Viruses are considered as obligate intracellular parasites due to the use of these ribosomes, as well as the need for molecules for biosynthesis (Dimmock et al., 2007).

1.5 *Apple chlorotic leaf spot virus* (ACLSV)

1.5.1 Nomenclature

Apple chlorotic leaf spot virus (ACLSV) is the type species of the *Trichovirus* genus from the family *Betaflexiviridae* of the order *Tymovirales* (King et al., 2012). It is a ssRNA+ virus with a flexible rod-shaped particle shape (Figure 2).

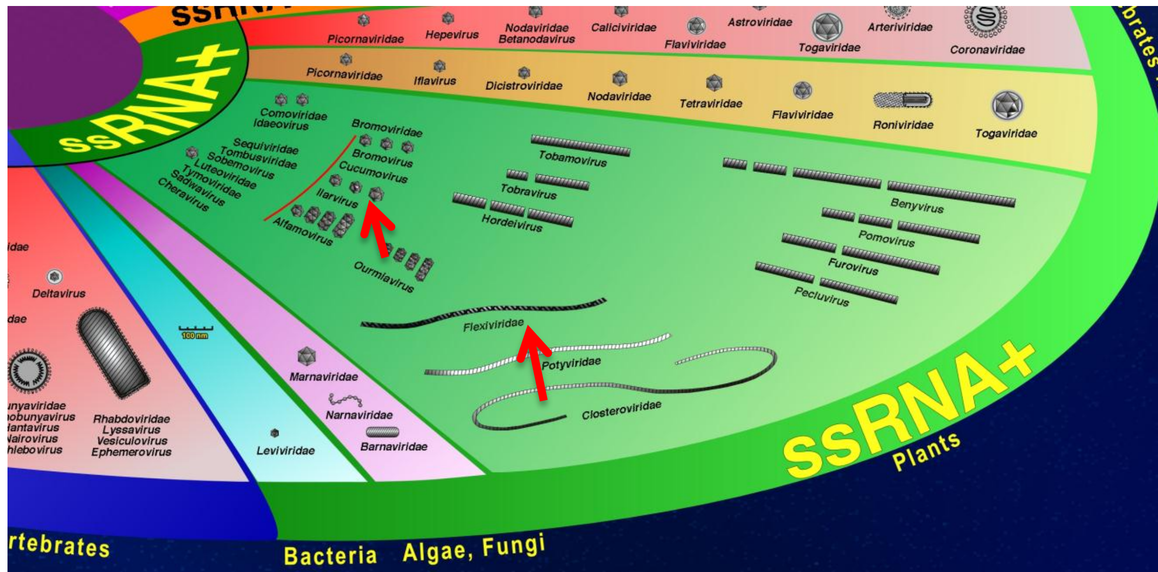

Figure 2. Particle morphology and relative size of ssRNA+ viruses with particular emphasis on *Ilarvirus* and *Flexiviridae* as indicated by arrows. RNA = ribonucleic acid, ss = single stranded, + = positive sense (derived from Fauquet, 2005).

The other members of the *Trichovirus* genus are *Cherry mottle leaf virus* (CMLV), *Grapevine berry inner necrosis virus* (GINV), *Peach mosaic virus* (PcMV), *Apricot pseudo-chlorotic leaf spot virus*, *Grapevine Pinot gris virus* and *Phlomis mottle virus* (International Committee on Taxonomy of Viruses (ICTV), 2012). Synonyms for ACLSV include *Pear ring pattern mosaic virus* (Cropley, 1969) and *Quince stunt virus*, *Plum pseudopox virus* and *Apple latent virus* type 1 (Brunt et al., 1996).

ACLSV is serologically unrelated to the other species in the genus *Trichovirus.* However the amino acid sequences of the conserved polymerase motif, putative movement protein and coat protein, show similarity with virus species in the genera *Capillovirus* and *Vitivirus* (Yoshikawa et al., 1997).

1.5.2 Host Range

ACLSV was first reported in *Malus* sp. from the USA by Mink and Shay (1959; 1962) (Lister et al., 1965). It was originally divided into a *Prunus* strain (CLSV-P from peach) and *a Malus* strain (CLSV-A from apple) (Chairez & Lister, 1973; Paunovic, 1988). It was reported that isolates from peach and apple trees consist of at least two or three variants that differ considerably from each other in nucleotide sequence (Candresse et al., 1995). However, other research suggests that

strains do not classify into categories based merely on their host range (Marenaud et al., 1976), (Kinard et al., 1996).

ACLSV is known to infect most fruit tree species, including apricot (*Prunus armeniaca*), cherry (*P. avium*), peach (*P. persica*), prune (*P. domestica*), pear (*Pyrus* sp.), plum (*P. domestica, P. salicina*), apple (*Malus* sp.) (Lister, 1970; Nemeth, 1986; Desvignes & Boyé, 1989; German-Retana et al., 1997), quince (*Cydonia oblonga*) (Rana et al., 2008) and almond (*Prunus dulcis*) (Spiegel et al., 2005). The symptoms on stonefruit are presented as pseudopox (Figure 3) or false plum pox (Jelkman & Kunze, 1995) or as "butteratura" in apricot and cherry. The latter was presented as severe fruit necrosis symptoms in Hungary (German-Retana et al., 1997).

Figure 3. Pseudopox symptoms of *ACLSV* detected on plums in Paarl, Western Cape, South Africa.

Other symptoms of ACLSV include severe mottling and pitting on cherry and peach leaves and fruits (Desvignes & Boyé, 1989) and symptoms of "viruela" on apricot (Peña-Iglesias & Ayuso, 1973). Severe graft incompatibilities may also occur due to ACLSV infection in some rootstock-scion combinations (Candresse et al., 1995). In some cultivars, like Bulida, fruit show irregular sinking, grooves, spots and important malformations that make them unmarketable (Peña-Iglesias, 1988). Plum bark split (Lister, 1970) and peach dark green sunken mottle (Salmon et al., 2002) were also reported. The Balaton 1 isolate (ACLSV-Bal1) caused very severe leaf distortion and discoloration symptoms on GF305 peach seedling indicators, which were very different from the mild, dark green mottle caused on peaches by most ACLSV isolates (Desvignes & Boyé, 1989; German-Retana et al., 1997; Nemeth, 1986). Fruit showing circular, sunken black necrotic

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lesions and circular ring symptoms were also reported from *Prunus domestica* 'Victoria' (Bénédicte et al., 2004).

On pomefruit ACLSV is mostly symptomless (German-Retana et al., 1997), but it can cause russet ring on apple fruits and in Japan it caused top-working disease and induced lethal decline in apple trees propagated on Maruba kaido (*Malus prunifolia* var. *ringo*) rootstocks (Salmon et al., 2002) within a few years (Kinard et al., 1996). On *Malus platycarpa* it caused line patterns and chlorotic rings, and on *Malus sylvestris* cv. R12740-7A it caused chlorotic leaf spots and stem pitting. On *Malus sylvestris* cultivar Spy, chlorotic spots and stem pitting, chlorosis and stunting occurred, whilst on *Malus sylvestris* cultivar Virginia Crab, stem pitting occurred (Brunt et al., 1996). On quince, at least for some host-cultivar/virus isolate combinations, ACLSV may be asymptomatic (Mathioudakis et al., 2007). However, on quince variety C7/1, chlorotic rings and spots occurred, whilst on *Pyronia veitchii* chlorosis and stunting occurred. Pears showed ring patterns and mosaic symptoms (Brunt et al., 1996).

1.5.3 Transmission

Grafting is known for transmitting ACLSV between woody hosts (Cosba et al., 1986). There is currently no certainty as to how ACLSV is transmitted naturally. Seed transmission has been reported (Poul & Dunez, 1998), but another study concluded that the virus was not transmitted from seed to seedling or by pollen (Garcia-Ibarra et al., 2010b).

1.5.4 Geographical distribution

ACLSV has been reported nearly worldwide (and occurs probably wherever apples are cultivated) (Brunt et al., 1996). It has been reported in the Eastern Asian region, including Korea (Park et al., 2006), the Eurasian region, including Greece (Mathioudakis et al., 2007), Spain (Casallo et al., 1988), Italy (Pasquini et al., 1998), Turkey (Ulubas & Ertunc, 2005), Serbia (Mandic et al., 2007) and Latvia (Pupola et al., 2011). It has also been reported in North America, and the Pacific region. Furthermore is has been reported from Australia (Constable et al., 2007), New Zealand (Richmond et al., 1998), China (Wu et al., 1998) and South Africa where it is tested as part of the South African Deciduous Fruit Plant Certification Scheme.

1.5.5 Economic Importance

ACLSV decreases the growth, mean fruit weight and yield of trees (Cosba et al., 1986). ACLSV, ApMV, ASGV and *Apple stem pitting virus* (ASPV) alone or in combination can cause significant yield reduction of fruit and reduced growth (Campbell, 1963; Campbell, 1981; Lemoine & Michelesi, 1990; Meijneke et al., 1975; Wood, 1974). A yield reduction of 12% (Meijneke et al., 1975) to 30% (Van Oosten et al., 1982) was reported on the apple cultivar Golden Delicious infected with ACLSV, ASGV and ASPV.

1.5.6 Genome

ACLSV is a filamentous particle of approximately 640-760 nm (Yoshikawa & Takahashi, 1988). The ACLSV genome consists of a linear positive-sense single strand poly-adenylated RNA molecule of 7545-7555 bp (Bar-Joseph et al., 1979; German et al., 1990; German-Retana et al., 1997; Sato et al., 1993), excluding the poly-A tail at the 3'-end (Sato et al., 1993).

The ACLSV genome contains three open reading frames (ORFs 1, 2 and 3). ORF 1 extend from bp 152 to 5803, ORF 2 from bp 5715 to 7097, and ORF 3 from bp 6781 to 7362 (Niu et al., 2012) (Figure 4). The ORF's encode for proteins with molecular masses of 216.5, 50.4 and 21.4 kDa respectively (Salmon et al., 2002). The 21.4 kDa protein is the viral coat protein (German et al., 1990; German-Retana et al., 1997; Sato et al., 1993). The 50.4 kDa protein is possibly the movement protein, as is indicated by observations that the protein fused to green fluorescent protein can move into bordering cells from the cells that produced it in the leaf epidermis (Satoh et al., 2000). It was further observed that the systemic spread of mutants of an infectious complementary DNA (cDNA) clone that is defective in ORF2, can be complemented in transgenic plants that express the protein (Satoh et al., 1999; Yoshikawa et al., 2000). The 50.4 kDa protein is located on the plasmodesmata in infected and transgenic plant leaves (Satoh et al., 1999; Yoshikawa et al., 2000). The 216.5 kDa protein contains the consensus motifs of methyltransferase, papain-like protease, nucleotide triphosphate-binding (NTP) helicase (German et al., 1990; German et al., 1992; German-Retana et al., 1997; Sato et al., 1993) and RNA-dependent RNA polymerase (Yoshikawa, 2008). The nucleotide sequence encoding the CP is the most conserved region of the ACLSV genome, whilst the MP is the least conserved (Niu et al., 2012).

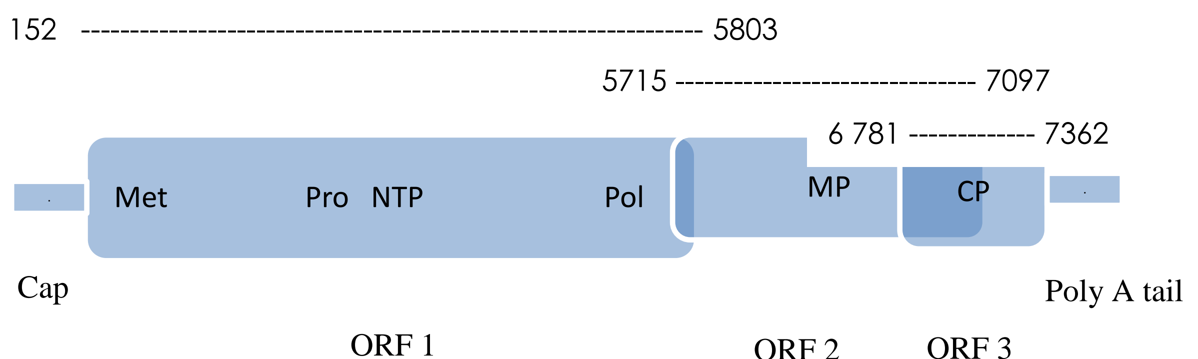

Figure 4. Genome organization of ACLSV; nucleotide sequence of ORF1 extends from bp 152 to 5803; ORF 2 sequence extends from bp 5715 to 7097; ORF 3 extends from nr 6781 to 7362; Met = methyltransferase; Pro = papain-like protease; NTP = nucleotide triphosphate-binding helicase; Pol = RNA-dependent RNA polymerase; CP= Coat protein; MP = movement protein.

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The complete nucleotide sequences of the genomes of ten ACLSV isolates were determined. The isolates were obtained from cherry (Hungarian ACLSV-Bal1 isolate) (German-Retana et al., 1997), plum (French isolate P863 and isolate PBM1) (German et al., 1990; Jelkmann, 1996), apple (Japanese P205, A4, B6 and MO-5 isolates) (Sato et al., 1993; Yaegashi et al., 2007), and peach (isolates Ta Tao5, Z1 and Z3) (Marini et al., 2008; Niu et al., 2012) host plants. Comparisons of the whole genome sequences from different isolates indicate large molecular variability, i.e. sequence conservation rates vary between 76.2% and 99.4%, with most of the isolates differing by 10-20% from one another (Candresse et al., 1995; German-Retana et al., 1997). Similarities of the nucleotide sequences were 79.8% (P863/P205), 76.2% (P863/Bal1), 81.5% (P863/PBM1), 76.5% (P205/Bal1), 79.6% (P205/PBM1), and 76.5% (Bal1/PBM1) (Yoshikawa, 2001). Another study found similarity of 69-84% at nucleotide levels, when performing multiple alignments of sequences of the coat protein genes of samples from India in comparison to other isolates of ACLSV originating from peach trees (Rana et al., 2009).

One study compared 35 isolates of ACLSV from apple (9 isolates), pear (1 isolate), almond (2 isolates), apricot (10 isolates), peach (9 isolates) and plum (4 isolates) hosts and originating from various countries (Italy, Spain, Jordan, Turkey, Albania, China, Hungary and Lebanon) by comparing sequences of a coat protein (CP) gene fragment of 500 bp (Al Rwahnih et al., 2004). The study found most of the variability in the N-terminal part of the CP gene, with the C-terminus significantly less variable. Two clusters were observed during their phylogenetic analysis (see Figure 5) - a large group containing 31 similar isolates and a small group of four showing very high variability throughout the CP gene. The apple isolates clustered into two subgroups. The one subgroup had isolates from Albania and from Turkey, while the other subgroup contained isolates from Albania, China and Italy, as well as the pear isolate from Italy (Al Rwahnih et al., 2004).

1.6 *Apple mosaic virus* (ApMV)

1.6.1 Nomenclature

Apple mosaic virus (ApMV) is one of the most common pathogens of apples worldwide (Desvignes, 1999; Rana, et al., 2010). ApMV belongs to the genus *Ilarviru*s, subgroup III, family *Bromoviridae* with non-enveloped isometric virions (Rybicki, 1995) (Figure 2). The ilarviruse*s* consists of 19 virus species and is the largest genus of the family *Bromoviridae* [International Committee on Taxonomy of Viruses (ICTV), 2012]. Other genera belonging to *Bromoviridae* include *Alfamovirus***,** *Cucumovirus*, *Bromovirus, Anulavirus* and *Oleavirus***.** ApMV is most closely related to PNRSV (Rybicki, 1995). It was reported that ApMV is often found in conjunction with PNRSV and PDV, but at a lower frequency than these two viruses (Petrzik & Lenz, 2002).

There has been some confusion in the virus nomenclature of ApMV. An isolate of PNRSV (GenBank AC no. U03857) was published as an ApMV isolate (Sanchez-Navarro & Pallas, 1994) and was consequently later re-classified as a PNRSV isolate (Sanchez-Navarro & Pallas, 1997). Isolate G of ApMV was published as PNRSV (Guo et al., 1995) according to Petrzik (2005). In

addition, the first two published ApMV sequences of the CP gene (designated as AMQOATPA and AMU15608), differed to a significant extent from the third sequence (designated as S78319) at the amino acid level due to frame-shift mutations identified later (Petrzik & Lenz, 2002).

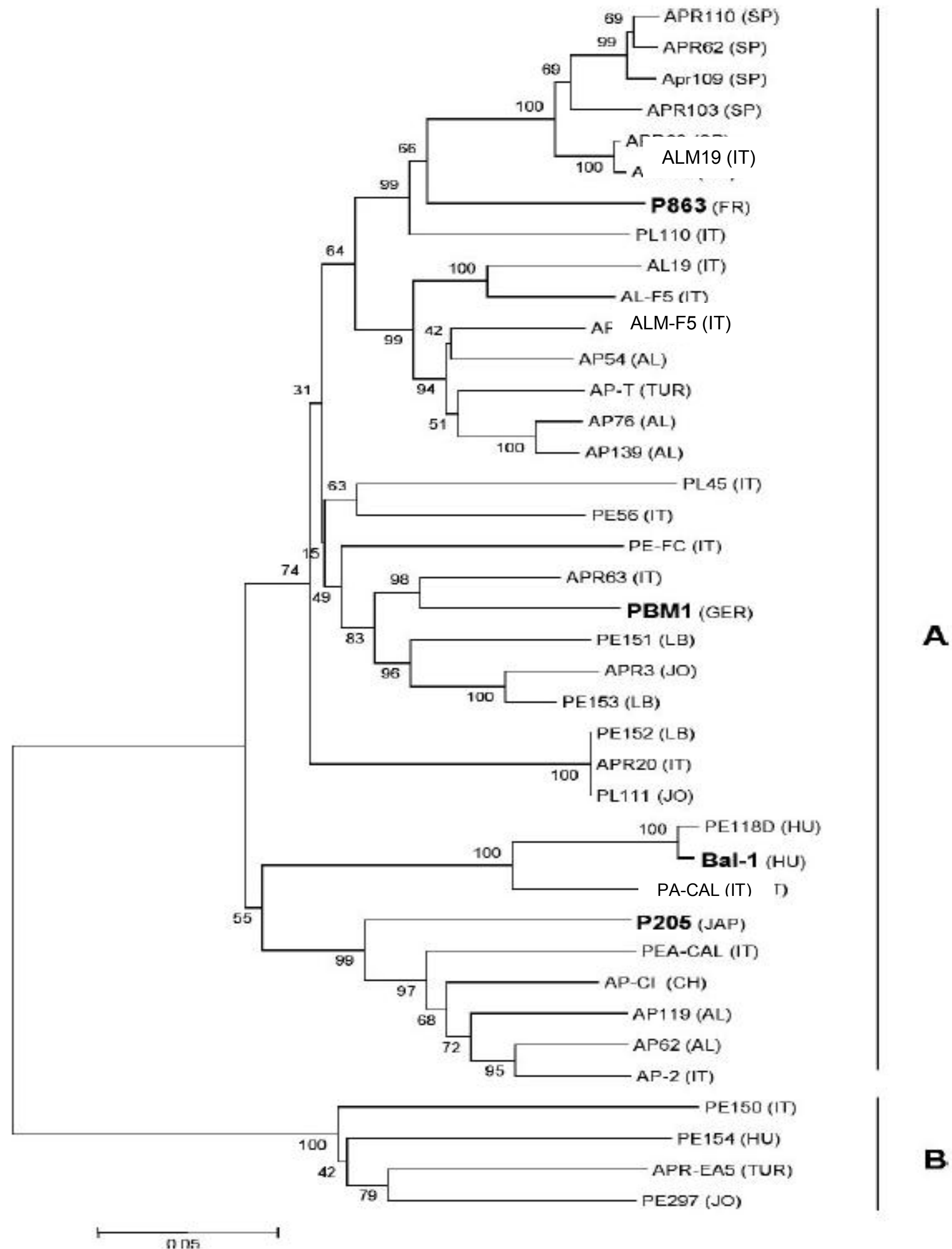

Figure 5. Phylogenetic tree generated using coat protein gene sequences showing the relationships of ACLSV isolates relative to a highly divergent group of four isolates by the minimum evolution method with 10 000 bootstrap replicates. Previously published sequences are in bold (PMB1, P-863, P205 and Bal-1). Group A: the majority of isolates. Group B: four diverging isolates. The following abbreviations were used to indicate hosts and countries: ALM = almond; APR = apricot; AP = apple; PE = peach; PA = pear; PL = plum; AL = Albania; CH = China; FR = France; GER = Germany; HU = Hungary; IT = Italy; JAP = Japan; JO = Jordan; SP = Spain; TUR = Turkey (adapted from Al Rwahnih et al., 2004)

ApMV showed a close relationship to PNRSV isolates with two pathotypes and three serotypes of ApMV closely related to PNRSV isolates (Mink et al., 1987). The isolates were antigenically related and clustered together with ApMV in the same subgroup (Mink et al., 1987), although the CP gene sequences of ApMV and PNRSV only show a sequence similarity of 47.7%. (Candresse et al., 1998). This similarity is highlighted by the reclassification of the Korean isolate ApMV-PV-32 which was later reclassified as a strain of PNRSV (Lee et al., 2002).

1.6.2 Host range

ApMV is a pathogen with a diverse natural host-range consisting primarily of woody plants. Hosts include apple, apricot, cherry, almond, rose (*Rosa* sp.) (Fulton, 1972), hazelnut (*Corylus avellana*), blackberry (*Rubus fructicosus*), raspberry (*Rubus idaeus*), hops (*Humulus lupulus*) (Petrzik & Lenz, 2002) and strawberry (*Fragaria* sp.) (Tzanetakis & Martin, 2005). ApMV was also reported in horse chestnut (*Aesculus hippocastanum*), mountain ash (*Sorbus aucuparia*), red horse chestnut (*A.x carnea*) and silver birch (*Betula pendula*) (Pol´ak & Zieglerov´a, 1997). Pear was not considered a host of ApMV until recently (Petrzik & Lenz, 2002). ApMV has various synonyms such as Hop virus, Hop virus A, Hop virus C, Rose mosaic virus, Horsechestnut yellow mosaic virus, Hop A virus, Dutch plum line pattern virus, Birch ringspot virus, birch line pattern virus, Birch ringspot virus, Birch line pattern virus, Mountain ash variegation virus, European plum line pattern virus, Mild apple mosaic virus and Severe apple mosaic virus (Petrzik, 2005).

1.6.3 Transmission

ApMV is transmitted by grafting (Postnette, 1963) and infected propagation material (Mink, 1992; Petrzik & Lenz, 2002). No insect vectors for ApMV are known to occur. ApMV has spread through pollen in hazelnut (Aramburu & Rovira, 2000), enhancing speculation that ApMV could possibly be spread naturally by pollen in apple (Petrzik & Lenz, 2002). In hazelnuts in Spain, the incidence of ApMV in virus-free trees planted between grafted trees, was 15% after 10 years of growth indicating natural spread of the virus (Aramburu & Rovira, 2000). However, the spread could not be attributed to a specific factor. Pollen and seed transmission was investigated, but results were inconclusive, as infected trees gave some healthy seeds and infected seeds did not always produce infected seedlings (Aramburu & Rovira, 2000). In another study, it was reported that only 6.2% of seedlings collected from hazelnut trees infected with ApMV, tested positive for the virus which could indicate seed transmission (Postman & Mehlenbacher, 1994).

1.6.4 Symptom expression

ApMV causes leaf symptoms including pale to bright yellow spots, mosaic and yellowing on leaves of apples (see Figure 6) in the spring (Nemeth, 1986). ApMV also cause line-pattern symptoms in plum trees and mosaic symptoms in roses (Fulton, 1972).

Symptom expression can vary between apple cultivars. Apple cultivar ‘Fuji’ displayed systemic yellow leaf spots between leaf veins, whilst cultivar ‘Golden Delicious’ displayed vein necrosis (Lee et al., 2002). These symptoms appeared clearly in late spring to early summer, but later

diffused to mild symptoms (Lee et al., 2002). Most commercial cultivars are affected by the virus, but cultivars 'Golden Delicious' and 'Jonathan' are more sensitive to the disease and significant yield losses could occur in some regions on these varieties (Desvignes, 1999; Nemeth, 1986).

Figure 6. Pale to bright yellow spot symptoms of *ApMV* infection on apple leaves.

Symptom expression can be very low, as was reported in Spain where less than 10% of ApMV infected hazelnut trees, showed symptoms (Aramburu & Rovira, 2000).

1.6.5 Genome

ApMV has a positive-sense single-stranded RNA genome, divided into three components designated RNA 1, 2 and 3, and a subgenomic messenger for the coat protein, designated RNA 4, like all *Bromoviridae* (Rybicki, 1995). Ilarviruses and *Alfalfa mosaic virus* (AMV) have the common biological trait of genome activation, where RNAs 1, 2 and 3 are non-infectious unless either the coat protein or subgenomic RNA 4 is added to the mixture. RNA 4 or coat protein of heterologous as well as homologous viruses of these genera can perform this function, but not RNA or coat protein from other genera (Gonsalves & Fulton, 1977). Assays, including biological and chemical RNA protection assays, indicate that both the AMV and ilarvirus 3' non-translated region (NTR) specifically bind to coat protein (Ansel-McKinney & Gehrke, 1998). Studies using the peptides from the coat protein of *Tobacco streak ilarvirus* (TSV) and the TSV 3' NTR showed that specific binding occurs between these two viruses despite only limited sequence homology (Swanson, et al., 1998).

RNA 3 encodes the putative movement protein and is bicistronic (Shiel et al., 1995). The movement protein is directly translated from RNA 3, whereas the coat protein is translated from the subgenomic mRNA, RNA 4 (Alrefai et al., 1994). RNAs 1 and 2 encode the virus replicases in the *Bromoviridae* (Rybicki, 1995).

ApMV differs from the other ilarviruses for which sequence data are available, as the ilarviruses contain a second ORF on RNA 2, which is expressed similarly to the 2b ORF present in cucumoviruses, although it encodes a larger peptide and has no sequence similarity to the 2b ORF of cucumoviruses (Shiel & Berger, 2000; Xin et al., 1998). There is no corresponding 2b ORF in ApMV (Shiel & Berger, 2000) and AMV (Xin et al., 1998).

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The complete nucleotide sequences of apple mosaic virus RNA 1, 2 (Shiel & Berger, 2000), 3 and 4 (Shiel et al., 1995) have been characterized. ApMV RNA 1 is 3476 bp in length and encodes a single large ORF which is similar to the methyltransferase-like and helicase-like domains that have been demonstrated to act as methyltransferases and helicases. ApMV RNA 2 is 2979 bp in length and also encodes a single ORF. The amino acid sequences encoded for by RNA 1, 2 and 3 show stronger similarity to AMV than to other ilarviruses. The coat protein is, however, more closely related to the ilarviruses, than to AMV (Shiel & Berger, 2000). Studies on AMV, which also applies to ilarviruses, showed that, in addition to forming the shell for the RNA 1, RNA 2 and RNA 3 genome components, the coat protein gene plays an important role in initiation and propagation of infection (Bol, 1999). Furthermore there is a motif around an arginine at the basic N-terminus of the coat protein that is very important for RNA-binding activity (Ansel-McKinney & Gehrke, 1998). This binding has an effect on genome activation, asymmetric plus strand RNA accumulation and cell-to-cell spread (Van der Vossen et al., 1994).

Shiel & Berger (2000) found that the presumed movement protein on RNA 3 of ApMV showed the greatest similarity to rosaceous-infecting ilarvirus subgroup III, followed by similarity to AMV, and only then the other ilarvirus subgroups, however the coat protein of ApMV showed little homology to AMV, with greater homology to the other ilarviruses. However, variation in ApMV coat protein gene sequences has been found (Lakshmi et al., 2011) and has been used to study the phylogenetic relationships of different isolates of ApMV from different parts of the world (Figure 7).

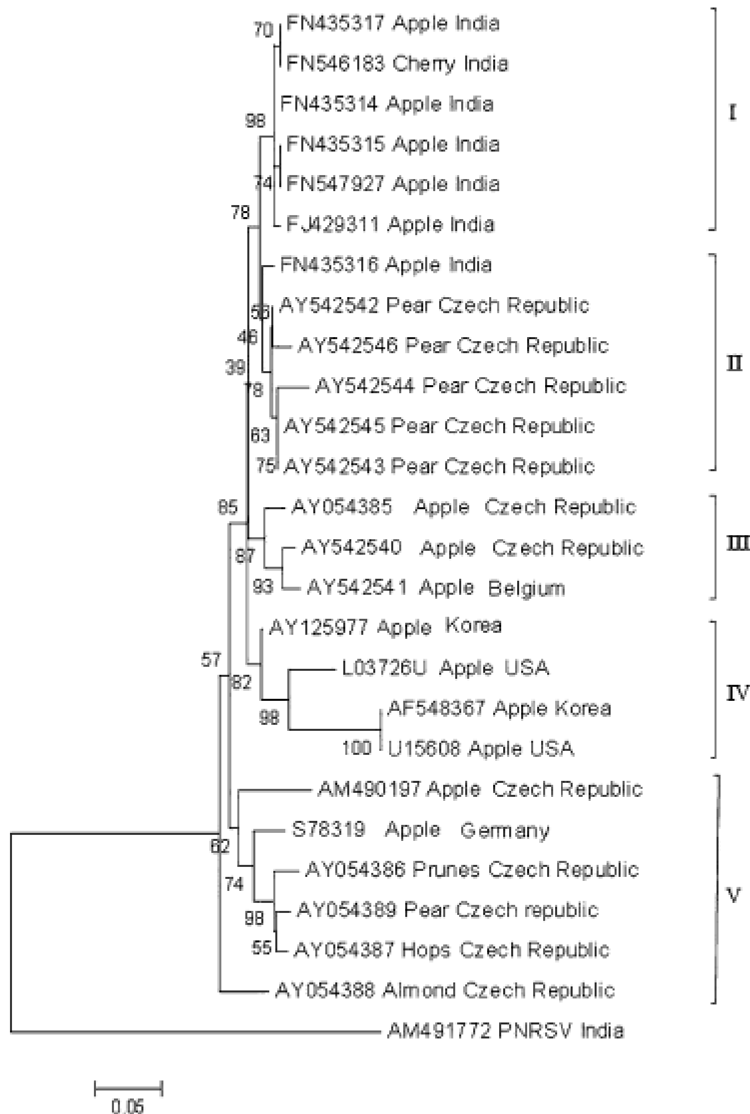

Figure 7. Phylogenetic tree of CP gene sequences of ApMV using neighbour-joining method with 1 000 bootstrap replicates. Branch lengths represent phylogenetic distances determined by distance matrices of nucleotide sequences. Bootstrap values above 75% are indicated above applicable branches. For each sequence, corresponding isolate and country of origin are indicated (Lakshmi et al., 2011).

1.7 Detection of viral infection in plants

1.7.1 Detection methods

Different diagnostic techniques are used to test for the presence of viruses including enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). These diagnostic techniques should be reliable, with high specificity and sensitivity, especially when mother trees are marketed as ‘virus free’, as these trees will be used as source of vegetative propagation material sold to producers.

ELISA incorporates the use of enzyme-linked antibodies binding to microplates for the detection of viruses (Clark & Adams, 1977). The colour intensity of the product formed by the enzyme reaction can be used to determine the presence of the virus using photometric measurement (Clark & Adams, 1977), but also the amount of pathogen occurring in the specific sample (Ward et al., 2004). A variation of the ELISA commonly used for the detection of plant viruses, is the double antibody sandwich ELISA (DAS-ELISA) which uses specific antibodies coated to a microtiter plate (see Figure 8). The antibodies trap the target antigen/viral particles in the test sample. An enzyme-linked specific antibody conjugate is finally used for detection (Ward et al., 2004). The substrate (such as *p*-nitrophenylphosphate) of the indicator enzyme is added and it is hydrolysed by the enzyme (often alkaline phosphatase). The hydrolysis releases *p*-nitrophenol, which changes the colour of the reaction mixture from colourless to yellow (Kokoskova & Janse, 2009). ELISA has the benefits of being a safe, inexpensive and rapid method for the detection of viruses. In addition, large numbers of samples can be processed, it can be mechanised and, due to the relative simplicity, can be carried out by technicians with relative low levels of training. The disadvantage, however, is that the assay requires more time to complete than for instance RT-PCR (Yang et al., 2012). Another disadvantage is that coupling of the enzyme to the antibody may result in stearic hindrance which in turn may result in a loss in function of the antibody and/or enzyme (Blake & Gould, 1984). Another disadvantage is that changes at the coat protein gene level may have an influence on the ability of the antibodies to recognize the antigen to be detected. Seasonal fluctuations of viral concentrations, which are particularly low at temperatures over 38ºC, and uneven distribution of viruses (Heleguera et al., 2001), can cause infected plants to test negative when tested by ELISA (Mekuria et al., 2003).

PCR enables *in vitro* amplification of DNA or RNA whereby an enzyme uses a target segment in a strand of DNA as a template for a complementary primer strand (Schochetman et al., 1988). PCR involves a three-step cycling process: (1) denaturation of double-stranded DNA, (2) annealing of the applicable primers, and (3) primer extension. RNA sequences require the manufacturing of a DNA copy (cDNA) of the RNA using a reverse transcriptase (RT) enzyme before the PCR begins and the complete process is called RT-PCR (Schochetman et al., 1988). The disadvantages of using RT-PCR are that the technique is technically more difficult to perform and more expensive than ELISA, as well as requiring more expensive equipment to complete the tests. The benefits, however, are the ability of PCR (or RT-PCR) to allow for the detection of low viral loads - it is considered to be 10^2 to 10^5 fold more sensitive than traditional ELISA (Dietzgen, 2002; Spiegel & Martin, 1993). It also has the additional benefits of high specificity and the ability to detect several pathogens in a single reaction, reducing cost, time and labour (Pallas et al., 2009).

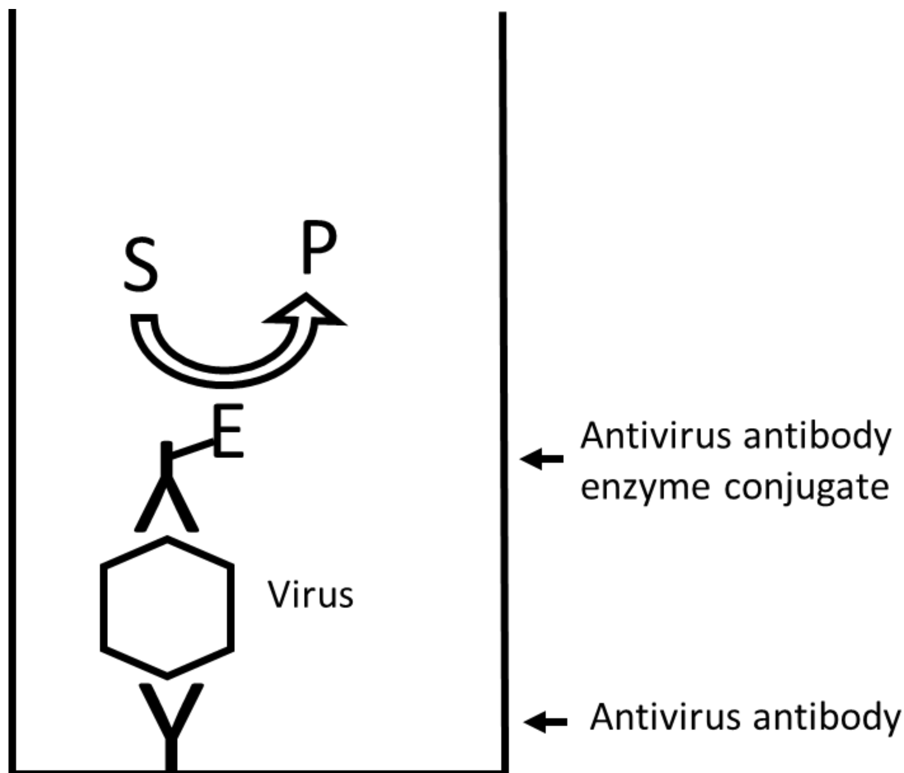

Figure 8. Schematic diagram illustrating the DAS-ELISA technique in a well of a microtiter plate. E = enzyme, S = substrate, P = Product.

1.7.2 Detection of ACLSV

ELISA (Poul & Dunez, 1998) and RT-PCR (Nemchinov et al., 1995) were developed for the detection of ACLSV. ELISA is efficient with its advantages as listed above, but has disadvantages for the detection of ACLSV. ACLSV occurs in low titres (Kinard et al., 1996; Poul & Dunez, 1998) and has diverse isolates which could increase false negative results (Kinard et al., 1996). The ACLSV particles are also highly unstable (Poul & Dunez, 1998). Generally, the optimal tissue type (Torrance, 1984) and season (Candresse et al., 1995; Svoboda & Polak, 2010) limits testing to a very restricted period during the year. Inhibition by polysaccharides or phenolic compounds in woody plant material, could also inhibit ELISA efficiency (Hassan et al., 2006; Kinard et al., 1996; Menzel et al., 2003; Wu et al., 1998). Furthermore no internal controls are available to prevent false negatives caused by such inhibitions (Menzel et al., 2003).

Viral disease intensity can change both temporally and spatially (Thresh, 1983). In a study conducted in Poland the general conclusion was reached that detection of ACLSV was less effective after a “long period of high temperatures” (Cieslinska et al., 1995). It could be attributed to a reduction in the virus titre caused by the higher temperatures experienced in the orchards and in general it limits ELISA to about three months after budbreak in the spring (Candresse et al., 1995). It was found that in mid-summer only 10% of known infected trees tested positive by ELISA, compared to 80% during mid-spring when taking the average of various fruit groups (Candresse et al., 1995).

