An evaluation of the efficacy of antimicrobial peptides against grapevine pathogens

by

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Abstract

This study investigated the use of antimicrobial peptides (AMPs) as possible source of resistance against a range of pathogens in grapevine. Whilst the ultimate aim would be to express AMPs in grapevine, the development of transgenic grapevine is time consuming and therefore pre-screening of potential AMPs is necessary. These small molecules, of less than 50 amino acids in length, are expressed by almost all organisms as part of their non-specific defence system. *In vitro* pre-screening of AMP activity is valuable but is limited since the activity on artificial media may differ from the AMP activity *in planta*. These tests are also restricted to pathogens which can be cultured *in vitro*. These limitations can be overcome by using transient expression systems to determine the *in planta* activity of AMPs against pathogens of interest. In this study transient systems were used to express AMPs in developed plant tissue to test their efficacy against grapevine pathogens such as *Agrobacterium vitis*, *Xylophilus ampelinus* and aster yellows phytoplasma. Aster yellows phytoplasma, which was recently discovered in local vineyards, is known to cause extensive damage and therefore pose a great threat to the South African grapevine industry.

To study the *in planta* effect of AMPs against the abovementioned pathogens, transient expression vectors were constructed expressing either of the AMPs D4E1 or Vv-AMP1. D4E1 is a synthetically designed AMP known to be active against bacteria and fungi, while Vv-AMP1, isolated from grapevine berries, has already shown activity against fungi. In a transient approach in grapevine, the expression of foreign genes from viral and non-viral vectors was confirmed by expression of the marker genes β-glucuronidase and Green Fluorescent Protein, while tissue-printing immunoassays confirmed viral replication and systemic spread in *Nicotiana benthamiana*. The viral vectors were based on the phloem-limited virus grapevine virus A. Only Agrobacterium-mediated 35S transient expression vectors were used for AMP *in planta* activity screening since the viral-mediated expression in grapevine was insufficient for screening against *A. vitis* and *X. ampelinus* as it was restricted to phloem tissues after whole-leaf infiltration. No phytoplasma-infected material could be established and as a result AMP activity screening was only performed against the *A. vitis* and *X. ampelinus*. Quantification of the bacteria was performed by qPCR. Vv-AMP1 did not show activity against either of the two bacteria *in planta* while D4E1 was found to be active against both. The observed *in planta* activity of D4E1 correlated with the *in vitro* activity as measured in an AMP plate bioassay. In contrast to *in vitro* screenings, the *in planta* AMP activity

screening might give a more accurate representation of the potential antimicrobial activity of the peptide in a transgenic plant environment.

This study proved that transient expression systems can be used as a pre-screening method of AMP activity *in planta* against grapevine pathogens, allowing the screening of various AMPs in a relatively short period of time before committing to transgenic grapevine development.

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Opsomming

Hierdie studie het die gebruik van antimikrobiese peptiede (AMPe) as 'n moontlik bron van weerstand teen 'n reeks van patogene in wingerd ondersoek. Alhoewel die uiteindelike doel sal wees om AMPe uit te druk in wingerd, is transgeniese wingerd ontwikkeling tydrowend en daarom is vooraf evaluering van potensiële AMPe nodig. Hierdie klein molekules, van minder as 50 aminosure in lengte, word uitgedruk deur amper alle organismes as deel van hul nie-spesifieke verdedigingsisteem. *In vitro* vooraf evaluering van AMP aktiwiteit is van waarde, maar is beperk aangesien die aktiwiteit op kunsmatige media mag verskil van die AMP-aktiwiteit *in planta*. Hierdie toetse is ook beperk tot patogene wat *in vitro* gekweek kan word. Hierdie beperkinge kan oorkom word deur gebruik te maak van tydelike uitdrukkingsisteme om die *in planta* aktiwiteit van AMPe te bepaal teen patogene van belang. In hierdie studie is tydelike uitdrukkingsisteme gebruik om AMPe uit te druk in ontwikkelde plantweefsel om hul effektiwiteite te toets teen wingerdpatogene soos *Agrobacterium vitis*, *Xylophilus ampelinus* en aster yellows fitoplasma. Aster yellows fitoplasmas, wat onlangs in plaaslike wingerde ontdek is, is bekend vir die uitgebreide skade wat hul aanrig en hou daarom 'n groot bedreiging in vir die Suid-Afrikaanse wingerd industrie.

Om die *in planta* effek van AMPe teen die bogenoemde patogene te bestudeer is tydelike uitdrukkingsvektore ontwikkel wat die AMPe D4E1 of Vv-AMP1 uitdruk. D4E1 is 'n sinteties-ontwerpte AMP wat aktief is teen bakterieë en fungi, terwyl Vv-AMP1, wat uit druiwekorrels geïsoleer is, alreeds aktiwiteit teen fungi getoon het. In 'n tydelike uitdrukkingsbenadering in wingerd is die uitdrukking van transgene, vanaf virus of nie-virus gebaseerde vektore, bevestig deur die uitdrukking van die merker gene β-glukuronidase en die Groen Fluoresserende Proteïen, terwyl weefsel afdrukkings-immunotoetse virus replisering en sistemiese beweging in *Nicotiana benthamiana* bevestig het. Die virusvektore was gebaseer op die floëem-beperkte virus, wingerdvirus A. Slegs Agrobacterium-bemiddelde 35S tydelike uitdrukkingsvektore is gebruik om die AMP *in planta* aktiwiteit te bepaal aangesien die virus-bemiddelde uitdrukking in wingerd onvoldoende was vir evaluering teen *A. vitis* en *X. ampelinus* weens die beperking tot die floëem weefsel na infiltrering van die totale blaar. Geen fitoplasma geïnfekteerde materiaal kon gevestig word nie, en daarom is AMP aktiwiteitsevaluering slegs teen *A. vitis* en *X. ampelinus* uitgevoer. Kwantifisering van die bakterieë is deur middel van qPCR uitgevoer. Vv-AMP1 het geen aktiwiteit getoon teen enige van die bakterieë *in planta* nie, terwyl D4E1 aktief was teen beide. Die waargenome *in planta* aktiwiteit van D4E1 het ooreengestem met die *in vitro* aktiwiteit soos bepaal deur 'n AMP

plaat bio-toets. In kontras tot *in vitro* evaluering kan die *in planta* AMP-aktiwiteit evaluering 'n meer akkurate voorspelling bied van die potensiële antimikrobiese aktiwiteite van die peptied in 'n transgeniese plant omgewing.

Hierdie studie het bewys dat tydelike uitdrukkingsisteme gebruik kan word as 'n voorafgaande evalueringsmetode vir AMP *in planta* aktiwiteit teen wingerdpatogene, wat die evaluering van 'n verskeidenheid AMPe in 'n relatiewe kort tydperk toelaat voor verbintenis tot die ontwikkeling van transgeniese wingerd.

Abbreviations

AMP(s)	Antimicrobial peptide(s)
ARC	Agricultural Research Council
ATP	Adonine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
CaMV	Cauliflower mosaic virus
cDNA	Complementary deoxyribonucleic acid
cfu	Colony forming units
CP	Coat protein
Ct	Threshold cycle
CTAB	N-Cetyl-N,N,N-trimethyl Ammonium Bromide
dpi	Days post infiltration (inoculation)
DTT	1,4-Dithiothreitol
E	qPCR reaction efficiency
EDTA	Ethylene Diamine Tetra-Acetic Acid di-sodium salt
ELISA	Enzyme-linked immunosorbent assay
EmGFP	Enhanced Green Fluorescent Protein
GFP	Green Fluorescent Protein
GOI	Gene of interest
GUS(i)	β- glucuronidase
GVA	Grapevine virus A
LTP	Lipid transfer protein
MCS	Multiple cloning site
MIC	Minimum inhibition concentration
MLOs	Mycoplasma-like organisms
MS	Murashige and Skoog
NA	Nutrient agar
NBT	Nitroblue tetrazolium
OD	Optical density
ORF	Open reading frame
PLRV	Potato leafroll virus
PTGS	Post-transcriptional gene silencing
PVDF	Polyvinylidene fluoride
PVX	Potato virus X

PVY	Potato virus Y
qPCR	Quantitative real-time PCR
R^2	Correlation coefficient
RCA	Rolling circle amplification
rDNA	Ribosomal DNA
REST	Relative Expression Software Tool
RNA	Ribonucleic Acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription - polymerase chain reaction
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Poli-acrylamid gel electrophoresis
S.E.	Standard error
sgRNA	Subgenomic RNA
SOC	Super optimal broth with catabolite repression
T-DNA	Transfer DNA
Ti-plasmid	Tumour inducing plasmid
TMV	Tobacco mosaic virus
TPIA(s)	Tissue-print immuno-assay(s)
VIGS	Virus-induced gene silencing

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Chapter 1

Introduction

1.1 Research background and motivation

The importance of grapevine (*Vitis vinifera*) as an agricultural commodity can not be over emphasised. In South Africa the wine industry alone contributed R2.6 billion to the country's gross domestic product in 2008 (http://www.sawis.co.za). More than 275000 people are employed by the wine industry and more than 66000 by the table grape industry (http://www.satgi.co.za). Altogether more than 115000 hectares of agricultural land in South Africa are planted under grapevine. The many grapevine pathogens therefore play an important agricultural role as they can be, and have been in the past, held responsible for great economic losses (Purcell, 1997; Lee *et al*., 2000; Hadidi *et al*., 2003). No other single crop has as many intracellular pathogens as grapevine (Martelli and Boudon-Padieu, 2006). They are targeted by phytoplasmas, bacteria, fungi, viroids and more than 60 viruses. Despite their almost omnipresent nature, grapevine viruses are not the cause of the most devastating diseases in these plants. Bacterial and especially fungal infections have on countless occasions led to vastly destructive disease outbreaks (Yamamoto *et al*., 2000). Of all grapevine diseases powdery mildew, caused by the fungus *Erysiphe necator*, is considered to be economically the most important (Winterhagen *et al*., 2008). In Europe and Australia, phytoplasma alone have destroyed large regions of vineyards (Lee *et al*., 2000). This gave local farmers reason for alarm after the first report of phytoplasma infections in South Africa in 2006 (Burger, 2008). It is therefore of high significance to find an approach to control these disastrous grapevine diseases before the further spread thereof.

To combat plant pathogens, scientists are now employing short peptides, known as antimicrobial peptides (AMPs). These antimicrobial agents are usually produced by organisms as part of their natural defence system (Montesinos, 2007). They can, when expressed through genetic transformation in host plants, offer a form of tolerance against a range of pathogens. The development of transgenic grapevine is, however, a tedious and time

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consuming process. It can take up to three years to develop a transgenic line before it can be tested for resistance against a pathogen. To prevent the development of transgenic grapevine lines that show no form of resistance, due to expression of a specific AMP, against pathogens, an efficient procedure for AMP activity pre-screening is desirable. *In vitro* pre-screening for the effect of candidate AMPs against pathogens may still lead to negative results as peptides may react differently in an artificial than in a plant environment (Florack *et al*., 1993). It would therefore be ideal to first screen the activity of AMPs *in planta* against pathogens before considering starting the process of developing transgenic plants expressing these peptides.

In this study, transient systems, in which plants were inoculated with vectors expressing the AMP sequences, were developed. These plants were used to test the *in planta* activity of two AMPs against grapevine pathogens. The use of a transient expression system will not circumvent the subsequent production of transgenic plants, but can be a useful *in planta* pre-selection system for AMP efficacy. It can be performed in a relative short time period for a large number of AMPs.

1.2 Project proposal

This study aimed to establish a transient expression system for the investigation of AMP efficacy against grapevine pathogens. The effect of AMPs would be tested against *Agrobacterium vitis* (*A. vitis*) and *Xylophilus ampelinus* (*X. ampelinus*) *in vitro* and *in planta* and against aster yellows phytoplasma *in planta* only.

To achieve this goal it was necessary to reach the following objectives:
- o AMPs needed to be obtained from available sources and their efficacy tested *in vitro* against *A. vitis* and *X. ampelinus*.
- o Transient expression vectors, both virus-based and Agrobacterium-mediated, containing control genes, needed to be constructed and the expression of the foreign genes needed to be tested in *Nicotiana benthamiana* and grapevine.
- o Transient expression vectors, viral and Agrobacterium-mediated, containing AMP genes needed to be constructed and the infectivity of the viral vectors needed to be tested in *Nicotiana benthamiana* and grapevine.
- o Healthy grapevine plants needed to be Agrobacterium vacuum-infiltrated with the AMP expressing vectors and tested for their activity against *A. vitis* and *X. ampelinus* infection.
- o Phytoplasma-infected plants needed to be identified and established *in vitro*.

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- o Phytoplasma-infected plants needed to be Agrobacterium vacuum-infiltrated with the AMP expressing vectors.
- o The effects of the AMPs needed to be tested by measuring microbial titres and disease development.