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ACLSV levels were also found to vary between different tissue and fruit types. For apple, flower petals showed highest virus levels in spring, whilst for leaf material early spring was the optimal sampling time and the values decreased later in the season (Cieslinska et al., 1995). Phloem tissue was also useful for detection during spring, but was more reliable later in the season (Cieslinska et al., 1995). Uneven distribution of ACLSV in infected trees was observed (Fridland, 1973; Poul & Dunez, 1998). It was found that in short budsticks (5-10 buds long) infection was usually systemic, whilst in longer budsticks (20-40 buds long) many uninfected buds were detected, especially towards the tips (Fridland, 1983). In *in vitro* plants, the highest concentration of virus was found at the base of stem, with decreasing levels of virus to the tip of the shoot (Knapp et al., 1995). Furthermore ACLSV in *in vitro* plants occurred in the epidermis, cortex and vascular bundles, but rarely in the pith of the stems (Knapp et al., 1995).

Pears are known for showing low detection rates for ACLSV (Candresse et al., 1995). Ripening fruits were found to be the best tissue type for ELISA when working with pears (Cieslinska et al., 1995). *In vitro* plants showed highest virus concentration at the base and tip of shoots, with lower titer in the middle of shoots (Wang et al., 2010).

Dissimilar results were obtained regarding the optimal time for testing cherry leaves using ELISA, as one report obtained positive results only from mid-summer, whilst another report indicated spring until summer to be effective (Cieslinska et al., 1995). Plums are notorious for low detection rates of ACLSV (Candresse et al., 1995). Early in the season phloem gave the same or better results than leaves, whilst during autumn, tissue from ripe fruit or phloem from young shoots gave the best ELISA results (Cieslinska et al., 1995). A much higher concentration of ACLSV in fruit than leaves of apricots was also reported (García-Ibarra et al., 2010).

Candresse et al. (1995) also found that RT-PCR results from April to November (Northern Hemisphere) indicated that ACLSV could be detected throughout the year, although the effect of fruit groups and tissue type influenced the efficiency (Candresse et al., 1995). However they found that in mid-summer only 65% of known infected trees tested positive by Immuno-Capture-PCR (IC-PCR), compared to 85% during mid-spring (Candresse et al., 1995).

RT-PCR, especially where different viruses are combined in one multiplex PCR, coupled with quick and simple RNA extraction can potentially be used for pome fruit virus certification programs, as it is much more sensitive than woody indexing (Hassan et al., 2006) and ELISA (Hassan et al., 2006; Menzel et al., 2003). However, RT- PCR assay has the risk of giving false negative results caused by RNA degradation or due to the presence of inhibitors of the reverse transcriptase or polymerase (Park et al., 2006).

1.7.3 Detection of ApMV

ELISA and RT-PCR (Alrefai et al., 1994) have been developed for the detection and the analysis of ApMV. ELISA is routinely used for detecting the virus worldwide (Choi & Ryu, 2003). The viral concentration of ApMV varies seasonally, in a similar fashion as observed with other viruses such as ASGV and ACLSV (Svoboda & Polak, 2010). It is higher during the first six months of the year

in the Northern Hemisphere (Fuchs, 1982; Matic et al., 2008; Svoboda & Polak, 2010) and detectable using ELISA from April to June in this region (specifically England) (Torrance & Dolby, 1984). In Mediterranean climatic conditions, only 10% of the ApMV samples from stonefruit trees detected during spring were detected during a hot summer season (Matic et al., 2008). ApMV ELISAs showed higher absorbance values in young leaves than in mature leaves (Torrance & Dolby, 1984). Young leaves showed a 1.9, 41 and 81 times higher average absorbance value than flower petals, dormant buds and bark phloem respectively for the ApMV using ELISA (Svoboda & Polak, 2010). Furthermore the highest concentrations were found in middle April, with a definite decline until no virus was detectable by ELISA in July in the Northern Hemisphere (Svoboda & Polak, 2010). In hazelnuts, ELISA results from leaves during spring indicated 100% detection of known-infected trees, with optical density values of about 20 times higher than negative controls (Aramburu & Rovira, 2000). In comparison ELISA from bark samples detected only 74% of infected samples and the optical density values were only three to eight times higher than the negative controls (Aramburu & Rovira, 2000). During summer, detection using leaves was considerably lower than during spring, detecting only 13% of positive samples (Aramburu & Rovira, 2000). In bark samples, detection levels were the same during spring and summer (Aramburu & Rovira, 2000).

Difficulties exist in experimenting with ilarviruses, as they are difficult to manipulate and transmit compared to other viruses. Most of the ilarviruses primarily infect woody perennial hosts, where they often occur in low titer. Futhermore they are difficult to isolate from their woody hosts and are poorly transmitted to such hosts by mechanical means (Shiel & Berger, 2000).

RT-PCR has been used more widely for research on ApMV in recent years (Lakshmi et al., 2011; Shiel et al., 1995; Shiel & Berger, 2000). Detection is usually carried out using DAS-ELISA, and further analysis is done using RT-PCR with consequent sequencing (Ferretti et al., 2010; Lakshmi et al., 2011). Phylogenetic trees of the CP gene sequence were also constructed, and five different clades retrieved (Lakshmi et al., 2011).

CHAPTER 2

Comparative detection of *Apple chlorotic leafspot virus* (ACLSV) and *Apple mosaic virus* (ApMV) by DAS-ELISA and RT-PCR in South Africa

2.1 Abstract

DAS-ELISA and RT-PCR are two of the methods used for detection of ACLSV and ApMV. Four replicates of twenty and seventeen leaf samples were collected for testing for ACLSV and ApMV, respectively. The samples were divided equally for each test and a dilution series ranging from undiluted to 1/400 and undiluted to 1/50 000 were prepared for ACLSV and ApMV, respectively. The DAS-ELISA and RT-PCR tests were performed for each virus and the results were compared. Results revealed that RT-PCR was significantly more sensitive than DAS-ELISA for detection of both viruses and numerically it was approximately 70 and 71 fold more sensitive for ACLSV and ApMV, respectively. Detection of ApMV in pears was additionally attempted and 10 samples tested positive for the virus by RT-PCR, whilst DAS-ELISA detected no infections.

2.2 Introduction

The two techniques by which viruses in fruit trees are detected in general, are enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR) (Chairez & Lister, 1973; Nemchinov et al., 1995; Poul & Dunez, 1998). However, according to the literature, ELISA is not sufficiently sensitive to allow detection of low concentrations of viruses in fruit tree tissues (Menzel et al., 2002). The basic principle of the ELISA technique is the proficiency of antibodies to recognize the proteins of the coat proteins which are unique for each virus. ELISA occasionally fails due to low virus titer or inhibitory effects of polysaccharides or phenolic compounds in tissue extracts of woody plants (Nemchinov et al., 1995; Kinard et al., 1996; MacKenzie et al., 1997; Menzel et al., 2003). The amount of virus at any given moment in a specific type of tissue is a dynamic relationship between protein synthesis and protein degradation by the plant’s natural defense system (Delaure et al., 2008).

The sensitivity of RT-PCR makes the detection of low levels of virus possible even in the presence of inhibitors. It therefore has the potential to be the most sensitive method for virus detection in some woody plants even during seasons of low virus titers (Rowhani et al., 1995; Kinard et al., 1996) such as during hot summer months when ELISA becomes unreliable (Candresse et al., 1995).

Apple chlorotic leaf spot virus (ACLSV) is the type species of the genus *Trichovirus* from the family *Betaflexiviridae* (King et al., 2012). ACLSV is known to infect most fruit tree species, including pomefruit such as apple (*Malus* sp.), pear (*Pyrus* sp.) (Desvignes & Boyé, 1989) and quince (*Cydonia oblonga*) (Rana et al., 2008). Stonefruit such as apricot (*Prunus domestica*), cherry (*P.* avium), peach (*P. persica*), prune (*P. domestica*) and almond (*Prunus dulcis*) can also

be infected (Lister, 1970; Nemeth, 1986; Desvignes & Boyé, 1989; German-Retana et al., 1997; Spiegel et al., 2005). ACLSV decreases the growth, mean fruit weight and yield of trees (Cosba et al., 1986).

Apple mosaic virus (ApMV) belongs to the genus *Ilarviru*s, subgroup III, family *Bromoviridae* with non-enveloped isometric virions (Rybicki, 1995). ApMV is a pathogen with a diverse natural host-range consisting primarily of woody plants. Hosts include apple, apricot, cherry, almond (*Prunus dulcis*), rose (*Rosa* sp.) (Fulton, 1972), hazelnut (*Corylus avellana*), blackberry (*Rubus fruticosus*), raspberry (*Rubus idaeus*), hops (*Humulus lupulus*) (Petrzik & Lenz, 2002) and strawberry (*Fragaria* sp.) (Tzanetakis & Martin, 2005). Infection by ApMV results in yield reduction and tree decline (Desvignes, 1999).

Pears have for many years not been considered a primary or secondary host of ApMV (Petrzik, 2005). There was only one report of the transmission of ApMV to pear cultivar Beurré Hardy by inoculation where faint yellow-green ringspot symptoms developed on the leaves, but since the authors could not retransmit the virus, they were doubtful as to the origin of the symptoms (Kristensen & Thomsen, 1963). ApMV was, however, reported in 2002 in a single pear tree in the Czech Republic (Petrzik & Lenz, 2002) and in a further 22 samples from Italy and the Czech Republic in 2005 (Petrzik, 2005).

The pome- and stonefruit industry in South Africa is governed under the South African Deciduous Fruit Plant Certification Scheme with the aim of improving plant material established in the country. This includes routine testing of plant material for certain economically important viruses, including ACLSV and ApMV. Currently the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) technique is prescribed by the South African Deciduous Fruit Plant Certification Scheme for routine testing of plant material for ACLSV and ApMV status. Testing of planting material of pears for ApMV is currently not required under the South African Deciduous Fruit Plant Certification Scheme, as it is not considered a host of ApMV.

In this study, DAS-ELISA and RT-PCR was used to test leaf samples for the presence of ACLSV and ApMV in South African pome- and stonefruit trees from a wide range of sources. The detection limits of DAS-ELISA were compared to that of RT-PCR. Pear samples were furthermore tested by means of DAS-ELISA and RT-PCR for the presence of ApMV.

2.3 Materials and Methods

2.3.1 Sample collection for ACLSV detection

Young leaf samples (approximately two weeks old) were collected from pome- and stonefruit plants in the ACLSV infected collection maintained at the SAPO Trust facility, located at Fleurbaix premises, Stellenbosch, South Africa. No symptoms were observed on the leaves of any of the trees. Leaves were used as this is the tissue type currently used for routine DAS-ELISA testing in the South African deciduous fruit industry. During the first few months of 2011 leaf samples stored at -80ºC from spring 2010, and fresh leaves collected during the hot summer months of January

and February 2011 were used. During the spring seasons of 2011, 2012 and 2013, leaf samples were tested fresh without storage.

The samples used for the comparison of DAS-ELISA and RT-PCR detection, are listed in Table 1.

Table 1. Fruit group, fruit type and variety of samples used for ACLSV detection.

Sample	Fruit group	Fruit type	Variety
A1	Pomefruit	Apple	Qin Yang
A4	Pomefruit	Apple	Royal Beaut
W5	Pomefruit	Apple	Rubens
V3	Pomefruit	Apple	Mahanna Red Braeburn
C4	Stonefruit	Peach	Fire Rich
D5	Pomefruit	Apple	Rubens
E3	Pomefruit	Apple	Unknown
11A	Stonefruit	Peach	Prof Malherbe
12A	Pomefruit	Apple	Red Gravenstein
13A	Pomefruit	Apple	Royal Beaut Prosser
14A	Stonefruit	Peach	Fire Rich
15A	Pomefruit	Apple	Rubens
16A	Pomefruit	Apple	Apple15
17A	Pomefruit	Apple	Mahanna Red
18A	Pomefruit	Apple	Golden Joy
19A	Pomefruit	Apple	SAPO Early Gold
20A	Pomefruit	Apple	Earligran

2.3.2 Sample collection for ApMV detection

Leaf samples were collected from pomefruit plants from various sources. Samples K3, J3, K2 and J1 were collected from plants established using buds from trees that tested positive using DAS-ELISA for ApMV in the Grabouw and Villiersdorp regions, Western Cape. Samples D6 and A168 tested positive during routine testing of plant material during 2012. Samples AP1-15 were collected from infected orchards in the Ceres area during 2013. The plants tested all expressed symptoms of yellow spots on their leaves. The samples used in this study are listed in Table 2. Leaf samples were tested without storage.

Table 2. Fruit group and origin of apple samples used for ApMV detection.

Sample	Fruit Group	Region of origin	Variety
K3	Apple	Grabouw	Granny Smith
J3	Apple	Villiersdorp	Golden Delicious
K2	Apple	Villiersdorp	Early Red One
J1	Apple	Grabouw	Golden Delicious
D6	Apple	Stellenbosch	Golden Delicious
A168	Apple	Stellenbosch	Granny Smith
AP1	Apple	Ceres	Early Red One
AP2	Apple	Ceres	Early Red One
AP3	Apple	Ceres	Granny Smith
AP4	Apple	Ceres	Early Red One
AP5	Apple	Ceres	Granny Smith
AP6	Apple	Ceres	Granny Smith
AP7	Apple	Ceres	Golden Delicious
AP8	Apple	Ceres	Granny Smith
AP9	Apple	Ceres	Granny Smith
AP10	Apple	Ceres	Golden Delicious
AP11	Apple	Ceres	Golden Delicious
AP12	Apple	Ceres	Early Red One
AP13	Apple	Ceres	Golden Delicious
AP14	Apple	Ceres	Golden Delicious
AP15	Apple	Ceres	Early Red One

2.3.3 Sample collection for ApMV detection in pears

Randomly selected leaf samples were collected for detection of ApMV from the uninfected collection of pear trees maintained at the Fleurbaix premises of the SAPO Trust facility, Stellenbosch. The samples were tested by both DAS-ELISA and RT-PCR. The fifteen samples included: P1 to P10, AP24, AP25, AP26, AP27 and AP28.

2.3.4 Sample preparation for DAS-ELISA and RT-PCR

Fifteen to twenty leaves of each tree were stacked on each other and subsequently cut in halve vertically and divided equally for DAS-ELISA and RT-PCR detection of ACLSV and ApMV. A sample was considered as the leaves collected from one tree.

2.3.5 Plant sample homogenization for DAS-ELISA

DAS-ELISA kits (Bioreba, Switzerland) were used for ACLSV and ApMV detection. Leaf samples of 1 g were weighed and placed in a bag containing a mesh unit. Extraction buffer was added according to the manufacturer’s instructions of 10 ml per 1 g leaf tissue. The material was ground

and homogenized using a Homex homogenizer (Bioreba, Switzerland). Homogenates were also prepared from uninfected plants as negative controls.

2.3.6 Plant sample homogenization for RT-PCR

Leaf samples were prepared according to the method of Visser (2008) which was modified from the method of La Notte et al. (1997). One hundred milligrams of leaf tissue of each sample was pulverized using an autoclaved mortar and pestle. Two milliliter of grinding buffer [15 mM Na_2CO_3, 35 mM $NaHCO_3$, 2% w/v PVP40 (Sigma-Aldrich, USA), 0.2% (w/v) BSA (Fluka, USA), 0.05% (v/v) Tween 20, 1% (w/v) sodium meta bisulphite, pH 9.6] was added to the leaf material and the grinding process continued until ground to a fine pulp. One milliliter of the ground sample was added to a 1.5 ml Eppendorf tube and it was centrifuged in a Picofuge (Stratagene, USA) for 15 seconds forming a pellet of remaining plant debris. Four microliters of supernatant was added to 25 µl of GES buffer (50 mM NaCl, 0.1 M glycine-NaOH, pH 9.0, 1 mM EDTA, pH 98.0, 0.5% (v/v) Triton X-100). The solution was incubated for 10 minutes at 95ºC in a digital dry bath (Labnet International, Inc., USA) followed immediately by placing the samples on ice for at least 5 minutes after which they were immediately used in the RT-PCR.

2.3.7 Total RNA extraction for RT-PCR

Total RNA was extracted from the plant samples using 100 mg of leaf tissue following the instructions as described by the manufacturer of the RNeasy Plant Mini Kit (Qiagen, Germany) and eluted in a final volume of 40 µl.

2.3.8 Dilution series prepared for comparative ACLSV detection by DAS-ELISA and RT-PCR

During initial screening of plant samples in 2012, samples were tested by DAS-ELISA and RT-PCR undiluted and at dilutions of 1/10, 1/100, 1/500, 1/1000, 1/5 000, 1/10 000 and 1/50 000. These dilutions were made by adding extraction buffer to the homogenate for DAS-ELISA and water provided by the manufacturer to the extracted RNA for RT-PCR. The samples were subsequently retested using the dilutions 1/5, 1/10, 1/50, 1/100, 1/200 and 1/400 since virus levels were found to be low. Each sample was tested in duplicate, i.e. two leaf samples were sampled independently by collecting leaves from the same plant tested previously. Each determination was also repeated i.e. performed in duplicate by using the same processed sample (homogenate for DAS-ELISA and extracted RNA for RT-PCR) and the DAS-ELISA and RT-PCR was therefore performed four times per sample.

During the testing season in 2013, the samples were tested by ELISA and RT-PCR without dilution and at 1/5, 1/10, 1/50, 1/100, 1/200 and 1/400 dilution. The dilutions were made by adding homogenate from a negative control plant to the homogenate of test samples for DAS-ELISA and RNA dilutions were made by adding RNA extracted from the negative control plant. The DAS-ELISA and RT-PCR was also performed four times per sample as described above for 2012.

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2.3.9 Dilution series prepared for comparative ApMV detection by DAS-ELISA and RT-PCR

In 2012 plant samples were tested without dilution and with DAS-ELISA and RT- PCR at the following dilutions: 1/5, 1/10, 1/50, 1/100, 1/500, 1/1000, 1/10 000. DAS-ELISA dilutions were performed by adding extraction buffer to the homogenate. Extracted RNA was diluted with water provided with the RNeasy Plant Mini Kit. Replicate samples were collected and duplicate determinations were performed for each sample.

During 2013 samples were tested without dilution by DAS-ELISA and RT-PCR and at the following dilutions: 1/10, 1/100, 1/1000, 1/10 000 and 1/50 000. DAS-ELISA dilutions were performed by adding homogenate from the negative control plant to the homogenate of test samples. Dilutions for RT-PCR were made by adding RNA isolated from the negative control plant to the RNA extracted from the samples. Replicate samples were collected and duplicate determinations were performed for each sample.

2.3.10 DAS-ELISA procedure

ELISA kits manufactured by Bioreba, Switzerland, were used for virus detection. ELISA plates were coated with polyclonal antibodies specific for each of the viruses respectively in coating buffer at a dilution of 1:10 000. They were incubated at 4ºC overnight and subsequently washed with washing buffer (3 x phosphate buffered saline solution containing 0.1% Tween 20). An amount of 200 µl of the homogenized sample was added to respective wells, incubated at 4ºC overnight and washed with washing buffer. An antibody-enzyme conjugate was diluted to 1:10 000 in conjugate buffer and 200 µl was added to each well. Plates were incubated at 30ºC for 5 hours, followed by a washing step. Subsequently substrate (p-Nitrophenylphosphate in citrate buffer, 1 mg/ml) was added (200 µl/well) and absorbance was measured at 405 nm after 60 minutes using a Multiskan FC (Thermo Scientific) multititer microplate reader. Two positive controls (one obtained from the manufacturer (Bioreba) and one from a local, infected plant source maintained in the SAPO Trust infected collection) and two negative controls (obtained from local plant sources) were included on every ELISA plate for each virus determination respectively. The cut-off value used to indicate the presence of virus was calculated as three times the mean value of the negative controls on each plate respectively.

2.3.11 RT-PCR procedure for ACLSV detection

The RT-PCR for the detection of ACLSV was carried out in thin-walled 0.2 ml PCR tubes. A reaction mixture was prepared of 2.5 µl 10 x PCR-buffer, 1.25 µl 0.1 M DTT, 2 µl 25 mM $MgCl_{2,}$ 0.625 µl 20 µM forward primer, 0.625 µl 20 µM reverse primer, 1 µl 5 mM dNTPs (Bioline, Germany), 0.25 µl 5 U/µl Super-Therm DNA Polymerase mixture (Bioline, Germany), 0.125 µl SuperScript^{TM} III (Invitrogen™, Life Technologies™, USA) and 14.625 µl Milli-Q® water. The primers used had the following sequences: forward primer (ACLSV-ORF-2-f), 5'-GAAGATCGCAGAAGGGGATATTC-3', and reverse primer (ACLSV-ORF-2-r), 5'-

GTCTACAGGCTATTTATTATAAG-3’ (Nakahara et al., 2001). Two microliters of plant isolated homogenate or RNA solution was added to 23 µl of the PCR reaction mixture. The RT-PCR amplifications were performed using one reverse transcription step at 48ºC for 30 minutes, followed by 35 cycles of the following steps: denaturation at 94ºC for 30 seconds, annealing at 55ºC for 45 seconds and extension at 72ºC for 90 seconds; and a final elongation step at 72ºC for 10 minutes. The amplifications were performed in a Veriti ™ Thermal Cycler (Life Technologies™, USA). Viral infection was indicated by the amplification of an amplicon of 1510 bp in size corresponding to position 5750 to 7512 bp of the genome sequence of ACLSV, which includes the complete sequence of the coat protein gene of 6784 to 7365 (581 bp).

2.3.12 RT-PCR procedure for ApMV detection

The RT-PCR for the detection of ApMV was performed in 0.2 ml PCR tubes. The RT-PCR mixture contained 2.5 µl 10 x PCR-buffer, 1.25 µl 0.1 M DTT, 1.5 µl 25 mM $MgCl_2$, 0.625 µl 20 µM forward primer, 0.625 µl 20 µM reverse primer, 1 µl 5 mM dNTPs (Bioline, Germany), 0.25 µl 5 U/µl Super-Therm DNA Polymerase mixture (Bioline, Germany), 0.125 µl SuperScript™ III (Invitrogen™, Life Technologies™, USA) and 15.125 µl Milli-Q® water. The first primer pair evaluated had the following sequences: forward primer (92D9up), 5’-GGCCATTAGCGACGATTAGTC-3’, and reverse primer (92E0re), 5’-ATGCTTTAGTTCCCTCTCGG-3’ (Petrzik, 2005). Two microliters of isolated RNA was added to 23 µl of the PCR reaction mixture. The RT-PCR amplifications were performed using one reverse transcription step at 48ºC for 30 minutes, followed by 40 cycles of the following parameters: denaturation at 94ºC for 30 seconds, annealing at 58ºC for 45 seconds and extension at 72ºC for 90 seconds. It was followed by a final elongation step at 72ºC for 10 minutes. The amplifications were performed in a Veriti ™ Thermal Cycler (Life Technologies™, USA). The amplicon amplified by the primer set was 822 bp in size corresponded to position 1044-1866 bp of the genome sequence of ApMV. The amplicon included the complete coat protein gene of 1126-1794 bp (668 bp).

Optimization of this protocol was performed and included varying the annealing temperature between 55ºC and 60ºC, varying the number of cycles and changing the $MgCl_2$ concentrations. A second primer pair was also evaluated consisting of forward primer PAPCP3-1: 5'-CTAACAAATCTTCATCGATAAG-3', and reverse primer PAPCP5: 5’-TCTAACATGGTCTGCAAGTAC-3' (Lee et al., 2002). The RT-PCR amplifications were performed using the same protocol as above, but after optimization, an annealing temperature of 56ºC for 45 seconds was chosen as it gave optimal amplification. Viral infection was indicated by the amplification of an amplicon of 668 bp in size which represents the complete sequence of the coat protein gene.

2.3.13 Electrophoretic analysis and quantification of RT-PCR-amplified products

RT-PCR products were detected by agarose gel electrophoresis. To this end, 1% agarose gel containing 1 µg/ml ethidium bromide was cast in a 20 cm x 10 cm or a 20 cm x 20 cm casting tray

and immersed in a 1 x TAE electrophoresis buffer (0.48% (w/v) Tris, 0.11% (v/v) glacial acetic acid, 0.5 M EDTA, pH 8.0) in an electrophoresis apparatus (Cleaver Scientific Ltd, England). A total of 20 µl of PCR product was mixed with a loading buffer (0.25% (w/v) bromophenol blue, 57.5% (v/v) glycerol, 0.5 M EDTA, 1 M Tris, pH 8.0, Milli-Q® water), and loaded into wells in the gel. A 100 bp DNA size marker ladder with a maximum marker of 1500bp (Promega, USA) was loaded to determine the size of the amplified RT-PCR products of ApMV isolates. A DNA size marker ladder with a maximum marker of 2000bp (Bioline, Germany) was loaded to determine the size of the amplified RT-PCR products of ACLSV isolates. Electrophoresis was performed at 100 V for 60-80 minutes using a Cleaver Scientific Ltd power source. The RT-PCR products were visualized using a UV transilluminator and portable darkroom (Cleaver Scientific Ltd, England).

2.3.14 Statistical analysis of DAS-ELISA and RT-PCR comparative results

In order to compare the sensitivities of the DAS-ELISA and RT-PCR the difference in the relative quantities used in each of the assay methods was calculated using the following formulae:

RT-PCR: 100 mg of leaf material was used for extraction of which the isolated RNA was made up in 40 µl water provided by the manufacturer. Of this RNA solution, 2 µl was used in the RT-PCR with final volume of 25 µl, and 20 µl was loaded onto the gel for detection: The equivalent amount of plant material used can be calculated as follows: (2 µl/40 µl) x (20 µl/25 µl) x 100 mg ≡ 4 mg i.e. if a positive result is obtained this reflects the presence of the virus in an amount of 4 mg of undiluted plant sample.

For the DAS-ELISA the following calculation was made: 1 g of leaf material was homogenized in 10 ml of buffer of which 200 µl was used in the DAS-ELISA. The equivalent amount of plant material used can be calculated as follows: 200 µl/10 000 µl x 1 000 mg = 20 mg per well i.e. if a positive result is obtained this reflects the presence of the virus in an amount of 20 mg of undiluted plant sample.

Thus the RT-PCR determination is based on the amount of virus present in 4 mg of plant material compared to 20 mg in the case of DAS-ELISA. The RNA equivalent value was calculated as follows: RT-PCR value x 4 mg / 20 mg.

The DAS-ELISA and RT-PCR results obtained were listed in columns in an Excel spreadsheet showing the rank order of whether the virus was detected by each of the methods or only one. The highest dilution of plant sample in which virus was detected by each technique was also indicated for each sample. Subsequently a rank order value of 1 was indicated for each sample in a column of the method which detected the highest dilution of virus, and a rank order value of 2 was indicated to the method which detected at a lower dilution of the sample. If both methods detected the virus at the same dilution, a value of 1.5 was indicated in both columns. These results were then analysed using the Friedman test which calculated Chi square and p-values.

The factor whereby RT-PCR was more sensitive than DAS-ELISA or *vice versa* was calculated for each sample replicate. The RT-PCR equivalent value was divided by the highest dilution of the

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DAS-ELISA value for each sample replicate to obtain this factor. The factors for each sample were converted to log values. The geometric mean of the factors was then calculated in order to express the differences between the sensitivities of the two methods numerically.

2.3.15 Detection of ApMV in pears using DAS-ELISA and RT-PCR

A total of 249 pear leaf samples were tested by DAS-ELISA for ApMV. From these samples, 15 samples were randomly chosen and used for RT-PCR detection of ApMV using the PAPCP3-1 and PAPCP5 primers as described before. Samples were arbitrarily numbered so that they would not be traced as this has confidentiality implications.

2.4 Results

2.4.1 RT-PCR and DAS-ELISA detection of ACLSV

2.4.1.1 RT-PCR detection of ACLSV

When leaf samples, known to be infected, were homogenized by the GES method and used in RT-PCR, very faint or no bands were visible when products were separated by agarose gel electrophoresis. The conclusion from these results was that this method of sample preparation was not suitable and therefore the RNA of all samples was isolated using the RNeasy Plant Mini Kit and used in the RT-PCR determination. In positively infected samples this consistently gave positive RT-PCR results indicating that the RNA isolation procedure gave reliable results. For this reason only isolated RNA was used for subsequent RT-PCR detection in all samples.

As a typical result an image showing the RT-PCR products of samples 11A to 20A is depicted in Figure 9. All RT-PCR products were sequenced (see Chapter 3) confirming their identities as ACLSV fragments.

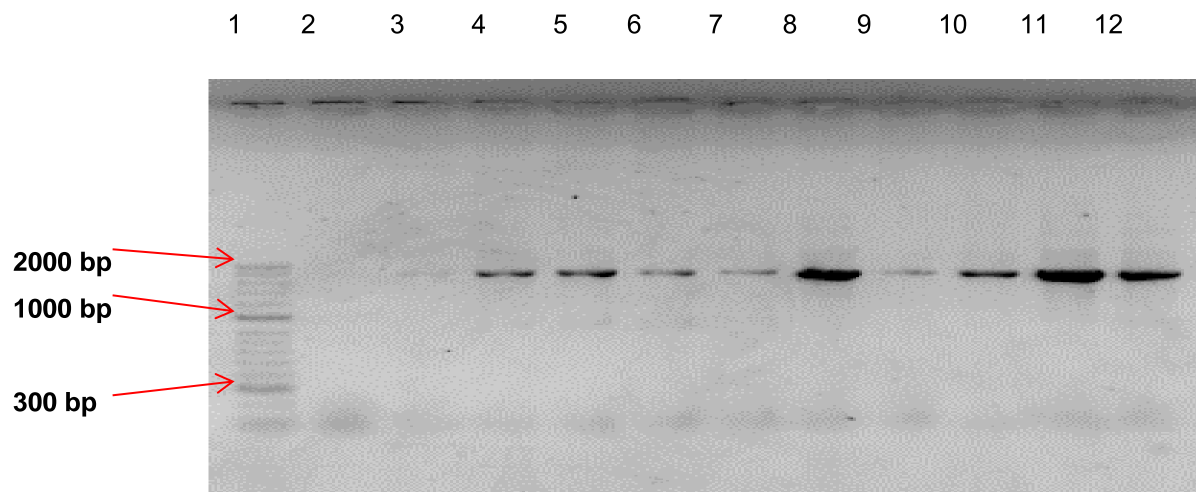

Figure 9. An image of the RT-PCR products of a 1/5 dilution of samples tested for ACLSV. Lane 1: Bioline 100 bp ladder. Lane 2: Negative control. Lane 3: Sample 11A. Lane 4: Sample 12A. Lane 5: Sample 13A. Lane 6: Sample 14A. Lane 7: Sample 15A. Lane 8: Sample 16A. Lane 9: Sample 17A. Lane 10: Sample 18A. Lane 11: Sample 19A. Lane 12: Sample 20A.

The highest dilution of the samples which tested positive by RT-PCR for ACLSV is listed in Table 3.

2.4.1.2 DAS-ELISA detection of ACLSV

The positive controls on all ELISA plates gave absorbance values of more than three times the mean absorbance values of the negative controls. The negative controls had low absorbance values as expected for negative controls. The highest dilution of the samples which tested positive by DAS-ELISA for ACLSV is listed in Table 3.

2.4.1.3 Diagnostic assay comparison: DAS- ELISA and RT-PCR detection of ACLSV

The results obtained with duplicate samplings and duplicate determinations of DAS-ELISA and RT-PCR at different dilutions are indicated in Table 3. The factor whereby one technique was more sensitive than the other technique, is also indicated. The highest dilution detected by DAS-ELISA for different samples was 1/10. The highest dilution detected by RT-PCR was 1/1 000. The factor whereby RT-PCR was more sensitive than DAS-ELISA varied between 25 – 1000 fold and the factor converted to a log value ranged between$10^{1.4}$ to $10^{3.0}$ fold.

Table 3. Results of duplicate samplings and duplicate determinations per plant sample by RT-PCR and DAS-ELISA. The highest dilution of sample in which ACLSV infection is detectable is indicated. The factor was calculated by dividing the RT-PCR equivalent value of the highest dilution by the DAS-ELISA value of the highest dilution for each sample replicate.

Sample	Duplicate sample	Duplicate determination	DAS-ELISA	RT-PCR	RT-PCR Equivalent Value	Factor	Factor converted to a log value
A1	1	1	1/1	1/10	1/50	50 X	$10^{1.70}$ X
		2	1/1	1/10	1/50	50 X	$10^{1.70}$ X
	2	1	1/1	1/10	1/50	50 X	$10^{1.70}$ X
		2	1/1	1/10	1/50	50 X	$10^{1.70}$ X
A4	1	1	1/5	1/25	1/125	25 X	$10^{1.4}$ X
		2	1/5	1/25	1/125	25 X	$10^{1.4}$ X
	2	1	1/5	1/25	1/125	25 X	$10^{1.4}$ X
		2	1/5	1/25	1/125	25 X	$10^{1.4}$ X
C4	1	1	1/10	1/5	1/25	2.5 X	$10^{0.4}$ X
		2	1/10	1/5	1/25	2.5 X	$10^{0.4}$ X
	2	1	1/10	1/5	1/25	2.5 X	$10^{0.4}$ X
		2	1/10	1/5	1/25	2.5 X	$10^{0.4}$ X
V3	1	1	Not detected	1/5	1/25	25 X*	$10^{1.4}$ X
		2	Not detected	1/5	1/25	25 X*	$10^{1.4}$ X
	2	1	Not detected	1/5	1/25	25 X*	$10^{1.4}$ X
		2	Not detected	1/5	1/25	25 X*	$10^{1.4}$ X
W5	1	1	1/10	1/50	1/250	25 X	$10^{1.4}$ X
		2	1/10	1/50	1/250	25 X	$10^{1.4}$ X
	2	1	1/10	1/50	1/250	25 X	$10^{1.4}$ X
		2	1/10	1/50	1/250	25 X	$10^{1.4}$ X

Table 3- cont. Results of duplicate samplings and duplicate determinations per plant sample by RT-PCR and DAS-ELISA. The highest dilution of sample in which ACLSV infection is detectable is indicated. The factor was calculated by dividing the RT-PCR equivalent value of the highest dilution by the DAS-ELISA value of the highest dilution for each sample replicate.

Sample	Duplicate sample	Duplicate determination	DAS-ELISA	RT-PCR	RT-PCR Equivalent Value	Factor	Factor converted to a log value
11A	1	1	1/1	1/5	1/25	25 X	$10^{1.4}$ X
		2	1/1	1/5	1/25	25 X	$10^{1.4}$ X
	2	1	1/1	1/5	1/25	25 X	$10^{1.4}$ X
		2	1/1	1/5	1/25	25 X	$10^{1.4}$ X
12A	1	1	Not detected	1/50	1/250	250 X*	$10^{2.4}$ X
		2	Not detected	1/50	1/250	250 X*	$10^{2.4}$ X
	2	1	Not detected	1/50	1/250	250 X*	$10^{2.4}$ X
		2	Not detected	1/50	1/250	250 X*	$10^{2.4}$ X
13A	1	1	1/1	1/50	1/250	250 X	$10^{2.4}$ X
		2	1/1	1/50	1/250	250 X	$10^{2.4}$ X
	2	1	1/1	1/50	1/250	250 X	$10^{2.4}$ X
		2	1/1	1/50	1/250	250 X	$10^{2.4}$ X
14A	1	1	1/1	1/50	1/250	250 X	$10^{2.4}$ X
		2	1/1	1/50	1/250	250 X	$10^{2.4}$ X
	2	1	1/1	1/50	1/250	250 X	$10^{2.4}$ X
		2	1/1	1/50	1/250	250 X	$10^{2.4}$ X
15A	1	1	1/1	1/50	1/250	250 X	$10^{2.4}$ X
		2	1/1	1/50	1/250	250 X	$10^{2.4}$ X
	2	1	1/1	1/50	1/250	250 X	$10^{2.4}$ X
		2	1/1	1/50	1/250	250 X	$10^{2.4}$ X

Table 3- cont. Results of duplicate samplings and duplicate determinations per plant sample by RT-PCR and DAS-ELISA. The highest dilution of sample in which ACLSV infection is detectable is indicated. The factor was calculated by dividing the RT-PCR equivalent value of the highest dilution by the DAS-ELISA value of the highest dilution for each sample replicate.

Sample	Duplicate sample	Duplicate determination	DAS-ELISA	RT-PCR	RT-PCR Equivalent Value	Factor	Factor converted to a log value
16A	1	1	1/5	1/200	1/1000	200 X	$10^{2.3}$ X
		2	1/5	1/200	1/1000	200 X	$10^{2.3}$ X
	2	1	1/5	1/200	1/1000	200 X	$10^{2.3}$ X
		2	1/5	1/200	1/1000	200 X	$10^{2.3}$ X
17A	1	1	Not detected	1/5	1/25	25 X*	$10^{1.4}$ X
		2	Not detected	1/5	1/25	25 X*	$10^{1.4}$ X
	2	1	Not detected	1/5	1/25	25 X*	$10^{1.4}$ X
		2	Not detected	1/5	1/25	25 X*	$10^{1.4}$ X
18A	1	1	1/1	1/5	1/25	25 X	$10^{1.4}$ X
		2	1/1	1/5	1/25	25 X	$10^{1.4}$ X
	2	1	1/1	1/5	1/25	25 X	$10^{1.4}$ X
		2	1/1	1/5	1/25	25 X	$10^{1.4}$ X
19A	1	1	1/1	1/200	1/1000	1000 X	$10^{3.0}$ X
		2	1/1	1/200	1/1000	1000 X	$10^{3.0}$ X
	2	1	1/5	1/200	1/1000	200 X	$10^{2.3}$ X
		2	1/5	1/200	1/1000	200 X	$10^{2.3}$ X
20A	1	1	1/1	1/100	1/500	500 X	$10^{2.7}$ X
		2	1/1	1/100	1/500	500 X	$10^{2.7}$ X
	2	1	1/1	1/100	1/500	500 X	$10^{2.7}$ X
		2	1/1	1/100	1/500	500 X	$10^{2.7}$ X

*The virus was not detected by DAS-ELISA, thus the lowest possible factor is indicated.

2.4.1.4 Statistical analyses: DAS- ELISA and RT-PCR detection of ACLSV

The results of DAS-ELISA *versus* RT-PCR detection of ACLSV were analyzed using the Friedman test. The Friedman test indicated that RT-PCR is more sensitive than DAS-ELISA for the detection of ACLSV at a 5% significance level ($p < 0.0001$).