Chapter 2

Literature review

2.1 Introduction

The grapevine industry makes a substantial contribution to the South African economy. This contribution is, however, threatened by the constant exposure of vineyards to potentially harmful pathogens. In general, combating these pathogens requires either the breeding of resistant cultivars or controlling the spread of the disease through vector control and sanitary measures. Vector control is costly and up until now the breeding of resistant cultivars, against bacterial and viral infections, has not been successful. Due to the presence of economically important pathogens in local vineyards it is important to establish an effective way of combating these pathogens. This chapter will give an overview of antimicrobial peptides, molecules used for inducing pathogen resistance in plants, as well as on transient expression systems. It will focus on what they are, how they function and what they are used for. A brief background will be provided on three grapevine pathogens, namely *A. vitis* and *X. ampelinus* and aster yellows phytoplasma, and also on the diseases they cause in their hosts.

2.2 Antimicrobial peptides

Plants, mammals, insects and fungi naturally produce short peptides, of less than 50 amino acids, known as antimicrobial peptides (Montesinos, 2007). These peptides often play an important role in an organism's non-specific defence systems. Organisms can produce AMPs in response to microbial infections or they can produce AMPs constitutively and store it in large quantities for later use when infection occurs (Rydlo *et al.*, 2006). Their production is at low metabolic cost. Currently, several hundred AMPs have been identified from diverse origins (Fjell *et al.*, 2007). Most AMPs, although not all, are cationic and amphipathic (Bowman, 2003; Yeaman and Yount, 2003), and based on their structural diversity, can be grouped into different classes (Montesinos, 2007). Due to their non-specificity they offer the host organism a broad spectrum of protection against a variety of microorganisms (Bals,

2000). This non-specificity also lessens the possibility of the development of resistance in microorganisms (Yeaman and Yount, 2003).

2.2.1 AMPs originating from plants

Similar to most other living organisms, plants express AMPs as part of their defence systems. Antimicrobial peptides expressed by plants can be divided into three main groups namely, the plant defensins (Terras *et al*., 1995), thionins (De Caleya *et al*., 1972) and lipid transfer proteins (LTP) (Kader *et al*., 1984). Other groups include the cyclotides, hevein- and knottin-type of AMPs (Forrokhi *et al*., 2008). The mostly antifungal activity of plant AMPs point towards the critical role fungi play as plant pathogens.

Plant defensins are small (45-54 amino acids in length), basic peptides that are rich in cysteine residues and have previously been classified as γ-thionins (Collila *et al*., 1990). Each peptide contains four disulfide bridges formed by eight cysteine residues (Thomma *et al*., 2002). Within the group the tertiary structure is conserved, while the amino acid homology is low (Castro and Fontes, 2005). Although different plant regions express defensins, they have largely been identified in the seeds (Lay and Anderson, 2005). They are thought to be expressed by all plant species. The first plant defensin isolated from *Vitis vinifera* (*V. vinifera*) was Vv-AMP1, and was shown to be expressed during berry ripening (De Beer and Vivier, 2008). The exact mechanism of plant defensin *in vivo* action against pathogens still needs to be elucidated; they do however show a broad range of antimicrobial activity specific to either fungi or a certain bacterial group (Castro and Fontes, 2005). One group of plant defensins, that has no antifungal activity, has an α-amylase inhibiting reaction which provides protection against insects that might feed on the plants (Shade *et al*., 1994). Only a few plant defensins show any form of antibacterial activity (Thomma *et al*., 2002). An example of crop improvement through transgenic plant development is the expression of an oat defensin in rice resulting in better resistance to *Burkholderia plantarii* and *Burkholderia glumaeby* (Segura *et al*., 1998). An increase in resistance to *Botrytis cinerea*, *Erwinia carotovora* and *Magnaporthe grisea* was observed in transgenic potatoes and rice expressing a defensin isolated from wasabi (Lay and Anderson, 2005).

Thionins are also small, basic peptides that are 45-47 amino acids in length (Forrokhi *et al*., 2008). They consist of sulphur-containing amino acids such as arginine, cysteine and lysine, and other basic residues (Castro and Fontes, 2005). They possess a β-sheet with an antiparallel double α-helix centre (Oard *et al*., 2010). The secondary structure is held together by three or four disulfide bridges and is conserved amongst thionins. They have been isolated

from cereals such as wheat (Balls *et al*., 1942) and oat (Bekes and Lasztity, 1981), as well as from mistletoe (Samuelsson, 1973). Based on their amino acid sequences or disulfide-bond structure they can be divided into five or three groups respectively (Garcia-Olmedo *et al*., 1998). Besides crambin, all thionins are known for their toxicity to a variety of different organisms as well as different cells, including mammalian and plant cells (Castro and Fontes, 2005). They offer their hosts resistance to fungal (Bohlmann *et al*., 1988) as well as bacterial (Garcia-Olmedo *et al*., 1998) infections.

Two subgroups of plant LTP exist, LTP1 and LTP2, which are 10 and 7 kDa in size, respectively (Carvalho and Gomes, 2007). Both families contain eight cysteine residues, connected by 4 disulfide bridges, at conserved regions of the primary structure. These peptides are able to move lipids from one membrane to another (Kader, 1996). As for most AMPs the method of pathogen inhibition still needs to be elucidated for LTPs. It has been suggested that they may cause membrane permeability due to the high value of their isoelectric point. However, not all LTPs play a role in plant defence reactions. Lipid transfer proteins with antimicrobial activity have been isolated from various regions of the plant, especially from leaves and seeds (Carvalho and Gomes, 2007), the most deadly being *Ace-AMP1* from onion seeds (Cammue *et al*., 1995). In general, fungi have been found to be more susceptible to LTPs than bacteria (Kader, 1996). Wheat and barley are two sources of LTPs and the genes isolated from these plants had been applied to the development of disease resistance in other plants (Jayaraj and Punja, 2007; Molina and Garcia-Olmedo, 1997). A pepper LTP, over-expressed in Arabidopsis, increased the plants' resistance to *Pseudomonas syringae* pv. *tomato* and *Botrytis cinerea* (Jung *et al*., 2005). A decrease in symptom development was observed as well as a 100-fold reduction in *Pseudomonas syringae* pv. *tomato* concentration.

2.2.2 Mechanisms of action

There are many proposed mechanisms of cell death induced by AMPs. Primarily, pathogen cell membranes are targeted, because of the peptide's high affinity for membranes, due to the fact that most AMPs are positively charged and have amphipathic qualities (Yeaman and Yount, 2003). The phospholipids, lipopolysaccharides and teichoic or teichuronic acids found in bacterial membranes give the membranes a net negative charge that attract cationic peptides. Most AMPs which come into contact with their target membranes take on amphipathic conformations, making them able to interact with both the inner and outer part of the microbial membranes. Approximately 50% of peptide residues are hydrophobic, allowing binding to the lipid bilayer. Lysine and arginine forms strong interactions with the lipid

bilayer phosphate groups (Mavri and Vogel, 1996). The bound AMPs affect the permeability of the target membrane and can form pores in it, resulting in leakage of metabolites and ions through these pores. The disruption of the membrane potential and depolarisation thereof in turn lead to cell death (Hancock and Chapple, 1999). Although, membrane permeability may lead to cell death, this mechanism on its own is not in all instances adequate to cause cell death, and often other target sites are required. In addition to cell membrane disruption, some AMPs have also been shown to target intracellular components of the invading pathogens (Park *et al.*, 1998), while others may inhibit the synthesis of biopolymers (Chitnis and Prasad, 1990). Figure 1 illustrates the two main mechanisms of AMP action, firstly, their interaction with the cell membrane and secondly, targeting intracellular molecules. When more than one mechanism is applied independently or in cooperation with others, and leads to cell death, it is described as a "multi-hit process" (Zhang *et al.*, 2000). Some AMPs also interacts with others and the resulting heterologous peptide has been shown to be even more effective against microbes than the independent peptide alone (Westerhoff *et al.*, 1995).
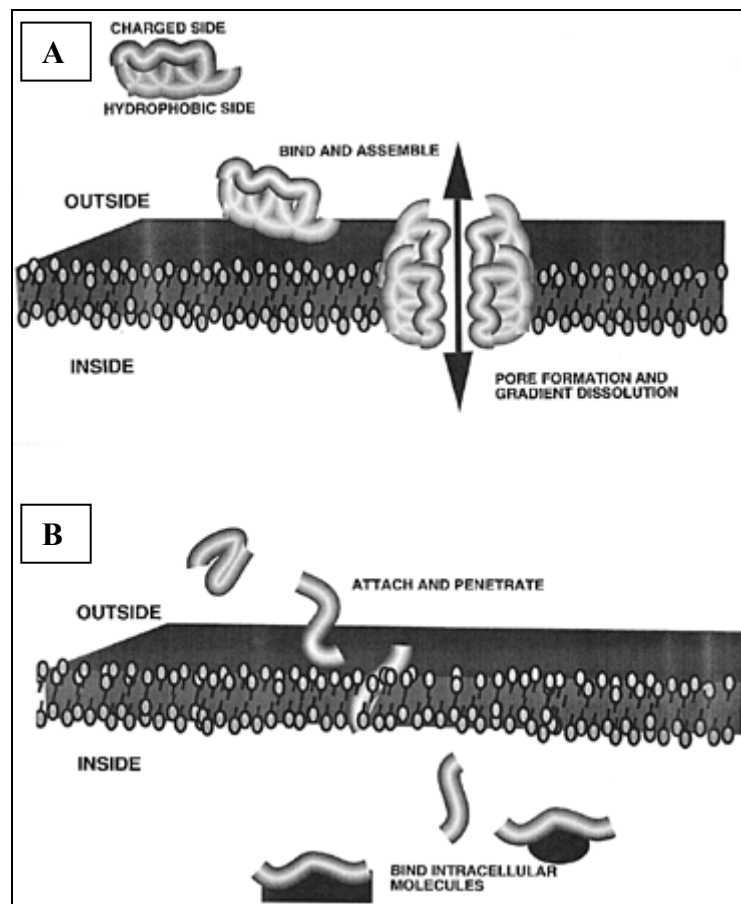
Figure 1: Mechanisms of antimicrobial activity. A) AMP forming pores in the cell membrane. B) The targeting of intracellular molecules by AMPs (Gallo and Huttner, 1998).

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2.2.3 Factors influencing AMP activity

Despite the many structural and chemical characteristics of AMPs that play a role in the efficacy of a peptide against a certain pathogen, many external factors play an additional role in the overall activity of a specific peptide. Fassi Fehri *et al*. (2007) showed that the *in vitro* action of peptides is dependent on factors such as the concentration of the AMP, the time of exposure as well as the density of the bacteria. The effect of temperature has been proven to vary for different AMPs. Some AMPs become insoluble at lowered temperatures (Maisnier-Patin and Forni, 1996) while others seem to have a more stable structure at these temperatures (Kaur *et al*., 2004). Other factors that have an effect on different AMPs activities are the pH as well as salt concentration (Rydlo *et al*., 2006). The cationic nature of the medium was also shown to have a strong influence on the antifungal activity of plant defensins (Osborn *et al*., 1995). All these factors are important to keep in mind when working with AMPs, as they may vary between *in vitro* and *in planta* states, which may in turn lead to a difference in observed peptide efficacy.

2.2.4 Target specificity

All antimicrobial peptides show activity against a wide variety of microbes. They often, however, tend to have a selected target organism specificity, for example only antifungal but no antibacterial activity. The questions therefore arise; why are AMPs only active against specific targets and why are they selectively toxic and do not attack their host cells? Many theories exist and much research has been done in support of the different theories. These studies mainly focus on the differences in membrane composition between the organisms, especially between prokaryotic and eukaryotic cells, their different membrane structures, the differences in hydrophobicity and their differences in charge and membrane-potential. Another factor that plays a role is the specific structure that the peptide adapts to before and when binding to a membrane. For a more detailed discussion on target specificity the reader is referred to the review article of Yeaman and Yount (2003).

2.2.5 Pathogen resistance against AMPs

The growing number of bacteria resistant against antibiotics has shift the focus to using AMPs as a measure to overcome these resistances. Despite their mostly non-specific action and the fact that AMPs have been shown to be highly effective against microorganisms, some microbes have, however, managed to developed mechanisms of resistance against these attacks.

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Figure 2 illustrates how microbes have developed parallel mechanisms to counter AMPs' mechanisms of attack. *Salmonella typhimurium*, for example, has developed a mechanism of losing the negative charge of its surface through the addition of aminoarabinose to the 4' phosphate of the lipid, a region of the lipopolysaccharide, preventing numerous AMPs to adhere to its outer membrane (Gunn *et al*., 1998). Other examples include *Escherichia coli* (*E. coli*) strains that have the ability to proteolytically cleave AMPs (Stumpe *et al*., 1998) and the ability of *Yersinia enterocolitica* to export AMPs from their cells via an energy-dependent pump (Bengoechea and Skurnik, 2000). Despite these microbial counter-strategies for resistance, there are still many AMPs to which resistance has not been developed yet. Caution has to be taken to prevent microbes from developing new resistant strains. Although these examples are based on pathogens of the animal kingdom, plant pathogens also have the potential of implementing these strategies to defend themselves against the activity of AMPs.
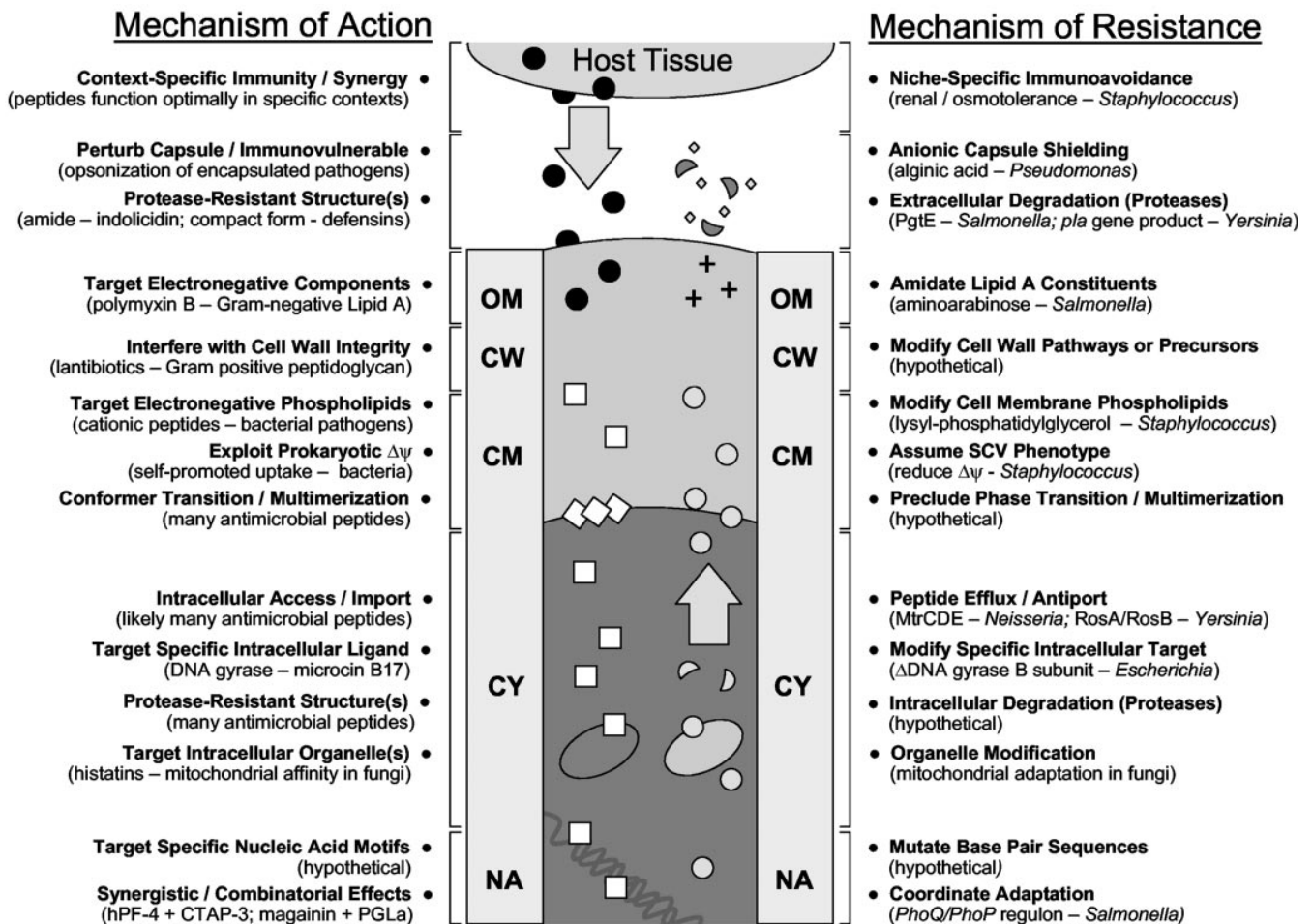
Figure 2: Diagram showing the mechanisms of resistance that microorganism developed to overcome antimicrobial peptide activity. Active peptides (squares) are broken down or inactivated (light circles) and released from the cells. Key: OM, outer membrane; CW, cell wall; CM, cytoplasmic membrane; CY, cytoplasm; NA, nucleic acid; CMV, small colony variant (Yeaman and Yount, 2003).

2.2.6 Synthetic AMPs

The development of synthetic AMPs has the potential to play an important role in improving naturally occurring AMPs and reducing the development of resistance to them. When designing new AMPs several strategies are followed. Some make use of naturally occurring peptides, shortening their sequences or hybridising fragments thereof (Montesinos and Bardaji, 2008). Examples of synthetic AMPs include the magainin analogues MSI-99 (De Gray *et al.*, 2001) and Myp30 (Li *et al.*, 2001). Myp30 has provided resistance in transgenic *Nicotiana tabacum* (*N. tabacum*) against bacterial and fungal infection. Two cecropin B analogues found to be active against a wide range of plant pathogens, fungal and bacterial, are D4E1 (De Lucca and Walsh, 1999) and MB39 (Owens and Heutte, 1997). As technology advances, it has also become possible to synthesise AMPs *de novo*. Newly designed peptides should agree to a few guidelines (Montesinos and Bardaji, 2008). They should be less toxic to plant and mammalian cells and have resistance to enzymatic proteases. They also have to comply with characteristics of natural peptides, such as amphipaticity, that results in binding and interacting with membranes. Though many peptides may be designed, the ones that show the least intrinsic toxicity and most activity against pathogens are chosen to be used in developing applications. Peptide length also plays an important role and shorter peptides are preferred. Numerous studies have focused on identifying structural or sequence characteristics that have an effect on either the antimicrobial activity or the toxicity to eukaryotic cells. Factors that had no effect on the antimicrobial activity of AMPs, but increased the toxicity thereof, are an increase in helical or β-sheet structures as well as increasing amphipaticity and hydrophobicity (Kondejewski *et al.*, 1999). It was also found that all-D-amino acid magainin peptides had the same activity as their all-L-amino acid counterparts, but are less toxic to eukaryotic cells (Bessalle *et al.*, 1990). Researchers have designed and tested computational software for designing new AMPs based on existing knowledge of peptide groups (Juretic *et al.*, 1009). These algorithms focused on improving the selectivity of the AMPs. This study was successful in developing a synthetic AMP, called Adaptin 1, that was highly selective and effective against *E. coli* and less than 50% identical to all other peptides. In a study by Hao *et al.* (2008) four cecropin analogues, based on the conserved sequence of this peptide family, were designed. These peptides were more active against Gram-positive than Gram-negative bacteria. ESF1 (Ali and Reddy, 2000) and PEP6 (Reed *et al.*, 1997) are examples of completely synthetic AMPs. PEP6 is only 6 amino acids in length.

2.2.7 Application in science

In an effort to decrease pesticide toxicity and harmful environmental impacts over the last few years, the potential of AMPs as protecting agents against plant pathogens, increased substantially. AMPs isolated from microorganisms tend to be phytotoxic, therefore their synthetic analogues have been produced (Montesinos, 2007). Numerous examples exist for the successful implementation of AMPs, natural and synthetic, in transgenic plants to induce pathogen resistance. Transgenic tobacco, expressing a magainin analogue, is an example displaying both bacterial and fungal resistance (De Gray *et al*., 2001). The peptide Shiva-1 has been expressed in transgenic *Paulownia,* resulting in an improved resistance to Witches' Broom disease in these plants (Du *et al*., 2005). A patent held by Smith *et al*. (2001) describes the *in planta* activity of Magainin and PGL AMP classes against phytoplasma in transgenic poinsettia. The effect of different AMPs against other member of the *Mollicute* class (Béven *et al*., 2003; Borth *et al*., 2001) illustrates the potential use of AMPs to be active against phytoplasma. Amphipathic peptides are generally active against mollicutes (Béven and Wroblewski, 1997).

The use of AMPs in important agricultural crops is of high value to induce pathogen resistance. The expression of DRR206, offering pathogen resistance in transgenic canola (Wang *et al*., 1999), is an example of such an application. A study has also shown increased resistance to powdery mildew and crown gall development in transgenic grapevine expressing the synthetic AMP, MSI-99 (Vida *et al*., 2006). This study was recently extended to include transgenic grapevine expressing the AMPs Cecropin B, Shiva-1 and EsF-12 (Rosenfield *et al*., 2010). These plants showed different levels of resistance against *Agrobacterium tumefaciens* (*A. tumefaciens*) and *A. vitis*, as well as the fungus *Botrytis cinerea,* but not against *Erysiphe necator*. Transgenic rice expressing the AMP, Rs-AFP2, is yet another example of an important agricultural crop with an increased resistance to threatening fungi (Jha and Chattoo, 2010). Although these are examples of AMPs expressed *in planta* most studies were only performed *in vitro* and only a few have been developed and tested in plant systems. Concerns are often raised against the development of genetically-modified plants. Studies have however indicated that some peptides are degraded during human or animal consumption (Osusky *et al*., 2004).

2.3 Transient gene expression and viral expression vectors

Technology has advanced in such a way that the breeding of disease resistant crops through selection and hybridisation of resistant plants may in the future be replaced by the

development of transgenic plants expressing foreign genes that enhances their ability to combat pathogen attacks. The development of transgenic plants is a labour intensive and time-consuming process. Transgenic grapevine lines take months or even several years to establish before AMP efficacy screening can even be performed. One method of supporting this route is by pre-screening possible candidates by means of expression vectors used to transform plant cells to allow the transient expression of foreign genes. During Agrobacterium-mediated transient expression of foreign genes the transfer-DNA (T-DNA) containing the foreign gene is not incorporated into the plant host genome (Hellens *et al*., 2005), as is the case for stably transformed cells, and expression of the foreign gene occurs only for a limited time period (Voinnet *et al*., 2003). Transient expression systems have the advantage, amongst others, that they can be applied to fully differentiated plant tissue (Fischer *et al*., 1999). They are much faster and more flexible (Voinnet *et al*., 2003).

Transient expression in plants can be achieved mainly through three different ways. Firstly, the introduction of "naked" DNA into the cells by way of particle bombardment can result in the transient expression thereof. This method has its limitations as the DNA reaches only a few cell nuclei (Christou, 1997). Secondly, recombinant Agrobacterium cells containing the foreign gene in the T-DNA region of a disarmed tumour-inducing plasmid (Ti-plasmid) or binary vector can be used to infiltrate the plant tissue in order to deliver the DNA to the plant nucleus. Lastly, recombinant viruses can be used to infect the plants, resulting in expression of the foreign gene(s) inserted in the viral genome. The latter two pathways will be described in more detail below.

2.3.1 Agrobacterium-mediated transient expression systems

The gene of interest can simply be brought under the control of a plant-functional promoter and inserting it into the T-DNA region of an *A. tumefaciens* disarmed Ti or binary plasmid (Kapila *et al*., 1997). Plants can then be inoculated with these recombinant bacteria which will lead to the expression of the gene of interest in specific plant tissues. An example of a widely used plant-functional promoter is the cauliflower mosaic virus (CaMV) 35S promoter. The transient expression, however, seems to decrease due to post-transcriptional gene silencing (PTGS), after about 3 to 4 days (Voinnet *et al*., 2003). This problem can be overcome by co-infiltration of a vector expressing a viral RNA silencing suppressor gene. More plant cells are accessed by the Agrobacterium-mediated introduction of the foreign gene than by particle bombardment (Kapila *et al*., 1997). Transient expression of the transgene is found only in the region of infiltration (largely in all the cells) and is not of systemic nature, as is the case with viral vectors. Agrobacterium-mediated vectors are, however, more stable in comparison to

viral vectors when larger gene inserts are required (Porta *et al*., 1996). Another important aspect of this system is its ability to facilitate the expression of more than one transgene from the same cell (Rybicki, 2009).

Many gene functional and pathogen resistance studies made use of Agrobacterium-mediated transient expression systems. In a study by Bendahmane *et al*. (2000) Agrobacterium-mediated transient expression of candidate resistant genes were implemented to isolate the gene from potato involved in potato virus X (PVX) resistance. Based on similar principles, the same group screened various potato virus Y (PVY) genes in order to determine the viral protein that activates *Ry*-mediated resistance to this virus in potatoes (Mestre *et al*., 2000). By expressing antibodies in lettuce, Negrouk *et al*. (2005) illustrated that this system can be applied for fast protein production of pharmaceutical products in a plant system. Agrobacterium-mediated transient expression systems were also established in grapevine. Santos-Rosa *et al*. (2008) implemented such a transient expression system to examine the function of stilbenes in a grapevine leaf environment against the fungus, *Plasmopara viticola* (downy mildew) through over-expression of stilbene synthase. The defence role of glyoxal oxidase from *Vitis pseudoreticulata* (*VpGLOX*) against the grapevine pathogen powdery mildew, was also investigated in a recent study (Guan *et al*., 2010). This was achieved by applying Agrobacterium-mediated transient expression of *VpGLOX* in susceptible plants.