The geometric mean of the factors is $10^{1.85}$ X i.e. the RT-PCR is 70.54 fold more sensitive than the DAS-ELISA.

2.4.2 RT-PCR and DAS-ELISA detection of ApMV

2.4.2.1 RT-PCR detection of ApMV

The amplicons of the RT-PCR products of ApMV using the primer pair 92D9up and 92E0re were of the expected size of ~822 bp, but very faint or no bands were observed after electrophoresis for undiluted samples except for one sample known to be severely infected. Attempts to optimize the RT-PCR conditions did not improve the results (results not shown).

RT-PCR using the primers PAPCP3-1 and PAPCP5 gave much darker bands upon electrophoresis at the expected size of ~668 bp. It was thus used for ApMV detection in all samples.

As a typical result an image showing after the RT-PCR products of samples AP1 to AP15 using PAPCP3-1 and PAPCP5 primers is depicted in Figure 10. All RT-PCR products were sequenced (see Chapter 4) confirming their identities as ApMV fragments.

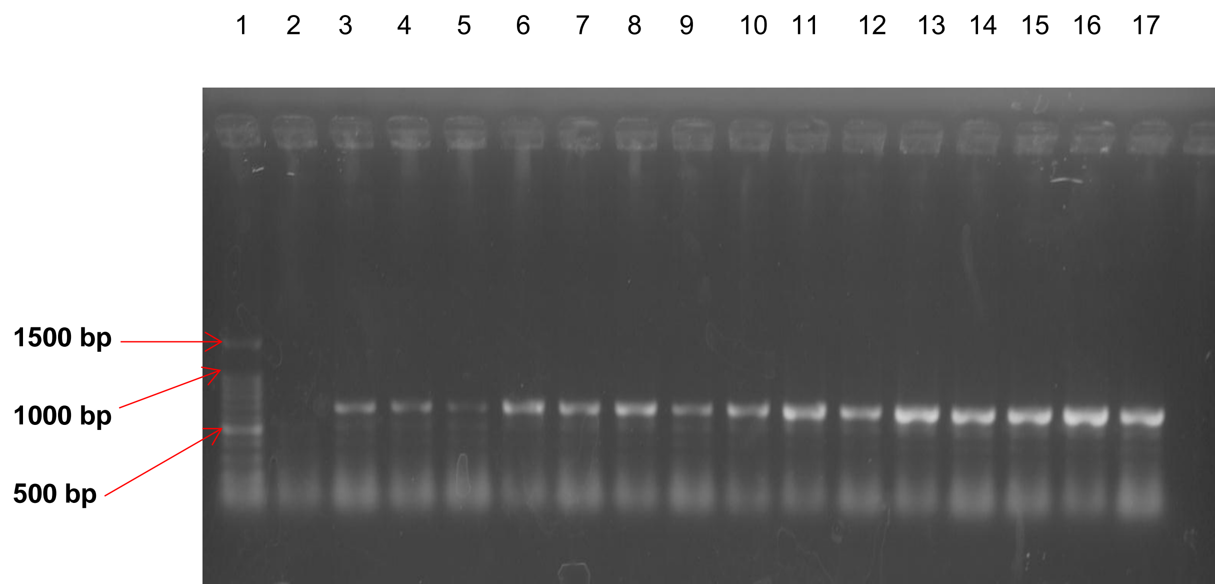

Figure 10. An image of the RT-PCR products of one duplicate of a 1/10 dilution of samples tested for ApMV. Lane 1: Promega 100bp ladder (USA). Lane 2: Negative control. Lane 3: Sample AP1. Lane 4: Sample AP2. Lane 5: Sample AP3. Lane 6: Sample AP4. Lane 7: Sample AP5. Lane 8: Sample AP6. Lane 9: Sample AP7. Lane 10: Sample AP8. Lane 11: Sample AP9. Lane 12: Sample AP10. Lane 13: Sample AP11. Lane 14: Sample AP12. Lane 15: Sample AP13. Lane 16: Sample AP14. Lane 17: AP15.

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The highest dilution of the samples which tested positive by DAS-ELISA for ApMV is listed in Table 4.

2.4.2.2 DAS-ELISA detection of ApMV

The positive controls on all ELISA plates gave absorbance values of more than three times the mean absorbance values of the negative controls. The negative controls had low absorbance values as expected for negative controls. The highest dilution of the samples which tested positive by DAS-ELISA for ApMV is listed in Table 4.

2.4.2.3 Diagnostic assay comparison: DAS- ELISA and RT-PCR detection of ApMV

The results obtained with duplicate samplings and duplicate determinations of DAS-ELISA and RT-PCR at different dilutions are indicated in Table 4. The factor whereby one technique is more sensitive than the other technique, is also indicated. The highest dilution detected by DAS-ELISA for different samples varied between undiluted to 1/1 000. The highest dilution detected by RT-PCR varied between 1/250 and 1/50 000. The factor whereby RT-PCR was more sensitive than DAS-ELISA varied between 50 – 500 fold and the factor converted to a log value ranged between$10^{1.7}$ to $10^{2.7}$ fold.

Table 4. Results of duplicate samplings and duplicate determinations per plant sample by RT-PCR and DAS-ELISA. The highest dilution of sample in which ApMV infection is detectable is indicated. The factor was calculated by dividing the RT-PCR equivalent value of the highest dilution by the DAS-ELISA value of the highest dilution for each sample replicate.

Sample	Duplicate sample	Duplicate determination	DAS-ELISA	RT-PCR	RT-PCR Equivalent Value	Factor	Factor converted to a log value
K3	1	1	1/1 000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
		2	1/1 000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
	2	1	1/1 000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
		2	1/1 000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
J3	1	1	1/5	1/500	1/2 500	500 X	$10^{2.7}$ X
		2	1/5	1/500	1/2 500	500 X	$10^{2.7}$ X
	2	1	1/5	1/500	1/2 500	500 X	$10^{2.7}$ X
		2	1/5	1/500	1/2 500	500 X	$10^{2.7}$ X
K2	1	1	1/10	1/100	1/500	50 X	$10^{1.7}$ X
		2	1/10	1/100	1/500	50 X	$10^{1.7}$ X
	2	1	1/10	1/100	1/500	50 X	$10^{1.7}$ X
		2	1/10	1/100	1/500	50 X	$10^{1.7}$ X
J1	1	1	1/1	1/50	1/250	250 X	$10^{2.4}$ X
		2	1/1	1/50	1/250	250 X	$10^{2.4}$ X
	2	1	1/1	1/50	1/250	250 X	$10^{2.4}$ X
		2	1/1	1/50	1/250	250 X	$10^{2.4}$ X
D6	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X

Table 4- cont. Results of duplicate samplings and duplicate determinations per plant sample by RT-PCR and DAS-ELISA. The highest dilution of sample in which ApMV infection is detectable is indicated. The factor was calculated by dividing the RT-PCR equivalent value of the highest dilution by the DAS-ELISA value of the highest dilution for each sample replicate.

Sample	Duplicate sample	Duplicate determination	DAS-ELISA	RT-PCR	RT-PCR Equivalent Value	Factor	Factor converted to a log value
A168	1	1	1/10	1/100	1/500	50 X	$10^{1.7}$ X
		2	1/10	1/100	1/500	50 X	$10^{1.7}$ X
	2	1	1/10	1/100	1/500	50 X	$10^{1.7}$ X
		2	1/10	1/100	1/500	50 X	$10^{1.7}$ X
AP1	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
AP2	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
AP3	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
AP4	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X

Table 4- cont. Results of duplicate samplings and duplicate determinations per plant sample by RT-PCR and DAS-ELISA. The highest dilution of sample in which ApMV infection is detectable is indicated. The factor was calculated by dividing the RT-PCR equivalent value of the highest dilution by the DAS-ELISA value of the highest dilution for each sample replicate.

Sample	Duplicate sample	Duplicate determination	DAS-ELISA	RT-PCR	RT-PCR Equivalent Value	Factor	Factor converted to a log value
AP5	1	1	1/1 000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
		2	1/1 000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/10 000	1/50 000	500 X	$10^{2.7}$ X
		2	1/100	1/10 000	1/50 000	500 X	$10^{2.7}$ X
AP6	1	1	1/100	1/10 000	1/50 000	500 X	$10^{2.7}$ X
		2	1/100	1/10 000	1/50 000	500 X	$10^{2.7}$ X
	2	1	1/1 000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
		2	1/1000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
AP7	1	1	1/1 000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
		2	1/1 000	1/10 000	1/50 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/10 000	1/50 000	500 X	$10^{2.7}$ X
		2	1/100	1/10 000	1/50 000	500 X	$10^{2.7}$ X
AP8	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
AP9	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X

Table 4- cont. Results of duplicate samplings and duplicate determinations per plant sample by RT-PCR and DAS-ELISA. The highest dilution of sample in which ApMV infection is detectable is indicated. The factor was calculated by dividing the RT-PCR equivalent value of the highest dilution by the DAS-ELISA value of the highest dilution for each sample replicate.

Sample	Duplicate sample	Duplicate determination	DAS-ELISA	RT-PCR	RT-PCR Equivalent Value	Factor	Factor converted to a log value
AP10	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
AP11	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
AP12	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
AP13	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
AP14	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X

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Table 4- cont. Results of duplicate samplings and duplicate determinations per plant sample by RT-PCR and DAS-ELISA. The highest dilution of sample in which ApMV infection is detectable is indicated. The factor was calculated by dividing the RT-PCR equivalent value of the highest dilution by the DAS-ELISA value of the highest dilution for each sample replicate.

Sample	Duplicate sample	Duplicate determination	DAS-ELISA	RT-PCR	RT-PCR Equivalent Value	Factor	Factor converted to a log value
AP15	1	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
	2	1	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X
		2	1/100	1/1 000	1/5 000	50 X	$10^{1.7}$ X

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2.4.2.4 Statistical analysis: DAS- ELISA and RT-PCR detection of ApMV

The results of DAS-ELISA *versus* RT-PCR detection of ApMV were analyzed using the Friedman test. The Friedman test indicated that RT-PCR is more sensitive than DAS-ELISA for the detection of ApMV at a 5% significance level ($p < 0.0001$). The geometric mean of the factors is $10^{1.85}$ X i.e. the RT-PCR is 71.01 fold more sensitive than the DAS-ELISA.

2.4.3 Detection of ApMV in pears

The DAS-ELISA positive controls indicated absorbance values of more than three times the mean of the negative controls. The negative controls gave absorbance readings of low values as expected from negative controls. The 249 samples tested using DAS-ELISA, all tested negative for the virus. Ten of the fifteen samples tested positive by RT-PCR for ApMV (which was confirmed by sequencing as described in Chapter 5 of this thesis).

2.5 Discussion

Reliable, rapid and more sensitive diagnostic assays for the detection of ACLSV and ApMV in pome- and stonefruit trees would be beneficial for improving the phytosanitary status of fruit tree planting material in South Africa as viral infections have major production and therefore economic implications. Two diagnostic assays, DAS-ELISA and RT-PCR were evaluated in this study to determine the relative sensitivity of the techniques. Although RT-PCR is generally considered to be more sensitive in the detection of viruses, DAS-ELISA is routinely used in commercial laboratories as it is cost-effective, has a high through-put and can be operated by personnel with lower levels of training than is required for RT-PCR. However if RT-PCR is significantly more sensitive than DAS-ELISA, the benefit of this high sensitivity may outweigh the advantages of DAS-ELISA.

RT-PCR is in general considered to be more sensitive in the detection of viruses than DAS-ELISA and this is confirmed by dilution studies. RT-PCR was able to detect *Tobacco mosaic virus* (Family *Virgaviridae*, genus *Tobamovirus)* in *Nicotina tabacum* leaf plant sap dilutions of up to $1{:}10^6$ in comparison to DAS-ELISA detection of up to $1{:}10^3$ (Yang et al., 2012.) In *Saccharaum* spp (sugarcane), duplex-immunocapture-RT-PCR (D-IC-RT-PCR) detected 10% more of samples infected with *Sugarcane streak mosaic virus* (Family *Potyviridae*, genus *Poacevirus)* and 20% more of samples infected with *Sugarcane mosaic virus* (Family *Potyviridae*, genus *Potyvirus)* than ELISA (Subba Reddy et al., 2011).

The first step in ensuring that comparisons between DAS-ELISA and RT-PCR for a particular sample are valid, is to ensure that plant material is uniformly sampled when making the comparison. In order to reach this goal within this study, leaves were halved vertically – one half was used in DAS-ELISA and the other half for RT-PCR.

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The effectiveness of DAS-ELISA and RT-PCR are influenced by various factors. DAS-ELISA's success is influenced by factors such as the structure of the virus particle, the stability of the virus particles and the number of particles present in a sample. The quality of antibodies and the "immunogenicity" of the virual proteins can also influence the DAS-ELISA. In general, geminivirus coat proteins have a low affinity for antibodies with negative consequences for DAS-ELISA detection. The influence of these different factors can also vary considerably between viruses.

With regard to viral structure, ACLSV is a filamentous particle of approximately 640-760 nm (Yoshikawa & Takahashi, 1988). The particles consist of many individual molecules of a single coat protein of 21.4 kDa encapsidating a positive-sense single strand poly-adenylated RNA molecule of 7545-7555 bp (Bar-Joseph et al., 1979; German et al., 1990; German-Retana et al., 1997; Sato et al., 1993). The flexuous, elongated particles of ACLSV have coat protein molecules aligned on the outside, which are easily detected by DAS-ELISA. Most of the variability in the CP gene, is in the N-terminal part of the CP gene, with the C-terminus significantly less variable (Al Rwahnih et al., 2004). Comparisons of the coat protein sequences from different isolates indicate some variability with isolates differing by as much as 10-20% from one another (Candresse et al., 1995; German-Retana et al., 1997; Yoshikawa, 2001; Rana et al., 2009). This molecular variability could influence DAS-ELISA, if the amino acid sequences of coat proteins also differ significantly from those used to produce the antibodies used in the DAS-ELISA. ApMV has a positive-sense single-stranded RNA genome, divided into three components designated RNA 1, 2 and 3, and a subgenomic messenger for the coat protein, designated RNA 4. The CP plays an important role in forming the shell for the RNA 1, RNA 2 and RNA 3 genome components and in initiation and propagation of infection (Bol, 1999). Variation in ApMV coat protein gene sequences has been found (Lakshmi et al., 2011) which could influence the effectiveness of detection by DAS-ELISA, if these sequences translate into differences in the protein structure.

The stability of the virus particle influences the ability of antibodies to capture the virus particle in the DAS-ELISA and to allow binding by the detecting antibody. ACLSV has a long filamentous structure which may be unstable, breaking down before ELISA antibodies can bind. The ApMV particle has coat proteins encapsulating nucleic acids and if the virus particle breaks up this may not allow DAS-ELISA detection, although this may also expose protein subunits, which may improve antibody binding. To this end, leaf material used was not stored, but used immediately to prevent any possible degradation during storage. Future studies could focus on investigating the effect of storage on detection of the virus, but in my experience it has a definite negative effect as was observed with DAS-ELISA tests conducted previously in the SAPO Trust laboratories (results not presented in this thesis).

The concentration of fruit tree viruses was determined to be approximately 1 ng/ml (Clark & Adams, 1977). Thus at low viral titers, DAS-ELISA might not detect viral infection. Experiments were limited to spring to ensure that the highest possible virus levels were present in the samples as has been done in other studies (Candresse et al., 1995).

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Factors which could influence the effectiveness of RT-PCR include the primer sequences, instability of RNA, the lack or presence of inhibitors and the number of particles required for positive identification of the virus.

The use of primers with the right target sequence homology and correct length is essential for the detection of different isolates of a virus. If the primers were designed based on a less-conserved region of the genome or if the amplicon is too big, the RT-PCR may be less successful in detection of certain isolates. This was experienced in this study with the ApMV primers. RT-PCR for ApMV was initially done using the primer pair 92D9up and 92E0re. Agarose gel analysis showed faint amplification products in most cases and sequencing of all but one sample was unsuccessful. The protocol was therefore adjusted and another set of primers (PAPCP3-1 and PAPCP5) was used (Lee et al., 2002). This was very effective in amplification of the extracted nucleic acids. This set of primers was subsequently used for all ApMV RT-PCR reactions. A possible explanation is that the first primer pair results in the amplification of a larger amplicon than the latter and that the RT-PCR gave better amplification of the shorter amplicon.

Instability of RNA can result in it being degraded before RT-PCR can take place. Storage of samples has been known to cause this. It is also possible that the extraction method influences the stability of RNA. Crude virus extraction using the GES method has the benefit of being less time-consuming since no nucleic acid extraction is required and plant sap can be used directly in a RT-PCR reaction. It thus also has the benefit of being useful for large scale extractions and screening of plant material, as is required within a certification scheme. However, this study showed that the GES method was unfortunately not effective for sample preparation for routine RT-PCR detection of ApMV and ACLSV viruses in pome- and stonefruit tissue. This could be due to instability of the viral RNA in the GES homogenate, compared to purified viral RNA using the RNA extraction method. It is probable that the isolated viral RNA is more stable as the extraction procedure would eliminate RNAse enzymes. Extraction was thus performed by nucleic acid extraction - a technique which proved to be very successful and more efficient in yielding RNA suitable for RT-PCR amplification.

The method whereby a viral template is isolated for the RT-PCR, can influence the presence of inhibitors. For instance isolated RNA has no inhibitors present, whilst performing RT-PCR using crude plant sap, has the risk of negative influences by inhibitors. This could also be a possible explanation as to why the crude virus extraction using the GES method was not effective for sample preparation for routine RT-PCR detection of ApMV and ACLSV viruses in pome- and stonefruit tissue. PCR-inhibitors could be included and non-specific amplification is also possible with the GES method (Noach, 2008). Again, this may be the reason why the extracted RNA gave better results in the RT-PCR.

RT-PCR is influenced by the number of virus particles present in a sample. It is estimated that around 10-100 virus particles can be detected by PCR (Candresse et al., 1995). This is a much lower virus titer than detectable by DAS-ELISA. RT-PCR offers the potential of more reliable detection of viruses for longer periods, since it detects viruses at lower viral titer (Candresse et al.,

1995). This could also be another possible reason why crude virus extraction using the GES method was not effective for sample preparation for routine RT-PCR detection of ApMV and ACLSV due to low viral RNA concentration, since when using the crude extract the amount of virus is considerably less than when RNA is purified using the RNA extraction method (Noach, 2008).

This study found that RT-PCR was significantly more sensitive in detection of ACLSV than DAS-ELISA. This was also reported previously where trees known to be infected with ACLSV, were detected with more success by RT-PCR than DAS-ELISA (Ulubas & Ertunc, 2005; Caglayan et al., 2006; Rana et al., 2011). Candresse et al. (1995) reported a hundred-fold increase in sensitivity of PCR compared to ELISA for detection of ACLSV. This is similar to the 70.5-fold increase in sensitivity of RT-PCR *versus* DAS-ELISA found in this study. ACLSV is the type species of the *Trichovirus* genus from the family *Betaflexiviridae.* Other members of the family *Betaflexiviridae* include the genera *Capillovirus* and *Carlavirus*. Similar results were reported from these genera. RT-PCR detected 12% more *Apple stem grooving virus* (ASGV) (Genus *Capillovirus*) infected samples than DAS-ELISA in 550 apple samples in China (Liu et al., 2013). Caglayan et al. (2006) also reported that 1.25% more samples tested positive by RT-PCR than ELISA for ASGV. Peiman & Xie (2006) reported that RT-PCR detected 3.2% more infections of *Potato virus S (*Genus *Carlavirus)* infections in *Solanum tuberosum* (potato) samples, than ELISA.

RT-PCR was also significantly more sensitive in detection of ApMV than DAS-ELISA in this study. This was also reported previously where DAS-ELISA detected only 2.1% of ApMV infections in apple trees and none in pears in comparison to 22% and 20.2% in apple and pear trees by RT-PCR (Pupula et al., 2011). Caglayan et al. (2006) also reported that between 4.25-10% more samples tested positive by RT-PCR than ELISA for ApMV. ApMV belongs to the genus *Ilarvirus*, of the family *Bromoviridae* (Rybicki, 1995). Other virus species of the ilarviruses include PDV and PNRSV (Pupola et al., 2011), whilst other genera belonging to *Bromoviridae* include *Cucumovirus* (including species *Cucumber mosaic* virus)*.* ApMV is most closely related to PNRSV (Rybicki, 1995). It was reported that RT-PCR can surpass ELISA in sensitivity for the detection of PNRSV (Spiegel et al., 1996, Kolber et al., 1998, Sanchez-Navarro et al., 1998, Moury et al., 2000), although it has also been reported that no difference in detection rates between the two methods were found (Mekuria et al., 2003). No significant difference in the sensitivity of ELISA in comparison to RT-PCR was reported for PDV, but when IC-RT-PCR was used for detection instead of RT-PCR, it was much more sensitive than ELISA (Mekuria et al., 2003). PCR-based methods were reported as the most sensitive method for detection of *Cucumber mosaic virus* (CMV) (De Blas et al., 1994, Hu et al., 1995, Raj et al., 2002, Wylie et al., 1993). Comparison of DAS-ELISA to RT-PCR for the detection of CMV indicated that RT-PCR was at least 100 times more sensitive than ELISA (Berniak et al., 2009). This is similar to the 71-fold increase in sensitivity of RT-PCR *versus* DAS-ELISA for the detection of ApMV found in this study.

Currently all techniques for detection of the viruses are dependent on virus titre and if levels are below detection levels, as observed with new infections and seasonal variation, this can result in

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false negative results. This can, however, be limited by the use of more sensitive techniques such as RT-PCR as a substitute for DAS-ELISA. Another alternative would be to use an alternative technique for detection which is not dependent on the virus titer. Virus infection usually leads to modulation of gene expression, regulated by endogenous, small RNA (sRNA) molecules (Singh et al., 2012). The plant's response to ACLSV and ApMV infection as detected by sRNA profiles of targeted genes can result in a marker signature which may lead to earlier and more reliable detection of virus infection, which would be extremely useful. However this may not be cost effective for routine diagnostics in Certification Schemes.

A major concern arising from this study, is that DAS-ELISA could not be used to detect ApMV in pears, yet RT-PCR showed that some pear tree samples were infected by ApMV. This is the first report of ApMV infection of pears in South Africa. It is not clear why DAS-ELISA cannot be used to detect ApMV in pear leaf material, but this may be due to the presence of inhibitors. Since DAS-ELISA is routinely used for detection of ApMV in stonefruit and apples under the South African Deciduous Fruit Plant Certification Scheme, but not pears, pears infected by ApMV are not detected. ApMV has been reported previously in pears in the Czech Republic and Italy (Petrzik, 2005). The detrimental effects of such an undetected infection could include economic losses in the pears specifically, but also infection of surrounding pome- and stonefruit orchards through the use of infected pruning equipment.

In conclusion RT-PCR was found to be significantly more sensitive than DAS-ELISA in the detection of both ACLSV and ApMV. The results where DAS-ELISA did not detect 17 of 26 randomly selected samples tested for ApMV, indicate that RT-PCR should replace DAS-ELISA as detection method in the South African Deciduous Fruit Plant Certification Scheme, due to the sensitivity and accuracy of the method.

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CHAPTER 3

Determination of genetic variation of *Apple chlorotic leafspot virus* (ACLSV) in South Africa

3.1 Abstract

Leaf samples were screened by DAS-ELISA for ACLSV infection. RT-PCR of the coat protein gene of the 47 samples which tested positive, was performed, followed by sequencing using the same primers, as well as one additional forward primer, as for the RT-PCR. Following sequence alignment of the 47 CP gene sequences with 47 coat proteins sequences from GenBank and *Cherry mottle leaf virus* (CMLV) as outgroup, phylogenetic analysis using parsimony was performed. The phylogenetic analysis retrieved 131 trees with a tree length of 1961. Isolates from South Africa clustered into three of the four retrieved clades, with isolates from stonefruit in all three clades, but South African pome fruit isolates restricted to clade four. This indicates that ACLSV is not fruit type specific and that cross-infection between pome- and stonefruit is possible. Geographic analysis of the phylogenetic tree indicates that South African isolates were most likely imported from other parts of the world. Symptom expression did not correlate with the phylogenetic analysis, as symptomless and isolates obtained which showed severe symptoms clustered together. Sequence similarity of South African ACLSV isolates of between 78.8 - 97.9% at nucleotide level and 83 - 100% at amino acid level were determined.

3.2 Introduction

Apple chlorotic leaf spot virus (ACLSV) is the type species of the *Trichovirus* genus of the family *Betaflexiviridae* (King et al., 2012). ACLSV is known to infect most fruit tree species, including pomefruit [such as apple (*Malus* sp.), pear (*Pyrus* sp.) (Desvignes & Boyé, 1989) and quince (*Cydonia oblonga*) (Rana et al., 2008)] and stonefruit [such as apricot (*Prunus armeniaca*), cherry (*P.* avium), peach (*P. persica*), prune (*P. domestica*) and plum (*P. domestica, P. salicina*)] (Lister, 1970; Nemeth, 1986; Desvignes & Boyé, 1989; German-Retana et al., 1997). ACLSV slows the growth, and decreases the mean fruit weight and yield of trees (Cosba et al., 1986). The symptoms caused by ACLSV vary considerably from one host to another and between different viral isolates. It can vary from symptomless (German-Retana et al., 1997) to russet ring on apple fruits and lethal decline in pomefruit (Salmon et al., 2002).

The ACLSV genome consists of a linear positive-sense single strand poly-adenylated RNA molecule of 7545-7555 bp, excluding the poly-A tail (Bar-Joseph et al., 1979; German et al., 1990; Sato et al., 1993; German-Retana et al., 1997). The ACLSV genome contains three open reading frames (ORFs 1, 2 and 3). The ORF encode proteins with molecular masses of 216.5, 50.4 and 21.4 kDa, respectively (Salmon et al., 2002). The nucleotide sequence encoding the coat protein (CP) is the most conserved region of the ACLSV genome (Niu et al., 2012). The N-terminal part of the CP gene contains the most variability, with the C-terminus significantly less variable (Al

Rwahnih et al., 2004). The coat protein sequences from different isolates shows variability with isolates differing by as much as 10-20% from one another (Candresse et al., 1995; German-Retana et al., 1997; Yoshikawa, 2001; Rana et al., 2009). Previous phylogenetic studies of nucleotide sequences of the CP gene of ACLSV isolates indicated various clusters. Song et al. (2011) reported two clusters, I and II, with two subclusters in cluster I (A and B). Niu et al. (2012) also reported two clusters, designated the Z1-type and Ta Tao5 type, but used different isolates to those used by Song et al. (2011).

In order to determine the genetic variation of ACLSV isolates in South Africa, RT-PCR amplification of the coat protein gene of different isolates was performed. The resulting products were sequenced and these sequences were analysed phylogenetically in order to determine the phylogenetic relationships and variation between different isolates.

3.3 Materials and Methods

3.3.1 Sample collection and DAS-ELISA detection

A total of 12 364 pome- and stonefruit leaf samples were collected from 2011 to 2013 from the Western Cape and North West Provinces in South Africa.

Leaf samples were stored at 4°C or were tested immediately without storage. Leaves were tested by DAS-ELISA (Clark & Adams, 1977) using commercially available DAS-ELISA reagents for ACLSV (Bioreba, Switzerland).

Leaf samples from trees which tested positive using DAS-ELISA were subsequently retested using RT-PCR. A number of samples which tested negative using DAS-ELISA were randomly selected and also tested using RT-PCR.

3.3.2 RNA isolation

Total RNA was isolated from 100 mg of leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Germany) as described by the manufacturer. The kit uses a silica gel-based membrane to bind the total RNA selectively in combination with microspin technology (Nakahara et al., 2011).

3.3.3 RT-PCR amplification of CP genes

The total RNA was dissolved in 40 µl elution buffer and used directly in the for RT-PCR or stored at -80ºC. RT-PCR was carried out in thin-walled 0.2 ml tubes. The RT-PCR reaction mixture consisted of 2.5 µl 10 x PCR-buffer, 1.25 µl 0.1 M DTT, 2 µl 25 mM $MgCl_2$, 0.625 µl 20 µM forward primer, 0.625 µl 20 µM reverse primer, 1 µl 5 mM dNTPs (Bioline, Germany), 0.25 µl 5 U/µl Super-Therm DNA Polymerase mixture (Bioline, Germany) and 0.125 µl SuperScript^{TM} III (Invitrogen™, Life Technologies™, USA) and 14.625 µl Milli-Q® water. The primers used had the following sequences: forward primer (ACLSV-ORF-2-f), 5’- GAAGATCGCAGAAGGGGATATTC-3’, and reverse primer (ACLSV-ORF-2-r), 5’-GTCTACAGGCTATTTATTATA AG-3’ (Nakahara et al., 2001). Two microliters of plant isolated homogenate or RNA solution was added to 23 µl of

the PCR reaction mixture. The RT-PCR amplifications were done using one reverse transcription step at 48ºC for 30 minutes, followed by 35 cycles of the following parameters: denaturation at 94ºC for 30 seconds, annealing at 55ºC for 45 seconds and extension at 72ºC for 90 seconds; and with a final elongation at 72ºC for 10 minutes. The amplifications were done using a Veriti ™ Thermal Cycler (Life Technologies™, USA). The amplicon of the primer set is 1510 bp in length corresponding to nt 5750 to nt 7512 of the genome sequence of ACLSV, thus including the complete length of the coat protein gene of nt 6784 to nt 7365 (581 bp long).

3.3.4 Electrophoretic analysis of RT-PCR amplification products

A 1% agarose gel containing 1 µg/ml ethidium bromide was used for electrophoresis and immersed in a 1 x TAE electrophoresis buffer (0.48% (w/v) Tris, 0.11% (v/v) glacial acetic acid, 0.5 M EDTA, pH 8.0). The total RT-PCR reaction mixture was mixed with a loading buffer (0.25% (w/v) bromophenol blue, 57.5% (v/v) glycerol, 0.5 M EDTA, 1 M Tris, pH 8.0, Milli-Q® water), and loaded onto the gel. A 100 bp DNA size markers ladder (Bioline, Germany) was used to determine the size of the amplified RT-PCR products. Electrophoresis was performed at 100 V for 60-80 minutes using a Cleaver Scientific Ltd power source (England). The RT-PCR products were visualized using a UV transilluminator and portable darkroom (Cleaver Scientific Ltd, England).

3.3.5 Purification of RT-PCR amplification products

The RT-PCR products that were obtained were excised using a sterile scalpel and eluted from the gel by the Wizard® SV and PCR Clean-up System (Promega, USA) according to the manufacturer's specifications. A total of 40 µl of Milli-Q® water was added to the purified products. In order to assess the amount of purified RT-PCR product a 1 % agarose gel containing 1 µg/ml ethidium bromide was loaded with 2 µl of the eluted DNA and separated by electrophoresis and visualized as described above. The DNA products were stored in nuclease-free Eppendorf tubes at -20ºC until nucleotide sequencing.

3.3.6 Sequencing of RT-PCR amplification products

The RT-PCR products were sequenced with the Cycle Sequencing kit (Applied Biosystems®, Life Technologies™, USA) and the cycle sequencing products were analyzed using an ABI®3730xl Genetic Analyser in the Central Analytical Facility, University of Stellenbosch. The following sequencing mix was used: 5 µl 5 x sequencing dilution buffer (Applied Biosystems®, Life Technologies™, USA), 2 µl Terminator Dye (BigDye® Terminator v3), 1 µl Milli-Q® water, 1 µl (0.8 µM) primer and 1 µl of the cDNA sample. A set of three primers was used in each respective sequencing reaction, the two ACLSV-specific primers used in the RT-PCR, as well as another intermediate-forward primer that binds within the existing amplicon. The intermediate-forward primer used had the following sequence: (ACLSV-ORF-2-int.f) 5'- AGAGAGTTTCAGTTTGCTCG-3'. The samples were submitted to 35 cycles of the 96ºC for 10 seconds, 52ºC for 30 seconds and 60ºC for 4 minutes in a Veriti ™ Thermal Cycler (Life Technologies™, USA). This was followed by

a final step of 60ºC for 10 minutes. The cycle sequencing products were analyzed at the Central Analytical Facility, University of Stellenbosch.

3.3.7 Nucleotide sequence analysis and alignment

The electropherograms obtained from the sequencing facility were edited using Chromas (v 2.23, Technelysium, Pty., Ltd.) and the generated ACLSV nucleotide sequences were aligned with ACLSV sequences obtained from GenBank using the BioEdit v 7.0.5.2 software package. Overlapping regions were identified and contiguous sequences were generated for each isolate thereby generating a complete CP gene sequence as well as a region upstream of the start of the coat protein gene. The identity of the generated sequences as ACLSV coat protein genes was verified using BLASTn searches on Genbank.

An automated alignment of the ACLSV coat protein gene sequences and the sequence from cherry mottle leaf virus (CMLV) (AF 170028), chosen as outgroup, was performed using the Clustal W v 1.4 alignment function of the BioEdit package. Further alignment was performed manually. Sequence similarity of South African isolates at nucleotide level and at amino acid level was calculated using BioEdit software.

3.3.8 Phylogenetic analysis

Phylogenetic analysis of the obtained sequences was done using the program Phylogenetic Analysis Using Parsimony (PAUP) (4.0b10) (Swofford, 2003). A total of 47 CP sequences obtained from GenBank (Table 5) and 47 coat protein gene sequences of South African isolates (Table 6) were used in the phylogenetic analysis with CMLV as the outgroup. A heuristic search (1 000 replicates) using TBR (tree bisection and reconnection) branch swopping was used with all characters weighted equally to establish the shortest possible trees from the data matrix. A bootstrap analysis (1 000 replicates) using TBR branch swopping was performed to determine clade support. Branches with bootstrap values less than 50% were considered weakly supported and were not indicated on phylograms. Values from 50% to 74% were considered as moderately supported, whilst values of 75% and more were considered as well supported.

Table 5. Details of ACLSV-CP isolates obtained from GenBank used for phylogenetic analysis and Cherry mottle leaf virus (CMLV) used as outgroup.

GenBank Accession no	Isolate	Host	Country of origin	Reference
AF170028 (CMLV)	SA1162-21	*Prunus avium*	-	(James, 2000)
AB326223	A4	Apple	Japan	(Yaegashi et al., 2007)
AB060957	B81	Apple	Japan	Unpublished
AB060962	MK9	Apple	Japan	Unpublished
AB060964	MO41	Apple	Japan	Unpublished
AB326224	B6	Apple	Japan	(Yaegashi et al., 2007)
AB326225	MO-5	Apple	Japan	(Yaegashi et al., 2007)
APCCOMS	P 205	Apple	Japan	(Sato et al., 1993)
DQ329160	P1R9D9	Sweet cherry	Bulgaria	Unpublished
DQ329161	R1D2P-L	Peach	Bulgaria	Unpublished
EU223295	Ta Tao 5	Peach	USA	(Marini et al., 2008)
FJ952167	TK4	Apple	Turkey	(Gadiou et al., 2010)
FJ952176	5Be	Apple	Belgium	(Gadiou et al., 2010)
FJ752494	-	Apple	Ukraine	Unpublished
FR750247	Hatkoti	Apple	India	Unpublished
FR873735	-	Apple	India	Unpublished
GQ334188	Malus0545	Apple	Canada	(Wang et al., 2011)
GQ334204	Pyrus0212	Pear	Canada	(Wang et al., 2011)
GQ334206	CL	Pear	China	Unpublished
GQ334216	CS	Pear	China	Unpublished
GQ334219	Malus0375	Apple	Canada	(Wang et al., 2011)
GU327982	PL1	Pear	China	(Song et al., 2011)
GU328003	PP63	Pear	China	(Song et al., 2011)
HQ398359	LV-m312	Apple	Latvia	(Pupola et al., 2011)
HQ398357	LV-py120	Pear	Latvia	(Pupola et al., 2011)
JN544924	hz-48	Pear	China	Unpublished
JN544921	hz-11	Pear	China	Unpublished
JN848979	HS6	Peach	China	(Niu et al., 2012) *
JN848982	HB3	Peach	China	(Niu et al., 2012) *
JQ320100	MB-CH	Apple	China	Unpublished
JQ392545	YL2	Apple	China	Unpublished
JQ392544	YL1	Apple	China	Unpublished
JN849009	S5	Peach	China	(Niu et al., 2012) *
JN849007*	S3	Peach	China	(Niu et al., 2012) *
JN849005*	Y3	Peach	China	(Niu et al., 2012) *

Table 5- cont. Details of ACLSV-CP isolates obtained from GenBank used for phylogenetic analysis and Cherry mottle leaf virus (CMLV) used as outgroup.

GenBank Accession no	Isolate	Host	Country of origin	Reference
JN849003*	Y1	Peach	China	(Niu et al., 2012) *
JN849001*	G4	Peach	China	(Niu et al., 2012) *
JN848999*	Z3	Peach	China	(Niu et al., 2012) *
JN848997*	Z1	Peach	China	(Niu et al., 2012) *
JN848995*	ST4	Peach	China	(Niu et al., 2012) *
JN848993*	ST2	Peach	China	(Niu et al., 2012) *
JN848991*	SQ8	Peach	China	(Niu et al., 2012) *
JN848989*	SQ5	Peach	China	(Niu et al., 2012) *
JN848987*	SQ2	Peach	China	(Niu et al., 2012) *
JN848985.1*	HL1	Peach	China	(Niu et al., 2012) *
JN848983.1*	HB4	Peach	China	(Niu et al., 2012) *
JN848981.1*	HB2	Peach	China	(Niu et al., 2012) *
NC_001409.1	-	*Prunus domestica*	France	(German et al., 1990)

* According to GenBank the published reference is Niu et al. (2012), but no reference is made in the article to this Accession number.

3.4 Results

3.4.1 DAS-ELISA screening

Fourty seven samples of the 12 364 pome- and stonefruit samples tested for ACLSV tested positive (0.38%) (Table 6). The samples that tested positive were collected from Stellenbosch as well as other areas of South Africa.

Table 6. Origin, variety, and fruit classification of samples which tested positive for ACLSV by DAS-ELISA and RT-PCR.