2.3.2 Viral transient expression systems

A form of transient expression, that also offers systemic expression of foreign genes, is by means of viral expression vectors. These viral vectors are becoming increasingly popular due to their more rapid expression, relative to those of transgenic plants, as well as their relatively high yield of foreign protein product (Gleba *et al*., 2004). Expression of proteins via a viral vector system reduce not only time and money spent on testing such proteins, but can also lead to a much higher throughput of protein screening. The development of successful virus expression vectors has not been without any difficulties. One of the problems which needed to be overcome was the instability of viral vectors (Gleba *et al*., 2007). First-generation viral vectors consisted of the entire viral genome. The first of these vectors proved to be highly unstable when large inserts were incorporated (Dawson, 1989). The development of second-generation virus expression vectors has however overcome this problem. These vectors are designed to be deconstructed, incorporating only essential viral sequences, and are often a combination of different components from different viruses (Gleba *et al*., 2004). They showed improved stability leading to a desired higher expression level of the protein of interest in their host organisms.

Expression vectors have been constructed from many different plant viruses of which tobacco mosaic virus (TMV) (Escobar *et al*., 2003) and PVX (Chapman *et al*., 1992) are two of the viruses most often exploited. Both these viruses have positive sense RNA genomes that are single stranded. Viral expression vectors have been developed for a wide variety of plants that include both dicotyledonous and monocotyledonous plants. In 2000, *Choi et al*. became the first to develop a viral expression vector that could not only express foreign genes in monocotyledonous plants, but also move systemically through these plants. Their vector was based on the wheat streak mosaic virus and was used to express foreign proteins in cereals. This study showed that vector stability is dependent both on the nature of the insert as well as the host plant species. In the above case, expression of an antibiotic resistance gene was still observed after 18-30 days post-inoculation, displaying the stability of the vector.

The study of virus-induced gene silencing (VIGS), the production of proteins for biomedical purposes and gene functional analysis are amongst the applications utilising viral vectors. A previous study by Zhao and Hammon (2000) illustrated the use of viral vectors in disease resistance studies. They showed bacterial sensitivity to Snakin-1 and the defensin PTH1 by expressing these AMPs using a vector based on PVX. Today, viral expression vectors are already used in commercial applications. An example of their industrial use is the biotechnology company Large Scale Biology Co., in the United States of America, which uses viral vectors for the commercial production of proteins in plants (Gleba *et al*., 2005).

Viral vectors have also been constructed from grapevine infecting viruses. The most successful grapevine-derived viral vectors presently available were constructed from grapevine virus A (GVA) (Galiakparov *et al*., 1999). Grapevine virus A*,* a single-stranded positive-sense RNA virus, belongs to the genus *Vitivirus* in the family *Flexiviridae* (Martelli et *al*., 1997) and its ~7.4 kb genome encodes for five major open reading frames (ORFs) (Galiakparov *et al*., 1999). In 2006, Haviv *et al*. described a viral vector based on the GVA genome that was able to infect *Nicotiana benthamiana* (*N. benthamiana*). This vector, pGVA-118 (Figure 3), made use of an independent additional subgenomic RNA (sgRNA) to express foreign proteins in plants. Subgenomic RNA promoters are used to restrict gene expression to only occur as a result of viral replication. Expression can only occur once the viral RNA-dependant RNA polymerase has been expressed. This vector was found to be far less efficient than the TMV viral vector of Shivprasad *et al*. (1999) and the PVX viral vector of Chapman et *al*. (1992). Another limitation of its applications is the fact that GVA is restricted to the phloem tissue of the plant. Muruganatham *et al*. (2009) modified pGVA-118 and placed the cDNA under control of a duplicated CaMV 35S promoter and 35S termination signal. This

cassette, when transferred into a binary vector, was used to effectively infect *N. benthamiana* and also *in vitro* cultured *V. vinifera* plants.
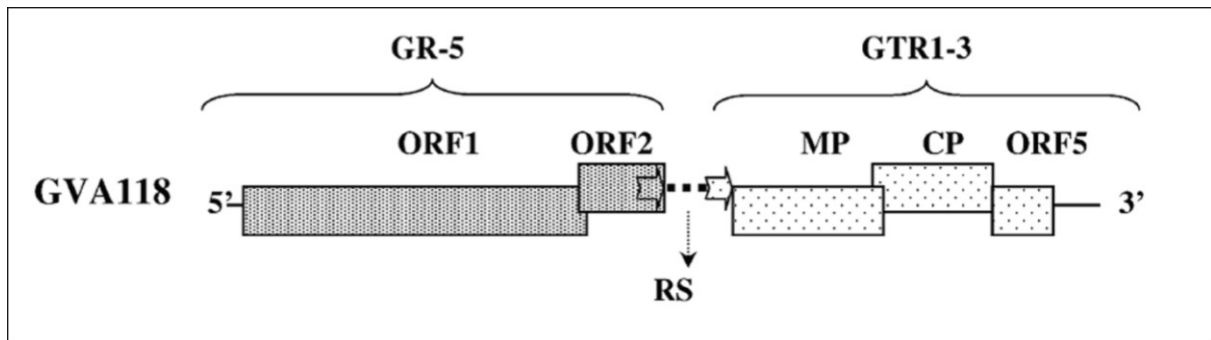
Figure 3: Schematic representation of the GVA118 viral vector compiled from GR-5 and GRT1-3 variants. The broad arrows indicate MP-subgenomic RNA promoters. Key: ORF, open reading frame; RS, restriction enzyme site; MP, movement protein; CP, coat protein (Haviv *et al*., 2006).

2.4 Quantification of plant pathogens

Monitoring disease resistance in plants mainly focus on symptom development in the plants after exposure to the pathogen of interest. To determine whether the resistance observed resulted from decreasing pathogens titres, techniques have been established to measure the pathogens titres in the plants. In the past, the colony forming units (cfu) for specific pathogens were determined for a dilution series of plant extracts (Morris *et al*., 1998; Tornero and Dangl, 2001). This method could only be implemented in the quantification of culturable microorganisms. To overcome this limitation and to extend the ability of quantification also to include unculturable plant pathogens such as viruses and phytoplasmas, competitive PCR analysis was developed (Berges *et al*., 2000). In this method the products of conventional endpoint PCRs for bacteria are compared to an internal control after gel electrophoresis. Competitive PCR analysis is however labour intensive and is not highly accurate.

Quantitative real-time PCR (qPCR) analyses were developed for, amongst other applications, the highly sensitive detection and accurate quantification of plant pathogens (Dorak, 2006). This method is based on the same principle as conventional PCR, but differs in that it is able to quantify the amount of DNA in a reaction after each PCR cycle, enabling the monitoring of the increasing PCR product in real-time. During qPCR a fluorescence signal is measured which gives an indication of the amount of amplicons. This signal could either arise from a reporter molecule being bound to the amplicon or being released from it during the PCR cycle. Three different fluorescence-based systems will briefly be explained below.

TaqMan®-based qPCR analysis requires the used of a probe in addition to the normal primers for PCR amplification (Heid *et al*., 1996). The probe is an oligonucleotide that anneals to the target DNA in the region spanned by the primers. It contains a fluorescent dye (reporter) on its 5' end and a fluorescence quencher on its 3' end. The reporter and quencher molecule are in close proximity resulting in the fluorescence of the reporter molecule being quenched. During primer annealing, the probe binds to the target DNA. Taq DNA polymerase's 5' exonuclease activity results in the reporter molecule being removed from the quencher during DNA strand elongation. The separation of the two molecules enables the reporter to emit a fluorescent signal. Each cycle releases more fluorescent molecules leading to an increase in intensity with every cycle.

Another qPCR detection system also implementing the use of a probe is the Molecular Beacon-based system (Tyagi and Kramer, 1996). It works in a way similar to the TaqMan® system. When unbound to the target DNA the probe forms a hairpin structure which brings the quencher and the reporter together in order to quench the signal before annealing. Annealing of the probe to the DNA results in the linearization thereof, with the quencher not close enough to the reporter in order to quench the fluorescent signal. This results in an increase in fluorescence during primer annealing of each cycle.

Not all qPCR systems require the use of a probe, some systems only make use of fluorescent molecules like SYBR Green (Dorak, 2006). This molecule only emits a fluorescent signal when bound to double-stranded DNA. During qPCR it therefore binds to the double stranded product at the end of each elongation step. As the amount of product amplifies the amount of bound SYBR Green in the reaction increases, resulting in an increase in the total fluorescent signal detected. Despite the fact that the use of a probe may seem to bring about more specificity to the reaction, studies have found the SYBR Green-based system to be as specific as its TaqMan® counterpart (Andersen *et al*., 2006).

Regardless of the qPCR system, all implement an increase in fluorescence which is observed after each cycle (Dorak, 2006). This increase in fluorescence could in turn be applied to quantify the amount of starting template in the reaction. During the first few cycles the amount of fluorescence in the reaction resulting from the template is shielded by the amount of background fluorescence. The threshold cycle (Ct) is the fractional cycle at which the fluorescence from the reaction rises above the background (Wilhelm *et al*., 2001). The more initial template there is in the reaction the smaller the Ct value will be (Gibson *et al*., 1996),

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and based on this principle the concentration of the DNA source (the microorganism) can be deducted.

Studies have used qPCR to quantify microorganisms in various environments. Ruppel *et al*. (2006) determined, by means of qPCR, the amount of the plant growth-promoting bacteria, *Enterobacter radicincitans*, in the leaves and roots of *Brassica oleracea* plants. Quantitative real-time PCR was also used to monitor the *in planta* growth of the oomycete *Phytophthora infestans* in potato plants (Llorente *et al*., 2010). An example of phytoplasma quantification using qPCR is the study performed by Christensen *et al*. (2004) where phytoplasma titres were measured in *Catharanthus roseus* (*C. roseus*) and *Euphorbia pulcherrima* plants. Plant 18S ribosomal-DNA (rDNA) was used to normalise the qPCR data. The use of plant DNA as an internal control for normalisation improves the accuracy of the quantification (Llorente *et al*., 2010). As an example of viral quantification in plants Jarosova and Kundu (2010) illustrated the use of quantitative RT-PCR to quantify prune dwarf virus titres in stone fruit. As is clear from all these examples, qPCR can be implemented to determine the titres of different kinds of plant pathogens.

2.5 Grapevine infecting pathogens

2.5.1 *Xylophilus ampelinus* the casual agent for grapevine bacterial blight

Xylophilus ampelinus is a bacterial pathogen which exclusively infects *V. vinifera* (Panagopous, 1969). It is the causative agent of bacterial blight in grapevine. They are Gram-negative bacteria that belong to the Beta-proteobacteria family *Comamonadaceae* and infect primarily the xylem tissue of grapevines (Willems *et al*., 1987). Infection of grapevine with *X. ampelinus* results in canker development as well as bacterial necrosis. They can however persist for many years inside grapevines without showing any symptoms (Ridé and Marcelin, 1983).

Bacterial blight in grapevines, known as "vlam siekte" in South Africa, was first described by Panagopous in 1969 in Greece. He found that the disease causing bacteria was *X. ampelinus*, which was at that time known as *Xanthomonas ampelina*. Later studies found, based on DNA and RNA sequence analysis, that these bacteria belong to a separate phylogenetic branch and forms the new genus *Xylophilus* (Willems *et al*., 1987). The disease, bacterial blight, has the potential of causing severe losses in vineyards as it can lead to the decay of the complete plant. Symptoms are found on the canes, roots, leaves and flowers (OEPP/EPPO, 2009). During early spring, diseased plants show reddish-brown lines on the shoots which later turn

into cracks and cankers on the canes (Figure 4). Vines are stunted due to the fact that infected shoots are much shorter than uninfected shoots. Flowers turn black before they reach maturity. Symptoms are easily observed on the leaves, and vary from pointed, reddish-brown lesions (Figure 4) and discolorations of the whole leaf or tips, to light-yellow regions and necrosis of the leaf, complete or one-sided. These symptoms depend on the route of leaf penetration either through the veins, stomata or hydathodes. Monitoring infection is difficult as plants can be symptomless for years after infection. It has been shown that up to half of the seemingly uninfected vines in a single vineyard could be infected with *X. ampelinus* (Panagopous, 1987). The disease occurs in the Mediterranean region as well as in South Africa where it has caused great losses in the past (Du Plessis, 1940). The disease is still prominent in France (Manceau *et al*., 2005) and in South African table grape vineyards (Pers. Com. Y. Petersen, ARC Infruitech-Nietvoorbij, Stellenbosch).
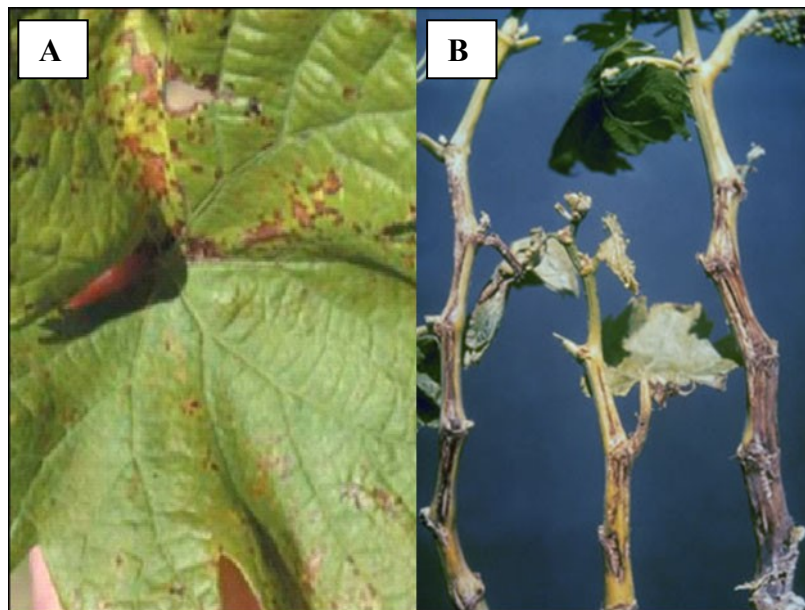
Figure 4: *Xylophilus ampelinus* infected grapevine showing (A) leaf lesions (Dreo *et al*., 2007); (B) canker development on the shoots (http://www.eppo.org/QUARANTINE/bacteria/Xylophilus_ ampelinus/XANTAM_images.htm).

Currently there are no insect vectors associated with the spread of bacterial blight caused by *X. ampelinus*. Warm and moist conditions have been shown to favour disease spread, and the use of overhead sprinklers have to a great extent been discouraged (Ridé *et al*., 1977). During spring, *X. ampelinus* emerges from infected vines from where moisture is thought to spread the bacteria from one plant to another, infecting the healthy plants through wounds. Wounding aids infection but is however not absolutely necessary (Bradbury, 1991).

Propagating material as well as grafting and pruning can also lead to spread of the disease (Ridé *et al*., 1977). Within a vineyard it seems that infection extends along the rows. This might be as a result of pruning methods or due to moist spread between adjacent plants. Over long distances bacterial blight is spread through propagation material and the illegal importation of infected plant material.

Detection of *X. ampelinus* is based on symptom recognition on infected plants. Techniques using bacterial isolation and selective culturing procedures are used to identify *X. ampelinus*. Other identification methods include examining their morphological aspects and also investigating the biological aspects of the bacteria through the use of standard bacteriological tests and serological techniques (Erasmus *et al*. 1974). The latter two techniques are not very sensitive (Manceau *et al*., 2000). The bacteria cells are rod-shaped (straight to somewhat bent) and have only one flagellum (Willems *et al*., 1987s). *Xylophilus ampelinus* growth is very slow on artificial media, forming colonies of only 0.4-0.8 mm in diameter after around 5 days. The colonies are smooth, round and non-mucoid. Their normally yellow colour may vary depending on the media on which they are grown.

In recent years identification shifted more to a molecular approach using a nested-PCR assay for the detection of *X. ampelinus* in the stem sap of grapevine plants (Botha *et al*., 2001). These PCRs amplify part of the 16S-23S rDNA intergenic spacer region. Even more recently a real-time PCR protocol was introduced for the reliable detection of *X. ampelinus* in grapevines (Dreo *et al*., 2007). This method was developed to avoid inaccuracy surfacing from handling PCR products, such as cross-contamination between samples.

No chemical treatment is available for the control of bacterial blight in grapevine. Infected vines are pulled out and removed immediately after disease detection. Preventative measures are currently the only form of control. Hot water treatment of canes is an efficient way of preventing spread by eliminating *X. ampelinus* from propagation material (Manceau, 2006). Plants are quarantined in areas where *X. ampelinus* infection occurs.

2.5.2 Crown gall disease causing pathogen *Agrobacterium vitis*

Crown gall disease is a severe grapevine infection found in many countries across the world including South Africa (Burr *et al*., 1998). In South Africa it is endemic and frequent outbreaks occur. This disease occurs primarily as a result of infection by *A. vitis* (Burr and Katz, 1984), previously known as *Agrobacterium tumefaciens* biovar or biotype 3 (Ophel and Kerr, 1990). *Agrobacterium vitis* (Figure 5) belong to the Alpha-proteobacteria, family

Rhizobiaceae. These Gram-negative bacteria are found systemically in grapevines (Burr and Katz, 1984). They are commonly spread through propagation material.
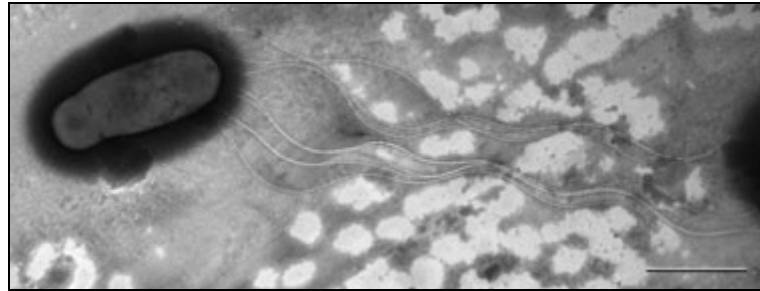
Figure 5: Electron microscopy of an *Agrobacterium vitis* F2 / 5 cell (scale bar, 1 µm) (Süle *et al*., 2009).

Survival of *A. vitis* can occur either in living vines or in grape tissue debris in the soil (Figure 6) (Burr *et al*., 1998). It is thought that during winter *A. vitis* survives in the grapevine roots and that root pressure during spring pushes the bacteria up into the plant (Lehoczky, 1968). Here the cells are drawn to sites of wounding, leading to gall development. An uneven distribution of bacterial cells is found in infected plants, with the highest concentration in shoots and at sites of infection (near wounding sites) (Bauer *et al*., 1994). Vasculature development between the canes and the shoots determines the point in time at which shoots are infected (Lehoczky, 1989). It is not clear what all the sites of survival of *A. vitis* in grapevines are, but evidence exists for their presence in the area directly below the bark of vines, in the cortex (Jäger *et al*., 1990). However, most studies are centred round their continued existence in the xylem of the plants. No *A. vitis* strains have been found living in soil (Bouzar *et al*., 1993). They have however, as mentioned previously, been found in grape tissue debris where they can survive for years (Burr *et al*., 1995). Although these cells are still tumorigenic they are in a saprophytic phase.

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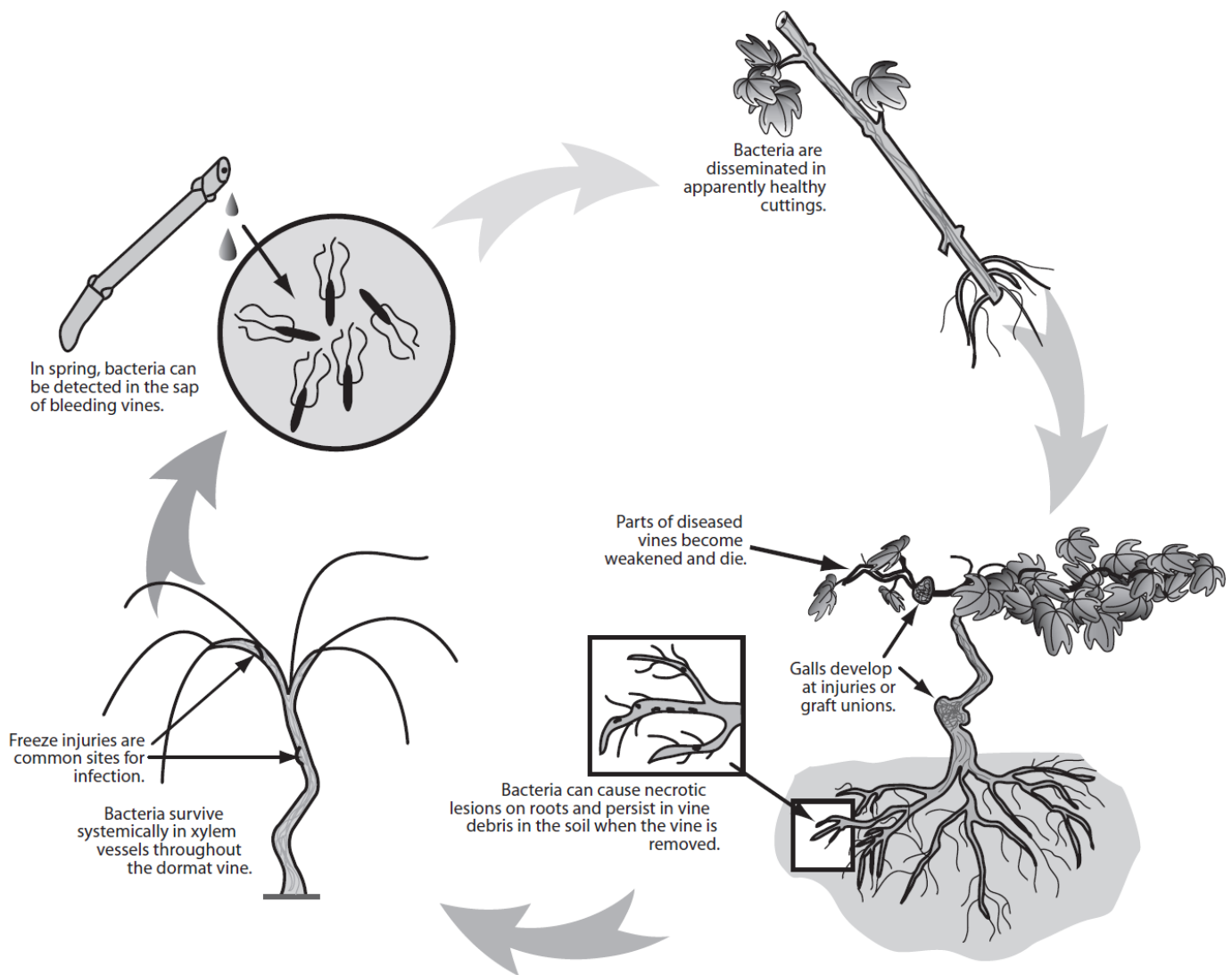
Figure 6: Disease life cycle of *Agrobacterium vitis*. Propagation of seemingly healthy material leads to spread of the disease. This process is enhanced by freeze injuries. Galls develop at site of grafting and cane injuries. The bacteria survive in the xylem of the plants as well as in the plant debris left in the soil after uprooting of the infected vines (http://www.nysipm.cornell.edu/factsheets/grapes/ diseases/crown_gall.pdf).

The high temperature and humidity in some parts of South Africa is responsible for cane injuries locally, as are freeze injuries in other parts of the world (Burr *et al*., 1998). Crown galls often develop at grafting sites and at the bottom of rooted cuttings. Agrobacterium cells are attracted to injured cells through chemotaxis. Injured cells release compounds, including phenolics, which trigger the agrobacterial virulence genes starting a sequence of events leading to the transfer and incorporation of the T-DNA of the bacteria into the genome of the plant. This will in turn lead to the expression of genes responsible for inducing gall formation in grapevines (Figure 7). The infection of a plant with *A. vitis* occurs in a way similar to that of *A. tumefaciens*, although less information about the first process of infection is available.

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Figure 7: Grapevine trunk showing crown gall development as a result of *Agrobacterium vitis* infection (http://www.nysipm.cornell.edu/factsheets/grapes/diseases/crown_gall.pdf).

Strategies to detect *A. vitis* in grapevine have progressed over the years. Screening for *A. vitis* infection includes symptom identification and describing the morphology of bacterial colonies from candidate plant extracts. To confirm the nature of the disease causing agent, the first tests relied on enzyme-linked immunosorbent assays (ELISA); this was followed by PCR-based screening methods of plants. Haas *et al*. (1995) designed primers based on the *virD2* gene sequence that could detect *A. vitis*, but could not unambiguous discriminate it from *A. tumefaciens* strains. More recently, a qPCR method was developed for *A. vitis* strain detection, as well as for differentiation between subgroups (Bini *et al*., 2008). Strains are grouped together on the basis of having the same arrangement of T-DNA oncogenes on their Ti-plasmids (Szegedi *et al*., 1988). Three types exist; those having the octopine, nopaline or vitopine-type opine genes on their Ti-plasmids, with the first two types grouping together according to the study by Bini *et al*. (2008). This qPCR method could distinguish *A. vitis* strains from *A. tumefaciens*.