Sample	South African area of origin	Import date	Origin	Fruit group	Fruit type
A1	Stellenbosch	2006	China	Pomefruit	Apple
A3	Stellenbosch	2007	New York State , USA	Pomefruit	Apple
A4	Stellenbosch	2001	California, USA	Pomefruit	Apple
A5	Stellenbosch	1998	Italy	Pomefruit	Apple
A9	Stellenbosch	1981	South Africa	Stonefruit	Peach
A119	Stellenbosch	Unknown	Unknown	Pomefruit	Apple
B1	Stellenbosch	1996	New Zealand	Pomefruit	Apple
C1	Stellenbosch	1984	USA	Stonefruit	Peach

Table 6- cont. Origin, variety, and fruit classification of samples which tested positive for ACLSV by DAS-ELISA and RT-PCR.

Sample	South African area of origin	Import date	Origin	Fruit group	Fruit type
C4	Stellenbosch	2000	USA	Stonefruit	Peach
C5	Stellenbosch	1993	USA	Pomefruit	Apple
C6	Stellenbosch	2004	Belgium	Pomefruit	Apple
D3	Stellenbosch	Unknown	South Africa	Pomefruit	Apple
E1	Stellenbosch	1997	New Zealand	Stonefruit	Peach
E2	Stellenbosch	1969	England	Pomefruit	Apple
U2	Unknown	Unknown	Unknown	Stonefruit	Peach
U3	Stellenbosch	Unknown	South Africa	Pomefruit	Apple
U5	Stellenbosch	Unknown	Unknown	Stonefruit	Peach
U6	Unknown	Unknown	Unknown	Pomefruit	Apple
U7	Unknown	Unknown	Unknown	Pomefruit	Apple
U8	Stellenbosch	Unknown	Unknown	Stonefruit	Peach
U9	Stellenbosch	Unknown	Unknown	Stonefruit	Peach
U12	Unknown	Unknown	Unknown	Pomefruit	Apple
U15	Stellenbosch	Unknown	Unknown	Pomefruit	Apple
Y1	Stellenbosch	Unknown	Unknown	Pomefruit	Apple
Y10	Stellenbosch	Unknown	Unknown	Pomefruit	Apple
AP1	Ceres	Unknown	Unknown	Pomefruit	Apple
AP2	Ceres	Unknown	Unknown	Pomefruit	Apple
AP3	Ceres	Unknown	Unknown	Pomefruit	Apple
AP4	Ceres	Unknown	Unknown	Pomefruit	Apple
AP5	Ceres	Unknown	Unknown	Pomefruit	Apple
AP6	Ceres	Unknown	Unknown	Pomefruit	Apple
AP7	Ceres	Unknown	Unknown	Pomefruit	Apple
AP8	Ceres	Unknown	Unknown	Pomefruit	Apple
AP9	Ceres	Unknown	Unknown	Pomefruit	Apple
AP10	Ceres	Unknown	Unknown	Pomefruit	Apple
AP11	Ceres	Unknown	Unknown	Pomefruit	Apple
AP12	Ceres	Unknown	Unknown	Pomefruit	Apple
AP13	Ceres	Unknown	Unknown	Pomefruit	Apple

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Table 6- cont. Origin, variety, and fruit classification of samples which tested positive for ACLSV by DAS-ELISA and RT-PCR.

Sample	South African area of origin	Import date	Origin	Fruit group	Fruit type
AP14	Ceres	Unknown	Unknown	Pomefruit	Apple
AP15	Ceres	Unknown	Unknown	Pomefruit	Apple
AP16	Grabouw	Unknown	Unknown	Pomefruit	Apple
AP17	Grabouw	Unknown	Unknown	Pomefruit	Apple
AP19	Grabouw	Unknown	Unknown	Pomefruit	Apple
AP20	Op-die-berg	Unknown	Unknown	Pomefruit	Apple
AP21	Op-die-berg	Unknown	Unknown	Pomefruit	Apple
AP22	Ceres	Unknown	Unknown	Pomefruit	Apple
AP23	Ceres	Unknown	Unknown	Pomefruit	Apple

Twenty-six randomly collected samples which tested negative for ACLSV by DAS-ELISA were also tested by RT-PCR for the virus and the results of all tests were negative.

3.4.2 Electrophoretic analysis of RT-PCR-amplified products of ACLSV

The amplicons of the RT-PCR products of all samples were of the expected size (~1510 bp). The purification of the RT-PCR products was confirmed by agarose gel electrophoresis and showed a single band of ~1510 bp (data not shown).

3.4.3 Nucleotide sequence analysis and alignment

The RT-PCR products of 47 samples were successfully sequenced using the three primers in a cycle sequencing reaction. The intermediate and reverse primers resulted in a sequence overlap covering the CP gene. Contiguous sequences of the CP gene were generated. The forward primer resulted in sequences that were 5' to the CP gene and all sequences were truncated to only include the CP gene sequence. The generated sequences were verified as ACLSV CP gene sequences by BLASTn on GenBank which indicated high degrees of similarity to other deposited ACLSV CP gene sequences. The aligned sequence matrix is shown in Addendum A.

3.4.4 Phylogenetic analysis of ACLSV CP gene sequence

A total of 131 trees were retrieved using the heuristic search with a tree length of 1961. According to the analysis, 93 of the 464 characters were constant. A total of 135 characters (29.1%) were parsimony uninformative and 236 characters (50.9%) were parsimony informative. The tree statistics obtained gave a consistency index (CI) of 0.299 and a retention index (RI) of 0.637. One of the shortest trees of the heuristic search is presented in Figure 11.

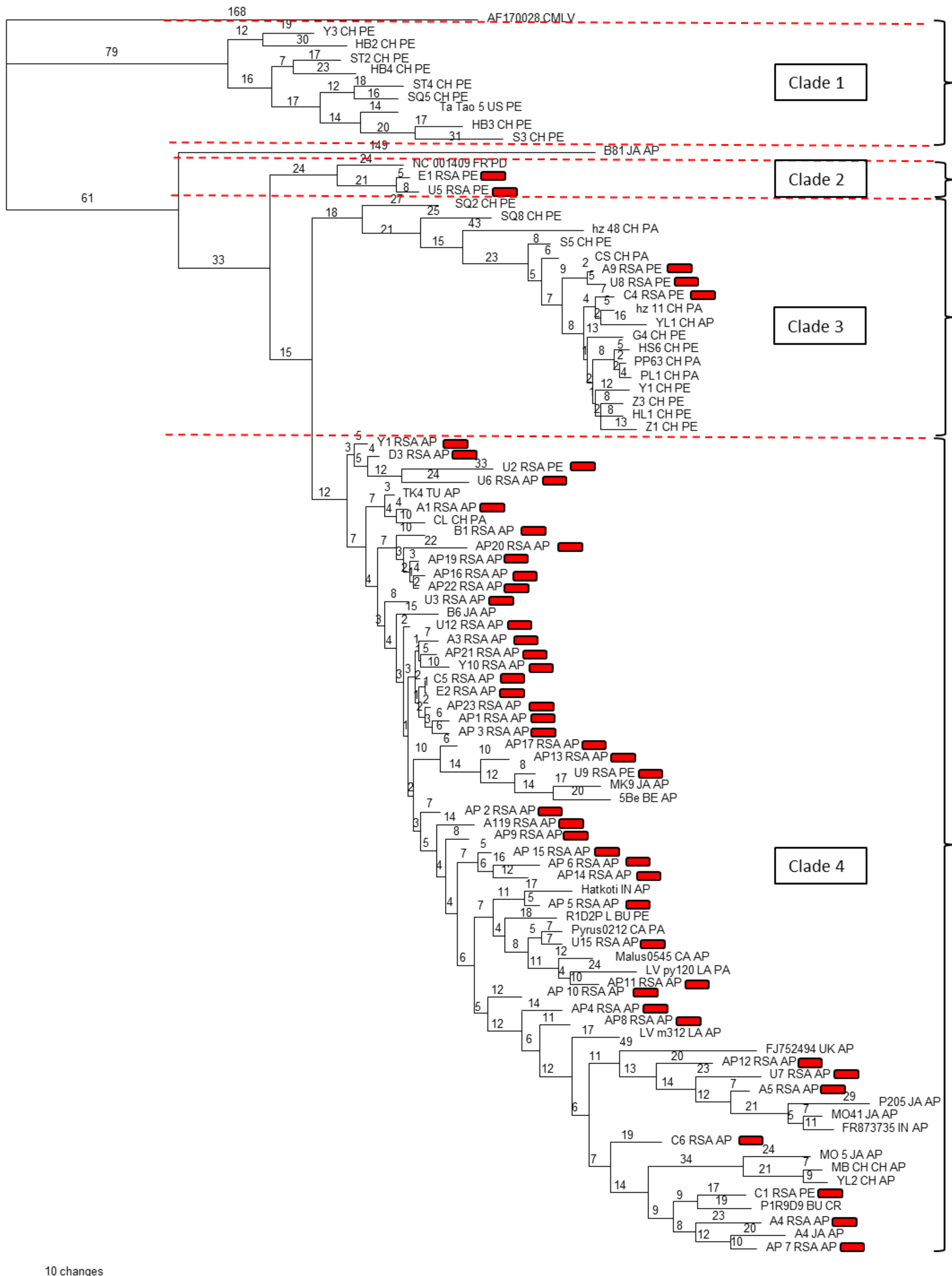

Figure 11. One of the shortest trees of the heuristic search performed on the ACLSV CP gene sequence data matrix. Branch lengths are indicated above branches. Red rectangles indicate South African isolates. AP=apple, CY = cherry, PA = pear, PE = peach, PD = *Prunus domestica,* JA = Japan, CH= China, IN = India, RSA = South Africa, LA = Latvia, CA = Canada, BU = Bulgaria, UK = Ukraine, BE = Belgium, TU =Turkey, FR = France.

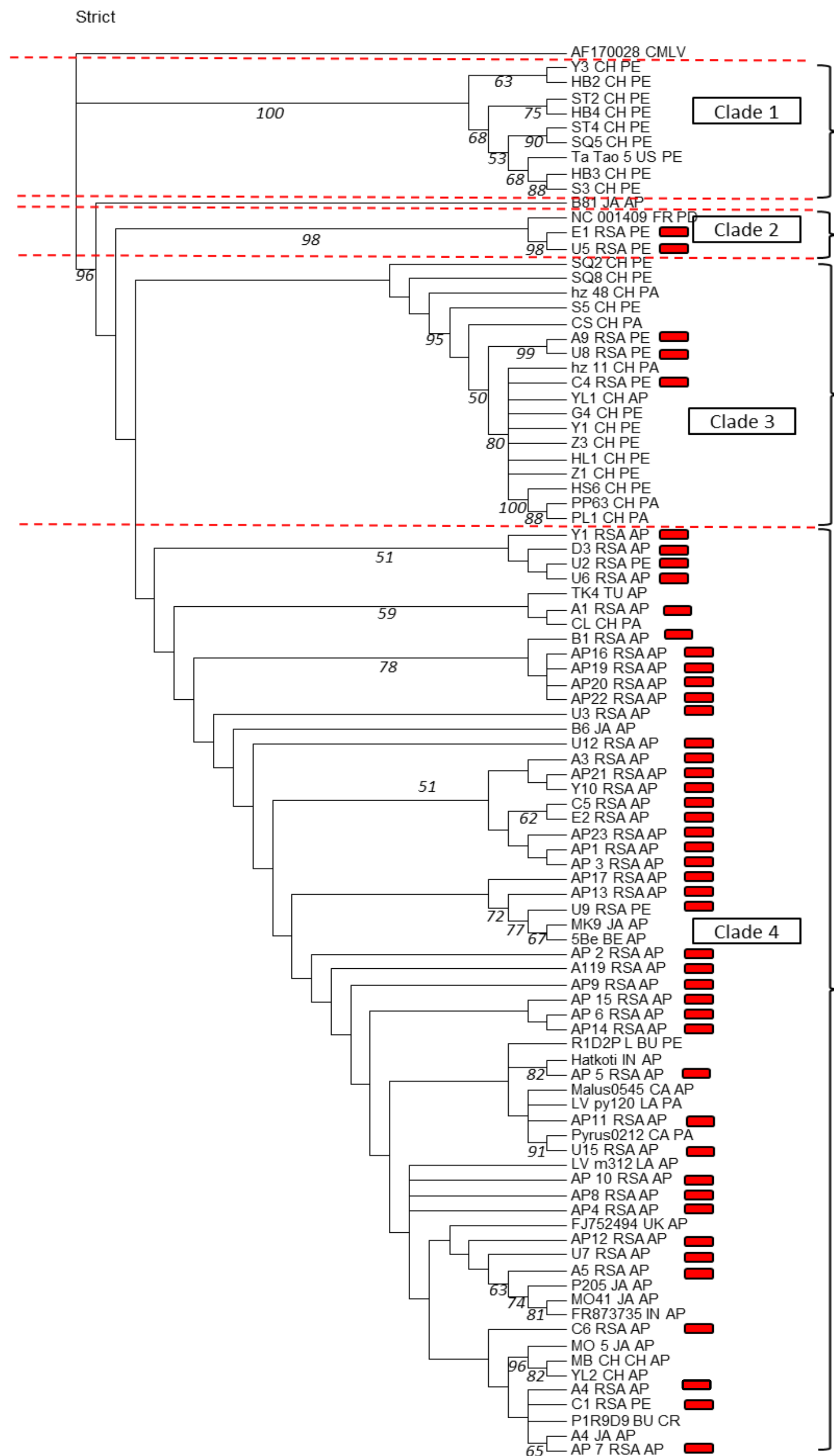

Figure 12: The strict consensus tree generated from the 42 trees retrieved in the heuristic search performed on the ACLSV CP gene sequence data matrix. Bootstrap values are indicated below branches in italics. Red rectangles indicate South African isolates. AP=apple, CY = cherry, PA = pear, PE = peach, PD = *Prunus domestica,* JA = Japan, CH= China, IN = India, RSA = South Africa, LA = Latvia, CA = Canada, BU = Bulgaria, UK = Ukraine, BE = Belgium, TU =Turkey, FR = France.

The strict consensus tree (Figure 12) showed that the phylogenetic analysis revealed two well-supported (100% and 96% bootstrap support respectively) major clades. The clade within which the SA ACLSV CP genes grouped showed additional variation among sequences with three subclades. Even though clades 3 and 4 formed monophyletic clades, there was no bootstrap support for them. The phylogenetic position of the 47 SA isolates showed that they were distributed amongst all the subclades of the second major clade. Sequence similarity of South African isolates of between 78.8-97.9% at nucleotide level (Addendum B) and 83-100% at amino acid level (data not shown) were obtained during this study.

3.5 Discussion

The purpose of the current phylogenetic study, was to determine the variation of ACLSV isolates in South Africa in relation to other regions of the world. Initial detection for isolates of ACLSV was done using DAS-ELISA, since the aim of the study was to detect as many different variants of the virus within South Africa as possible. As very low infection rates were expected from previous personal experience (data not shown), it would not have been cost effective to use RT-PCR to screen large numbers of samples. Thus the initial screening was done using DAS-ELISA and positive samples were confirmed by RT-PCR. From the 12 364 pome- and stonefruit samples screened for ACLSV by DAS-ELISA, only 47 were positive.

The coat protein gene of these 47 isolates was successfully sequenced. Eight of the isolates successfully sequenced were from stonefruit and 39 isolates were from pomefruit hosts. The phylogenetic analysis performed in this study further confirms the presence of a variety of different ACLSV isolates in South Africa.

The South African isolates show considerable variation and clustered into clades 2, 3 and 4 of the 4 clades that were retrieved. The large genetic variance amongst some of the South African isolates of ACLSV, most likely indicates different introduction sources, similar to reports from Latvia (Pupola et al., 2011). Sequence similarity of South African ACLSV isolates of between 78.8 -97.9% at nucleotide level (Addendum B) and 83-100% at amino acid level is similar to other reports. Song et al. (2011) reported sequence similarity of Chinese isolates of ACLSV ranging between 87.3-100% and 92.7-100% at nucleotide and amino acid levels respectively. Rana et al. (2010) reported similarity of between 91-100% at amino acid level of Indian ACLSV-CP gene sequence isolates from commercial pome-and stonefruit orchards, whilst Wang et al. (2011) reported 82.6-100% sequence similarity at nucleotide level and 91-100% similarity at amino acid level for various samples in the Canadian Clonal Genebank. Similarity of between 81-100% was previously reported for PNRSV isolates (Hammond, 2003). This indicates that the sequence similarities obtained in this study, correlate well with results from other regions of the world for ACLSV and other viruses. It also indicates that the sequence variation obtained during this study, represents an acceptable illustration of isolate variation within the country.

The phylogenetic analysis presented in this study includes all of the ACLSV CP gene sequences included by previous authors and the CP gene sequences of South African isolates. Previous

phylogenetic analyses revealed only two clades and some non-assigned isolates due to the limited number of isolates studied. This is the first study in which sufficient isolates from all of the clades were included in a phylogenetic analysis which allowed the identification of 4 clades. It was possible to confirm that all the phylogenetic clades in previous reports, fit into the phylogenetic clades retrieved in this study. Yaegashi et al. (2007) reported two clades in their phylogenetic analyses of CP gene sequences, whilst additional ACLSV isolates which grouped outside these two clades were also reported by other authors (Song et al., 2011; Niu et al., 2012; Gadiou et al., 2010). Clade 1 of this study was designated as the Ta Tao 5-type by Niu et al. (2012) and clade 3 of this study, was designated as the Z1 type by Niu et al. (2012). It was also reported by Song et al. (2011) that isolate NC 001409 grouped in a different clade to clade 1 of this study and this isolate with two South African isolates was retrieved in the current phylogenetic analysis in clade 2. The grouping of the isolates PP63 and PL1, as well as the grouping of isolates A4 (Japan), MO 5 and P 205, as reported by Song et al. (2011) were retrieved in this analysis in clades 3 and 4 respectively. The Z1 type of Niu et al. (2012) was found to group with the PP63 and PL1 grouping of Song et al. (2011) in this study. It was also reported that isolates 5Be and TK4 were in a separate cluster to isolate Ta Tao (Gadiou et al., 2010) as was also confirmed in this study. Al Rwahnih et al. (2004) reported two groups namely A and B, however, group B was later confirmed as isolates of *Apricot pseudo chlorotic leaf spot virus* (APCLSV) (Liberti et al., 2005) and the isolates were thus not included in this study.

The phylogenetic clustering of this study indicated that isolates of the ACLSV are not restricted to either stone- or pomefruit varieties, but that clades contain isolates from both fruit groups, except clade 1 and 2, which only contains isolates from stonefruit varieties. Previously it was reported that ACLSV isolates are restricted to either *Prunus* or *Malus/Pyrus* (Rana et al., 2008), but the authors later reported that clustering of both pome- and stonefruit isolates into one group do in fact occur (Rana et al., 2010) and that clustering is thus not host specific (Ferretti et al., 2010). It was also reported that ACLSV isolates in Latvia indicated low divergence between different host species based on pairwise distance calculations and phylogenetic analysis (Pupola et al., 2011). Similarly low divergence of virus isolates between hosts was also reported for ASGV (Liu et al., 2013), PNRSV (Hammond, 2003, Scott et al., 1998, Fiore et al., 2008), *Yam mosaic virus* (Bousalem et al., 2003), *Lettuce mosaic virus* (Krause-Sakate et al, 2002), *Turnip mosaic virus* (Ohshima et al., 2002, Tomimura et al., 2004) and *Cucumber mosaic virus* (Roossinck et al., 1999) where no correlation was found between viral and host groups. This appears to be the case with many plant viruses, with *Cherry leaf roll virus* being one of the exceptions (Rebenstorf et al., 2006). In the case of *Cherry leaf roll virus* it was postulated that ecological barriers could exist which prevent transmission between the host species and that rapid genetic isolation of viral variants and evolutionary divergence within hosts and over time could occur as a consequence (Rebenstorf et al., 2006).

Geographically, the isolates in clade 1 of this study mostly originate from China, with the exception of one isolate from the United States of America (USA). The isolates in clade 2 of this study occur in South Africa and France. The majority of isolates in clade 3 occur from China with

the exception of three isolates from South Africa (A9, C4 and U8). Clade 4 contains isolates from around the world, including Bulgaria (R1D2P L), Latvia (LV m312), Canada (Pyrus0212) and India (FR873735). Thirty-eight of the South African isolates belong to this clade.

One of the key questions, is whether different genetic variants of the same virus spread from one region to another. In the natural distribution of biological organisms, one would expect to find that a group of isolates would occur in one geographic region only. However where human mediated transport of biological organisms has occurred, such as in fruit trees, this geographic pattern will disintegrate. An analysis of where South African isolates appear in the phylogenetic tree of this study, shows that the geographical origin of viral isolates is not reflected in their phylogenetic clustering, as was also reported in the literature for isolates from other countries (Ferretti et al., 2010). For example, in clade 4 of this study, South African isolates C6, A4, C1, U7 and A5 show strong homology. The host plant of C6 was imported into South Africa in 2004 from Belgium, whilst the host plant of A4, was imported to South Africa from California, USA, in 2001. The host plant of C1 has been in the country since 1984 after import from the USA, whilst the host plant A5 was imported from Italy in 1998. The origin of U7 is unknown. Isolates A119, U9, B1, U3, U12, C5, E2, A1, D3, U6 and U15 in clade 4 also show homology. Their host plants, however, originate from various locations in South Africa, USA, England, China and New Zealand and were imported or locally selected at various times. The phylogenetic relationships described here suggest that these isolates were introduced by humans and that a geographic clustering of isolates can no longer be identified, since isolates from various distinct geographical regions are almost genetically identical. Similarity of about 99% (Addendum B) for isolates U12, C5 and E2 were obtained, originating from respectively a field sample in South Africa (U12), a host plant originally imported from USA (C5) and a host plant originally imported from England (E2) (Table 6). The South African isolate E1 in clade 2, which was imported from New Zealand (1997) clusters with a GenBank NC 001409 isolate from France. The host plants of A9 and U8 were bred in South Africa between 1980 and 1985 and show the strongest homology with C4 - the host plant of which was imported from the USA in 2000. Cross contamination of South African isolates used for this study, is highly unlikely since the trees from which these isolates were obtained, are maintained in insect proof gauze houses, preventing spread of the viruses amongst trees by insects. Pruning shears are also sterilized between use on different trees to prevent transmission of viruses. Furthermore individual trees are not situated directly next to each other, other plants are planted between these trees to prevent tree to tree transmission thus preventing cross-contamination of the virus to adjacent fruit trees plants. Geographic origin also did not affect the phylogenetic clustering of other viruses such as *Apple stem grooving virus* (Liebenberg et al., 2012, Liu et al, 2013) and *PNRSV* (Scott et al., 1998, Hammond, 2003, Fiore et al., 2008). This could be explained by the countrywide and worldwide exchange in plant material, as was also reported previously (Liebenberg et al., 2012, Rana et al., 2010). The virus mode of transmission and methods whereby plant propagation occur may also play a role (Hammond, 2003, Liebenberg et al., 2012).

It could be expected that virulent isolates or isolates showing similar symptoms could possibly cluster together, whilst symptomless isolates could cluster into separated clades, as the CP gene could be a possible region affecting virulence or symptom expression. This did not seem to correlate with phylogenetic clustering, as the symptomless isolates from South African and other parts of the world grouped together with isolates which did show severe symptoms. Isolates from GenBank used in this study that did show noticeable symptoms were isolates Ta Tao 5, causing severe necrosis, NC 001409, causing bark split disease, and A4 (Japan) showing fruit russet ring symptoms. South African isolates that grouped with necrosis-forming isolate NC 001409 in clade 2 and russet ring-forming isolate A4 in clade 4, were symptomless. The other isolates obtained from GenBank for this study, were also reported as symptomless. For example, isolates Pyrus0212, Malus0545 and Malus0375 originated from a study of varieties maintained at the Canadian Clonal Genebank and 46.9% of samples tested positive for ACLSV although they were mostly symptomless (Wang et al., 2011). Isolates TK4 and 5Be originated from a study of apple varieties in commercial orchards in Europe and Asia and were also reported as mostly symptomless (Gadiou et al., 2010). In general, all infected samples were reported symptomless in the survey from which isolates HQ398359.1 and HQ398357.1 were collected (Pupola et al., 2011). No reference could be found in the literature whether the samples used to obtain isolate Z3 (Niu et al., 2012), PP63 and PL1 (Song et al., 2011) exhibited any symptoms. The other GenBank isolates were lodged without published articles and thus no data is available as to their symptom expression. The conclusion from this study and from the published data, is that lack of symptom expression does not relate to a particular phylogenetic clade. It was also reported that no correlation could be found between symptom expression in apples, pears and quince, and the presence of ApMV or ACLSV in combination with other viruses (Constable et al., 2007). The same lack of correlation of genetic variation with symptom expression was also reported with different isolates of PNRSV (Scott et al., 1998; Aparicio et al., 1999; Vascova et al., 2000; Aparicio & Pallas, 2002; Fiore et al., 2008).

The most worrying outcomes of this study are that South African ACLSV isolates do not appear to be host specific between pome- and stonefruit groups and that viral isolates do not appear to come from geographically limited areas, but rather from throughout the world. If the isolates from South Africa had grouped into just one clade, it could have been concluded that the isolates were not imported from different sources from different countries, but had possibly originated locally or that such an isolate had originated from a single importation. However, as the isolates from South Africa group into various clades containing isolates from other countries it can be concluded that these ACLSV isolates were in all likelihood imported to South Africa from more than one source and from more than one country. The history of the importation of the original tree planting material from different countries, supports this deduction.

In conclusion, a wide diversity of South African isolates as revealed, grouping into 3 of the 4 worldwide clades retrieved in this study. The current study prompts further investigation into the genetic diversity of ACLSV within South Africa. Examining more samples could contribute towards a better understanding of spread of the virus within the country. The whole-genome sequences of

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South African isolates were not determined during this study and for more reliable diversity analysis, the complete genome of ACLSV isolates should be compared. This could provide more insight into the geographical origin(s) of the South African isolates and sub-population evolution. This could also provide insight into the pathogenicity of isolates and if genetic composition correlates with pathogenicity. The extent of transmission of the virus using pruning equipment and propagation material, could also be investigated in future.

CHAPTER 4

Determination of genetic variation of *Apple mosaic virus* (ApMV) in South Africa

4.1 Abstract

Leaf samples were screened by DAS-ELISA for ApMV. RT-PCR amplification of the coat protein gene of 33 samples which tested positive by DAS-ELISA and 26 which tested negative, was performed. Seventeen of the sample which tested negative by DAS-ELISA tested positive by RT-PCR. Sequencing using the same primers as for the RT-PCR was performed on all samples which gave RT-PCR amplification products. Following sequence alignment of the 50 CP gene sequences with 28 coat proteins sequences from GenBank and *Prunus necrotic ringspot virus* (PNRSV), phylogenetic analysis using parsimony was performed. Isolates from South Africa clustered into two clades. The clusters contained isolates from stone- and pomefruit, indicating that the virus is not fruit type specific and that cross-infection between pome- and stonefruit is possible. Geographic analysis of the phylogenetic tree indicates that South African isolates were most likely imported from other parts of the world. Symptom expression did not correlate with the phylogenetic analysis, as symptomless and isolates obtained which showed severe symptoms clustered together.

4.2 Introduction

Apple mosaic virus (ApMV) is one of the most common pathogens of apples worldwide (Nemeth, 1986; Desvignes & Boyé, 1989). ApMV belongs to the genus *Ilarvirus*, subgroup III, family *Bromoviridae* (Rybicki, 1995). ApMV has a positive-sense single-stranded RNA genome, divided into RNA 1, 2 and 3, and RNA 4, a subgenomic messenger for the coat protein (Rybicki, 1995). RNAs 1, 2 and 3 are non-infectious unless either the coat protein (CP) or subgenomic RNA 4 is present.

Lakshmi et al. (2011) sequenced the CP genes of ApMV isolates in India and distinguished five clades using phylogenetic analysis. Pupola et al. (2011) reported various degrees of genetic diversity within respective virus isolates of ApMV in Latvia during sequence analyses targeting the 3'-terminal region of the CP gene. Lee et al. (2002) reported heterogeneity in the CP size and gene sequence variability in Korea, with most amino acid residue differences located in the N'-termini of the isolates, and conserved regions in the middle and C-termini. Thockchom (2009) studied molecular variation of CP genes of ApMV isolates in India and reported that Indian isolates clustered together with Korean isolates. Petrzik (2005) reported that ApMV isolates of pears were phylogenetically closely related to isolates infecting apple and that infected pears were mostly symptomless (Petrzik, 2005).

In order to assess the genetic variation of ApMV isolates in South Africa, the CP gene of different isolates was amplified by RT-PCR. The resulting products were sequenced and these sequences

were analysed phylogenetically in order to establish the relationships and determine the variation between different isolates and to correlate this with host specificity.

4.3 Materials and Methods

4.3.1 Sample collection

Initial detection of ApMV isolates was done by testing a total of 11 969 pome- and stonefruit leaf samples collected during 2011 to 2013 from the Western Cape and North West Provinces of South Africa with DAS-ELISA.

Leaf samples were stored at 4 °C or were tested immediately without storage. Leaves were tested by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark & Adams, 1977) using commercially available ELISA reagents with polyclonal antibodies for ApMV (Bioreba, Switzerland) according to the manufacturer's instructions. Absorbance was measured at 405 nm after 60 minutes.

Samples which tested positive using DAS-ELISA were subsequently tested using RT-PCR. A random collection of samples which tested negative using DAS-ELISA for ApMV, were also tested by RT-PCR for the virus (see Table 7).

Table 7. Origin, fruit type and fruit group of samples which tested negative for ApMV using DAS-ELISA and was tested by RT-PCR for ApMV.

Sample	Region of Origin	Fruit Group	Fruit Type
P1	Stellenbosch	Pomefruit	Pear
P2	Stellenbosch	Pomefruit	Pear
P3	Stellenbosch	Pomefruit	Pear
P4	Stellenbosch	Pomefruit	Pear
P5	Stellenbosch	Pomefruit	Pear
P6	Stellenbosch	Pomefruit	Pear
P7	Stellenbosch	Pomefruit	Pear
P8	Stellenbosch	Pomefruit	Pear
P9	Stellenbosch	Pomefruit	Pear
P10	Stellenbosch	Pomefruit	Pear
T1	Stellenbosch	Pomefruit	Apple
T2	Stellenbosch	Pomefruit	Apple
T3	Stellenbosch	Pomefruit	Apple
T4	Riviersonderend	Stonefruit	Prune
T5	Riviersonderend	Stonefruit	Prune
T6	Riviersonderend	Stonefruit	Peach
T7	Stellenbosch	Pomefruit	Apple
T8	Stellenbosch	Pomefruit	Pear
T9	Stellenbosch	Pomefruit	Apple

Table 7-cont. Origin, fruit type and fruit group of samples which tested negative for ApMV using DAS-ELISA and was tested by RT-PCR for ApMV.

Sample	Region of Origin	Fruit Group	Fruit Type
T10	Stellenbosch	Pomefruit	Apple
T10	Stellenbosch	Pomefruit	Apple
T11	Stellenbosch	Pomefruit	Apple
T12	Stellenbosch	Pomefruit	Apple
T13	Stellenbosch	Pomefruit	Apple
T14	Stellenbosch	Pomefruit	Apple
T15	Stellenbosch	Pomefruit	Pear
T16	Riviersonderend	Stonefruit	Nectarine

4.3.2 Sample preparation for RT-PCR

Total RNA was isolated from 100 mg of leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Germany) as described by the manufacturer. RT-PCR was performed using 2 µl of the isolated RNA solution added to 23 µl of a PCR-mixture. The mixture consisted of 2.5 µl 10 x PCR-buffer, 1.25 µl 0.1 M DTT, 1.5 µl 25 mM $MgCl_2$, 0.625 µl 20 µM forward primer, 0.625 µl 20 µM reverse primer, 1 µl 5 mM dNTPs (Bioline, Germany), 0.25 µl 5U/µl Super-Therm DNA Polymerase mixture (Bioline, Germany) and 0.125 µl SuperScript™ III (Invitrogen™, Life Technologies™, USA) and 15.125 µl Milli-Q® water. The primers used had the following sequences: forward primer PAPCP3-1: 5' – CTAACAAATCTTCATC GATAAG-3', and reverse primer PAPCP5: 5'-TCTAACATGGTCTGCAAGTAC-3' (Lee et al., 2002).
The RT-PCR amplifications were performed as follows: a single step of 48ºC for 30 minutes, followed by 40 cycles of 94ºC for 30 seconds, 56ºC for 45 seconds and 72ºC for 90 seconds. Finally elongation was performed at 72ºC for 10 minutes. The amplifications were done using a Veriti ™ Thermal Cycler (Life Technologies™, USA) and the amplicon of the primer set was 668 bp in length encompassing the whole length of the CP gene of ApMV.

4.3.3 Electrophoretic analysis of RT-PCR-amplified products

A 1% agarose gel containing 1 µg/ml ethidium bromide was used for electrophoresis and immersed in a 1 x TAE electrophoresis buffer (as described in Chapter 3). The total PCR product was mixed with a loading buffer and loaded onto the gel. A 100 bp DNA size markers ladder (Promega, USA) was used to determine the size of the amplified RT-PCR products. Electrophoresis was performed at 100 V for 60-80 minutes using a Cleaver Scientific Ltd power source (England). The RT-PCR products were visualized using a UV transilluminator and portable darkroom (Cleaver Scientific Ltd, England). The RT-PCR products that were obtained were excised using a sterile scalpel with visualization on the UV transilluminator and eluted from the gel by the Wizard® SV and PCR Clean-up System (Promega, USA) according to the manufacturer's specifications and the final product was eluted in 40 µl of Milli-Q® water. A 1% agarose gel was

loaded with 2 µl of the cDNA and electrophorized and visualized as described above in order to assess the concentration of the purified RT-PCR products prior to sequencing. The cDNA products were stored in nuclease free Eppendorf tubes at -20ºC.

4.3.4 Sequencing of RT-PCR amplification products

The RT-PCR products were directly sequenced with the Cycle Sequencing kit (Applied Biosystems®, Life Technologies™, USA). The RT-PCR products were sequenced using one of the following sequencing mixes. Mix 1 contained 5 µl of 5 x sequencing dilution buffer (Applied Biosystems®, Life Technologies™, USA), 2 µl Terminator Dye (BigDye® Terminator v3 Cycle Sequencing kit, Applied Biosystems®, Life Technologies™, USA), 1 µl Milli-Q® water, 1 µl (0.8 µM) primer and 1 µl of the cDNA sample. Mix 2 contained 3 µl Half Dye Mix (Bioline, Germany), 2 µl Terminator Dye (BigDye® Terminator v3 Cycle Sequencing kit, Applied Biosystems®, Life Technologies™, USA), 3 µl Milli-Q® water, 1 µl (0.8 µM) primer and 2 µl of the cDNA sample. The same primers used for RT-PCR was also used for sequencing. The samples were submitted to 35 cycles of the 96ºC for 10 seconds, 52ºC for 30 seconds and 60ºC for 4 minutes in a Veriti ™ Thermal Cycler (Life Technologies™, USA). This was followed by a final step of 60ºC for 10 minutes. The cycle sequencing products were analyzed using an ABI®3730xl Genetic Analyser at the Central Analytical Facility, University of Stellenbosch.

4.3.5 Nucleotide sequence analysis and alignment

The electropherograms obtained from the sequencing were edited using Chromas v 2.23 (Technelysium, Pty., Ltd.) and the generated ApMV nucleotide sequences were compared to ApMV sequences obtained from GenBank using the BioEdit v 7.0.5.2 software package. An automated alignment of the ApMV coat protein gene sequences and the outgroup sequence from *Prunus necrotic ringspot virus* (PNRSV) (NC004362) was performed using the Clustal W v 1.4 alignment function of the BioEdit package. Further manual alignment was also performed. The percentage nucleotide and amino acid sequence similarities were determined using the BioEdit software.

4.3.6 Phylogenetic analysis

Phylogenetic analysis of the obtained and GenBank sequences was done using the program Phylogenetic Analysis Using Parsimony (PAUP) (4.0b10) (Swofford, 2003). A total of 28 ApMV CP gene sequences from GenBank (Table 8) and 50 CP gene sequences (Tables 9 and 10) from South African isolates of ApMV were used in the phylogenetic analysis. A heuristic search (1 000 replicates) using TBR branch swopping was used with all characters weighted equally to establish the shortest possible trees from the data matrix. A bootstrap analysis (1 000 replicates) using TBR branch swopping was performed to determine clade support. Branches with bootstrap values less than 50% were considered weakly supported and are not indicated on the phylogram. Values from 74% to 50% were considered as moderately supported, whilst values of 75% and more were considered as well supported.

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Table 8. Details of ApMV CP gene isolates obtained from GenBank used for phylogenetic analysis and *Prunus necrotic ringspot virus* (PNRSV) used as out-group in phylogenetic analysis.

GenBank accession number	Isolate	Host	Country of origin
NC004362	PNRSV	Unknown	Unknown
AF548367	-	Apple	Korea
AM490197	-	Apple	Czech Republic
AMU15608	-	Apple	USA
AY125977	-	Apple	Korea
AY542540	B1	Apple	Czech Republic
AY542541	C3a	Apple	Belgium
AY542542	Iv10	Pear	Czech Republic
AY542543	Kravare	Pear	Czech Republic
AY542544	Cerin	Pear	Czech Republic
AY542545	Roz144	Pear	Czech Republic
AY542546	It1	Pear	Czech Republic
AY054385	-	Apple	Czech Republic
AY054386	-	Prune	Czech Republic
AY054387	-	Hops	Czech Republic
AY054388	-	Almond	Czech Republic
AY054389	-	Pear	Czech Republic
FJ429311	-	Apple	India
FM178274	Shimla	Unknown	Unknown
FN435314	-	Apple	India
FN435315	-	Apple	India
FN435316	-	Apple	India
FN435317	-	Apple	India
FN546183	-	Cherry	India
FN547927	-	Apple	India
FN564150	Tc-3	Unknown	Unknown
HE574164	-	Apple	Unknown
L03726 U	AMQCOATPA	Apple	USA
S78319	-	Apple	Germany

4.4 Results

4.4.1 DAS-ELISA and electrophoretic analysis of RT-PCR-amplified products of ApMV

All the samples which tested positive by DAS-ELISA for ApMV, also tested positive by RT-PCR and are listed in Table 9. A number of samples which tested negative for ApMV by DAS-ELISA, tested positive by RT-PCR (Table 10).