The strategies used to control *A. vitis* outbreaks are mostly focused on prevention rather than cure. Some grapevine cultivars are more susceptible to crown gall development than others. Highly susceptible cultivars grown in South Africa include Merlot, Cabernet Sauvignon, Riesling and Chardonnay (Burr *et al*., 1998). Planting resistant cultivars does not supply a solution for farmers who unfortunately need to grow the susceptible cultivar due to market demands. Other control strategies, described by Burr *et al*. (1998), include the production of

vines free of *A. vitis*, monitoring *A. vitis* infection in existing vineyards as well as methods of biological control by means of non-tumorigenic Agrobacterium strains. Disease-free grapevine production is possible through the use of hot water treatment, shoot tip propagation and application of efficient diagnostic methods.

2.5.3 Aster yellows phytoplasma

Phytoplasmas are plant pathogens responsible for causing the grapevine disease grapevine yellows. These phloem-limited organisms, previously known as mycoplasma-like organisms (MLOs) because of their resemblance to these pathogens that infect animals and humans, were first reported in 1967 (Doi *et al*., 1967). They were first identified in mulberry, aster, potato and paulownia plants showing yellows disease symptoms. They have a reduced genome (530-1350 kb) and are host dependent due to the lack of genes such as those involved in ATP-synthesis or sugar utilisation (Christensen *et al*., 2005). This host dependence makes them unsuitable for *in vitro* culturing. Due to their minimal genome, phytoplasmas have been described as "the simplest natural self-replicating organism" and to be on the "border of living cellular organisms and viruses" (Christensen *et al*., 2005).

Diseases associated with phytoplasma infection were first described in the beginning of the 1900's. In those days however, the diseases were thought to be caused by viruses as they were unable to culture the disease causing agent (Kunkel, 1926). The causative agent was later found to be MLOs living in the phloem of diseased plants (Doi *et al*., 1967). Since their discovery, these MLOs, now known as phytoplasmas, have been held responsible for causing yellows disease in more than 1000 different plant species (ICSBSTM, 1997). These plant species belong to 98 different families (Gasparich, 2009) and infections were reported to occur in more than 85 countries (McCoy *et al*., 1989). Different phytoplasmas have been shown to infect *V. vinifera*. They are proven to be destructive, leading to immense economical losses in the grapevine industry. In 2006, phytoplasma was first reported to be found in South African vineyards (Botti and Bertaccini, 2006). Later, the phytoplasma strain causing yellows disease in infected vines observed in these vineyards was found to be aster yellows phytoplasma (Engelbrecht *et al*., 2010). Aster yellows phytoplasma infects more than 200 plant species around the world (Gundersen *et al*., 1996). To date, aster yellows infections have been observed in vineyards in the Waboomsrivier area near Rawsonville and in the Olifants River area in the Vredendal district of South Africa. Extensive damage has been caused to plants in these areas, affecting the grape yield negatively. Whole vineyards had to be removed and replace with healthy vines.

Classification of different phytoplasmas has been complicated due to the inability to culture them outside their host. The different phytoplasmas were once grouped according to their insect vectors, host range and the symptoms they induce in their hosts (Gasparich, 2009). Symptoms may vary between hosts and the same symptoms may be caused by different phytoplasmas. To solve these problems, focus has shifted to a molecular approach. Phytoplasmas are now classified and grouped into different subgroups according to the sequence of their 16S ribosomal-RNA (rRNA) genes (Seemüller *et al.*, 1998). Aster yellows phytoplasma falls into the 16SrI group and along with other phytoplasmas belong to the order *Acholeplasmatales* in the class *Mollicutes* (Lee *et al.*, 2000), genus "*Candidatus Phytoplasma*" ("*Ca. Phytoplasma*") (IRPCM Phytoplasma/ Spiroplasma Working Team-Phytoplasma Taxonomy Group, 2004). They are classified as "*Candidatus*" because they are still unculturable (Murray and Schleifer, 1994). They group together in a monophyletic clade (Lee *et al.*, 2000).

Phytoplasma has a dual host cycle (Figure 8). They can replicate in both their plant hosts and their insect vectors (Gasparich, 2009). They are known to spread from one plant to others by means of insect vectors that feed on the sap of these plants (Lee *et al.*, 2000). These vectors include planthoppers but are primarily leafhoppers belonging to the Cicadellidea family. Some phytoplasmas can only be transmitted by one specific insect vector (Seemüller *et al.*, 2002), while others may have multiple vectors, as is the case for aster yellows phytoplasma, which is transmitted by more than 24 different leafhoppers (Lee *et al.*, 2003). The leafhopper *Mgenia fuscovaria* has recently been identified as a possible vector for aster yellows phytoplasma in South African vineyards (Pers. Com. K. Kruger, Department of Zoology and Entomology, University of Pretoria). Phytoplasma can also spread between plants through vegetative propagation and grafting (Lee and Davis, 1992).

Insects acquire phytoplasma when feeding on the sap of infected plants. Inside the insects the phytoplasmas are relocated to the hemolymph from where it spreads to different tissues (Gasparich, 2009). After multiplication it becomes part of the saliva via the saliva glands. Fifteen to twenty days after infection of the insect, the phytoplasma titres are high enough for plant infection to occur. Infected insects maintain their infectivity, even during winter. When feeding on plants, they then release the phytoplasmas, along with saliva, into the sieve elements of the plants (Christensen *et al.*, 2005). Subsequently, they spread through the phloem. Systemic spread of the pathogen from the leaves to other organs is thought to occur via the assimilate flow. Multiplication occurs inside the phloem of the plant and symptoms develop when sufficient phytoplasma titres are reached.
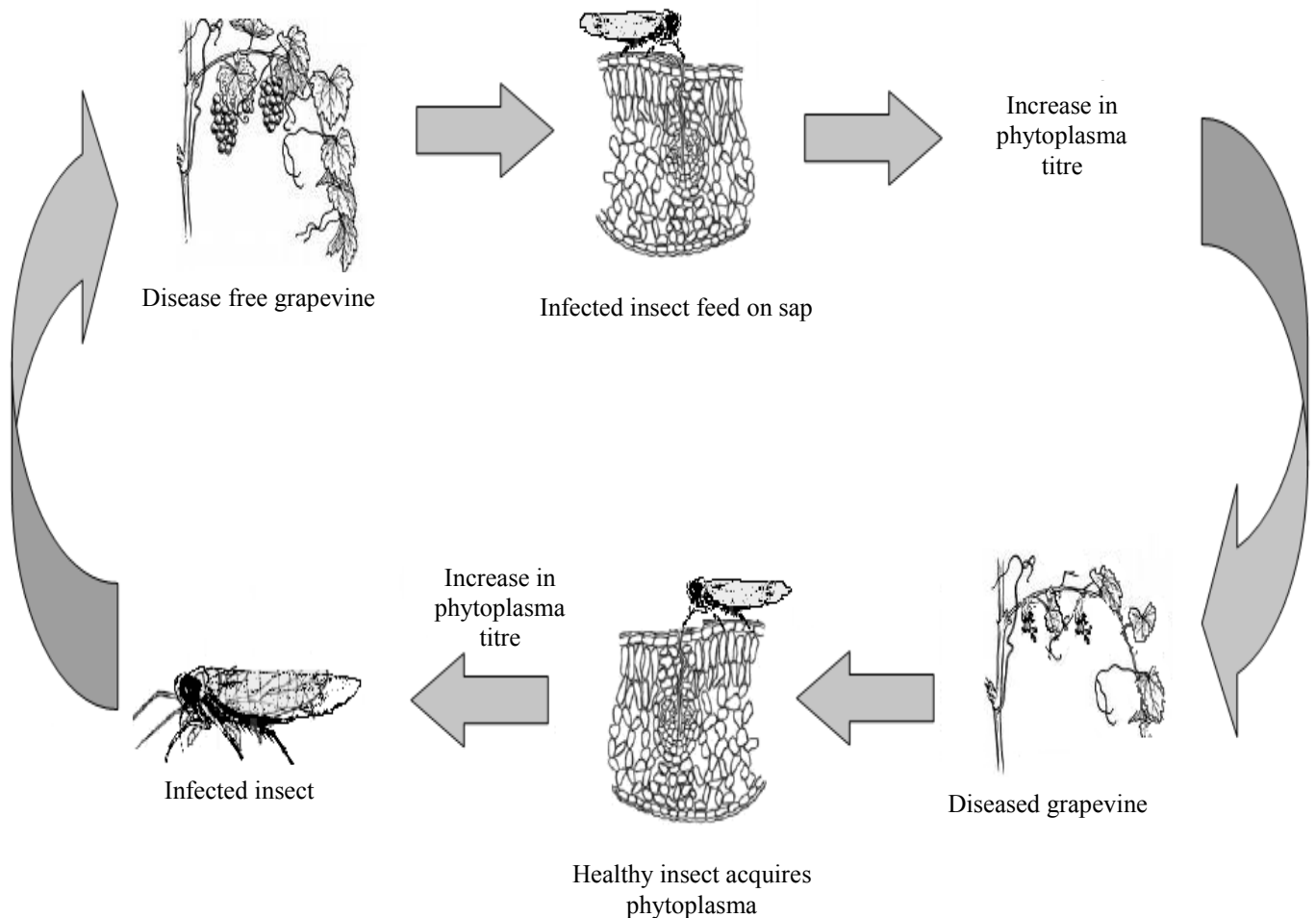
Figure 8: Dual host life cycle of phytoplasma. Infected insects feeding on healthy grapevine infect these plants with phytoplasma. The titres accumulated in the phloem of the infected plants and healthy insects feeding on the infected plants acquire phytoplasma. When titres inside the insects become adequate they are able to infect plants when feeding on their phloem sap (Adapted from Christensen *et al*., 2005).

Infected plants show a wide range of symptoms (Figure 9). In grapevines the symptoms can be observed on leaves, canes and bunches (Lee *et al*., 2000). The leaves curl downward and stunting of the vines can be observed. Bunches seem to dry out and auxiliary buds are inhibited. The exact interaction of the pathogen with the host plant is still unknown. However, the symptoms point to unbalanced hormone levels, dysfunctional phloem transport or changes in composition of the phloem sap. A recent study has identified a protein secreted by the aster yellows phytoplasma strain Witches'-Broom that targets the host plant nuclei (Bai *et al*., 2009). This may lead to a change in host plant cell physiology.
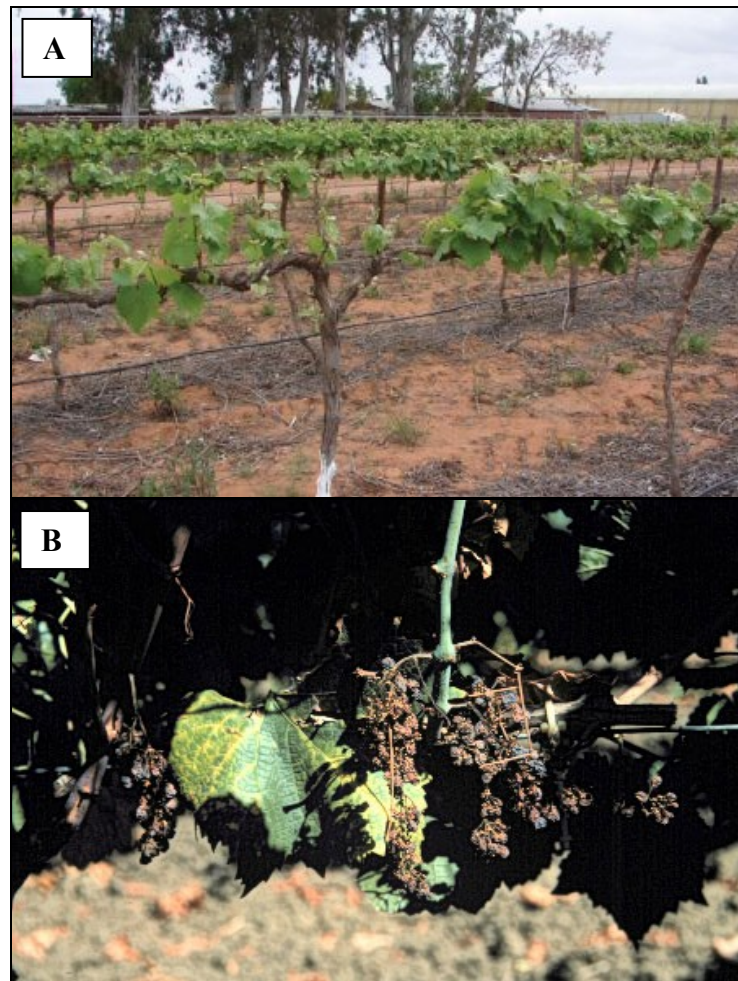
Figure 9: Phytoplasma associate symptoms in grapevine. A) Vine with delayed budding in variable parts of the plant (http://www.wynboer.co.za/recentarticles/200808aster.php3). B) Grapevine plant showing bunch abortion and no lignification of the canes (http://www.ars.usda.gov/pandp/people/ people.htm?personid =1289).

Phytoplasma are small (diameter of 0.1-0.8 μm) cell wall-less microorganisms (Doi *et al*., 1967; Hoshi *et al*., 2007). They are either pleimorphic or filamentous in shape (Gasparich, 2009). Previously, the observation of symptoms in plants and the use of ultra-thin sections of plants were used to detect phytoplasmas (Haggis and Sinha, 1978). As with most diagnostic procedures, the detection methods have shifted to molecular approaches, using antibodies in ELISA-based systems (Lin and Chen, 1985). Later PCRs followed by analysing restriction fragment length polymorphisms or highly sensitive nested-PCRs were used. The newest editions to detection methods are microarrays (Nicolaisen and Bertaccini, 2007) and real-time PCR methods (Christensen *et al*., 2004). Christensen *et al*. (2004) designed primers for the universal detection of the phytoplasma 16S rRNA region. Angelini *et al*. (2007) designed a qPCR assay specific to aster yellows phytoplasma also based on their 16S rRNA gene sequence. This real-time approach has proven to be as sensitive as a nested-PCR method

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using the primers R16(I)F1/R1 (Lee et al., 1994) following the amplification with the primers R16F2n/R2 (Gundersen and Lee, 1996).

In agricultural areas where phytoplasmas are found, plants are quarantined and the removal of plants is strictly regulated (Lee *et al*., 2000). There have also been reports of vines showing recovery after years of infection (Osler *et al*., 1993). Antibiotics such as tetracycline have shown efficacy against phytoplasma infection in *in vitro* cultured plants (Wongkaew and Fletcher, 2004). Symptoms reappeared, however, after plants were transferred to non-selective media. The use of antibiotics will also be too expensive for practical implementation and holds a potential human health risk as it may lead to the development of resistant pathogens. A study by Ćurković (2008) used auxins to induce the *in vitro* recovery of *Catharanthus roseus* shoots from phytoplasma infection. In grapevines, hot water treatment has been used on scions (Tassart-Subirats *et al*., 2003). Partial uprooting and pulling of vines to induce a stress response also lead to recovery of grapevines from phytoplasma infection (Romanazzi and Murolo, 2008). The same research group evaluated commercially available elicitor products' potential as resistance inducers against grapevine infecting phytoplasma in a field study (Romanazzi *et al*., 2009). They demonstrated the efficacy of the elicitors to induce resistance against grapevine phytoplasma Bois noir. D'Amelio *et al*. (2010) illustrated how systemic acquired resistance, by means of the commercially available plant resistance elicitor benzothiadiazole, reduced not only the development of symptoms in phytoplasma infected daisy plants but also the multiplication of these microbes in the plants. In recent years, genetic modification became an option for inducing disease resistance in host plants. Du *et al*. (2005) observed an increase in plant resistance against phytoplasma in greenhouse transgenic *Paulownia* plants expressing the antimicrobial peptide Shiva-1. Currently, no transgenic grapevine is available that show an increased resistance against aster yellows phytoplasma. Up until now, prevention has been more feasible than cure. These strategies include controlling the vector, producing disease-free plantlets, hot water treatment of propagation material, as well as uprooting of contaminated vines.

2.6 Conclusion

Antimicrobial peptides can offer plants protection against infecting agents. It has been proven effective against important grapevine pathogens (Rosenfield *et al*., 2010). This AMP-based defence system has little or no toxicity to plants and potential consumers (Yeaman and Yount, 2003). Due to the non-specific action of AMPs, microorganisms are less likely to develop resistance to them. Transient expression systems are a reliable and time-effective method of

expressing foreign proteins in plants. These systems have been validated in important agricultural crops including grapevine (Santos-Rosa *et al.*, 2008). They can be used for various purposes including gene functional analysis and plant pathogen resistance studies. The latter process is supported by the development of qPCR to facilitate the quantification of pathogen titres *in planta*. These applications can play an important role in the development of plant resistance to a range of pathogens to which the only form of control is currently preventative measures such as controlling the insect vector.

Chapter 3

Experimental Procedures

3.1 Transient expression vector construction and evaluation

3.1.1 Plant samples

Vitis vinifera cv. Sultana *in vitro* cultured plantlets were used for evaluation of gene expression from transient expression vectors in *V. vinifera* and for determining the *in planta* effect of the AMPs against *X. ampelinus* and *A. vitis* (see section 3.4). These plants were cultured in liquid Murashige and Skoog (MS) media containing perlite in tissue culture flasks, and kept in an incubation room under controlled conditions (Figure 10).
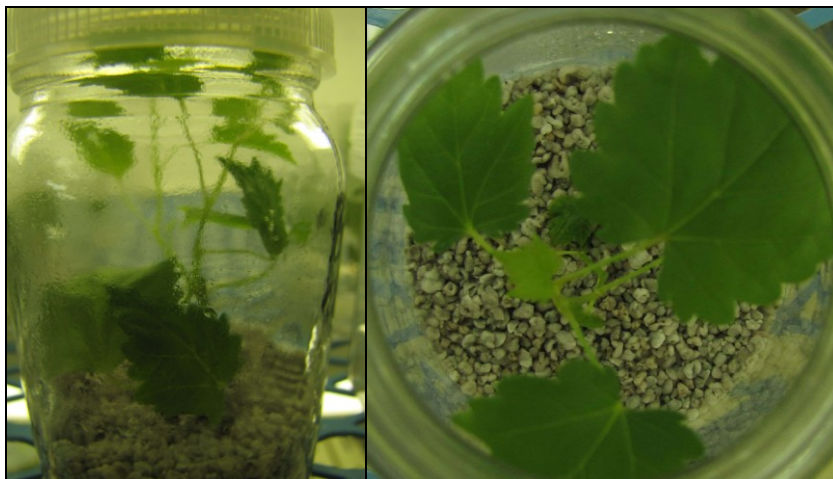
Figure 10: Tissue culture *Vitis vinifera* cv. Sultana plants, cultured in MS containing perlite.

3.1.2 Candidate antimicrobial peptides (AMPs)

The nucleotide as well as amino acid sequences of two AMPs were available for screening the AMP activity against grapevine pathogens. The first was Vv-AMP1 (RTCESQSHRFKGTCVR QSNCAAVCQTEGFHGGNCRGFRRRCFCTKHC), an AMP characterised and isolated from *V.*

vinifera by the Institute of Wine Biotechnology at Stellenbosch University (De Beer and Vivier, 2008). The second AMP was D4E1 (FKLRAKIKVRLRAKIKL), a synthetic peptide developed and kindly supplied by Professor J.M. Jaynes (AgroMed LLC., USA). Both AMPs were used in the construction of viral and Agrobacterium-mediated (or 35S) expression vectors. Only D4E1 was used for *in vitro* plate screening against *A. vitis* and *X. ampelinus*. Vv-AMP1 was not available in peptide form since the source was not able to produce the peptide in an amount sufficient for *in vitro* screening before the conclusion of this study.

3.1.3 Transformation of *Escherichia coli* cells

The *E. coli* strain, DH5α, was used to prepare competent *E. coli* cells according to a protocol described by Hanahan (1985). These cells were transformed using a method described by Sambrook *et al.* (2001).

3.1.4 AMP expression vector construction

Vectors were designed and constructed to express AMPs by way of an existing grapevine viral vector and also by means of an existing 35S binary vector. Table 1 lists the primers used for construction of these, as well as control vectors. The primers were designed to contain the restriction enzyme recognition sequences required for the cloning of the fragments into the respective vectors. During the cloning steps, competent *E. coli* cells were used for transformation and regeneration of the cloned plasmids. After construction, vector-plasmids were transferred to *A. tumefaciens* as described in section 3.1.5.

Table 1: List of primers used during expression vector construction and their sequences. The sequences of the restriction enzyme sites are underlined.

Primer name	Sequence
Kpn2I_VvAMP1 _as	ATCCGGATTAACAATGCTTAGTGC
ApaI_VvAMP1 _as	AGGGCCCTTAACAATGCTTAGTG
HpaI_VvAMP1_s	AGTTAACATGAGGACCTGTGAGAGTCA
ApaI_GFP_as	AGGGCCCTTACTTGTACAGCTCGT
HpaI_GFP_s	AGTTAACATGGTGAGCAAGGGCG
NotI_GUS_s	AAGCGGCCGCATGTTACGTCCTGTA
NotI_GUS_as	AAGCGGCCGCTCATTGTTTGCCTCC
HpaI_D4E1_F	AGTTAACATGTTTAAGTTGAGAGCTAAGATTAAGGTTAGATTG
ApaI_D4E1_R	AGGGCCCTTACAACTTAATCTTAGCTCTCAATCTAACCTTAAT
GVA118-6914-F	CAGTGAAAGGAGAACTTTTGGTTACA
SacI_35S_D4E1_s	AGAGCTCATCGATTAGGAGATATAACAATGTTTAAGTTGAGA
BamHI_35S_D4E1_as	AGGATCCTTACAACTTAATCTTAGCTCTCA
SacI_35S_VvAMP1_s	AGAGCTCATCGATTAGGAGATATAACAATGAGGACCTGTGAGAGT
BamHI_35S_VvAMP1_as	AGGATCCTTAACAATGCTTAGTGCAGAAG

Construction of GVA118 viral vectors expressing AMPs

Viral vectors expressing AMPs were constructed by making use of a GVA118 expression vector (Haviv *et al.*, 2006; Du Preez, 2010). Four GVA118-based viral vectors were constructed. Two containing the AMP genes for D4E1 and Vv-AMP1, namely pBinSN-GVA118-D4E1 and pBinSN-GVA118-VvAMP1, respectively, and two containing either the control gene Green Fluorescent Protein (GFP) or the intron containing β-glucuronidase gene (GUSi) called pBinSN-GVA118-GFP and pBinSN-GVA118-GUS respectively.

In order to obtain the D4E1 fragment coding sequence, a PCR with overlapping-primers was performed using the primer pair HpaI_D4E1_F and ApaI_D4E1_R. The resulting 71 bp amplicon was cloned into the pGem®-T Easy (Promega) cloning vector, from where it was excised by the restriction enzymes *Hpa* I (Fermentas) and *Apa* I (Fermentas). It was then cloned into the multiple cloning site (MCS) between ORF 2 and 3 of GVA118 of the plasmid pSKM-e35S-GVA118-pA (Du Preez, 2010) that was digested with the same enzymes. The GVA118 construct containing the D4E1 gene was digested with *Sna* BI (Fermentas) and *Sal* I (Fermentas) and cloned into the binary vector pBinSN digested with *Sna* BI and *Xho* I (Fermentas). pBinSN is a pBin19 derivative (Bevan, 1984) kindly supplied by Professor E. Maiss (Institute of Plant Diseases and Plant Protection, Hannover University, Germany). The final plasmid that resulted from this last cloning step, pBinSN-GVA118-D4E1, was used to infiltrate plants.

The construction of the GVA118 viral vectors containing the Vv-AMP1 and GFP genes were performed following a similar cloning strategy as mentioned above. The gene-containing fragments were, however, not obtained by primer-dimer formation, but rather by amplifying the genes from existing plasmids containing the respective sequences. In the case of Vv-AMP1 the primer pair HpaI_VvAMP1_s and ApaI_VvAMP1_as was used to amplify the AMP sequence, including the restriction enzyme recognition sequences, from pGEM-Vv-AMP1 (De Beer, 2008) which contained the Vv-AMP1 sequence. The primer pair HpaI_GFP_s and ApaI_GFP_as was used to amplify the enhanced Green Fluorescent Protein (EmGFP) sequence from a previously constructed vector, 35S:EmGFP (Ghazala *et al.*, 2008). The PCR products were cloned into the pDrive (Qiagen) cloning vector from where the same strategy, as used for D4E1, was followed to clone the respective genes first into GVA118 and then into pBinSN to obtain the final vectors pBinSN-GVA118-VvAMP1 and pBinSN-GVA118-GFP.

Vv-AMP1 was also cloned into a GVA (strain Gr5) viral vector from which ORF2 was removed, namely GR5-ΔORF2+sgMP-pA (Du Preez, 2010). The Vv-AMP1 gene was amplified with the primers HpaI_VvAMP1_s and Kpn2I_VvAMP1_as, using pGEM-Vv-AMP1 (De Beer, 2008) as

template. The amplicon was first cloned into pDrive from where it was excised with the enzymes *Hpa* I and *Kpn* 2I (Fermentas), and cloned into the *Sna* BI and *Kpn* 2I sites of GR5-ΔORF2+sgMP-pA. The vector containing the Vv-AMP1 gene was then cloned into the pBinSN by excising it with *Not* I and *Sal* I and cloning it into the complementary *Not* I and *Xho* I sites of the binary vector to form the vector pBinSN-GR5-ΔORF2-VvAMP1.