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Table 9. Origin and fruit group of samples which tested positive for ApMV using DAS-ELISA and RT-PCR.

Sample	Region of origin	Fruit group	Fruit type
K4	Grabouw	Pomefruit	Apple
J3	Villiersdorp	Pomefruit	Apple
K2	Villiersdorp	Pomefruit	Apple
D6	Stellenbosch	Pomefruit	Apple
J1	Stellenbosch	Pomefruit	Apple
AA1	Ceres	Pomefruit	Apple
A114	Ceres	Stonefruit	Nectarine
A115	Ceres	Stonefruit	Nectarine
A116	Ceres	Stonefruit	Nectarine
A120	Unknown	Pomefruit	Unknown
A131	Unknown	Pomefruit	Apple
A132	Unknown	Pomefruit	Apple
A133	Unknown	Pomefruit	Apple
A159	Unknown	Pomefruit	Unknown
A160	Unknown	Pomefruit	Unknown
A161	Unknown	Pomefruit	Unknown
A163	Riviersonderend	Stonefruit	Prune
A168	Stellenbosch	Pomefruit	Apple
A171	Unknown	Pomefruit	Apple
A172	Unknown	Pomefruit	Apple
AP1	Ceres	Pomefruit	Apple
AP2	Ceres	Pomefruit	Apple
AP3	Ceres	Pomefruit	Apple
AP4	Ceres	Pomefruit	Apple
AP5	Ceres	Pomefruit	Apple
AP7	Ceres	Pomefruit	Apple
AP8	Ceres	Pomefruit	Apple
AP9	Ceres	Pomefruit	Apple
AP10	Ceres	Pomefruit	Apple
AP11	Ceres	Pomefruit	Apple
AP12	Ceres	Pomefruit	Apple
AP13	Ceres	Pomefruit	Apple
AP14	Ceres	Pomefruit	Apple

Table 10. Origin, fruit group and fruit type of samples which tested negative for ApMV using DAS-ELISA and tested positive by RT-PCR for ApMV.

Sample	Region of Origin	Fruit Group	Fruit Type
P1	Stellenbosch	Pomefruit	Pear
P2	Stellenbosch	Pomefruit	Pear
P3	Stellenbosch	Pomefruit	Pear
P4	Stellenbosch	Pomefruit	Pear
P5	Stellenbosch	Pomefruit	Pear
P6	Stellenbosch	Pomefruit	Pear
P7	Stellenbosch	Pomefruit	Pear
P8	Stellenbosch	Pomefruit	Pear
P9	Stellenbosch	Pomefruit	Pear
P10	Stellenbosch	Pomefruit	Pear
T2	Stellenbosch	Pomefruit	Apple
T3	Stellenbosch	Pomefruit	Apple
T6	Riviersonderend	Stonefruit	Peach
T7	Stellenbosch	Pomefruit	Apple
T9	Stellenbosch	Pomefruit	Apple
T10	Stellenbosch	Pomefruit	Apple
T11	Stellenbosch	Pomefruit	Apple

The amplicons of the RT-PCR products of the 50 South African samples were of the expected size (~668 bp) (as shown Chapter 2 of this thesis in Figure 10). The products were purified using the Wizard® SV and PCR Clean-up System (Promega, USA). Two microliters of the eluted DNA samples were loaded on a 2% agarose gel containing ethidium bromide, subjected to electrophoresis and the subsequent visualization under UV-light confirmed that the RT-PCR product was a single product of ~668 bp and was used to assess DNA concentration for subsequent cycle sequencing.

4.4.2 Nucleotide sequence analysis and alignment

The RT-PCR products of 50 isolates of ApMV were sequenced. Use of the forward and reverse primers in the cycle sequencing reactions resulted in a sequence overlap covering the CP gene and contiguous sequences were generated. During sequencing alignment gaps had to be introduced to accommodate the outgroup of PNRSV and the ApMV CP gene sequences. All 50 isolates could be identified as ApMV by comparison to other sequences in the data matrix and reference sequences from GenBank. The aligned sequence matrix is shown in Addendum C. The percentage nucleotide sequence similarities (Addendum D) of South African isolates ranged from 96% to 100% and the amino acid sequence similarities ranged from 96% to 100% (data not shown).

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4.4.3 Phylogenetic analysis of ApMV CP gene sequence

Fifty South African sequences were included in the final phylogenetic analysis. A total of 1603 trees were retrieved using the heuristic search with a tree length of 746. A total of 139 characters were constant, 349 characters were parsimony uninformative and 133 characters were parsimony informative. A consistency index (CI) of 0.812 and a retention index (RI) of 0.823 were revealed by tree statistics. One of the shortest trees of a heuristic search is presented in Figure 13. The strict consensus tree generated from the 1603 trees retrieved in the heuristic search performed on the ApMV CP gene sequence is presented in Figure 14. The South African isolates clustered together into two groups (RSA Clade A and B). Four of the isolates grouped in Clade A with a bootstrap support of 98%. The remaining isolates clustered in Clade B with 62% bootstrap support.

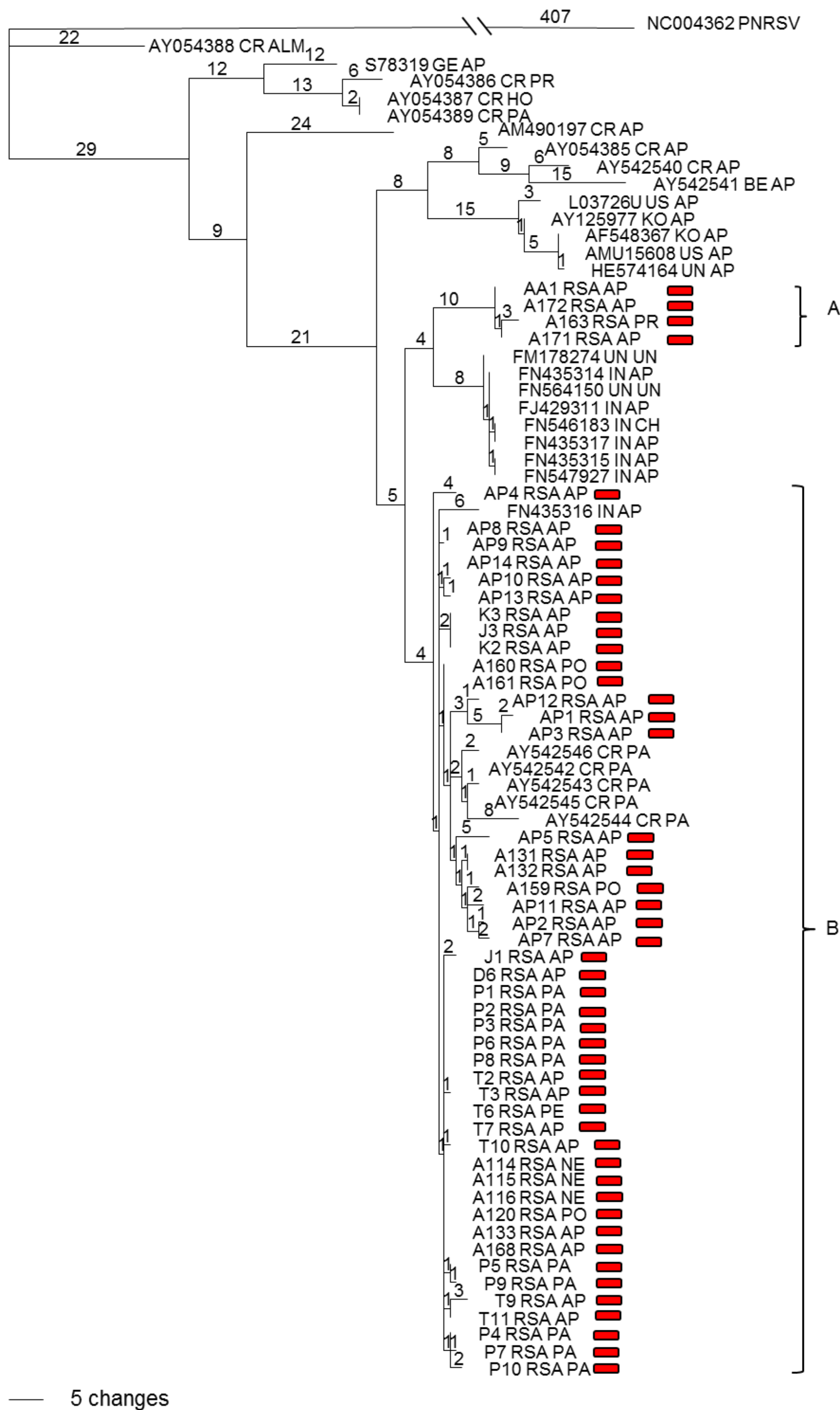

Figure 13. One of the shortest trees of heuristic search performed on the ApMV CP gene sequence data matrix. Branch lengths are indicated above branches. Branch length of isolate The branch of NC004362 was shortened. South African isolates are indicated by red rectangles. AP = apple, CY = cherry, PR = Prune, PA = pear, HO = hops, ALM = almond, NE = nectarine, PE = peach, PO = pomefruit, IN = India, UN = Unknown, GER = Germany, USA = United States of America, KO = Korea, CR = Czech Republic, BE = Belgium, RSA = South Africa.

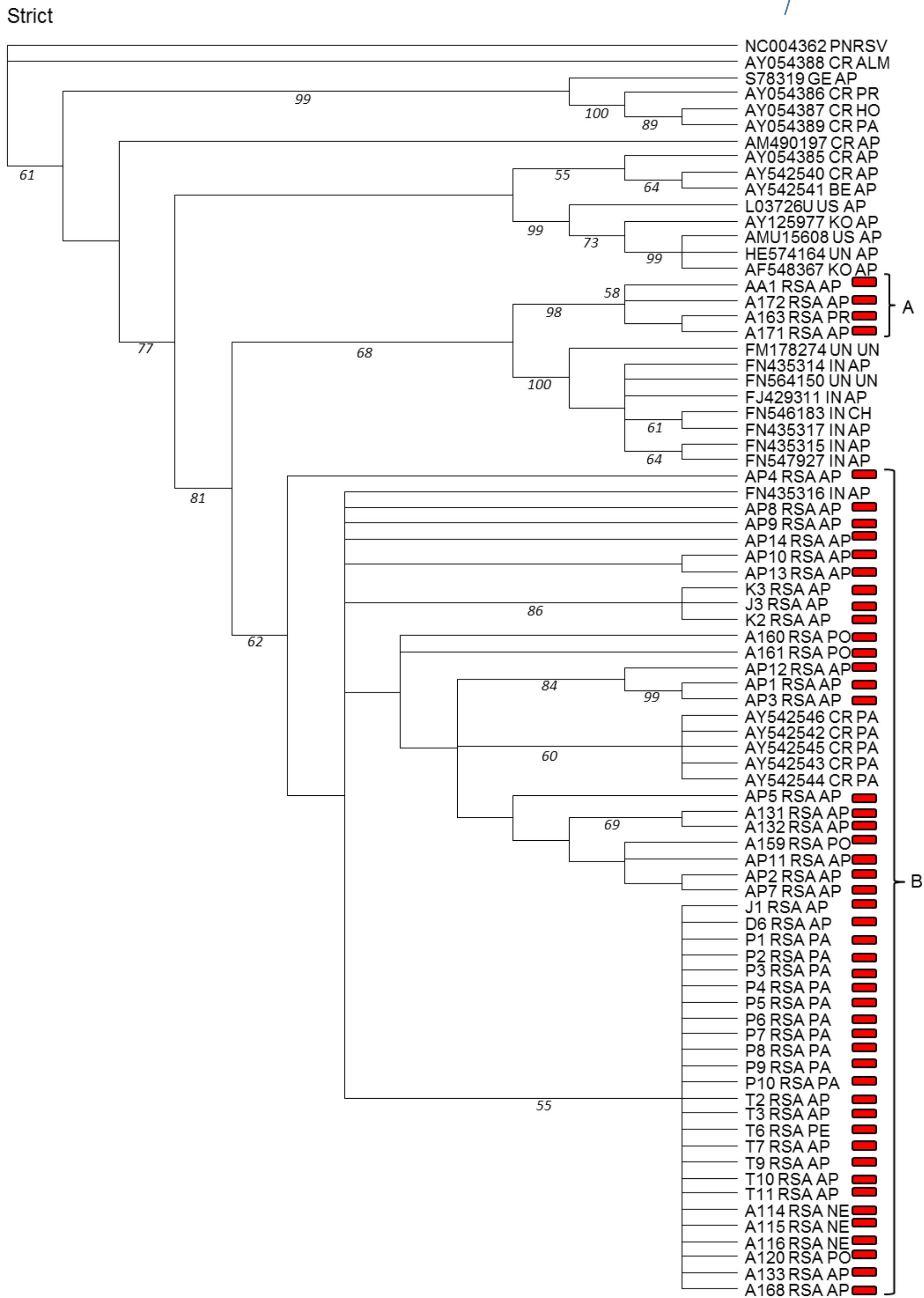

Figure 14. The strict consensus tree generated from the 1603 trees retrieved in the heuristic search performed on the ApMV CP gene sequence data matrix. Bootstrap values are indicated below branches in italics. South African isolates are indicated by red rectangles. AP = apple, CY = cherry, PR = Prune, PA = pear, HO = hops, ALM = almond, NE = nectarine, PE = peach, PO = pomefruit, IN = India, UN = Unknown, GER = Germany, USA = United States of America, KO = Korea, CR = Czech Republic, BE = Belgium, RSA = South Africa.

4.5 Discussion

Initial detection of isolates of ApMV for the determination of genetic variability with South Africa was performed using DAS-ELISA. Screening of large numbers of samples by RT-PCR was not considered economically feasible and DAS-ELISA was used to detect positive samples for a phylogenetic assessment of variability within ApMV in South Africa. Thus a total of 33 samples which tested positive for ApMV using DAS-ELISA were confirmed as positive by RT-PCR and sequencing. However, a total of 17 randomly chosen samples which tested negative by DAS-ELISA, did test positive by RT-PCR and sequencing for ApMV infection. Thus, the results indicate that a further investigation into determining the extent of ApMV infection in samples using RT-PCR should be made as DAS-ELISA gives an underestimate of infection levels.

The CP gene sequences of South African isolates of ApMV together with reference sequences were compared through phylogenetic analysis. The South African CP gene sequences grouped into two major clades. Four of the South African isolates formed a separate clade (designated clade A), sister to a clade containing isolates from India. This clade is strongly supported with a bootstrap value of 98%. It could indicate a unique South African group, which possibly originated from local hosts and migrated to the pome- and stonefruit plants.

The remaining 46 South African isolates grouped in a weakly supported clade with pear isolates from the Czech Republic and one apple isolate from India. The South African isolates in the latter clade showed little nucleotide variation as observed from the the short branch lengths in this clade (see Figure 13).

Pairwise sequence comparisons of the CP gene of South African ApMV isolates indicate relative high sequence homology of between 96% to 100% at both nucleotide (Addendum C) and amino acid level (data not shown). It could in all likelihood indicate common ancestors. This conclusion was also made by Pupola et al. (2011) for ApMV isolates from Latvia. It was previously reported that the CP gene of two ApMV isolates, M804 and M101, were exactly identical despite host plant genotype and geographical origin differences, indicating that the infection source could be due to clonal rootstocks (Pupola et al., 2011). Similar results were found with South African isolates from different geographical origins, which were identical at CP gene level. The reason for the identical CP gene sequences, could be the use of clonal rootstocks, which are commonly used in South Africa whereby viruses are transmitted from rootstocks to scions. Highly conserved sequences were also previously reported with nucleotide sequence homology of 91-100% of ApMV isolates in India on pome- and stonefruit (Lakshmi et al., 2011), 83-100% for Korean apple isolates (Lee et al., 2002), 83.2% for pomefruit isolates from various regions worldwide (Candresse et al., 1998) and 87-91% on strawberries (Tzanetakis & Martin, 2005). Nucleotide sequence similarity of between 81-100% was also reported for the ilarvirus PNRSV (Hammond, 2003) and between 86.5-100% for ASGV which belongs to the same virus family as ApMV (Liu et al., 2013).

It was reported that the CP gene sequences of ApMV isolates can be phylogenetically classified into three subclades (I, II, III) originating from apple, hop/pear and almond respectively (Lee et al., 2002). The phylogenetic analysis also indicated that the almond isolate of Lee et al. (2002) belongs to a separate group from the rest of the isolates. Similar to Lee et al. (2002), the phylogenetic analysis of their sequences indicated that their isolates of hops and one pear in subclade II show strong homology, with 90% bootstrap support. In contrast to the report of Lee et al. (2002), where apple and pear isolates were in different subclades (subclade I and II), the pear isolates other than the one in subclade II of Lee et al. (2002), were in a different clade together with apple, peach and nectarine isolates (RSA clade B). There was one clade that was strongly supported by a bootstrap value of 100% containing only apple isolates (as was also reported by Lee et al., 2002), but the other apple isolates grouped together with other fruit groups. The lack of correlation between sequences and host group, corresponds to the results of ACLSV in this study (Chapter 3) and was also reported for PNRSV (Vaskova et al., 2000; Aparicio et al., 1999; Aparicio & Pallas, 2002; Fiore et al., 2008) and ASGV (Liu et al., 2013). Reports on plant viruses generally indicate that genetic diversity is not influenced by the host species (Scott et al., 1998; Roossinck et al., 1999; Krause-Sakate et al., 2002; Ohshima et al., 2002; Hammond, 2003; Bousalem et al.; 2003; Tomimura et al., 2004; Fiore et al., 2008), with *Cherry leaf roll virus* being one of the exceptions (Rebenstorf et al., 2006).

The phylogenetic analysis of CP genes sequences of SA isolates in relation to overseas isolates has important practical implications for the South African deciduous fruit industry. RSA clade B contains isolates of ApMV originating from apple, pear, nectarine and peach trees, indicating that ApMV is cross-infective between pome- and stonefruit. This is a major concern, as it indicates that there is a risk of spread of the virus between different pome- and stonefruit. A number of pear and apple isolates and one peach isolate tested negative for ApMV by DAS-ELISA, but positive for the virus by RT-PCR. These isolates all fell within the RSA clade B into which most of the South African isolates grouped. These isolates which tested negative by DAS-ELISA were dispersed between the isolates that tested positive by DAS-ELISA. It is thus difficult to determine why they were not detected by DAS-ELISA, as there appears to be no clear sequence difference between isolates that tested positive by DAS-ELISA and isolates that tested negative. The undistinguishable difference between ApMV isolates testing positive or negative by DAS-ELISA, but positive using RT-PCR will require further investigation but, given that the CP gene sequence differences are minimal, this may indicate that the plant material in South Africa contains unique inhibitors of the DAS-ELISA that have not been identified before. The most serious implication of this study is that the DAS-ELISA which is currently used as the only method for the detection of ApMV is not suitable as detection technique for ApMV and should be replaced by the RT-PCR as method for detection of ApMV in South Africa. A more feasible reason why these samples tested negative by DAS-ELISA but positive by RT-PCR is most probably because RT-PCR is much more sensitive than DAS-ELISA as shown in Chapter 2.

The most worrying outcome of this study is the detection of ApMV by RT-PCR in pears in South Africa for the first time and the fact that the DAS-ELISA is unable to detect the virus. RT-PCR

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detected 20.6% of ApMV infections in plantations of pears, but no positive results were obtained from DAS-ELISA in these pear orchards (Pupola et al., 2011). In the same study ApMV was also reported in 21 of 36 pear orchards sampled in Latvia (Pupola et al., 2011). Within the South African certification scheme it is clear that the short term cost of accurate viral detection is worth the expense to prevent the long term costs of replacing a diseased orchard. ApMV is currently not tested under the South African Deciduous Fruit Plant Certification Scheme for pears, as it was considered not to occur in pears. Thus currently ApMV infections appear to go undetected in pears in South Africa and could have the consequence of unrivalled spread through the industry. As only a small number of samples were tested by RT-PCR, this survey needs to be expanded in order to assess the extent of the problem. The RT-PCR determinations were not false positives as this result was confirmed by coat protein gene sequencing of these isolates.

In conclusion, genetic analysis of ApMV isolates was performed and the South African isolates grouped into two clades with strong similarity within the two groups. Clade A contains exclusively South African isolates, which could indicate a local source of virus infection. Further research is needed to confirm the current phylogenetic analysis of South African isolates and the very close similarity of South African isolates of ApMV by investigating a larger number of samples. As with ACLSV, the full-length sequences of South African isolates were not determined during this study and more reliable diversity analysis would be possible if the complete genome of ApMV isolates are compared.

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CHAPTER 5

Conclusions and future perspectives

This study showed that RT-PCR is more sensitive for the detection of ACLSV and ApMV than DAS-ELISA by a factor of 70.5 fold and 71 fold respectively. Although DAS-ELISA is currently the method that is used within the South African Deciduous Fruit Plant Certification Scheme, this study shows that RT-PCR should be considered as a replacement, especially in the case of detection of ApMV, since it was found that some isolates are not detected by DAS-ELISA.

From the phylogenetic analysis and variation found within South African isolates of ACLSV and ApMV in this study, in comparison to the DAS-ELISA/RT-PCR comparative results, it appears that DAS-ELISA detects isolates from all of the South African clades. However, the effectiveness of DAS-ELISA is dependent on several factors, making it not as reliable as RT-PCR.

South African isolates of ACSLV (78.8 to 97.9%) and ApMV (96% to 100%) were genetically highly similar when their CP nucleotide sequences were compared. The phylogenetic analyses did, however, group the isolates into different clades with 3 of 4 possible clades for ACLSV and 2 clades for ApMV. The isolates within these clades do not correlate with specific geographic areas both within and outside of South Africa. It appears that human mediated transport assisted in the import of isolates into South Africa and that a number of different isolates were introduced into the country.

Phylogenetic analysis also indicated that the majority of South African isolates from individual clades of ACLSV and ApMV are not host specific and that cross-infection between pome- and stonefruit is thus possible. The important implication of this is that sanitation of equipment between fruit groups is essential to prevent spread of the viruses and that one fruit group can serve as a source of infection for another fruit group.

Both DAS-ELISA and RT-PCR are dependent on virus titre for detection of ApMV and ACLSV and virus titre levels below detection levels can result in false negative results, in cases such as with new infections and seasonal variation. Recent breakthroughs in the development of alternative techniques for detection have been made which are not dependent on the virus titer. Virus infection usually leads to modulation of gene expression. This is regulated by endogenous, small RNA (sRNA) molecules (Singh et al., 2012). It would be interesting to study the plant’s response to ACLSV and ApMV infection by comparing sRNA profiles and using differentially targeted genes so that a marker signature can then be obtained. This can lead to earlier and more reliable detection of the viruses, which would greatly benefit the Deciduous Fruit Plant Certification Scheme.

During this study, ApMV was detected in pears for the first time in South Africa by RT-PCR, whilst it could not be detected by DAS-ELISA. ApMV was also not detected by DAS-ELISA in some apple samples and one peach sample. This finding renders the technique not suitable as a

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diagnostic technique within the South African Deciduous Fruit Plant Certification Scheme, and it is recommended that it be replaced by RT-PCR for effective detection of infected samples.

Future studies which can benefit from the basis laid during this study include investigation as to why pear isolates were not detected by DAS-ELISA. It was found in Chapter 2 that ApMV from pears were not detected and it was considered that the reason is that the genome of ApMV isolates infecting pears differs from other hosts. However in Chapter 4 it was shown that the coat protein genes are very similar. This is thus not the reason- it could possibly be due to inhibitors and this needs to be investigated. The use of fruit tissue instead of leaf tissue could also be investigated in future, as ripening fruit was reported as the best tissue type to use for ELISA detection of ACLSV in pears and ApMV could show a similar tendency (Cieslinska et al., 1995). Furthermore optimization of RT-PCR techniques for the detection of ACLSV and ApMV, such as the use of multiplex primers similar to multiplex primers developed by Menzel et al. (2003) would benefit the use within commercial laboratories. Investigation of alternative techniques for detection of ACLSV and ApMV which is not titer dependent, such as the use of sRNA profiles, would be useful to limit the number of false negative results. However this is likely not to be cost-effective for routine testing and would thus not be suitable for a Certification Scheme. Whole-genome sequencing of more ACLSV and ApMV isolates from South Africa and from other regions of the world would be useful to obtain more information on genetic variability within these viruses.

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ADDENDUM A

Aligned coat protein gene sequences of the 47 South African isolates of ACLSV, 47 GenBank ACLSV reference sequences and one CMLV GenBank reference sequence. A dot indicates a nucleotide identical to the top sequence.

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                            10        20        30        40        50        60        70        80        90       100
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MK9 JA AP         ATGGCAGCAGTTCTGAATCTGCAACTAAAGGTAGACGCAGATTTGAAGGCTTTCCTGGCCGCGGAAGGCAGACCCCTTCATGGAAAGACAGGGGCAATCC
AF170028 CMLV     ...T.G..GCGAT.......AACGAAC...A..C.TA.C..G....TT..C..T..AT.TCAA.CGAATC.T..A..C...CA..GAT...A.C.GGAAA
Y3 CH PE          ....................A.....G..A..G...AAG...C...G...C.....A..G.AA..CAATC.T......................G..CAG
HB2 CH PE         .....C...ACCT....C..A..G..G.....G...AAG..CC...G..T...T.....A.A...CAATC.T..T...................G..CAG
ST4 CH PE         .....C...AC.T....C..A..G..G.....G...A.G..A....GA........A..A.AA.CCAATC.C..A..G................G..CAG
SQ5 CH PE         .....C...AC.T....C..A..G..G.....G...A.G..A....GA........A..G.AA.CCAATC.C.....G................G..CAG
HB3 CH PE         .........AC.T....C.....G..G.....G...AGG..G....G............G.A..GCAATC.T...T.G................G..CAG
S3 CH PE          .....G...AC.T....C........G.....G...AGG..G....G.........A..G.A..CCAATC.T...T.G................G..CAG
ST2 CH PE         .....C..TAC............G..G.....G.....G..A....G...C........A.AA.CCAATC.T...T.G................G..CAG
HB4 CH PE         .....C...AC......C........G.....G........A....G...C........G.A..CCAATC.T...T.G................G..CAG
NC 001409 FR PD   ..................T....G..C.....G......AG.C.A.....A..T....G...A...AA......T......................C..
E1 RSA PE         ..................T....G........G......TCAC.A.....A...........A...AA......G...................T..C..
SQ2 CH PE         ............T....C..A..G........G.....C...C...GA..G.....T.T...A.....................................
hz-48 CH PA       ----------------------------------------------------------------------------........................
SQ8 CH PE         .....G..G...T....CT....G........G.........C....A..G.......T...A...............................T.....
CS CH PA          ----------------------------------------------------------------------------........................
S5 CH PE          ..................T....T........G.....CA..C....A..G.......T...A...............................T.....
A9 RSA PE         .................CT....G........G.....GA..C....A..G.......T...A......................G..............
hz-11 CH PA       ----------------------------------------------------------------------------........................
C4 RSA PE         .......................G........G.....C...C....A..G.......TT..A.....................................
YL1 CH AP         --------------------------------------------------------------------------------------------.....C..
G4 CH PE          .......................GT.......G.....C...C....A..G.......T...A.....................................
HS6 CH PE         ........G...........A...........G.....C..CC....A..G.......T...A...............................T.....
PP63 CH PA        ....................A...........G.....C...C....A..G.......T...A...............................T.....
PL1 CH PA         ....................A...........G.....C...C...GA..G.......T...A...............................T.....
Y1 CH PE          .......................GT.......G...A.C...C....A..G.......T...A...............................T.....
Z3 CH PE          ............T..........G........G.....C..CC....A..G.......T...A...............................T.....
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ADDENDUM A- cont.

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                        10        20        30        40        50        60        70        80        90       100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
HL1 CH PE       .......................G........G..T..C...C....A..G.......T...A...............................T.....
Z1 CH PE        .......................G........G.....C.......G...G...T...T...A...............................T.....
MB-CH CH AP     ------------------------------------------------------------------------------......................
YL2 CH AP       --------------------------------------------------------------------------------------------.....C..
LV-m312 LA AP   ----------------------------------------------------------------------------------------------------
C6 RSA AP       .....G.....G..C.....T..G........G.........C....A..G..........GA...................................A.
A4 RSA AP       ...........G..C.....T..G........G.....G...C....A..G...........A...................................A.
C1 RSA PE       .....G.....G..C.....T........A..G.........C...GA..G..........GA...................................A.
P1R9D9 BU CR    ------------------------------------------------------------------------------------------.......CA.
FJ752494 UK AP  ----------------------------------------------------------------------------------------------------
B81 JA AP       .....C.TKC.RAACY~.BCARY~~....R..G.........C....A..G...........A.....................................
MO41 JA AP      .....G.....AT.......T...T.......G.........C.......G...........A...................................T.
FR873735 IN AP  .....G.....AT.......T...........G.........C....A..G...........A...................................T.
5Be BE AP       ------------------------------------------------------------------------------......................
Malus0375 CA AP ----------------------------------------------------------------------------------------------------
A5 RSA AP       ....................A..G........G.........C....A..G...........A.....................................
A3 RSA AP       ........G.........T.A..G........G.........C....A..G...........A......................G..............
C5 RSA AP       ........G.........T.A..G.....A..G.........C....A..G...........A......................G..............
E2 RSA AP       ........G.........T.A..G.....A..G.........C....A..G...........A......................G..............
TK4 TU AP       ------------------------------------------------------------------------------......................
A1 RSA AP       .................CT.A..G........G.........C....A..G...........A.....................................
CL CH PA        ----------------------------------------------------------------------------........................
B1 RSA AP       ............T.....T.A..G.....A..G.........C....A..G.................................................
Pyrus0212 CA PA ----------------------------------------------------------------------------......................T.
Malus0545 CA AP ----------------------------------------------------------------------------........................
LV-py120 LA PA  ----------------------------------------------------------------------------------------------------
R1D2P-L BU PE   ------------------------------------------------------------------------------------------..........
Hatkoti IN AP   --------------------------------------------------------------------..........................T.....
P 205 JA AP     .....G.....G.....C..C...T....A..G.........C....A..G...........A...................................T.
A4 JA AP        .....G.....G..C..C...........A..G.........C....A..G...........A...................................A.
B6 JA AP        ..................T.............G.........C....A..G...........A...................................T.
MO-5 JA AP      .....G........A.....T...........G.........C....A.TG...........A...................................T.
```

ADDENDUM A- cont.

```
                         10        20        30        40        50        60        70        80        90       100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Ta Tao 5 US PE  ........CAC.T....C.....G..G.....G...AGG..G....G............G.A..CCAATC.T...T.G................G..CAG
U5 RSA PE       ..................T....G........G......TCAC.A.....A...........a..................................C..
U8 RSA PE       ...............G.CT....G........G.....C...C....A..G.......T...A......................G..............
U12 RSA AP      ........G.........T.A..G..................C....A..G...........A.....................................
U9 RSA PE       ....................A..G.......................A..A.................................................
U15 RSA AP      ..................T.A..G.....A..G.........C....A..G...........A.....................................
U2 RSA PE       .................C..A..GT....A..G.........C....A..G...........A.....................................
D3 RSA AP       ...........G...G....A..G.....A..G.........C....A..G...........A.....................................
U6 RSA AP       .........T.......CT.A..GT....A..G.........C....A..G...........A...................................T.
U7 RSA AP       .........T.....G....T..G........G.........C.......G...........A.....................................
U3 RSA AP       ....................A..G..................C.......G...........A.....................................
A119 RSA AP     ..............CG.CT.A..G........G.........C....A..G...........A.....................................
AP1 RSA AP      ..................T.T..G.....A..G.........C....A..G...........A......................G..............
AP2 RSA AP      .................CT.C..G.....A..G.........C....A..G...........A.....................................
AP3 RSA AP      ..................T.A..G.....A..G.........C....A..G...........A.....................................
AP5 RSA AP      ........G........CT.A..G.C...A..G.........C....A..G...........A.....................................
AP6 RSA AP      ................TCT....G.....A..G.........C....A..G...........A.....................................
AP7 RSA AP       ...........G..A.....T..GT.......G.........C....A..G...........A...................................A.
AP10 RSA AP     ..................T.A..G........G.........C....A..G...........A.....................................
AP15 RSA AP     ................TCT.A..G........G.........C....A..G...........A.....................................
AP16 RSA AP     ..................T.A..G.....A..G.........C....A..G...........A.....................................
AP17 RSA AP     .................CT.A..G..................C....A..A.................................................
AP19 RSA AP     ..................T.A..G.....A..G.........C....A..G...........A.....................................
AP20 RSA AP     ..................G.A..G........G.........C....A..G.................................................
AP22 RSA AP     ..........C.......T.A..G.....A..G.........C....A..G...........A.....................................
AP23 RSA AP     ..................T.A..G.....A............C....A..G...........A......................G..............
AP21 RSA AP     .................CT.A..G......A................A..G...........A......................G..............
AP9 RSA AP      .................CT....GT.......G.........C....A..G...........A......................G..............
Y10 RSA AP      ................TCT.A..G........G.........C....A..G...........A......................G..............
Y1 RSA AP       .................CT.A..G........G.........C....A..G...........A.....................................
AP13 RSA AP     ........GA..........A..G.....A............C.......A.................................................
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ADDENDUM A- cont.

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                           10        20        30        40        50        60        70        80        90       100
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|

AP11 RSA AP       ................T.TAC..G.....A..G.........C....A..G...........A.....................................
AP14 RSA AP       ................TCT....G........G.........C....A..G...........A.....................................
AP12 RSA AP       ................TCT....G.....T..G.........C....A..G...............................................A.
AP8 RSA AP        ................TCTAC..G........G.........C....A..G...........A.....................................
AP4 RSA AP        .......................G.....A..G.........C....A..G...........A.....................................

                          110       120       130       140       150       160       170       180       190       200
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MK9 JA AP         TGGAACAGATGTTGGAGTCCATCTTCGCGAACATAGCGATACAGGGGACGTCGGAGCAAACGGAGTTCCTGGATCTGGTGGTGGAAGTGAAGTCAATGGA
AF170028 CMLV     A.A.GTT...TC..AG...A..T.....C.....T..A..C..A...........CTC.........TA.TTCAACCACT..TC.G....T.CGTCC.AC
Y3 CH PE          ......T...AC................A.....C..A..C..A..A..A..A..............T.....CG...AA..C.........ATGT.T.G
HB2 CH PE         ......T...AC................A.....C..A..T..A..A.....A...........A........CG...AA..C.........A..T.T.G
ST4 CH PE         ......T...AC................A.....C..A..T..A..A.....A........A..A..T..C..CG...AA..C..G.....AAGG.GT.G
SQ5 CH PE         ......T...AC................A.....C...G.T..A..A.....A........A..A..T..C..CG...AA..C..G.....AAGG.GT.G
HB3 CH PE         ......T...AC.....C..........A.....C...G.T.....A..C..A........A..A.....C..CG...AA..C........AATG.GT.G
S3 CH PE          ......T...AC................A.....C...G.T..A..A..C..A..A.G......A.....C..CG...A...C........AATG.GT.G
ST2 CH PE         ......T...AC................A.....C...G.T..A..A.....A.....................CG...AT..C........AATG.GT.G
HB4 CH PE         ......T...AC................A.....C...G.T..A..A..A..C........A.....T.....CG...AA..C........AATG.GT.G
NC 001409 FR PD   ..........AC............................C.....A.....A.....G........T.....C..A..............A........
E1 RSA PE         ......T...AC..................................A..A........G.........T....C..........................
SQ2 CH PE         ..........AC............................T.....A..A.....A.....A..A............ACA.....G..A...........
hz-48 CH PA       ..........AG.C...........T....G......A..C..A.TA..C..A..A..G.....A..T........C.CA.....G.....A........
SQ8 CH PE         ..........AC.........................A..C.....A..C........G.....A..T.........AC...A..G.....A........
CS CH PA          ..........AC.........................A..C.................G..A.....T....GC...A.......G.....A........
S5 CH PE          ..........AC.........................A..T.....A.....A.....G..A.....T.........A.......G.....A........
A9 RSA PE         ..........AC............................T..A........A.....G..A.....T.....C...A.......G.....A........
hz-11 CH PA       .....................................A..C..A........A.....G.....A..T.....C...A.......G.....A........
C4 RSA PE         .....................................A..C..A........A.....G..A.....T.....C...A.......G.....A........
YL1 CH AP         ........................................C..A........A.....G..............C...A.......G.....A........
G4 CH PE          ...............................T..T..A..C..A........A.....G..A...........CT..A..............A........
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ADDENDUM A- cont.

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                       110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
HS6 CH PE       .....................................A..C..A..A.....A.....G..A...........C...A.......G.....A........
PP63 CH PA      .....................................A..C..A.........A.....G..A...........C...A.......G.....A........
PL1 CH PA       .....................................A..C..A.........A.....G..A...........C...A.......G.....A........
Y1 CH PE        .....................................A..C..A.........A..A..G..A...........C...A.......G.....A........
Z3 CH PE        .....................................A..C..A.........A.....G..A...........C...A.......G.....A........
HL1 CH PE       .....................................A..C..A..A.....A.....G..A...........C...A.......G.....A........
Z1 CH PE        .....................................A..C...............A.....G..A...........C...A.......G..............
MB-CH CH AP     .........CAC.........................A..C........C........G.....A..........G..A.........A...........
YL2 CH AP       .........CAC.........................A..C........C.....A..G..............G..A......G..A..A..G.....
LV-m312 LA AP   --------------------C...........C.......T..A.....C........G........T.....CG..AC......G..............
C6 RSA AP       .........CAC.....G.......................C..A.....c........G.....A.........CG..ACA.....G..C...........
A4 RSA AP       .........CA......G..........A...........T..A.....C..............A..TT....CG..ACA.....G..C.....C.....
C1 RSA PE       ..........AC.....G.......................C..A.....T........G...........c..CG..AC......G..C...........
P1R9D9 BU CR    .........CAC.....G.......................C..A.........A.....G........T.....CG..ACA.....G..C...........
FJ752494 UK AP  ----------AC.....--...........CATAGCGATC.........C........G.................CG..ACT.....G......A..C.....
B81 JA AP       .........YAC.....K.......................W..R..R..S........R..R......Y....CS.RS....R..R......R..M.....
MO41 JA AP      .........CAC.....G.......MK..................A.....C.......................CG.AC.......G......A..C.....
FR873735 IN AP  .........CAC.....G..........................A..C.....A...................G.AC...............A..C.....
5Be BE AP       .........C....................................A...................A..T....................G..............
Malus0375 CA AP  ------------------------------------------------------TT..TGGAA..ACAGG..CAA.CC...AAC.GA..TT.GAGTCCAT
A5 RSA AP       .........CAC.....G...............................C.......................c...........G......A..c.....
A3 RSA AP       ..........AC................................A........A...............................G..............
C5 RSA AP       ..........AC................................A..A.........................................G..............
E2 RSA AP       ..........AC................................A..A.........................................G..............
TK4 TU AP       ..........AC................................A..A...........G.....A...........A.......G......A........
A1 RSA AP       ..........AC................................A..A...........G.....A...........A.......G......A........
CL CH PA        .........CAC...................T............A..A...........G.....A...........A.......G......A........
B1 RSA AP       .........CAC.........................A.....A..A........A........A.........T....A.....G......A........
Pyrus0212 CA PA .........CAC.............................C..A..A..A..A.....G.....A........C..A...........................G.....
Malus0545 CA AP .........CAC.........................A..T..A.....A........G..A..A........C..A........G...........G.....
LV-py120 LA PA  ----------------------..T.................T..A.........A...........A........C..A........G..............
R1D2P-L BU PE   ..........AC.............................C..A.....A........G.....A...........A.......G......A........
Hatkoti IN AP   ..........AC.........................A..C..A..A..A..A.....G.....A........C..A........G......A........
P 205 JA AP     .........CAC.....G...............................C.....A.....A...........CG..C........G......A..C.....
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ADDENDUM A- cont.

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                     110       120       130       140       150       160       170       180       190       200
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
A4 JA AP     .........CAC.....G.......................C..A.....C........G...........C..CG..A..........C.....C.....
B6 JA AP     ..........AC...............................A..A.....A.....G..................A.......G..............
MO-5 JA AP   ......T..CAC............................C.....A..C........G..............CT..........G..C...........
Ta Tao 5 US PE ......T...AC................A.....C..AG.T..A..A..C..............A.....C..CG...AA..C..G......AAG.GT.G
U5 RSA PE    ......T...AC..................................A..A........G.........T....C..........................
U8 RSA PE    ..........AC.............................T..A........A.....G..A.....T.....C...A.......G.....A........
U12 RSA AP   ..........Ac...............................A..A...........G..........................G..............
U9 RSA PE    ..........A..........................AG....A..A...........G........T.................G..............
U15 RSA AP   .........CAC.............................C..A.....C.....A..G.....A........C..A.................G.....
U2 RSA PE    ..........A..........................A...........T.....A..G.....A.........G.AA.......G..C..A........
D3 RSA AP    ..........AC...........................................A..G..A..A....................G.....A........
U6 RSA AP    .........CAC.....G...............................C.....A.....A...........CG.A........G.....A..C.....
U7 RSA AP    ..........Ac.....G.........................A.....C........G.....A..T.....CG..A.......G..C.....C.....
U3 RSA AP    ..........AC...............................A..A...........G.....A........C...........G.....A........
A119 RSA AP  .........CAC...............................A..A..........................C...........G..............
AP1 RSA AP   .........YAC...............................A..A.......................................G..C...........
AP2 RSA AP   ..........AC...............................A..............G..........................G..............
AP3 RSA AP   ..........AC...............................A..A...........G.........................................
AP5 RSA AP   ..........AC.............................C..A.....A..A.....G.....A........C..AA......................
AP6 RSA AP   .........CAC...............................A.....A........G..........................G..C..A........
AP7 RSA AP   .........CAC.....G.......................C..A.....C........G...........C..CG..AC......G..C.....C.....
AP10 RSA AP  ..........AC.............................C..A....................A........C...A.......G..............
AP15 RSA AP  ..........AC.............................T..A..............G..........................G.....A........
AP16 RSA AP  .........CAC...............................A..A........A..G.....A.........T..........G.....A........
AP17 RSA AP  .........CAC..........................AG....A..A...........G..........................G..............
AP19 RSA AP  .........CAC.............................C..A..A........A..G.....A..............A.....G.....A........
AP20 RSA AP  .........CAC...............................A..A...........G.....A.........G....A.....T..............
AP22 RSA AP  .........CAC...............................A..A........A..G.....A....................G.....A........
AP23 RSA AP  ..........AC...............................A..A..................A....................G..............
AP21 RSA AP  ..........AC...............................A.......................................A..G..............
AP9 RSA AP   ..........AC.............................T..A..............G..............C...........G........G.....
Y10 RSA AP   ..........AC.............................C..A.........................................G..............
Y1 RSA AP    ..........AC......................................A........G.....A........C...........G........G.....
AP13 RSA AP  .........CAC...........................G.C..A..............G..........................G.....A........
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ADDENDUM A- cont.

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                          110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AP11 RSA AP     .........CAC.........................A..T..A..............G.....A........C..A........G........G.....
AP14 RSA AP     ..........AC............................G..A..A...........G.....A........C...........G.....A........
AP12 RSA AP     .........CAC............................C..A.....C........G.....A.....A..CG..........G..C..A..C.....
AP8 RSA AP      .........CAC............................C..A.....C........G..............C...A.......G........G.....
AP4 RSA AP      ..........AC............................C..A..............G..............C...A.......G........G.....

                          210       220       230       240       250       260       270       280       290       300
                 ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MK9 JA AP       GGACCAAAAGGTGATCGGGTCATACAACCTGAGGGAGGTGGTCAATATGATCAAGGCCTTCAAGACTACATCTTCGGACCCAAACATCAGCAACATGACT
AF170028 CMLV   ..GTGCGG.A.C.T.A.T...C.....T.C....AC.A.T..TGGGT....A..ACTT...GC.T.A...AACGA...TGA...T..TTCT.GG.GC...
Y3 CH PE        C..T.CC.CA...C.GCA.AGG.....T....A..C...C..GG.GC........CTT...CG....G...........AAG.......AT.C.C.T..C
HB2 CH PE       ...T.CC.CA...C.GCA.AGG.....T....A.ACA..C..GG.GC........CTT..TCG...C............AAG.......A..C.C.A..C
ST4 CH PE       ...T.CC.CA...C.GCA.AA......T......AC...C..GG.GC........CTT..TCG...C.T..........AAG..T....AT.C.C.C...
SQ5 CH PE       ...T.CC.CA...C.GCA.AAG.....T......AC...C..GG.GC........CTT..TCG......G.........AAG.......A..T.C.T...
HB3 CH PE       ...T.C..CA...C.TCA.AAG.....T......AC...C..GG.GC....A...CTT..TCG...C............AAG.......ATGC.C.T...
S3 CH PE        .....CG.CA...C.TCA.AAG............AC...C..GG.GC....A..ACTT...CG............A...AAG.......AT.C.C.T...
ST2 CH PE       .A...CC.CA..AC.GCA.AAG.....T....A.ACA..C..GG.GC........CTT...CG................AAG.......AT.C.C.T..C
HB4 CH PE       .A.T.CC.CA...C.GCA.AA......T....A.ACA..C..GG.GT.......ACT....CG................AAG......GAT.C.C.T..C
NC 001409 FR PD ...T...TCA..CC.......T..........A...A........CT.......A..T...........C...........G.......A...A.....A
E1 RSA PE       ...T..G.CC..CC.......T..........A.........G..CC.......A..T...........C........T..G.......A...A......
SQ2 CH PE       ...T..G........A...........TT...A...AA.A.....C........A..............C........T..G.......A.........C
hz-48 CH PA     ...T..G........A....AT.....T....A......C.....C........A..T....G..T...C........T..G.......A...T..T..C
SQ8 CH PE       ......G........A.....T.................C.....C........A..T.....A.....C...........G..T....A...T.....C
CS CH PA        ......G........A...........T....A....A.A..............A..............C........T..G.......A.........C
S5 CH PE        ......G........A...........T.C..A....A.C..............A..............C........T..G.......A.........C
A9 RSA PE       ......G........A................A....A.C..............A.......G......C........T..G..T....A.........C
hz-11 CH PA     ......G........T...........T....A....A.C..............A.......G......C........T..G.......A.........C
C4 RSA PE       ......G........A...........T....A....a.C..............A..............C........T..G.......A...T.....C
YL1 CH AP       ......G........A...........T....A....ACC..............A.......G......C........T.....T..............C
G4 CH PE        ......G........A..A..G.....T....A....A.C..............A.......G......C.....A..T..G.......A.........C
HS6 CH PE       .....GG..A.....A...........T....A....A.C..............A..A....G..................G.......A.........C
PP63 CH PA      ......G........A...........T....A....A.C..............A..A....G..................G.......A.........C
PL1 CH PA       ......G.G......A...........T....A....A.C..............A..A....G.................AG.......A.........C
Y1 CH PE        ...............A...........T....A......C..............A.......G......C........TTTG.......A.........C
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ADDENDUM A- cont.

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                         210       220       230       240       250       260       270       280       290      300
                  ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Z3 CH PE         ......G........A...........T....A....A.C..............A.......G......C........T..G.......A.........C
HL1 CH PE        ......G........A...........T....A....ACC..............A..............C........T..G.......A..G......C
Z1 CH PE         ......G........A...........T....A....A.C......................G......C........T..G.................C
MB-CH CH AP      ...T..G........AA.T..C..........AATC......GG..C.T........A.......................G..T..A.A.GGG.....A
YL2 CH AP        ...T..G........AA.T..C...........ATC......GG..C.T........A.......................G.....A.A.GGG......
LV-m312 LA AP    ......G...........C..C.T...T....A...........G.........AA.............C........T..G..................
C6 RSA AP        ......G........A..c..C.T...t....A...........GCT........AT.....................T..G.......A...T......
A4 RSA AP        ...T..G........A..C..C.T...T....AA...........CT....A..AAT.....................T..G..T....A..........
C1 RSA PE        ......G........A..C..C.T...T....A...........G.T........AT.....................T..G.....A.A.........C
P1R9D9 BU CR     ...T..G........A.....C.T........A...A.......G.T........AT.....................T..G.....A.A..........
FJ752494 UK AP   ......G........A.....C.T...T....A.............T....A...A.A....................T..G..T..............C
B81 JA AP        ...T..G..R...R.R..R..S.W...T....AR..A........CW.R..M..RSYW....................Y..G.....M.....Y.....Y
MO41 JA AP       ......GM.....G.G.......T...T....A......A.....CT.......AATA....G......T...........G.....A.....T.....C
FR873735 IN AP   ......G......G.G.......T...T....A......A.....CT.......AATA....G......T........T..G.....A.....T.....C
5Be BE AP        ...T..G.....A...................A.....................A..............C........T..............T......
Malus0375 CA AP  CTT.GCG..CA.AGCGATACA.GGA.CGTC.GA.C..AC..AGTT.C..GATTT..TGG.GG.AGTG.A...AAT...GGATC.G.AAGTG.T.GG.T.A
A5 RSA AP        ......G..............C.T...T....A............CT...............G..................G...........T.....C
A3 RSA AP        ...T..G..A...........T.....TT...A............C........A..............C........T..G..T.......G.......
C5 RSA AP        ...T..G..............C.....TT...A............C........A..............C........T..G..........G.......
E2 RSA AP        ...T..G..............C.....TT...A............C........A..............C........T..G..........G.......
TK4 TU AP        ......G.....A........C.....TT...A............C........A..T...........C.....A..T..G..................
A1 RSA AP        ......G..A..A........C.....TT...A............C........A..T.....A.....C........T..G..................
CL CH PA         ......GG.A..A..A.....C.....TT...A............C........A..T.....A.....C........T..G..................
B1 RSA AP        ...T..G.....A........C.....TT...A.........G..C........A..T...........C........T..G..................
Pyrus0212 CA PA  ...T..G.....C..A..A..C..........A.....................A..A...........C........T..G..................
Malus0545 CA AP  ......G.....C........T.....T....A............C........A..T...........C........T..G..................
LV-py120 LA PA   ...T..G.....T...................A......A.....C........A..T...........C....T...T..G..................
R1D2P-L BU PE    ......G.....C..A.....C.....T....AA....................A..A.....A.....C........T..G..................
Hatkoti IN AP    ...T..G.....C........C.....T....T....................................C........T..G.....T............
P 205 JA AP      ...T..G......G.G.......T...T....A......A...GG.T.......AATA....G..................G..T..A....G......C
A4 JA AP         ......G........A..C..C.T...T....A...........G.T....A...AT........................G.....A.A.........C
B6 JA AP         ...T..G..A..A.....A..C.....TT...A...A........C........A..............C........T..G..................
MO-5 JA AP       ...T..G......G.GAAT..C.....T....A.TCA.....GG..C.C......AT......A.................G.....A.A.GGG......
Ta Tao 5 US PE   ...T.CC.CA...C.GCA.AAG.....T......AC...C..GG.GC........CTT..TCG...C............AA........AT.C.C.T..C
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ADDENDUM A- cont.

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                       210       220       230       240       250       260       270       280       290       300
                 ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
U5 RSA PE       ...T..G.CC..CC.......T..........A.........G..CC.......A..T...........C........T..G.......A..........
U8 RSA PE       ......G........A.................A......C..A...........A.......G......C........T..G..T....A.........C
U12 RSA AP      ...T..G................c.....tt...A............C.........A..................C........T..G..................
U9 RSA PE       ...T..G..A......................A.........G...........A.........................................c
U15 RSA AP      ......G.....C..A.....C.....T....A.......................A..A.....A.....C........T..G..................
U2 RSA PE       ......G.....A........C..........AA.....A...G.C..T.....a..T....G......C...........G.......A.GG.......
D3 RSA AP       ...T..G.....A........C.....TT...A............C.........A..T....A.....C........T..G...........G.......
U6 RSA AP       ...T..G......G.A.......t...T....A......A...G..T.......AATA....G.......................G..T..A.A.........C
U7 RSA AP       ...T..G...........C..C.T...T....AA...........CT....A..AAT....................T..G..T....A.GG.......
U3 RSA AP       ...T..G.....A........C.....TT...A............C.........A..T...........C........T..G..................C
A119 RSA AP     ......G........A.....C.....T....A............C.........A..................C........T..G.......A..........
AP1 RSA AP      ......G..A.....a.....C.....TT...A............C.........A..................C........T..G..................
AP2 RSA AP      ......G..A...........C.....TT...A............C.........A..................C........T..G.......C..........
AP3 RSA AP      ......G..A.....A.....C.....TT...A............C.........A..................C........T..G...........G.......
AP5 RSA AP      ...T..G.....C........T.....T....A.......................A..................C........T..G..................C
AP6 RSA AP      .....G........G.....C.T....T...A......................A..............T........T..G.................
AP7 RSA AP       ......G........A..C..C.T...T....A.............T.A..A...AT............C........T..G..T..A.A.........C
AP10 RSA AP     ......G..............C.....T....A.............T.......AA..................C........T..G..................
AP15 RSA AP     ......G..A...........C.....TT...A.........................................C........T..G..................
AP16 RSA AP     ...T..G...C.A........C.T...TT...A............C.........A..T...........C........T..G...........G.......
AP17 RSA AP     ...T..G..............C......T...A.........G..G.........A..T...........C...........G............A......
AP19 RSA AP     ...T..G...C.A........C.C...TT...A............C.........A..T...........C........T..G...........GA......
AP20 RSA AP     ...T..G.....A........C.....TT...A............CG........A..T...........C........T..G.....G.A..GA......
AP22 RSA AP     ...T..G...C.A........C.....TT...A............C.........A..T...........C........T..G...........GA......
AP23 RSA AP     ...T..G..A...........C.....TT...A............C.........A..................C........T..G...........G.......
AP21 RSA AP     ...T..G..............C.....TT...A............C.........A..................C........T..G...........G.......
AP9 RSA AP      ......G..A...........C.T...T....A............C.........A..................C........T..G..................
Y10 RSA AP      ...T..G..............C.....TT...A............C.........A..................C........T..G...........G.......
Y1 RSA AP       ...T..G.....R........C.....TT...A.......................A..T...........C........T..G...........G.......
AP13 RSA AP     ...T..G..A...........C.....T....A.......................A..................C...........G..................
AP11 RSA AP     ......G........T.....C.....T....AA...........C.........A..................C........T..G..................
AP14 RSA AP     ......G........G.....C.....TT...A............C...........TT...................T..G..................
AP12 RSA AP     ......G........A.....C.T........A...........T.T....A...AT...................T..G.....G...........
AP8 RSA AP      ......G..............C.T...T....A.............T.......AATT....G......C........T..G..................
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ADDENDUM A- cont.

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                         210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AP4 RSA AP      ......G..A...........C.T...T....A............CT.......AAT........................G..T...............

                         310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MK9 JA AP       TTCCGCCAGGTGTGTGAGGC-CTTCGCACCTGAGGCGAGGAACGGGTTGGTCAAACTGAAGTATAAAGGGGTTTTCACTAACCTCTTTACAACCATGCCG
AF170028 CMLV   ...A.G..AA.T.....ACA-T..T..TGAAT.T..C.AA..T..T...A.AGC........TACTCA...AA.A...A..T..G.A..GG.AGGCA..T
Y3 CH PE        ..TA.G..AA.A..C..A..~T..T..C..A.....C..AG.T..A............C..ATA.TT..T.....G..C..T..G.AC.A...A.....A
HB2 CH PE       ..TA.G..AA.A..C..A..~...T..T..G.....C..AG.T...C....T......C..ATG.TT..C...C.G..C..T..G.AC.A...A.....A
ST4 CH PE       ...A.A...A.A.....A..~......T..G..A..C..AG.T...C...........C..ACA.TT..T.....G..C..T..G.A..AG..A......
SQ5 CH PE       ..TA.G...A.A..C..A..~......T..G..A..C..AG.T...............C..ACA.TT..T..C..G..C..T..A.AC.AG.........
HB3 CH PE       ..TA.G..AA.A.....A..~...T..T..A.....T..AG.T...C....T..G..C..AACC.TT..T...C.......T..G.AC.A...T.....T
S3 CH PE        ...A.G..AA.A.....A..~T..T..C..G.....C...G.T...C....T..G..T..ACC.TT..C...C........T.G.AC.A.........T
ST2 CH E        ..TA.G...A.A..C.....~T..T..T..G.....T..AG.T..AC...........C..ACA.TT..T.....G.....T..A.AC.A...A.....T
HB4 CHPE        ..TA.A...A.T.....A..~T..T..T..G.....C..AG.T...C...........C.AACA.TT..T..C..A..C..T..G.AC.AG..A.....T
NC 00409 FR PD  .....T.....C.....A..~T........A.....A..A..T..AC........T....A..C...........T..A..............T......
E1 RA PE        .....T.....T........~T.....G..A.....C..A..T..T........G..T.....C...........T.................T.....A
SQ2CH PE        .....G...T.A........~......C..A..A............C.............A..C..............C........C..G..A.....T
hz48 CH PA      .....G.....A........~T..T....GCA.A........T..AC........T.A.....C............C.......T..C.....A..A..A
S8 CH PE        ..T..G.....A........~T.....G..C..A..A..A..T..AC....T..G........C..............A..............A......
CS CH PA        .....G..A..A........~G..T.....C.....A..A..T...........G..T....................A..T........G..A.....A
S5 CH PE        .....G..A..A........-T..T.....C.....A..A..T...........G..T....................A..T........G..A.....A
A9 RSA PE       .....G..A..A........-G..T..............A..T..............T.....C........C.....A..T........G..A.....A
hz-11 CH PA     .....G.....A........-G..T..G........A..A..T..............C....................A.....T.....T..A.....A
C4 RSA PE       .....G..A..A........-G..T........A..A..A..T........A.....C..A.................A.....T........A.....A
YL1 CH AP       .....G..A..A........-G..T.T...C.....A..A..T...........G..C..A.......................T..C..T..A.....A
G4 CH PE        .....G..A..A........-G..T...........A..A..T..............C.....C..............A.....T..C..T..A.....A
HS6 CH P        .....G..A..A........-G..T...........A..A..T..............C.....C..............A.....T.....T..A.....A
PP63 CHPA       .....G..A..A........-G..T...........A..A..T..............C.....C..............A.....T.....C..A.....A
PL1 CHPA        .....G..A..A........-G..T...........A..A..T..............C.....C..............A.....T.....T..A.....A
Y1 CHPE         .....G..A..A........-G..T...........A.....T..............C....................A...........C..A.....A
Z3 C PE         .....G..A..A........-G..T...........A..A..T..............T....................G.....T.....T..A.....A
HL1CH PE        .....A..A..A........-G..T...........A..A..T..A...........C....................G.....T.....T..A.....A
Z1 CH PE        .....G..A...........-G..T.....C.....A..A..T..............C....................G.....T.....T..A.....A
MB-CH CH A      ...........T........-...T..C........A..A..............G..A....................G...........G........T
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ADDENDUM A- cont.

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                         310       320       330       340       350       360       370       380       390       400
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
YL2 CH AP        ...........T........-...T..C........A..A..............G..A.....C..............G.....T..C..G........T
LV-m312 A AP     ....................-T.................A...............T.......C..........................T........A
C6 RSA P         ....................-T..t..........................A..GT......................A........C...........A
A4 RSAAP         ...........T.....A..-T.....T..G........A..T..A.....T...T.......C..............A.....T..CT.T..T.....A
C1 RS PE         ...........C........~A..T..T..g..A..A.....T........t..g........C..............A.....t...T.T........A
P1R99 BU CR      ........A..C........-A..T..T..A..A.....A...........T...T......................G.....A...T.T........A
FJ752494 UK AP   ..T.CG.CA.GTGTGTGA.GCT..T...........A..A..T...C....T....................................T.T........A
B81 JA AP        .....Y...........R..WT.CGCWC.TGAG.CMAGAA.YG..Y.S.TCA.GYTRA.GTAYA..G...T...CACWA.C.TYT..WCW.CYAYGC.K.
MO41 JA AP       .................A..-A..T..T..C..A..A..A..T...C.C.....G........C..............A.....T...T.T..A.....T
FR873735 IN AP   .................A..-A..T..T........A..A..T...C.C.....G........C.....T........A.........T.T..A.....T
5Be BE AP        ....................-T.................A....................A..............T..A.....................
Malus0375 CA AP  .A.AAT.T.AA.GAG.T..TCAACATG.T.AAG.C.TTCA.GACTACATC.TCGGACCC.AACATC..CAACA.GACT.TC.G..AGGTGTGTG.G..-T
A5 RSA AP        ....................-A..T..T..C.....A..A..T...C.......G........C..............A..T......T.T..A.....C
A3 RSA AP        ....................-T........G.....A..............T..........................C...........G.........
C5 RSA AP        ....................-T........G.....A..............T..........................C...........G........A
E2 RSA AP        ....................-T........G.....A..............T..........................C...........G........A
TK4 TU AP        ....................-T........G.....A..A....................A.......................T...............
A1 RSA AP        ....................-T........G.....A.......................A.................C...........G.........
CL CH PA         ....................-T........G.....A..A...........T......................................G.........
B1 RSA P         ....................-.........C.....A..............T...........c..............C.....................
Pyrus012 CA PA   ....................-T........G.....A..A...........T........A..............T.......................A
Malus545 CA AP   ....................-T..............A..A...........T...T.......C..............C...........G..A.....A
LV-p120 LA PA    ....C...............-T..T.....A.....A.........C....G...T....A................................A..T..A
R1DP-L BU PE     ....................-T........G........A......C....A...T.......C...........T..C........C..T........A
Hakoti IN AP     ....................-T........G..A.................G...T.A.....C........C..T..............G.........
P205 JA AP       ........A...........-A..T..C..G.....A..A..T...C.C..A..G..A.....C........C.....A.........T.T..T.C..T
A4 JA AP         ........A..T.....A..-T..T..C..G........A..T...C....T.......................T..A.....A...T.T..T.....A
B6 JA AP         ....................-T........G.....A..............T....................C..T..C...........G........A
MO-5 JA AP       .....T.....C........-T..T..T..G.....A..AG.....C..........A.....C........C.....A....................T
Ta Tao 5 USPE    ..TA.G...A.A.....A..-......C..G..A..C...G.T........T.....C...ACA.TT..T........C..T..G.A..A...A......
U5 RSA PE        .....T.....T........-T.....G..A.....A..A..T..T.....G..G..T.....C..G........T.................T.....a
U8 RSA PE        .....G..A..A........-G..T..............A..T..............T.....C........C.....A..T........G..G.....A
U12 RSA P        ....................-t........G.....A..............T..........................C...........G........A
U9 RSA E         .....t..............-T..............A..............T...T......................A.....................
U15 RS AP        .....t..............-T..............A..A...........T...T....A..............T..a....................A
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ADDENDUM A- cont.

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                  310       320       330       340       350       360       370       380       390       400
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
U2 RS PE     ........A...........-G........c.....A..a..............G..A....................C...........G........T
D3 RA AP     .....T..............-T........G.....A..A...........T....................C.....C..............A......
U6 SA AP     ........A...........-T........G.....A..A...........T....................C.....C..............A......
U7RSA AP     ...........A.....A..-G.....T..G........A..T........G..G..C.....C.....C........C.....T...T.T..A.....C
U RSA AP     .....T..............-T........G.....A..............T..........................C....................A
A119 RSA AP  ....................-T..T..G...........A...........G.......................T..A...........T........A
AP1 RSA AP   ....................-T........G.....A..A...........T...........G...........T..C...........G.........
AP2 RSA AP   ....................-T.....G..G.....A..............Y...T......................C...........G.........
AP3 RSA AP   ....................-T........G.....A..A...........T.......................T..C...........T........A
AP5 RSA AP   ....................-T........G.....A..............G...T.......C...........T..............T.........
AP6 RSA AP   ....................-T..T..G..G....................T...........C..............C...........T.........
AP7 RSA AP   ........A..T........-T..T..C..G........A..T...C....T...T.......C..............G.....A...T.T........A
AP10 RSA AP  .....T..............-T..T..G........A..............T...T......................C...........C.........
AP15 RSA AP  ....................-T..T.....G.....A..A...........T...........C...........T..C.........T.G.........
AP16 RSA AP  ....................-T........G.....A..............T...T......................C.....................
AP17 RSA AP  ....................-T........G.....A..............T................................................
AP19 RSA AP  ....................-T........G.....A..............T..........................C.....................
AP20 RSA AP  ........A..T........-T.....T..G.....A.....G........T...G......................C....................T
AP22 RSA AP   ....................-T........G....A..............T..........................C.....................
AP23 RSA AP   ....................T........G.....A..A...........T..........................C...........G.........
AP21 RSA AP   ...................-T........G.....A..A...........T..........................C...........G.........
AP9 RSA AP    ...................-T........G.....A..A...........T...A.......C..............C...........T.........
Y10 RSA AP    ...................-T..............A..............T..........................C...........G........A
Y1 RSA AP     ....T.............-T........G.....A..A...........T..........................C..............A......
AP13 RSA AP   ...................-T..............A..A...........T...T.......C..............C.....................
AP11 RSA AP   ...................-T.....G..G.....A..A...........T...T....A...........................T..........A
AP14 RSA AP   ...................-T..T..G..G.....A..A...........T...T......................C.........T...........
AP12 RSA AP   ...................-T..T.....G.....A..A..G............T.......C..............C.........T.T..T.....A
AP8 RSA AP    ...................-T..T..G........A..A..G............T...................T..............T.........
AP4 RSA AP    ...................-T........G.....A..............T...T.......C..............C...........T........A
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ADDENDUM A- cont.

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                         410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MK9 JA AP       GAAGTAGGAAGTAAGTACCCGGAGCTGATGTTCGACTTCAACAAGGGCCTTAATATGTTTATCATGAATAAGGCCAA~CAGAAGGTCATTACTAATATGA
AF170028 CMLV   AGTC.T..TG.A.....T......T.ATGC..T.....T..TGGA..T...G.....A.AGATC.......T.AAC.GAGA.....G..C..G...T.C.
Y3 CH PE         ..G..G..T.AC..A..T..C..A..C.....T...........A..G......CCAA.GC.A.........A..C.AAGAGTA..TG.......CC.T.
HB2 CH PE        ..G..G..T.AC..A..T..T.....C.....T..T...........G......CCAA.GC.A........AA..C.AAG.GTA...G....C..CC.C.
ST4 CH PE        .....G..C.AC..A..T..C.....C.....T...........A..G..G..CCCAA.GT.G.........A..C.GAGAGTT..TG.......CC.C.
SQ5 CH PE        ..G..G..C.AC..A..T..C..A..C.....T...........A..G.....CCC.A.GT.A.........A.TC.AAGAGT...TG....C..CC.T.
HB3 CH P         ..G..G..C.A...A..T..T.....C.....T..T........A..G.....CCC.A.GT.G........AA.TC.GAGAGTT..AG.A......C.A.
S3 CH P          ..G..G..T.AC........T.....T.....T..T........A..G.....CCC.A.GT.G........AA.TC.GAGAGTT..GG.G..C..CC.T.
ST2 CHPE        ..G..G..C.A...A..T..C.....C.....T.....T.....A..G.....CCC.A.GT.A.........A..C.AAG.GT...GG.C.....CC.C.
HB4 C PE         ..G..G..T.AC..A..T..C.....C.....T.....T.....A..G..C..CCCAA.GT.G.........A..C.AAG.GT...TG.C.....CC.C.
NC 01409 FR PD  .....G..G..C..............A.....T..T.....T.....T..G........C........C......C.G.....A..T..A..C.......
E1 SA PE         ..G..G.....C........A.....A.....T..T.....T.................C..............TC.A.....A..T..A..C.......
SQ CH PE         ..G..G........A..T.....A........T..T..T..T.....T.....C.....C........C......C.A.....A..A..A..G..C....
h-48 CH PA       ..G..G.....C..A..T..A...T.A.....T.....T..T..........TC......G..C..........TC.G..A.....A..A.A....C...
SQ8 CH PE        ..G.....G..C..A..T..A..AT..........T..............C..C.....C........C......C.A........G..A..C.......
CS CH PA         ..G..G..T..C..A.....A...........T..T..T..T........C..C.....C........C.....TC.G..A.....G..A..........
S5 CH PE         ..G..G.....C..A.....A...........T..T..T..T........C..C.....C........C.....TC.G..A.....A..A..C.......
A9 RSA PE        ..G..G..G..C..A.................T..T.....T........C..C..............C.....TC.G..A.....A..A..........
hz-11 CH P       ..G..T..G..C..A.................T........T........C..C.....C........C.....TC.G..A.....G..A..........
C4 RSA PE        ..G..T..GG.C..A.................T..T.....T........C..C.....C........C.....TC.G........G..A..........
YL1 CH AP        ..G..T..G.....A..........................T.....T..G-------------------------------------------------
G4 CH PE         ..G..T..G..C..A.................T..T..T..T..A.....C..C.....C........C.....TC.G..A.....A..A..C.......
HS6 CH PE        .....T..G..C..A.................T..T.....T........C..C.....C..A.....C.....TC.G........T..A..........
PP63 CH P        .....T..G..C..A.................T..T.....T........C..C.....CG.A.....C.....TC.G........T..A..C.......
PL1 CH PA        .....T..G..C..A.................T..T.....T........C..C.....C..A.....C.....TC.G........T..A..C.......
Y1 CH PE         ..G..T..G..C..A........A........T..T.....T...........C.....C........C.....TC.G........A..A..........
Z3 CH PE         .....T..G..C..A.................T..T..T..T........C..C..............C.....TC.G.....A..A.............
HL1 CH PE        ..G..T..G..C..A.................T..T..T..T........C..C.....C........C.....TC.G..A.....A..A..........
Z1 CH PE         ..G..T..G..C..A.................T..T..T..T...........C.....C........C.....TC.G........A..A..........
MB-CH C AP       .....G..T.....A.....A.....T...........T.....A..T..G...........A.....C.....TC.G..A..A..A..A..........
YL2 CH AP        .....G..T..C..A.....A.....T..............T.....T..G-------------------------------------------------
LV-m312 LA AP    ...........G.......................T.....T.....G..G..C........A.....C......C.G..A..A..T.....C.......
C6 RSA AP        .....T.....C..A.................T..T.....T...........C....................TC.A..A..A.....A..........
A4 RSA AP        .....T..TG.G..A..T..A..A..T.....T..T.....T..A..G..G...........A.....C.....TC.G..A..A..A..A..C.......
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ADDENDUM A- cont.

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                        410       420       430       440       450       460       470       480       490       500
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C1 RSA PE        .....T...G.G..a.....a..A..t.....T..T.....T..A..G..g..c.....C..A.....C......C.G..A.....g.....c.......
P1R9D9 BU CR     ..G..T..GG.G..A........A..C.....T.....T..T..A..G..G..C........A.....C..A..TC.G..A..A..G..A..A.......
FJ752494 UK AP   .....G.....A..A.....................T.....T.....G..G..C.....C........C......C.G.....A.....A..........
B81 JA AP        A.GTRG.WRGKA..TAC..G.AGYTKATGT..GATT.CA.TA.G..Y.TKA.CATGT..ATMATGA.YA.G.C...GCA.A...TMATWACTA.TATGA.
MO41 JA AP       .....G..CG.C..............T.....T..T.....T........A........C..A.....C..A..TC.G..A..A..A..A..........
FR873735 IN AP   .....G..CG.C..............T.....T..T.....T........A........C..A.....C......C.G..A..A..A..A..C.......
5Be BE AP        .....G........A....................T.................C.....................C.CA.A..A...........C....
Malus0375 CA AP  TTC.C.CCTGAGGC.AGAAAC.G.T..G..AAATTGAAGT.T..A..GG..TTC.CAAACC..T.T.CA.CCATGCCGG.AGTA.GA.G..AAT.CCC.G
A5 RSA AP        .....T..CG.C..............T.....T..T..T..T........A..c.....C..A.....C......C.G..A..A..A..A..C.......
A3 RSA AP        ..............A.................T..T.....T...........C....................TC.G..A..A.....A..........
C5 RSA AP        ..............A.................T..T.....T.....T.....C....................TC.G..A..A.....A..........
E2 RSA AP        ..............A.................T..T.....T.....T.....C....................TC.G..A..A.....A..........
TK4 TU AP        ...........C..A.................T..T.....T.....T.....C.....................C.G..A..A.....A..........
A1 RSA AP        ...........C..A.................T..T.....T.....T.....C.....................C.G..A........A..........
CL CH PA         .....T..T..C..A.................T.....T..T.....T.....C.....................C.G..A..A.....A..........
B1 RSA AP        .....G..........................T..T.....T.....T.....C..............C......C.A..A..A.....A..........
Pyrus0212 CA PA  ..............A.................T..T...........T.....C.....C................C.A..A..A.....A..........
Malus0545 CA AP  ...........C..A.................T..T.....T........C........C..A.....C.....TC.A..A..A................
LV-py120 LA PA   ...........C....................T....................C..............C.....TC.A..A..A.....A..A.......
R1D2P-L BU PE    .....G..G..C....................T.....T..T..A..G.....C..............C......C.G..A..A................
Hatkoti IN AP    .....T.....C....................T..T.................C.....C...............TC.G..A..A.....A..C.......
P 205 JA AP      .....G..GG.G.....T........C.....T........T........A........C..A.....C..A...C.G.....A..G..A..........
A4 JA AP         .....T...G..........A.....C.........T.....T..A..G..G..C........T.....C..A..TC.G..A..A..A.............
B6 JA AP         ..........A.....................T..T.....T.....T.....C.....C........C......C.A..A..A.....A..........
MO-5 JA AP       .....G..TG.C..A.....A.....C.................A..TT.G...........A.....C..A..TC.G..A.....A.............
Ta Tao 5 US PE    ..G..G..C.A.....T..T.....T.....T..T........A..G.....CCC.A.GC.G.....C...A..C.GAG.GT...TG.C.....CC.T.
U5 RSA PE        ..G..G.....C........A.....A.....T..T.....T.................C..............TC.G..A........A..C.......
U8 RSA PE        ..G..G..G..C..A.................T..T.....T........C..C..............C.....TC.G..A.....A..A..........
U12 RSA AP        ...............................T..T.....T.....T.....C....................TC.G..A..A.....A..........
U9 RSA PE        ..............A....................T..T..............C....................TC.A..A..A.....C..........
U15 RSA AP       ..............A.........T.......T..T...........T.....C.....C................C.a..A..A.....A..........
U2 RSA PE        .....G...G.G..A.................T..t..T..T.....T..............A.....C......C.G..a........A..........
D3 RSA AP        .....G.....C..A.................T..T.....T.....T.....C.....C................C.A..A..A.....A..........
U6 RSA AP        .....G.....C..A.................T..T.....T.....T.....C.....C................C.A..A..A.....A..........
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ADDENDUM A- cont.

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                       410       420       430       440       450       460       470       480       490       500
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
U7 RSA AP     .....T..CG.C..............T.....T..T..T..T........A..c.....C..A.....C......C.G..A..A..A..A..C.......
U3 RSA AP     ..............A.................T..T.....T........C..C..............C.....TC.A..A..A.....A..........
A119 RSA AP   ..............A.................T..T.....T.....T.....C.....................TC.G..A..A.....A..........
AP1 RSA AP    ..............A...........A.....T..T.....T.....T.....C.....................TC.G..A..A.....A..........
AP2 RSA AP    ...........C..A.................T..T.....T.....T.....C......................C.G..A..A.....A..........
AP3 RSA AP    ..............A.................T..T.....T.....T.....C......................C.G..A..A.....A..........
AP5 RSA AP    .....C.....C....................T..T..T..............C......................C.G..A..A.....A..........
AP6 RSA AP    ...........C....................T..T..T..T........C..C........T.....C.....YC.G..A..A.....A..........
AP7 RSA AP    .....C...G.A........A..A..C.....T..T........A..G..G...........T.....C..A..TC.G..A..A..G.....C.......
AP10 RSA AP   ...........C....................T..T..T..T.....T.....C......................C.A..A........A..........
AP15 RSA AP   ...........C....................T..T.....T........C..C..............C.....TC.G..A..A.....A..........
AP16 RSA AP   ................................T..T.....T.....T..C..C..............C......C.A..A..A.....A..........
AP17 RSA AP   ..............A.................T..T..T..T.....T.....C.....................TC.A..A..A.....A..........
AP19 RSA AP   ................................T..T.....T.....T..C..C..............C......C.A..A........A..........
AP20 RSA AP   ...........G..............T.....G..T..A..T.....T..C..C..............C......C.A..A..A.....C.....G....
AP22 RSA AP   ................................T..T.....T.....T..C..C..............C......C.T..A..A.....A..........
AP23 RSA AP   ..............A.................T..T.....T.....T.....C.....................TC.G..A..A.....A..........
AP21 RSA AP   ..............A.................T..T.....T.....T.....C.....................TC.G..A..A.....A..........
AP9 RSA AP    ................................T..T.....T.....T.....C.....................TC.G..A..A.....A..........
Y10 RSA AP    ...........C....................T..T.....T.....T.....C........T...........TC.C..A..A................
Y1 RSA AP     .....G.....C..A.................T..T.....T.....T.....C.....C................C.A..A..A.....A..........
AP13 RSA AP   ..............A.................T..T.....T...........C.....................TC.A..A...................
AP11 RSA AP   .....G.....C....................T..T..T..T...........C..............C.....TC.G..A..A.....A..........
AP14 RSA AP   .....G.....C..A.................T..T.....T........C..C..............C.....TC.G..A..A.....A..........
AP12 RSA AP   .....T...G.C..............T.....T..T..T..T..A..T..G........C........C......C.G..A..A..G..A..........
AP8 RSA AP    .....G...G.C....................T..T.....T........C..C..............C......C.G..A..A.....A..........
AP4 RSA AP    .....G...G......................T..T.....T...........C........T.....C.....TC.G..A..A.....A..........
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ADDENDUM A- cont.

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                      510       520       530       540       550       560       570       580
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
MK9 JA AP        ATCGGCGTCTTTTACAGACTGAATTTGCAAAAAGTGA-GAACGAGGCGAAACTGTCGTCTGTTACAACTGATCTTTGCGTTTAG
AF170028 CMLV    ...A.........G..A.....GA.C......G.C..-.GCT..A..A..CA....TG.G..CT.G..AA....C..T......
Y3 CH PE         ................A..............G.....-...T..A..A..GA.TG.T..A...T.T.AC...T.G...A.....
HB2 CH PE        ................A..............G.....-A..T..A..A..GA.TG.T..A...T.T.AC...T.G...A.....
ST4 CH PE        ....A..C....................G........-......A..A..GA.TG.T..A...T.T.AC...T.G...A....A
SQ5 CH PE        .C.....C.............................-.........A..GA.TG.T..A...T.T.AC...T.G...A....A
HB3 CH PE        .C..A....C......A....................-...T..A..A..GA.TG.T..G..CT.T.A....T.G...A.....
S3 CH PE         ................A........C...........-......A.....GA.TG.T..G...T.T.AC...T.G...A.....
ST2 CH PE        .....................................-...T..A..A..GA.TG.T..G...T.T.A......G...A.....
HB4 CH PE        .C...........G...........C...........-...T..A..A..GA.TG.T..G...T.T.A....T.G...A.....
NC 001409 FR PD  .........................C.....G..C..-.........A..G..C........GT.G............A.....
E1 RSA PE        .........................C.....G.....-A........T..G..C........AT..............A.....
SQ2 CH PE        ................A..............G.....-...T........G..C..A......T.G............A.....
hz-48 CH PA      .........C........................C.G-...T...........C.GT.TCA.GT..............A.....
SQ8 CH PE        ....A..........................G..C..-A..T.....A..G.....A.....GT.G...........TA....A
CS CH PA         .C..T................................-A..T...........C........CT.G............A.....
S5 CH PE         .C..T....C.C.G.......................-A..T...........C........CT..............A.....
A9 RSA PE        .C..T................................-A..T...........C........CT..............A.....
hz-11 CH PA      .C..T................................-A..T...........C........CT..............A.....
C4 RSA PE        .C..T................................-A..T...........C........CT..............A.....
YL1 CH AP        ------------------------------------------------------------------------------------
G4 CH PE         .C..T..........................G.....~A..T...........C........CT..............A.....
HS6 CH PE        .C..T................................-A..T...........C........CT..............A.....
PP63 CH PA       .C..T................................-A..T...........C........CT..............A.C...
PL1 CH PA        .C..T........................G.......-A..T...........C........CT..............A.C...
Y1 CH PE         .C..T................................-A..T...........C........CT..............A.....
Z3 CH PE         .C..T..C.............................-A..T...........C........CT..............A.....
HL1 CH PE        .C..T................................-A..T...........C........CT..............A.....
Z1 CH PE         .C..C....................C...........-A..T...........C........CT..............A.....
MB-CH CH AP      .C...........G..A....................-A..T.....A.....C...........G.................A
YL2 CH AP        ------------------------------------------------------------------------------------
LV-m312 LA AP    .C..............A....................-...T.....A..T..C..A.....................A.....
C6 RSA AP        .C..............A....................-...T....G...G.....A.....................A.....
A4 RSA AP        .C.....C........A..............G.....-...T..A..A...A....A.....................A.....
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ADDENDUM A- cont.

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                           510       520       530       540       550       560       570       580
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
C1 RSA PE          .C..............A..............G.....-.........A...A....A.....................A.....
P1R9D9 BU CR       .C..............A..............G.....-A..T.....A...A....A.....................A.....
FJ752494 UK AP     .C.............................G.....GA..T....GCG..A....AG......AC.....A.....GC.....
B81 JA AP          CCG.CGTCT...ACARACTGA.T..GCA..G.GTGARAATGA.GC.AARMTS.CRTC.G.TAC.ACTGATC.T.GC.TT.AATC
MO41 JA AP         .C.............................G..C..-...T.....A...A.............G.................A
FR873735 IN AP     .C.............................G..C..-.........A...A....A..........................A
5Be BE AP          .C..............A....................-...T..A.GC.....C..C..C..................A.....
Malus0375 CA AP    .G.T.ATGT.CGATTTC.ACA.GGGCCTT..C.TGTTTATCATGAA.A.GG..CAACAAAAAGT..TTACCAA.A..AACCGGC
A5 RSA AP          .C..............A..............G..c..-...T.....A...A.......G........................
A3 RSA AP          .C..............A.................C..-...T.....A..G..C..A.....................A.....
C5 RSA AP          .C..............A.................C..-...T........G..C..A.....................A.....
E2 RSA AP          .C..A...........A.................C..-...T........G..C..A.....................A.....
TK4 TU AP          .C..............A....................-...T.....A..G..CC.A.....................A.....
A1 RSA AP          .C..............A....................-A..T.....A..G..C..A........G............A.....
CL CH PA           .C..............A....................-...T.....A..G..C..A........G............A.....
B1 RSA AP          .C.......................C...........-...T........G..C..A.....................A.....
Pyrus0212 CA PA    .C..............A....................-...T........G..C..A.....................A.....
Malus0545 CA AP    .C...................................-...T........G..C..A.....................A.....
LV-py120 LA PA     .C......T.......A....................-...T........G..T..A.T...................A.....
R1D2P-L BU PE      .C..............A..............G.....-...T.....A..G..C..A.....................A.....
Hatkoti IN AP      ................A........C...........-A..T........G..C..A.....................A.....
P 205 JA AP        .C.............................G..C..-A..T.........A................................
A4 JA AP           ................A..............G..C..-A..T.....A..GA....A.....................A.....
B6 JA AP           ................A.................C..-...T........G..C..A.....................A.....
MO-5 JA AP         .C....................G........G.....-A..............T........C..G............A....A
Ta Tao 5 US PE     .C..A..C........A...........T........-...T.....A..GA.TG.T..G...T.T.AC...T.G...A....A
U5 RSA PE          .........................C.....G.....-A........a..G..C........AT..............A.....
U8 RSA PE          .C..T................................-A..T...........C........CT....................
U12 RSA AP         .C..............a.................C..-...T........G..c..A.....................A.....
U9 RSA PE          .C................................C..-.............T..........................A.....
U15 RSA AP         .C..............A....................-...T........G..C..A.....................A.....
U2 RSA PE          .C.............................G.....-A..T...---------------------------------------~
D3 RSA AP          .C..............A.....G........G..C..-...T........G..C..A.....................A.....
U6 RSA AP          .C..............A.....G........G..C..-...T........G..C..A.....................A.....
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ADDENDUM A- cont.

```
                          510       520       530       540       550       560       570       580
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
U7 RSA AP         .C..............A.................G..c..-...T.....A...A................................
U3 RSA AP         .C..............A....................C..-...T........G..C..A.....................A....
A119 RSA AP       .C..............A.................G.....-...T........G..C..A.....................A....
AP1 RSA AP        .C..............A.......................-...T........G..C..A.....................A....
AP2 RSA AP        .C..............A.......................-...T........G..C..A.....................A....
AP3 RSA AP        .C..............A....................C..-...T........G..C..A.....................A....
AP5 RSA AP        ................A.......................-A..T........G..C..A.....................A....
AP6 RSA AP        .C..............A....................G..-...T.....A........A.....................A....
AP7 RSA AP        .C..............A.................G..C..-A..T.....A...A....A.....................A....
AP10 RSA AP       .C..............A.......................-A..T.....A..GT.C..A.....................A....
AP15 RSA AP       .C..............A.................G.....-...T........GA.C..A.....................A....
AP16 RSA AP       .C..............A........C..............-...T........G..C..A.....................A....
AP17 RSA AP       .C..............A.......................-...T........G..C..A.....................A....
AP19 RSA AP       .C..............A........C..............-...T........G..C..A.....................A....
AP20 RSA AP       .C..............A........C..............-...T........G..C........................A....
AP22 RSA AP       .C..............A........C..............-...T........G..C..A.....................A....
AP23 RSA AP       .C..............A....................C..-...T........G..C..A.....................A....
AP21 RSA AP       .C..............A....................C..-...T........G..C..A.....................A....
AP9 RSA AP        .C..............A.......................-...T........G..C..A.....................A....
Y10 RSA AP        ................A....................C..---------------------------------------------
Y1 RSA AP         .C..............A.....G...........G..C..-...T........G..C..A.....................A....
AP13 RSA AP       .C..............A.......................-...T.........T..........................A....
AP11 RSA AP       .C..............A.......................-...T........G..C........................A....
AP14 RSA AP       .C..............A.......................-...T.....A..G..C..A.....................A....
AP12 RSA AP       .C.....C........A.................G..C..-...T.....A...A....A.....................A....
AP8 RSA AP        .C..............A.................G.....-...T.....A........A.....................A....
AP4 RSA AP        .C..............A.................G..C..-...T..............A.....................A....
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ADDENDUM B

Percentage nucleotide sequence similarities between the ACLSV CP gene sequences of isolates from South Africa as determined using BioEdit.

	E1	A9	C4	C6	A4	C1	A5	A3	C5	E2	A1	B1	U5	U8	U12	U9	U15	U2	D3	U6	U7	U3	A119	AP1
E1		86	85.2	84.3	82.3	82.1	85	86.9	87.1	86.9	86.9	87.1	97.7	85.9	87.6	86	86.9	78.8	87.1	84.1	84	87.9	87.9	87.2
A9			95	87.1	84.8	85	87.8	89.6	89.3	89.3	89.3	87.6	87.1	98.7	89.5	87.8	88.1	82.3	88.1	87.9	86.2	89.5	90.5	89.3
C4				87.8	84.5	86.5	87.6	88.4	88.4	88.4	88.6	87.2	86	94.5	89	88.3	88.8	81.7	88.1	86.5	86.4	89.6	89.5	88.3
C6					90.5	91.5	89	90.3	90.8	90.7	90	89.6	84.8	86.9	91.4	88.8	91.4	81.2	89.6	89.3	88.8	91.2	92.6	91
A4						91.4	88.4	86.5	86.5	86.4	85	85.3	83.3	84.5	86.7	84.3	85.9	78.8	85.2	85.9	91.7	86.4	88.1	87.1
C1							89.8	86.5	87.2	87.1	86.4	85.5	83.6	84.8	87.4	85.2	86.7	79.8	86	86.5	89.8	87.1	88.1	87.6
A5								89.3	89.3	89.1	88.8	88.6	85.9	87.8	89.5	87.9	88.3	82.6	89.1	89.3	92.7	89.6	90.2	89
A3									98.4	98.2	95.7	93.9	88.3	89.5	97.9	92.7	92.7	83.5	94.3	90.8	88.3	95.7	94.6	96.7
C5										99.8	95.8	95.1	88.3	89.1	99.1	92.7	93.9	84.3	95.1	91.2	87.9	96.5	95.8	97.5
E2											95.7	95	88.1	89.1	98.9	92.6	93.8	84.1	95	91	87.8	96.3	95.7	97.4
A1												94.5	88.4	89.1	96.3	91	93.9	86	94.8	90.3	86.9	95.5	94.3	95.5
B1													87.9	87.6	95	91.5	93.1	84.8	94.5	90.5	86.4	95.1	93.1	94.5
U5														86.9	88.8	86.9	87.8	80.2	87.9	85	85.2	88.8	89.1	88.4
U8															89.3	87.8	87.9	82.3	88.1	87.6	86.5	89.3	90.7	89.1
U12																93.6	94.1	84.1	95	91	88.1	97.4	96	97
U9																	91.2	81.9	90.7	88.1	85.7	93.4	91.2	91.7
U15																		83.1	93.9	91.5	86	93.9	94.5	94.1
U2																			85	83.5	81.9	84	83.6	83.8
D3																				93.1	88.1	95.5	92.9	93.8
U6																					87.6	90.7	91	90.7
U7																						88.3	88.1	87.4
U3																							94.3	94.8
A119																								96

ADDENDUM B- cont.

Percentage nucleotide sequence similarities between the ACLSV CP gene sequences of isolates from South Africa as determined using BioEdit.

	AP2	AP3	AP5	AP6	AP7	AP10	AP15	AP16	AP17	AP19	AP20	AP22	AP23	AP21	AP9	Y10	Y1	AP13	AP11	AP14	AP12	AP8	AP4
E1	87.2	87.6	87.6	85.7	82.3	86.9	86.9	86.5	87.8	86.5	86.5	86.9	86.9	86.4	87.4	80	88.4	86.2	86.9	86.9	83.6	86.4	86
A9	89.6	89.1	89.1	89.6	85.5	89.5	90.7	87.6	87.9	87.8	86.4	87.8	89	89.3	90.3	82.3	88.8	88.6	89.1	90	86.5	89.5	89
C4	88.8	88.8	88.3	87.9	85.3	89.3	89.3	87.4	87.6	87.9	86.5	87.8	88.1	88.1	88.8	81.2	88.4	88.6	89.3	89.3	86.2	88.6	88.6
C6	91.5	90.8	90	90.5	90	92.2	90.3	90.7	90.2	90.5	89.3	90	90.5	90.2	91.2	83.3	90.2	90.3	91	91.9	90.2	91.5	92.2
A4	86.4	86.4	85.2	86.9	92.2	86.9	86.7	86	84.8	85.5	85.9	85.3	86.5	86.2	87.4	78.8	85.9	85.7	87.4	87.4	90.2	88.1	89.5
C1	87.8	87.8	86.7	88.4	92.9	88.4	88.3	86.4	85.3	86.4	85.9	86	86.5	86.2	87.6	79.7	86.5	86.9	86.7	88.1	90.7	89	90.2
A5	89.3	89.5	88.4	90.8	89	90	91	88.8	88.3	88.4	87.8	88.8	89.1	89.1	90	82.4	89.8	89.1	89.8	90.2	91.4	91.5	91.4
A3	96.5	96.9	93.9	93.6	86.7	94.5	95.3	95	95	94.6	92.4	95.1	98.1	97.7	96	89.1	95	94.1	93.2	94.3	88.4	91.7	94.1
C5	97	98.1	93.8	93.8	86.7	94.6	95.1	96.2	95.8	95.8	93.4	96.3	98.9	98.2	96.2	89.8	95.5	94.3	94.1	94.5	89.1	91.5	94.3
E2	96.9	97.9	93.6	93.6	86.5	94.5	95	96	95.7	95.7	93.2	96.2	98.7	98.1	96	89.6	95.3	94.1	93.9	94.3	89	91.4	94.1
A1	96.9	96	93.8	93.4	85.5	95	95.1	95.5	94.8	95.5	92.7	95.7	96.2	95.5	95.1	87.6	95	92.9	93.4	95.1	89.3	92.2	92.2
B1	94.3	94.5	92.2	92.6	84.8	93.2	93.4	97.2	95.1	96.9	94.1	96.9	95.1	94.1	93.6	86	93.9	93.1	93.1	93.4	88.6	90.8	92
U5	88.4	88.8	89	86.9	83.3	88.3	88.1	87.4	88.3	87.4	87.1	87.6	88.1	87.6	88.6	81	89.3	87.4	88.1	88.3	84.7	87.6	87.2
U8	89.5	89	89	89.5	85.3	89.3	90.5	87.4	87.9	87.6	86.2	87.6	88.8	89.1	90.2	82.3	88.4	88.4	89	89.8	86.4	89.3	88.8
U12	97.2	97.9	93.9	93.9	87.2	94.8	95.7	96	96.7	95.7	93.6	96.2	98.4	98.1	96.3	89.3	95.7	94.8	94.3	95	89.5	92	94.5
U9	92.2	92.4	90.8	90.7	84.8	92	91.2	91.4	94.8	90.7	90.2	91	93.1	92.7	91.7	84.8	91.4	94.8	90.8	90.7	86.9	89.1	91.7
U15	94.1	95.3	94.1	91.4	86.7	94.3	92.7	94.3	93.1	93.9	91.2	93.9	93.9	93.2	94.1	85.3	94.8	93.1	94.5	92.7	89.6	92.6	92.2
U2	84.8	84.5	83.3	83.5	79.2	84	83.8	84.7	83.8	84.5	83.1	84.7	84.5	84.1	83.8	88.5	84.7	83.1	84	83.5	81.6	83.3	83.6
D3	94.6	95.1	91.9	91.7	85.2	93.1	93.4	95.5	93.4	95.1	92.2	95.5	95.5	94.8	93.2	86.2	97.4	92.6	92.4	93.4	88.6	90.7	92
U6	91.2	91.2	89.8	89.6	87.2	90.5	90.2	91.2	90.5	90.5	88.6	90.7	91.2	91.2	91.2	82.6	93.4	89.8	89.8	90.5	89.5	90.5	91
U7	87.9	88.1	87.4	89	89.5	88.4	88.3	87.6	85.9	86.9	87.4	87.2	88.1	87.8	88.4	80.7	88.8	87.1	88.4	88.3	90.3	89.5	90.8
U3	95	95.8	93.1	93.2	86.9	94.1	94.8	96.5	95.5	96.2	93.9	96.5	96.5	95.8	94.6	87.2	95.7	94.3	93.8	95.5	89	91.7	93.8
A119	95.7	96.3	92.9	93.4	88.3	94.5	94.8	93.6	94.5	93.2	91.7	93.8	95.5	95.5	95.5	87.1	93.9	92.7	93.9	94.5	89.8	93.6	92.9

ADDENDUM B- cont.

Percentage nucleotide sequence similarities between the ACLSV CP gene sequences of isolates from South Africa as determined using BioEdit.

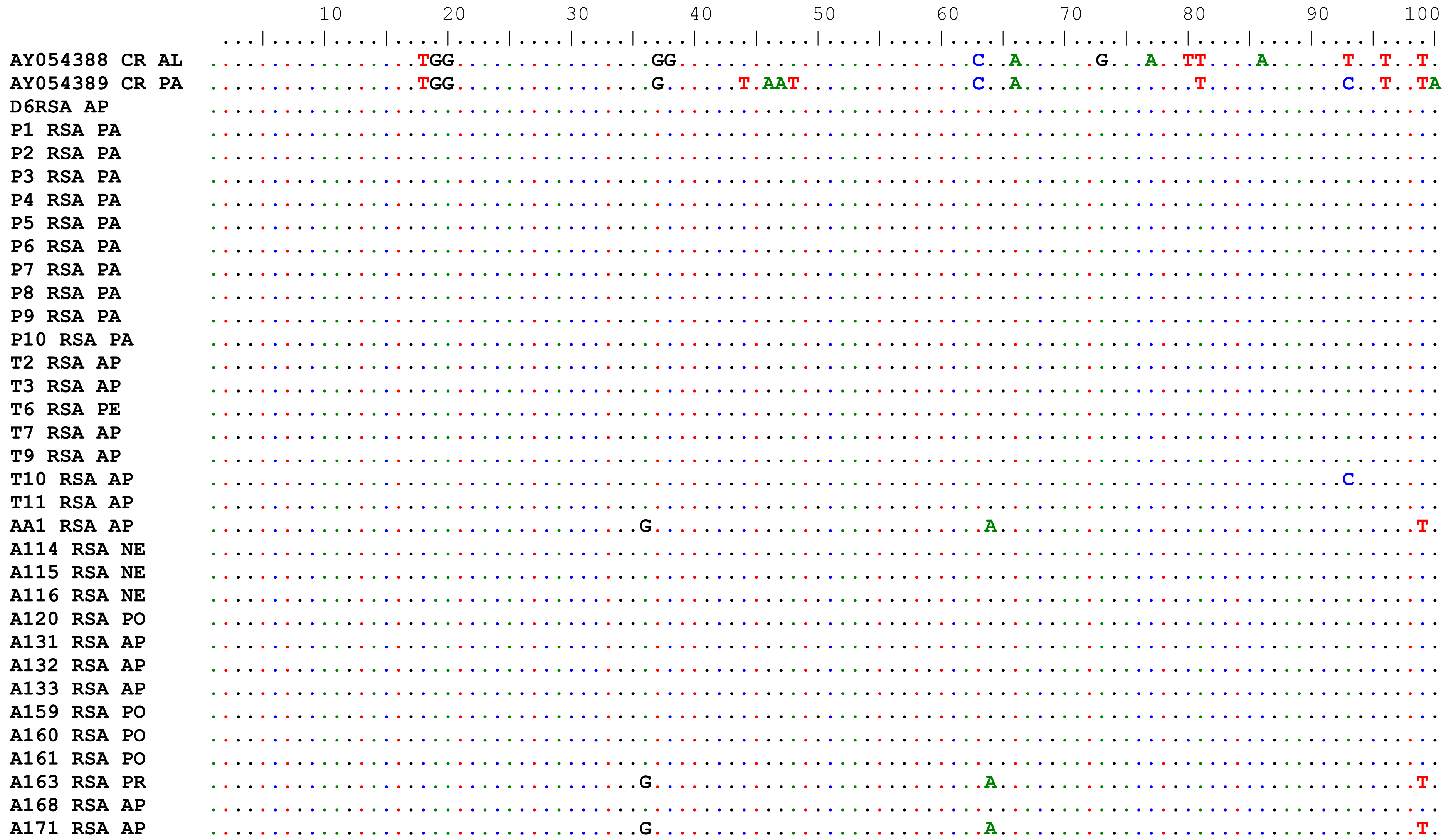
	AP2	AP3	AP5	AP6	AP7	AP10	AP15	AP16	AP17	AP19	AP20	AP22	AP23	AP21	AP9	Y10	Y1	AP13	AP11	AP14	AP12	AP8	AP4
AP1	96.9	97.7	92.9	93.9	87.2	93.9	95.7	95	95	94.6	92.2	95.1	97.9	96.9	96.5	87.8	94.1	93.9	93.9	94.5	89.5	91.9	93.6
AP2		97.2	94.5	94.8	86.9	95.5	96	95.7	95.3	95	93.1	95.5	97	96.7	96.7	88.3	95.3	94.1	95	95.5	90	93.6	94.3
AP3			93.9	93.9	87.2	94.3	95.7	95.8	95.1	95.5	93.2	96	98.1	97	96.3	88.3	95.5	93.9	94.3	94.6	90	92.4	94.5
AP5				92.7	87.1	94.5	93.6	93.1	92.7	92.7	90.5	92.9	93.4	93.1	94.1	85.9	93.6	92.4	93.6	92.6	89.3	92.7	92.7
AP6					88.6	93.4	95.3	93.9	93.2	93.4	90.8	93.8	93.4	93.4	94.3	86.5	92.7	92.6	93.4	95	92.4	93.9	93.8
AP7						88.3	88.4	86.2	85.2	85.5	85.9	85.5	86.4	86.4	88.4	80	86.2	86.7	87.4	87.9	91.4	89.8	90.7
AP10							94.3	94.1	93.6	94.1	92	93.8	94.6	94.3	94.8	87.6	94.5	93.6	93.9	94.3	90.8	94.3	93.6
AP15								94.5	93.9	94.3	91.7	94.6	95.5	95.5	96.2	87.8	94.5	93.9	94.5	96.3	91.7	94.1	93.8
AP16									95.1	98.7	95.3	98.9	96.2	95.1	94.8	86.9	95	93.6	93.8	94.8	89.6	92.4	93.1
AP17										95.1	93.4	95.5	95.8	95.8	94.6	86.9	94.6	94.8	93.4	93.8	88.6	91.5	92
AP19											95.3	98.9	95.8	94.8	94.3	86.9	94.6	93.6	93.2	94.3	89	91.9	92.6
AP20												95.3	93.2	92.6	91.9	85	92.6	91.2	91.7	92	88.3	90.2	90.2
AP22													96.3	95.3	94.5	87.1	95	93.2	93.6	94.6	89	91.9	92.6
AP23														98.6	96.5	89.1	95.8	94.6	94.1	94.8	89.1	91.5	93.9
AP21															96.5	89.5	95.8	93.9	93.4	94.5	89	91.9	93.2
AP9																87.9	95	94.1	95	95	90.8	93.8	95.7
Y10																	87.2	86.4	86	86.4	82.3	85	86.2
Y1																		92.6	93.4	94.5	90.2	92.9	92.7
AP13																			92.7	92.4	89	91.5	93.6
AP11																				95.3	90.5	93.9	93.8
AP14																					91.9	94.5	93.6
AP12																						92.9	92
AP8																							94.6
AP4																							

ADDENDUM C

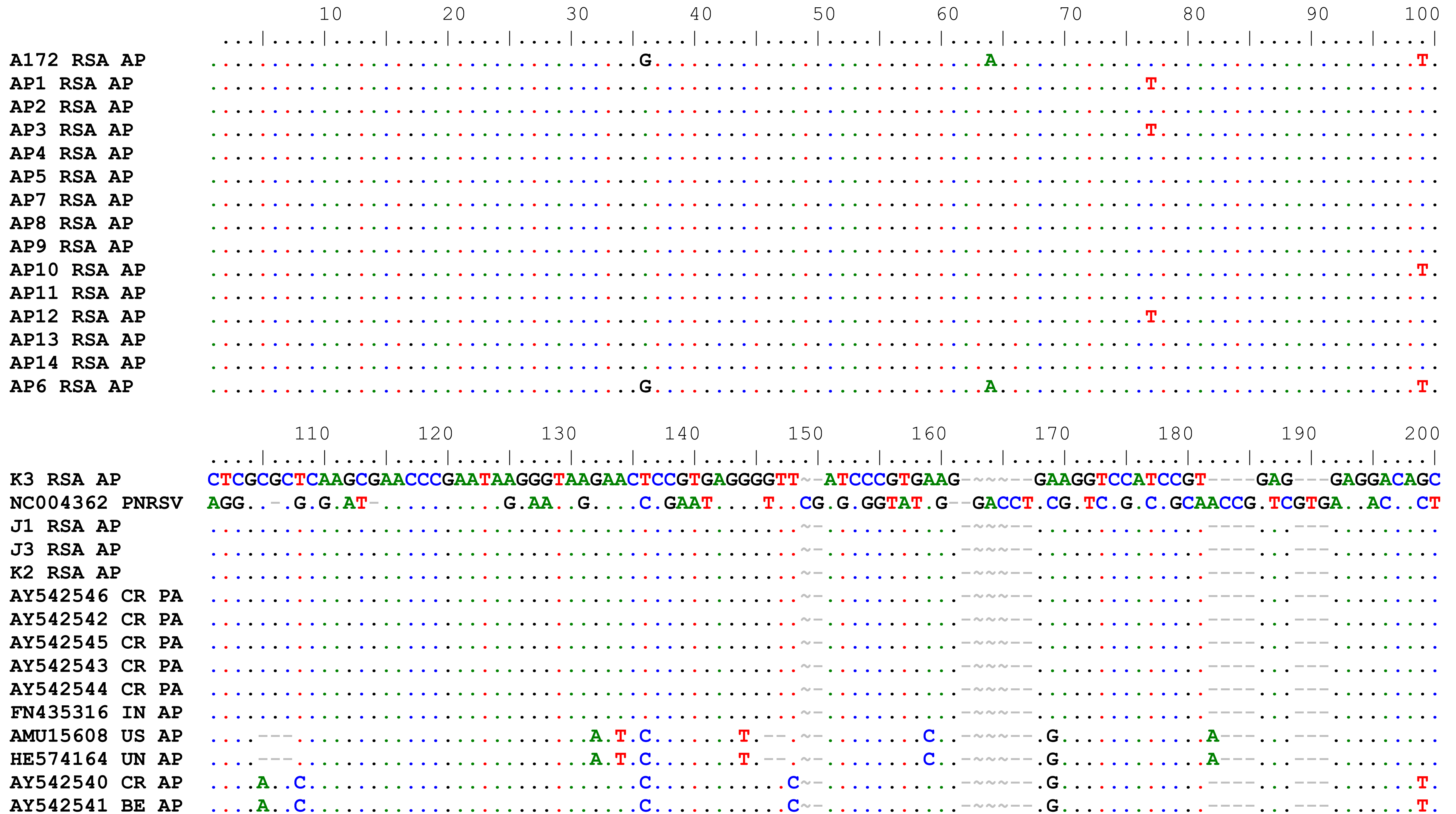
Aligned coat protein gene sequences of the 50 South African isolates of ApMV, 28 GenBank ApMV reference sequences and one PNRSV GenBank reference sequence used as an outgroup. A dot indicates a nucleotide identical to the top sequence.

```
                          10        20        30        40        50        60        70        80        90       100
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
K3 RSA AP         ATGGTCTGCAAGTACTGCAATCACACTCACCCTGGATCTTGCGCTGGATGCAAGTGGTGTCATGGTACGAATAGACCTGCACCTCCGAAGCGAGCGGTCG
NC004362 PNRSV    .....T...CGAATT........T..C...G....TGGA...CG.TCT......A....C...CCG.ATG..-.CT...GT..CA.TC..G.CT.AACAA
J1 RSA AP         ....................................................................................................
J3 RSA AP         ....................................................................................................
K2 RSA AP         ....................................................................................................
AY542546 CR PA    ....................................................................................................
AY542542 CR PA    ....................................................................................................
AY542545 CR PA    ....................................................................................................
AY542543 CR PA    ....................................................................................................
AY542544 CR PA    ....................................................................................................
FN435316 IN AP    ....................................................................................................
AMU15608 US AP    .................T.....T......................................CA...........TTC....................T.
HE574164 UN AP    .................T.....T............................--~~~CD...CA...........TTC....................T.
AY542540 CR AP    .................T...........................A......--~~~...TGC..C...................T..T....AA..T.
AY542541 BE AP    .................T...........................A..............TGC..C...................T..T..A..AA..T.
FN546183 IN CR    ...........................................................................T...................A..T.
FN435317 IN AP    ...........................................................................T...................A..T.
FN435315 IN AP    ...........................................................................T...................A..T.
FN547927 IN AP    ...........................................................................T...................A..T.
FN435314 IN AP    ...........................................................................T...................A..T.
FM178274 UN UN    ...........................................................................T...................A..T.
FN564150 UN UN    ...........................................................................T...................A..T.
S78319 GE AP      .................TGG................G......T.AAT..............C..A..............T...........C..C..T.
L03726U US AP     .................T.....T..................C...................CA...........TTC....................T.
AY054385 CR AP    ......G..........T.....T.....................A................C..C.......................T.....A...T.
AF548367 KO AP    .................T.....T......................................CA...........TTC....................T.
AM490197 CR AP    ........T....T...TGG................G..........T...........C.....A......C....CA.T.....A.....T..A..T.
AY125977 KO AP    .................T.....T......................................CA...........TTC....................T.
FJ429311 IN AP    ...........................................................................T...................A..T.
AY054386 CR PR    .................TGG................G......T.AAT..............C..A..............T...........C..T..TA
AY054387 CR HO    .................TGG................G......T.AAT..............C..A..............T...........C..T..TA
```

ADDENDUM C- cont.



ADDENDUM C- cont.



ADDENDUM C- cont.

```
                        110       120       130       140       150       160       170       180       190       200
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FN546183 IN CR   ...................................C..A.........~-...........-~~~--..............----...---.........
FN435317 IN AP   ...................................C..A.........~-...........-~~~--..............----...---.........
FN435315 IN AP   ...................................C..A.........~-...........-~~~--..............----...---.........
FN547927 IN AP   ...................................C..A.........~-...........-~~~--..............----...---.........
FN435314 IN AP   ...................................C..A.........~-...........-~~~--..............----...---.........
FM178274 UN UN   ...................................C..A.........~-...........-~~~--..............----...---.........
FN564150 UN UN   ...................................C..A.........~-...........-~~~--..............----...---.........
S78319 GE AP     T......C.T..................A..A.C-~~~~~~~~~~~~~~~-..........-~~~--.G............----...---......G..
L03726U US AP    ....TA.........................A.T.C.......---..~-........C..-~~~--.G............----...---.........
AY054385 CR AP   ....A..C.........................G.C............~-...........-~~~--.G.......C....----...---.........
AF548367 KO AP   ....---........................A.T.C.......T.--.~-........C..-~~~--.G............---A...---.........
AM490197 CR AP   TG..T......T.....................C-~~~~~~~~~~~~~~~-..T.......-~~~--.G............----...---......G..
AY125977 KO AP   ...............................A.T.C............~-........C..-~~~--.G............----...---.........
FJ429311 IN AP   ...................................C..A.........~-...........-~~~--..............----...---.........
AY054386 CR PR   T......C...........................~~~~~~~~~~~~~~~--.........-~~~--.G.......A....----...---.........
AY054387 CR HO   T......C...........................~~~~~~~~~~~~~~~--.........-~~~--.G.......A....----...---.........
AY054388 CR AL   T......G..............C...........T~~~~~~~~~~~~~~~--.......GACAAACT.G............----...---......G..
AY054389 CR PA   T......C...........................~~~~~~~~~~~~~~~--.........-~~~--.G.......A....----...---.........
D6RSA AP         ................................................~-...........-~~~--..............----...---.........
P1 RSA PA        ................................................~-...........-~~~--..............----...---.........
P2 RSA PA        ................................................~-...........-~~~--..............----...---.........
P3 RSA PA        ................................................~-...........-~~~--..............----...---.........
P4 RSA PA        ................................................~-...........-~~~--..............----...---.........
P5 RSA PA        ................................................~-...........-~~~--..............----...---.........
P6 RSA PA        ................................................~-...........-~~~--..............----...---.........
P7 RSA PA        ................................................~-...........-~~~--..............----...---.........
P8 RSA PA        ................................................~-...........-~~~--..............----...---.........
P9 RSA PA        ................................................~-...........-~~~--..............----...---.........
P10 RSA PA       ................................................~-...........-~~~--..............----...---.........
T2 RSA AP        ................................................~-...........-~~~--..............----...---.........
T3 RSA AP        ................................................~-...........-~~~--..............----...---.........
T6 RSA PE        ................................................~-...........-~~~--..............----...---.........
T7 RSA AP        ................................................~-...........-~~~--..............----...---.........
T9 RSA AP        ................................................~-...........-~~~--..............----...---.........
```

ADDENDUM C- cont.

```
                           110       120       130       140       150       160       170       180       190       200
                       ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
T10 RSA AP             ................................................~-...........-~~~--..............----...---.........
T11 RSA AP             ................................................~-...........-~~~--..............----...---.........
AA1 RSA AP             ..................................TC............~-...........-~~~--..............----...---......G..
A114 RSA NE            ................................................~-...........-~~~--..............----...---.........
A115 RSA NE            ................................................~-...........-~~~--..............----...---.........
A116 RSA NE            ................................................~-...........-~~~--..............----...---.........
A120 RSA PO            ................................................~-...........-~~~--..............----...---.........
A131 RSA AP            ..................................A............~-...........-~~~--..............----...---.........
A132 RSA AP            ...................................A............~-...........-~~~--..............----...---.........
A133 RSA AP            ................................................~-...........-~~~--..............----...---.........
A159 RSA PO            ...................................A............~-...........-~~~--..............----...---.........
A160 RSA PO            ................................................~-...........-~~~--..............----...---.........
A161 RSA PO            ................................................~-...........-~~~--..............----...---.........
A163 RSA PR            ..................................TC............~-...........-~~~--..............----...---......G..
A168 RSA AP            ................................................~-...........-~~~--..............----...---.........
A171 RSA AP            ..................................TC............~-...........-~~~--..............----...---......G..
A172 RSA AP            ..................................TC............~-...........-~~~--..............----...---......G..
AP1 RSA AP             ......................C.........................~-...........-~~~--..............----...---........T
AP2 RSA AP             ...................................A............~-...........-~~~--..............----...---.........
AP3 RSA AP             ......................C.........................~-...........-~~~--..............----...---........T
AP4 RSA AP             ................................................~-...........-~~~--..............----...---.........
AP5 RSA AP             ...................................A............~-...........-~~~--..............----...---.........
AP7 RSA AP             ...................................A............~-...........-~~~--..............----...---.........
AP8 RSA AP             ................................................~-...........-~~~--..............----...---.........
AP9 RSA AP             ................................................~-.C.........-~~~--..............----...---.........
AP10 RSA AP            ................................................~-...........-~~~--..............----...---.........
AP11 RSA AP            ...................................A............~-...........-~~~--..............----...---.........
AP12 RSA AP            ......................C.........................~-...........-~~~--..............----...---.........
AP13 RSA AP            ................................................~-...........-~~~--..............----...---.........
AP14 RSA AP            ................................................~-...........-~~~--..............----...---.........
AP6 RSA AP             ..................................TC............~-...........-~~~--....-.SGCGGG..----.G.---....GKG.T
```

ADDENDUM C- cont.

```
                      210       220       230       240       250       260       270       280       290       300
                   ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
K3 RSA AP          TTGGGAGGTTAGAGGCCCGAATGTTGAGCCGAAAAT-TCCGAAAGG-TCACAGGGTCCTGAGCAGTCGAGAAGTGACTGCCACGGTTGAAGGCAAGTTCG
NC004362 PNRSV     .G.ACC.TGAG.G.T..GA.TGTGCCTC..CG..T-TC.TA.GG.-T.ATGTA.CA.A.A.T..CCGAGAGGTGACGACGACA.AGGC.GT.A.GTACTT
J1 RSA AP          ....................................-.........-.....................................................
J3 RSA AP          ....................................-.........-.....................................................
K2 RSA AP          ....................................-.........-.....................................................
AY542546 CR PA     ....................................-.....G...-...................................T.................
AY542542 CR PA     ....................................-.....G...-...................................T.................
AY542545 CR PA     ....................................-.....G...-...................................T.................
AY542543 CR PA     ....................................-.....G...-...................................T.................
AY542544 CR PA     ....................................-.....G...-...................................T.................
FN435316 IN AP     ....................................-.........-.................................T...................
AMU15608 US AP     ............G.......................-...T.....GC..............................................GA..T.
HE574164 UN AP     ............G.......................-...T.....GC..............................................GA..T.
AY542540 CR AP     ............G..............A........-...T.....-...............T.............................T.....T.
AY542541 BE AP     ............G..............A........-...T.....-...................................T.................
FN546183 IN CR     ....................................-.....G...-C......................................C.............
FN435317 IN AP     ....................................-.....G...-C......................................C.............
FN435315 IN AP     ....................................-.....G...-C......................................C.............
FN547927 IN AP     ....................................-.....G...-C......................................C.............
FN435314 IN AP     ....................................-.....G...-C......................................C.............
FM178274 UN UN     ....................................-.....G...-C......................................C...........T.
FN564150 UN UN     ....................................-.....G...-C......................................C.............
S78319 GE AP       ........................G..A..A..G..-...A..G..-........CTT..........................................
L03726U US AP      ............G.............-.........-...T....--........................-......................GA..T.
AY054385 CR AP     ............G.......................-...T.....-C...............................................A..T.
AF548367 KO AP     ............G.......................-...T.....GC..............................................GA..T.
AM490197 CR AP     ........................G..A.....GG.-...A..G..-.........TT.......................G................T.
AY125977 KO AP     ............G.......................-...T.....-C..............................................GA..T.
FJ429311 IN AP     ....................................-.....G...-C......................................C.............
AY054386 CR PR     ........................G..A..C..G..-...A..G..-........CT...........T..G........T.................T.
AY054387 CR HO     ........................G..A..C..G..-...A..G..-........CT...........T..G........T.................T.
AY054388 CR AL     .........C.................A..A.G...-C.....G..-C..T.....TT.T...................................A....
AY054389 CR PA     ........................G..A..C..G..-...A..G..-........CT...........T..G........T.................T.
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ADDENDUM C- cont.

```
                  210       220       230       240       250       260       270       280       290       300
                ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
D6RSA AP       ....................................-.........-.....................................................
P1 RSA PA      ....................................-.........-.....................................................
P2 RSA PA      ....................................-.........-.....................................................
P3 RSA PA      ....................................-.........-.....................................................
P4 RSA PA      ....................................-.........-.....................................................
P5 RSA PA      ....................................-.........-.....................................................
P6 RSA PA      ....................................-.........-.....................................................
P7 RSA PA      ....................................-.........-.....................................................
P8 RSA PA      ....................................-.........-.....................................................
P9 RSA PA      ....................................-.........-.....................................................
P10 RSA PA     ....................................-.........-.....................................................
T2 RSA AP      ....................................-.........-.....................................................
T3 RSA AP      ....................................-.........-.....................................................
T6 RSA PE      ....................................-.........-.....................................................
T7 RSA AP      ....................................-.........-.....................................................
T9 RSA AP      ....................................-.........-.....................................................
T10 RSA AP     ....................................-.........-.....................................................
T11 RSA AP     ....................................-.........-.....................................................
AA1 RSA AP     ............G........C........A.....-.....G...-...................................................T.
A114 RSA NE    ....................................-.........-.....................................................
A115 RSA NE    ....................................-.........-.....................................................
A116 RSA NE    ....................................-.........-.....................................................
A120 RSA PO    ....................................-.........-.....................................................
A131 RSA AP    ....................................-.....G...-..............................C....T.................
A132 RSA AP    ....................................-.....G...-..............................C....T.................
A133 RSA AP    ....................................-.........-.....................................................
A159 RSA PO    ....................................-.....G...-...................................T.................
A160 RSA PO    ....................................-.........-...................................T.................
A161 RSA PO    ....................................-.........-...................................T.................
A163 RSA PR    ............G........C........A.....-.....G...-.....................................................
A168 RSA AP    ....................................-.........-.....................................................
A171 RSA AP    ............G........C........A.....-.....G...-.....................................................
A172 RSA AP    ............G........C........A.....-.....G...-...................................................T.
```

ADDENDUM C- cont.

```
                       210       220       230       240       250       260       270       280       290       300
                  ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AP1 RSA AP       ....................................-.....G...-......A............................T.................
AP2 RSA AP       ....................................-.....G...-...................................T.................
AP3 RSA AP       ....................................-.....G...-......A............................T.................
AP4 RSA AP       ....................................-.........-....................C..............................T.
AP5 RSA AP       ....................................-.....G...-........C...........c........................A.......
AP7 RSA AP       ....................................-.........-...................................T.................
AP8 RSA AP       ....................................-.........-.....................................................
AP9 RSA AP       ....................................-.........-.....................................................
AP10 RSA AP      ....................................-.........-.....................................................
AP11 RSA AP      ...............T....................-.....G...-...................................T.................
AP12 RSA AP      ....................................-.....G...-......A............................T............A....
AP13 RSA AP      ....................................-.........-................-....................................
AP14 RSA AP      ....................................-.........-.....................................................
AP6 RSA AP       .G.........WG..T....T.-.W...GTT.M.TCCG..YTGG..--..T-.WT..G..M.T-..TA.KG---.T.YC.TKT.C.CT.TAC.C~~~~~~

                       310       320       330       340       350       360       370       380       390       400
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
K3 RSA AP        TCAATATTGACTTTGCCGATGTCTTTCGTGATCTTTTGGAGAAGGATCTGAAGGTGTATACCTTCATAATCCGAGTGAACAGTCTATCCTCTAATGGATG
NC004362 PNRSV   GAGTAT.GACT.CACGACCAC...CC.TCAG.TGA.G..TC.GAAT.TGACCTTAT..ACTG.CATAGTC.GA.TGA.CTCTATG.GTTCGA.TG.TTG.
J1 RSA AP        .G...C............................C..................................................G..............
J3 RSA AP        ....................................................................................................
K2 RSA AP        ....................................................................................................
AY542546 CR PA   .......C.............................................................................G..............
AY542542 CR PA   .......C.............................................................................G..............
AY542545 CR PA   .......C.............................................................................G..............
AY542543 CR PA   .....................................................................................G..............
AY542544 CR PA   .......C................................A............................................G..............
FN435316 IN AP   .............C....................................................................C..G..............
AMU15608 US AP   ....C....................C.............................A............................................
HE574164 UN AP   ....C....................C.............................A............................................
AY542540 CR AP   .........................C.............................A.............................G..T...........
AY542541 BE AP   .......C.............................................................................G..............
FN546183 IN CR   ....C....................C........C..................................................G.....C........
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ADDENDUM C- cont.

```
                       310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FN435317 IN AP  ....C...............C........C..................................................G.....C........
FN435315 IN AP  ....C...............C....G...C..................................................G.....C........
FN547927 IN AP  ....C...............C....G...C..................................................G.....C........
FN435314 IN AP  ....C...............C........C..................................................G.....C........
FM178274 UN UN  ....C...............C........C..................................................G.....C........
FN564150 UN UN  ....C...............C........C..................................................G.....C........
S78319 GE AP    .G.....C............C..C.....GC.A.........T........C.C.......................................G..
L03726U US AP   ....C...............C.............................A............................................
AY054385 CR AP  ....................C.............................A.......................T.....G..............
AF548367 KO AP  ....C...............C.............................A............................................
AM490197 CR AP  ....................C........C............T.....A.T..C..........................G.....G........
AY125977 KO AP  ....C...............C.............................A............................................
FJ429311 IN AP  ....C...............C........C..................................................G.....C........
AY054386 CR PR  .G.....A............C...A....GC.A..A......T............................A.............A......G..
AY054387 CR HO  .G.....A............C...A....GC.A..A......T............................A....................G..
AY054388 CR AL  .G........T.........C.....C..GC.......A..T...........C.....T.....T..............G..............
AY054389 CR PA  .G.....A............C...A....GC.A..A......T............................A....................G..
D6RSA AP        .............................C..................................................G..............
P1 RSA PA       .............................C..................................................G..............
P2 RSA PA       .............................C..................................................G..............
P3 RSA PA       .............................C..................................................G..............
P4 RSA PA       .............................C..................................................G..............
P5 RSA PA       .............................C..................................................G..............
P6 RSA PA       .............................C..................................................G..............
P7 RSA PA       .............................C..................................................G..............
P8 RSA PA       .............................C..................................................G..............
P9 RSA PA       .............................C..................................................G..............
P10 RSA PA      .............................C..................................................G..............
T2 RSA AP       .............................C..................................................G..............
T3 RSA AP       .............................C..................................................G..............
T6 RSA PE       .............................C..................................................G..............
T7 RSA AP       .............................C..................................................G..............
T9 RSA AP       .............................C..................................................G..............
T10 RSA AP      .............................C..................................................G..............
```

ADDENDUM C- cont.

```
                     310       320       330       340       350       360       370       380       390       400
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
T11 RSA AP   ..................................C..................................................G..............
AA1 RSA AP   ....C....................C.....C.....................................................G..............
A114 RSA NE  ..................................C..................................................G..............
A115 RSA NE  ..................................C..................................................G..............
A116 RSA NE  ..................................C..................................................G..............
A120 RSA PO  ..................................C..................................................G..............
A131 RSA AP  .....................................................................................G..............
A132 RSA AP  .....................................................................................G..............
A133 RSA AP  ..................................C..................................................G..............
A159 RSA PO  .....................................................................................G..............
A160 RSA PO  .....................................................................................G..............
A161 RSA PO  .....................................................................................G..............
A163 RSA PR  .........................C.....C.....................................................G..............
A168 RSA AP  ..................................C..................................................G..............
A171 RSA AP  ....C....................C.....C.....................................................G..............
A172 RSA AP  ....C....................C.....C.....................................................G..............
AP1 RSA AP   .T...................................................................................G..............
AP2 RSA AP   ....C.....................................................C..........................G..............
AP3 RSA AP   .T...................................................................................G..............
AP4 RSA AP   ...................................................................T.................G..............
AP5 RSA AP   .....................................................................................G..............
AP7 RSA AP   ....C................................................................................G..............
AP8 RSA AP   .....................................................................................G..............
AP9 RSA AP   .....................................................................................G..............
AP10 RSA AP  ...............................C.....................................................G..............
AP11 RSA AP  ..........................................................................A..........G..............
AP12 RSA AP  .....................................................................................G..............
AP13 RSA AP  ...............................C.....................................................G.....C........
AP14 RSA AP  .....................................................................................G..............
AP6 RSA AP   ~~~CYCCCCC.CCCCS.C.C-C.CCCG.C.CC.CCCC--~~~~~~--.CCCCCCCCCC....CC.......M..MCCCC.CSC..G......CCC.-...
```

ADDENDUM C- cont.

```
                       410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
K3 RSA AP       GATTGGGTTAGTGGAGGATTACGATGAGAGTAATCCGAAAGGTCCGAATCCGATGGACCGAAAGGGTTTCAAAAAGGACCAACCGAGAGGTTGGCAATGG
NC004362 PNRSV  AT.G..A.GGTG.AG.AC.ATAAGGTG..TC..C.T..TG.TC.GA.TG..CTGTCTAG...G.....CTTG..G.AC.A.C.GAGAG.T.G.CAGT.C.
J1 RSA AP       ..................................................................................................G...
J3 RSA AP       ....................................................................................................
K2 RSA AP       ....................................................................................................
AY542546 CR PA  ...........................................................................A....................G...
AY542542 CR PA  ...........................................................................A....................G...
AY542545 CR PA  ...........................................................................A....................G...
AY542543 CR PA  ...........................................................................A....................G...
AY542544 CR PA  ......TC.......................................................A...........A....................G...
FN435316 IN AP  .........G.............................G........................................................G...
AMU15608 US AP  .........G.................A...................................A..G..T..G..A...............G.CATG...
HE574164 UN AP  .........G.................A...................................A..G..T..G..A...............G.CATG...
AY542540 CR AP  ...............................................................A..G..T..G...........................
AY542541 BE AP  ...........................................................................A....................G...
FN546183 IN CR  ........................C.......................................................................G...
FN435317 IN AP  ........................C.......................................................................G...
FN435315 IN AP  ........................C.......................................................................G...
FN547927 IN AP  ........................C.......................................................................G...
FN435314 IN AP  ........................C.......................................................................G...
FM178274 UN UN  ........................C.......................................................................G...
FN564150 UN UN  ........................C.......................................................................G...
S78319 GE AP    ...C..TC....A..................................................A..C.............................G...
L03726U US AP   .........G.................A...................................A..G..T..G..A....................G...
AY054385 CR AP  ..................................................................G..T..G...........................
AF548367 KO AP  .........G.................A...................................A..G..T..G..A...............G.CATG...
AM490197 CR AP  ......T..G..T..............A.............................................G..A........................
AY125977 KO AP  .........G.................A...................................A..G..T..G..A....................G...
FJ429311 IN AP  ........................C.......................................................................G...
AY054386 CR PR  ......TC.C..AC..................................................................................G...
AY054387 CR HO  ......TC....A.....................................................C.............................G...
AY054388 CR AL  ......TC..........................................................A.....G..A........................
AY054389 CR PA  ......TC....A.....................................................C.............................G...
```

ADDENDUM C- cont.

```
                        410       420       430       440       450       460       470       480       490       500
                  ...|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
D6RSA AP         ................................................................................................G...
P1 RSA PA        ................................................................................................G...
P2 RSA PA        ................................................................................................G...
P3 RSA PA        ................................................................................................G...
P4 RSA PA        ................................................................................................G...
P5 RSA PA        ................................................................................................G...
P6 RSA PA        ................................................................................................G...
P7 RSA PA        ................................................................................................G...
P8 RSA PA        ................................................................................................G...
P9 RSA PA        ................................................................................................G...
P10 RSA PA       ................................................................................................G...
T2 RSA AP        ................................................................................................G...
T3 RSA AP        ................................................................................................G...
T6 RSA PE        ................................................................................................G...
T7 RSA AP        ................................................................................................G...
T9 RSA AP        ................................................................................................G...
T10 RSA AP       ................................................................................................G...
T11 RSA AP       ................................................................................................G...
AA1 RSA AP       ................................................................................................G...
A114 RSA NE      ................................................................................................G...
A115 RSA NE      ................................................................................................G...
A116 RSA NE      ................................................................................................G...
A120 RSA PO      ................................................................................................G...
A131 RSA AP      ................................................................................................G...
A132 RSA AP      ................................................................................................G...
A133 RSA AP      ................................................................................................G...
A159 RSA PO      .................................................................................G..............G...
A160 RSA PO      ................................................................................................G...
A161 RSA PO      ................................................................................................G...
A163 RSA PR      ................................................................................................G...
A168 RSA AP      ................................................................................................G...
A171 RSA AP      ................................................................................................G...
A172 RSA AP      ................................................................................................G...
```

ADDENDUM C- cont.

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                          410       420       430       440       450       460       470       480       490       500
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AP1 RSA AP       .......................................G................G.......................................G...
AP2 RSA AP       ................................................................................................G...
AP3 RSA AP       ................................................................................................G...
AP4 RSA AP       ..........................................................G.....................................G...
AP5 RSA AP       ................................................................................................G...
AP7 RSA AP       ................................................................................................G...
AP8 RSA AP       ................................................................................................G...
AP9 RSA AP       ................................................................................................G...
AP10 RSA AP      ................................................................................................G...
AP11 RSA AP      ................................................................................................G...
AP12 RSA AP      ................................................................................................G...
AP13 RSA AP      ................................................................................................G...
AP14 RSA AP      ................................................................................................G...
AP6 RSA AP       ..C....................C....CC............C.....................................................G...

                          510       520       530       540       550       560       570       580       590       600
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
K3 RSA AP        GAAGCCCCTCCAAACACAACTTTTGATGACTTCGTGAGGAAGTTTAGGTTGGTATTGGAGTTTAAGACGAATTTCGCCGCTGGCGCGAAAGTCTTTATGA
NC004362 PNRSV   A.CCT......G.TTTAG.T..CGACACTT..GCGCGTACGCA.CGT..C.TC..C.A.T.CA.GAC....G.G.C.GCTG..GC.A.GGTCT.GGT.AG
J1 RSA AP        ....................................................................................................
J3 RSA AP        ....................................................................................................
K2 RSA AP        ....................................................................................................
AY542546 C PA    .....................................................................G.....T........................
AY542542 R PA    ....................................................................................................
AY542545CR PA    .....................................A..............................................................
AY54254 CR PA    .....................................A..............................................................
AY542544 CR PA   .....T.TCT...........................A..............................................................
FN435316 IN AP   ..................................C.................................................................
AMU15608 US AP   ...........G.........................................T.............................A................
HE574164 UN AP   ...........G.........................................T.............................A................
AY542540 CR AP   ...........G...........C.............A...............TC............................T................
AY542541 BE AP   ....................................................................................................
FN546183 IN CR   ..........................C..........................G.............................T.............C..
```

ADDENDUM C- cont.

```
                          510       520       530       540       550       560       570       580       590       600
                 ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
FN435317 IN AP   ..........................C..........................G.............................T.............C..
FN435315 IN AP   ..........................C..........................G.............................T................
FN547927 IN AP   ..........................C..........................G.............................T................
FN435314 IN AP   ..........................C..........................G.............................T................
FM178274 UN UN   ..........................C..........................G.............................T................
FN564150 UN UN   ..........................C..........................G.............................T................
S78319 GE AP     ..................T............................A.....T.............................T................
L03726U US AP    ...........G.........................................T.............................A................
AY054385 CR AP   ...........G.........................................TC............................T................
AF548367 KO AP   ...........G.........................................T.............................A................
AM490197 CR AP   ...........G.........................................TC............................T.....G..........
AY125977 KO AP   ...........G.........................................T.............................A................
FJ429311 IN AP   ..........................C..........................G.............................T................
AY054386 CR PR   ....................................................................................................
AY054387 CR HO   .....T...............................A...............T.............................T................
AY054388 CR AL   ..G..T...TTG........C..C..C..........................T..............................................
AY054389 CR PA   .....T...............................A...............T.............................T................
D6RSA AP         ....................................................................................................
P1 RSA PA        ....................................................................................................
P2 RSA PA        ....................................................................................................
P3 RSA PA        ....................................................................................................
P4 RSA PA        ....................................................................................................
P5 RSA PA        ...............................................................................G....................
P6 RSA PA        ....................................................................................................
P7 RSA PA        ....................................................................................................
P8 RSA PA        ....................................................................................................
P9 RSA PA        ..........................................................................A....G....................
P10 RSA PA       ...............................................................................G....................
T2 RSA AP        ....................................................................................................
T3 RSA AP        ....................................................................................................
T6 RSA PE        ....................................................................................................
T7 RSA AP        ....................................................................................................
T9 RSA AP        ...........................................................................A...G....................
T10 RSA AP       ....................................................................................................
```

ADDENDUM C- cont.

```
                       510       520       530       540       550       560       570       580       590       600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
T11 RSA AP      ...........................................................................A........................
AA1 RSA AP      .............G............C..........................G..............A..............T................
A114 RSA NE     ....................................................................................................
A115 RSA NE     ....................................................................................................
A116 RSA NE     ....................................................................................................
A120 RSA PO     ....................................................................................................
A131 RSA AP     .............................................................................A......................
A132 RSA AP     .............................................................................A......................
A133 RSA AP     ....................................................................................................
A159 RSA PO     .....................................................T.......................A......................
A160 RSA PO     ....................................................................................................
A161 RSA PO     ....................................................................................................
A163 RSA PR     .............G............C.........................................A...............................
A168 RSA AP     ....................................................................................................
A171 RSA AP     .............G............C..........................G..............A..............T................
A172 RSA AP     .............G............C..........................G..............A..............T................
AP1 RSA AP      .....................................................T.......................T..C...................
AP2 RSA AP      .....................................................T.......................A......................
AP3 RSA AP      .....................................................T.......................T..C...................
AP4 RSA AP      .........................................................A..........................................
AP5 RSA AP      .....................................................C..............................................
AP7 RSA AP      .....................................................T..............................................
AP8 RSA AP      ....................................................................................................
AP9 RSA AP      ....................................................................................................
AP10 RSA AP     ....................................................................................................
AP11 RSA AP     .....................................................T.......................A......................
AP12 RSA AP     ....................................................................................................
AP13 RSA AP     ....................................................................................................
AP14 RSA AP     ....................................................................................................
AP6 RSA AP      .............G............C..........................G..............A...............................
```

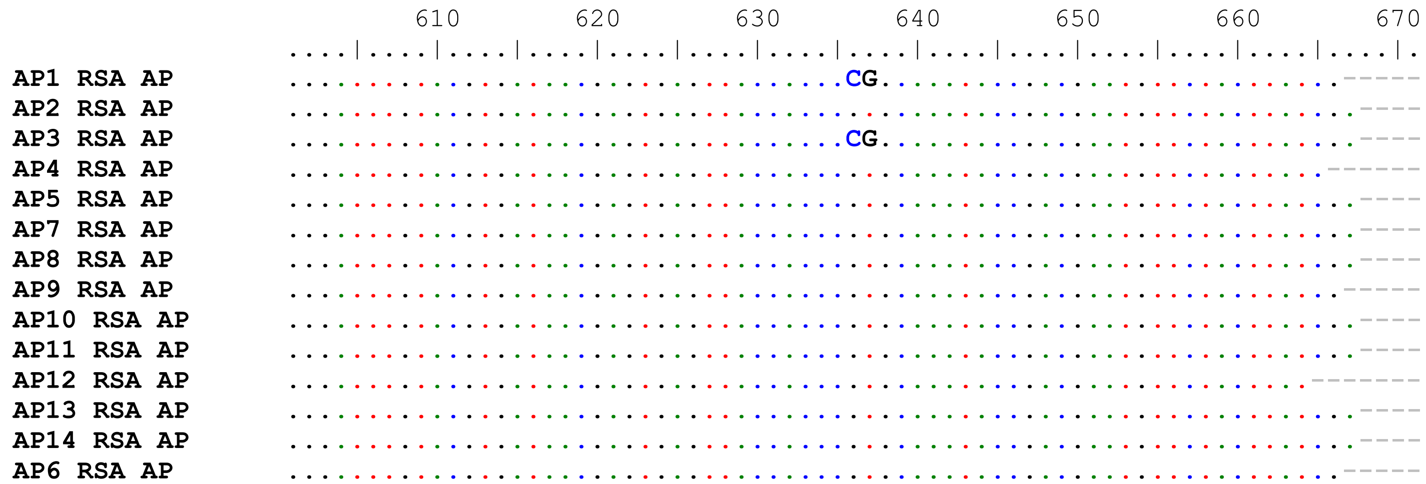
ADDENDUM C- cont.

```
                             610       620       630       640       650       660       670
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
K3 RSA AP           GGGATTTGTACGTGATAACGAGTGAGTTACCACCCGTGCAAATACCGACGAATGTTCTACTTATCGACGAA
NC004362 PNRSV       ..AT..GTACGTA.TGGTAAGTGACT.AC.ACGAGTGCA..T.C.GACT..TGTC.TGCTGGTCGATGAGG
J1 RSA AP           ..................................G....................................
J3 RSA AP           .......................................................................
K2 RSA AP           .......................................................................
AY542546 CR PA      ...........................................................T...........
AY542542 CR PA      .......................................................................
AY542545 CR PA      .......................................................................
AY542543 CR PA      .......................................................................
AY542544 CR PA      ...............................................TT..G..AC...............
FN435316 IN AP      .............................................................C.........
AMU15608 US AP      ..................................G................................T...
HE574164 UN AP      ..................................G................................T...
AY542540 CR AP      ..................................G................G...............T...
AY542541 BE AP      ......................................................C................
FN546183 IN CR      ..................................A....................................
FN435317 IN AP      ..................................A....................................
FN435315 IN AP      ..................................A....................................
FN547927 IN AP      ..................................A....................................
FN435314 IN AP      ..................................A....................................
FM178274 UN UN      ..................................A................................T...
FN564150 UN UN      ..................................A....................................
S78319 GE AP        ..................................G..A..........T..................T...
L03726U US AP       ..................................G................................T...
AY054385 CR AP      ..............G...................G.................C..............T...
AF548367 KO AP      ..................................G................................T...
AM490197 CR AP      ..................................T....G........T.G.C..............T..G
AY125977 KO AP      ..................................G................................T...
FJ429311 IN AP      ..................................A................................T...
AY054386 CR PR      ...............................................TT.............C.A.CT...
AY054387 CR HO      ..................................A............TT..G..........G....T...
AY054388 CR AL      .....................C............T.....G.....TT...................T...
AY054389 CR PA      ..................................G........T...TT..........T.......T...
```

ADDENDUM C- cont.

```
                     610       620       630       640       650       660       670
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
D6RSA AP    ..................................G....................................
P1 RSA PA   ..................................T............T...................T...
P2 RSA PA   ...................................................................T...
P3 RSA PA   .................................--------------------------------------
P4 RSA PA   .............C.....................................................T...
P5 RSA PA   ..............................................T....................T...
P6 RSA PA   .................................G.C.....C.........................T...
P7 RSA PA   .......C.....C.....................................................T...
P8 RSA PA   ...................................................................T...
P9 RSA PA   .................................G.C.....------------------------------
P10 RSA PA  .............C.......C...........--------------------------------------
T2 RSA AP   ..................................-------------------------------------
T3 RSA AP   .......C........................---------------------------------------
T6 RSA PE   ..................................-------------------------------------
T7 RSA AP   .................................--------------------------------------
T9 RSA AP   .......C...........C...........C....-----------------------------------
T10 RSA AP  .......................................--------------------------------
T11 RSA AP  .................................G.C.........G...----------------------
AA1 RSA AP  ..................................T....T.......T...................T...
A114 RSA NE ........................................C..............................
A115 RSA NE ...................................................................T...
A116 RSA NE ...................................................................T...
A120 RSA PO ...................................................................T...
A131 RSA AP ...............................................T...................T...
A132 RSA AP ..................................G............T...................T...
A133 RSA AP .........................................................--------------
A159 RSA PO ...................................................................T...
A160 RSA PO .................................T.................................T...
A161 RSA PO .................................T.............C...................T...
A163 RSA PR ...................................................................T...
A168 RSA AP ...................................................................T...
A171 RSA AP ..................................T....T.......T...................T...
A172 RSA AP ..................................T............T...................T...
```

ADDENDUM C- cont.



ADDENDUM D

Percentage nucleotide sequence similarities between ApMV CP genes of isolates from South Africa as determined using BioEdit.

	K3	J1	J3	K2	D6	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	T2	T3	T6	T7	T9	T10	T11	AA1	A114	A115	A116
K3		99.1	100	100	99.5	99.3	99.5	99.3	99.3	99.3	99.5	99.1	99.5	99	98.8	99.3	99	99.3	99.3	98.5	99.1	99.3	96.4	99.5	99.5	99.5
J1			99.1	99.1	99.6	99.5	99.6	99.5	99.5	99.5	99.6	99.3	99.6	99.1	99	99.5	99.1	99.5	99.5	98.7	99.3	99.5	96.2	99.6	99.6	99.6
J3				100	99.5	99.3	99.5	99.3	99.3	99.3	99.5	99.1	99.5	99	98.8	99.3	99	99.3	99.3	98.5	99.1	99.3	96.4	99.5	99.5	99.5
K2					99.5	99.3	99.5	99.3	99.3	99.3	99.5	99.1	99.5	99	98.8	99.3	99	99.3	99.3	98.5	99.1	99.3	96.4	99.5	99.5	99.5
D6						99.8	100	99.8	99.8	99.8	100	99.6	100	99.5	99.3	99.8	99.5	99.8	99.8	99	99.6	99.8	96.5	100	100	100
P1							99.8	99.8	99.6	99.6	99.8	99.5	99.8	99.5	99.3	99.8	99.5	99.8	99.8	99	99.6	99.6	96.7	99.8	99.8	99.8
P2								99.8	99.8	99.8	100	99.6	100	99.5	99.3	99.8	99.5	99.8	99.8	99	99.6	99.8	96.5	100	100	100
P3									99.6	99.6	99.8	99.5	99.8	99.6	99.5	100	99.6	100	100	99.1	99.8	99.6	96.5	99.8	99.8	99.8
P4										99.6	99.8	99.8	99.8	99.3	99.5	99.6	99.3	99.6	99.6	98.8	99.5	99.6	96.4	99.8	99.8	99.8
P5											99.8	99.5	99.8	99.6	99.5	99.6	99.3	99.6	99.6	99.1	99.5	99.6	96.4	99.8	99.8	99.8
P6												99.6	100	99.5	99.3	99.8	99.5	99.8	99.8	99	99.6	99.8	96.5	100	100	100
P7													99.6	99.1	99.3	99.5	99.5	99.5	99.5	99	99.3	99.5	96.2	99.6	99.6	99.6
P8														99.5	99.3	99.8	99.5	99.8	99.8	99	99.6	99.8	96.5	100	100	100
P9															99.5	99.6	99.3	99.6	99.6	99.1	99.5	99.3	96.2	99.5	99.5	99.5
P10																99.5	99.1	99.5	99.5	99	99.3	99.1	96.1	99.3	99.3	99.3
T2																	99.6	100	100	99.1	99.8	99.6	96.5	99.8	99.8	99.8
T3																		99.6	99.6	99.1	99.5	99.3	96.2	99.5	99.5	99.5
T6																			100	99.1	99.8	99.6	96.5	99.8	99.8	99.8
T7																				99.1	99.8	99.6	96.5	99.8	99.8	99.8
T9																					99	99.1	95.7	99	99	99
T10																						99.5	96.4	99.6	99.6	99.6
T11																							96.4	99.8	99.8	99.8
AA1																								96.5	96.5	96.5
A114																									100	100
A115																										100

ADDENDUM D- cont.

Percentage nucleotide sequence similarities between ApMV CP genes of isolates from South Africa as determined using BioEdit.

	A120	A131	A132	A133	A159	A160	A161	A163	A168	A171	A172	AP1	AP2	AP3	AP4	AP5	AP7	AP8	AP9	AP10	AP11	AP12	AP13	AP14
K3	99.5	98.7	98.7	99.5	98.7	99.5	99.3	97.2	99.5	96.5	96.4	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
J1	99.6	98.5	98.5	99.6	98.5	99.3	99.1	97	99.6	96.4	96.2	97.7	98.3	98	98.7	98.5	98.8	99.5	99.3	99.1	98.3	98.5	99	99.5
J3	99.5	98.7	98.7	99.5	98.7	99.5	99.3	97.2	99.5	96.5	96.4	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
K2	99.5	98.7	98.7	99.5	98.7	99.5	99.3	97.2	99.5	96.5	96.4	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
D6	100	98.8	98.8	100	98.8	99.6	99.5	97.4	100	96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8
P1	99.8	99	99	99.8	98.7	99.5	99.5	97.2	99.8	96.9	96.7	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
P2	100	98.8	98.8	100	98.8	99.6	99.5	97.4	100	96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8
P3	99.8	98.8	98.8	99.8	98.7	99.5	99.5	97.2	99.8	96.7	96.5	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
P4	99.8	98.7	98.7	99.8	98.7	99.5	99.3	97.2	99.8	96.5	96.4	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
P5	99.8	98.7	98.7	99.8	98.7	99.5	99.3	97.2	99.8	96.5	96.4	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
P6	100	98.8	98.8	100	98.8	99.6	99.5	97.4	100	96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8
P7	99.6	98.5	98.5	99.6	98.5	99.3	99.1	97	99.6	96.4	96.2	97.5	98.3	97.8	98.7	98.5	98.8	99.5	99.3	99.1	98.3	98.5	99	99.5
P8	100	98.8	98.8	100	98.8	99.6	99.5	97.4	100	96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8
P9	99.5	98.5	98.5	99.5	98.3	99.1	99.1	96.9	99.5	96.4	96.2	97.4	98.2	97.7	98.5	98.3	98.7	99.3	99.1	99	98.2	98.3	98.8	99.3
P10	99.3	98.3	98.3	99.3	98.2	99	99	96.7	99.3	96.2	96.1	97.2	98	97.5	98.3	98.2	98.5	99.1	99	98.8	98	98.2	98.7	99.1
T2	99.8	98.8	98.8	99.8	98.7	99.5	99.5	97.2	99.8	96.7	96.5	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
T3	99.5	98.5	98.5	99.5	98.3	99.1	99.1	96.9	99.5	96.4	96.2	97.4	98.2	97.7	98.5	98.3	98.7	99.3	99.1	99	98.2	98.3	98.8	99.3
T6	99.8	98.8	98.8	99.8	98.7	99.5	99.5	97.2	99.8	96.7	96.5	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
T7	99.8	98.8	98.8	99.8	98.7	99.5	99.5	97.2	99.8	96.7	96.5	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
T9	99	98	98	99	97.8	98.7	98.7	96.4	99	95.9	95.7	96.9	97.7	97.2	98	97.8	98.2	98.8	98.7	98.5	97.7	97.8	98.3	98.8
T10	99.6	98.7	98.7	99.6	98.5	99.3	99.3	97	99.6	96.5	96.4	97.5	98.3	97.8	98.7	98.5	98.8	99.5	99.3	99.1	98.3	98.5	99	99.5
T11	99.8	98.7	98.7	99.8	98.7	99.5	99.3	97.2	99.8	96.5	96.4	97.7	98.5	98	98.8	98.7	99	99.6	99.5	99.3	98.5	98.7	99.1	99.6
AA1	96.5	96.5	96.5	96.5	96.4	96.5	96.5	99.1	96.5	99.8	100	95.2	96.5	95.6	96.2	96.4	96.7	96.7	96.5	97	96.2	96.1	96.5	96.7
A114	100	98.8	98.8	100	98.8	99.6	99.5	97.4	100	96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8
A115	100	98.8	98.8	100	98.8	99.6	99.5	97.4	100	96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8

ADDENDUM D- cont.

Percentage nucleotide sequence similarities between ApMV CP genes of isolates from South Africa as determined using BioEdit.

	A120	A131	A132	A133	A159	A160	A161	A163	A168	A171	A172	AP1	AP2	AP3	AP4	AP5	AP7	AP8	AP9	AP10	AP11	AP12	AP13	AP14
A116	100	98.8	98.8	100	98.8	99.6	99.5	97.4	100	96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8
A120		98.8	98.8	100	98.8	99.6	99.5	97.4	100	96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8
A131			100	98.8	99.3	99.1	99.1	97	98.8	96.7	96.5	97.8	99.1	98.2	98.2	98.7	99	99	98.8	98.7	99.1	98.7	98.5	99
A132				98.8	99.3	99.1	99.1	97	98.8	96.7	96.5	97.8	99.1	98.2	98.2	98.7	99	99	98.8	98.7	99.1	98.7	98.5	99
A133					98.8	99.6	99.5	97.4	100	96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8
A159						99.1	99	97	98.8	96.5	96.4	98.2	99.5	98.5	98.2	98.8	99.3	99	98.8	98.7	99.5	98.7	98.5	99
A160							99.8	97.4	99.6	96.7	96.5	98.2	99	98.5	99	98.8	99.5	99.8	99.6	99.5	99	99.1	99.3	99.8
A161								97.2	99.5	96.7	96.5	98	98.8	98.3	98.8	98.7	99.3	99.6	99.5	99.3	98.8	99	99.1	99.6
A163									97.4	99.3	99.1	95.9	96.9	96.2	96.7	97	97	97.5	97.4	97.8	96.9	96.9	97.4	97.5
A168										96.7	96.5	97.8	98.7	98.2	99	98.8	99.1	99.8	99.6	99.5	98.7	98.8	99.3	99.8
A171											99.8	95.4	96.7	95.7	96.1	96.5	96.9	96.9	96.7	97.2	96.4	96.2	96.7	96.9
A172												95.2	96.5	95.6	96.2	96.4	96.7	96.7	96.5	97	96.2	96.1	96.5	96.7
AP1													98	99.6	97.2	97.5	98	98	97.8	97.7	98	98.7	97.5	98
AP2														98.3	98	98.7	99.5	98.8	98.7	98.5	99.3	98.5	98.3	98.8
AP3															97.5	97.8	98.3	98.3	98.2	98	98.3	99	97.8	98.3
AP4																98.5	98.5	99.1	99	98.8	98	98.2	98.7	99.1
AP5																	98.8	99	98.8	98.7	98.7	98.3	98.5	99
AP7																		99.3	99.1	99	99.1	98.7	98.8	99.3
AP8																			99.8	99.6	98.8	99	99.5	100
AP9																				99.5	98.7	98.8	99.3	99.8
AP10																					98.5	98.7	99.5	99.6
AP11																						98.5	98.3	98.8
AP12																							98.5	99
AP13																								99.5
AP14																								