Cloning of the GUSi gene was performed by firstly amplifying the gene from an existing plasmid 35S:GUSi (Vaucheret *et al.*, 1994; kindly supplied by P. Metre, University of Louis Pasteur Strasbourg, Colmar, France) with the primers NotI_GUS_s and NotI_GUS_as. The PCR fragment of 1957 bp was cloned into the pGem®-T Easy cloning vector from where it was introduced into a pBinSN vector already containing an infectious copy of GVA118. This was achieved by digesting both plasmids with *Not* I (Fermentas) and ligating the GUSi fragment into the GVA118 vector, resulting in the pBinSN-GVA118-GUS control vector. The primers GVA118-6914-F and NotI_GUS_s were used to confirm the insertion of the GUSi gene in the correct orientation into the GVA118 vector. The expected amplicon size was 2470 bp.

Construction of 35S vectors expressing AMPs

To construct a 35S expression vector for the expression of D4E1, the gene was amplified with SacI_35S_D4E1_s and BamHI_35S_D4E1_as using the D4E1 containing pGem®-T Easy plasmid, generated during the construction of the D4E1 viral vector, as template. The primers contained *Sac* I (Fermentas) and *Bam* HI (Fermentas) restriction enzyme sequences as well as a plant enhancer sequence (Lütcke *et al.*, 1987) between the *Sac* I site and the D4E1 sequence. The generated PCR fragment were again cloned into pGem®-T Easy from where it was excised and cloned into the *Sac* I and *Bam* HI sites of the binary vector pBin61S (Silhavy *et al.*, 2002), resulting in the vector pBin61S-D4E1. The same cloning strategy was followed to produce the 35S-based expression vector, called pBin61S-VvAMP1. The primer pair SacI_35S_VvAMP1_s and BamHI_35S_VvAMP1_as was used, where the first-mentioned primer again contained the enhancer sequence between the *Sac* I site and the AMP gene sequence. Lütcke *et al.* (1987) illustrated that a adenine at the position -3 relative to sequence of the start codon resulted in the highest translation efficiency and that the nucleotide sequence AAC<u>AUG</u>GC was conserved in plants. The control vector, 35S:GUSi (Vaucheret *et al.*, 1994) was already constructed during a previous study, and was readily available.

3.1.5 Transformation of Agrobacterium cells

Electrocompetent *A. tumefaciens* cells (strain C58C1), containing the helper plasmid pCH32 (Hamilton *et al.*, 1996; kindly supplied by P. Mestre, University of Louis Pasteur Strasbourg,

Colmar, France), were prepared according to the protocol by Annamalai and Rao (2006). Competent cells were electroporated setting the electroporator to 25 μF, 200 Ω, 1.5 kW.

3.1.6 Agro-infiltration of plants

Recombinant *A. tumefaciens* cells were grown on selective media (kanamycin/tetracycline). Overnight shaker cultures were pelleted by centrifugation for 5 min at 3200 *g*. The supernatant was discarded and the pellet resuspended in inoculation buffer (10 mM MgSO4, 10 mM MES, 100 μM acetosyringone) (Stephan and Maiss, 2006). After incubating for 2-3 h at room temperature, the bacterial suspension was used for plant infiltration.

Nicotiana benthamiana plants were agro-infiltrated with the expression vectors using a method by Llave *et al*. (2000). A 2 ml syringe with needle was used to aspirate an Agrobacterium suspensions (OD_{600} of 0.1-0.5), transformed with the vector, into the leaf of the plant. The needle was then removed and the syringe pressed against the lower surface of a leaf. The cell suspension was slowly injected into the leaf by applying constant, low pressure.

Infiltration of *V. vinifera* plants was conducted by means of Agrobacterium vacuum-infiltration. This was performed by making several small cuts using a scalpel on the leaves of *in vitro* cultured *V. vinifera* plants. The plants were then immersed completely in a cell suspension of Agrobacterium, which had an OD_{600} of 0.1-0.5, and placed in a vacuum chamber. To achieve infiltration, two successive vacuums (-90 kPa) of 2 min each were applied in the chamber. The vacuum was quickly released between the two vacuum steps. After restoration of atmospheric pressure, the leaves were rinsed with distilled water and the plantlets transferred into a container with perlite and watered with autoclaved water.

3.1.7 Testing of infectivity

To determine whether the expression of the AMPs had an influence on viral replication, disease-free *N. benthamiana* plants were agro-infiltrated with the viral vectors as described in section 3.1.6. Viral replication was tested by tissue print immuno-assays (TPIAs) (adapted from Franco-Lara *et al*., 1999) 6 days post-infiltration (dpi), as described below. An antibody specific to the coat protein of GVA was used as primary antibody. The expression of the GVA coat protein is an indication of viral replication. The coat protein will not be transcribed as part of the 35S transcript as it is transcribed from the viral sgRNA.

Methanol was used to wet a Hybond PVDF membrane for 2 s. The membrane was then rinsed in excess water for 15 min, and equilibrated for 15 min in 1X TBS buffer (0.02 M Tris-base, 0.5 M

NaCl, pH 7.4). This step was followed by drying the membrane on filter paper. The lower epidermis of infiltrated leaf patches was removed with tweezers or petioles were cut with a scalpel. After pressing the plant tissue on the dried membrane for 2 seconds, the membrane was incubated on a shaker, in 4.5% milk powder dissolved in 1X TBS. The blocking solution was discarded after 1 h and the membrane washed 3 times in TBS-T (1X TBS, 0.05% Tween 20) for 5 min. The TBS-T was removed and the membrane incubated on a shaker for 2 h in the first antibody solution [GVA-CP-antiserum diluted 1/400 in TBS-TPO (1X TBS, 2% PVP-40, 0.2% Bovine Serum Albumin Fraction V)]. After washing the membrane 3 times in TBS-T for 5 min, the TBS-T was discarded and the membrane incubated for 1 h on a shaker with the alkaline phosphatase-conjugated goat anti-rabbit secondary antibody in TBS-T (1/10 000 dilution). Incubation was again followed by washing of the membrane in TBS-T, 3 times for 5 min. The membrane was then incubated in the dark for 30 min in the AP-BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate / nitroblue tetrazolium) colouring solution (100 mM Tris, 100 mM NaCl; 5 mM $MgCl_2$, 167 µg/ml BCIP, 495 µg/ml NBT) without shaking, after which tap water was used to rinse the membrane. Filter paper was used to dry the membrane. Dark stained areas of leaf or petiole prints from agro-infiltrated plants in comparison to non-infiltrated plants indicated viral presence and therefore viral replication. Tissueprint results were captured with a Leica DFC 320 digital camera attached to a Leica MZ 7.5 microscope (C. Janion, Centre for Invasion Biology, Department of Botany and Zoology, Stellenbosch University).

3.1.8 Control expression of foreign protein

The control GUSi constructs, viral (pBinSN-GVA118-GUS and pBinSN-GVA118-GFP) and 35S (35S:GUSi), were used to determine the functionality of the vector constructs for gene expression. Six days after *N. benthamiana* and *V. vinifera* plants were infiltrated with the GFP-construct, pBinSN-GVA118-GFP, plants were visualised under a microscope containing a UV light for GFP expression, while GUS assays (section 3.1.9) were performed on the GUSi-construct infiltrated plants.

3.1.9 GUS assay

Small cuts were made on leaves after which they were placed in a clean small beaker. Approximately 6 ml of GUS substrate buffer (10 mM NaH_2PO_4, 0.5 mM K-Ferrocyamid, 0.5 mM K-Ferricyamid, 0.1% Triton X100, 100 mM Na_2EDTA), with freshly added X-Gluc (12.5 µl/ 100 ml of buffer), were added to the beaker. The leaves were then vacuum-infiltrated (see section 3.1.6). Parafilm was used to cover the beaker while incubating overnight at 37°C, with gentle shaking. The leaves were then decoloured by rinsing them for an extended period of time

in 100% ethanol, replacing the ethanol regularly. Areas of GUS expression were visually assessed as blue areas on the leaves.

3.1.10 Testing of systemic movement of virus and expression of foreign proteins

Nicotiana benthamiana plants were agro-infiltrated with the viral vectors pBinSN-GVA118-D4E1, pBinSN-GVA118-VvAMP1 and the control pBinSN-GVA118-GUS, as described in section 3.1.6. A TPIA (section 3.1.7) was performed at 17 dpi on the non-infiltrated petioles of plants infiltrated with the AMP vectors, while a GUS assay (section 3.1.9) was performed on non-infiltrated leaves of GUS vector-infiltrated plants, also at 17 dpi. Plant tissue of non-infiltrated plants served as negative controls in both the assays.

After a further 9 days (26 dpi) a one-tube RT-PCR was performed on systemic leaves showing symptoms of viral infection. Five millilitres of extraction buffer (0.5 M Tris-HCL, 137 mM NaCl, 2% PVP-40, 1% PEG 6000, 9.8 mM $MgCl_2 \cdot 6H_2O$, 0.05% Tween 20; pH 8.2) were added to 0.3 g of leaf material. After grinding with a mortar and pestle, 10 μl of material were added to 100 μl of GES buffer (0.1 M Glycine-NaOH (pH 9), 50 mM NaCl, 1 mM EDTA). The samples were then incubated for 10 min at 95°C and immediately placed on ice for a minimum incubation time of 2 min. Of this crude RNA extract, 2 μl were directly used as template in a 25 μl RT-PCR reaction (1X KapaTaq buffer A, 0.625 μM forward and reverse primer, 0.2 μM dNTPs, 5 mM DTT, 2 U AMV, 1 U KapaTaq DNA polimerase). The PCR cycling conditions consisted of a 30 min incubation period at 48°C, followed by 35 cycles of 30 s at 95°C, 45 s at 55°C and 2 min at 72°C. A final step of 7 min at 72°C was included and afterwards the amplified DNA was visualised on a 1% agarose gel. Negative control reactions were included which did not contain any AMV in the reaction mixture. The primer GVA118-6914-F, annealing to ORF2, was used along with ApaI_D4E1_R, ApaI_VvAMP1 _as or NotI_GUS_as for detection of the recombinant viruses GVA118-D4E1, GVA118-Vv-AMP1 and GVA118-GUS, respectively (Table 1). The expected amplicon lengths of the RT-PCR reactions were 206 bp, 296 bp and 2070 bp, respectively.

3.1.11 Peptide expression

Protein extractions were performed on *N. benthamiana* plants infiltrated with the pBinSN-GVA118-VvAMP at 6 dpi, in order to determine peptide expression. Plants infiltrated with pBinSN-GVA118-D4E1 served as negative control. Western blot analysis was then use to visualise the expressed peptide. The expected size of the Vv-AMP1 peptide was ~5.5 kDa. The

antibody for Vv-AMP1 detection was raised in mice against the Vv-AMP1 peptide (De Beer, 2008). No antibody was available for D4E1, therefore no expression test could be performed on the D4E1 peptide.

Protein extraction

Using a mortar and pestle, 0.3 g of fresh plant material was ground in 750 μl Berger buffer (Berger *et al*., 1989; 750 mM Tris-base (pH 8.8), 4% SDS, 4% 2-Mercaptoethanol, 40% Sucrose) which was pre-heated to 95°C. The mixture was pipetted into clean 2 ml reaction tubes with safe locks and incubated at 95°C. After 10 min of incubation, the samples were centrifuged for 15 min at 15700 *g* and the supernatant transferred into clean reaction tubes. These crude extracts were stored at -20°C until needed.

Western blot

Crude protein extracts were fractionated on polyacrylamide gels specific for small proteins. These gels were 8-16% Ready Gels® from Biorad. All runs were performed by Dr. G. George at the Institute for Plant Biotechnology, Stellenbosch University.

3.2 Tagging of pathogens of interest

3.2.1 Bacterial strains

Agrobacterium vitis (strain A39) and *X. ampelinus* (strain VS9) cultures were provided by Dr. T. Goszczynska (ARC – Plant Protection Research Institute, Pretoria, South Africa). To confirm that colonies which were grown on mannitol media (54.89 mM mannitol, 2.87 mM K_2HPO_4, 0.81 mM $MgSO_4·7H_2O$, 1.71 mM NaCl, 0.4 g/l Yeast extract) plates were indeed *A. vitis*, a single colony-PCR was performed, while a nested-PCR procedure was performed to confirm that colonies grown on Difco nutrient agar (NA; 8 g/l) plates were *X. ampelinus*.

3.2.2 Diagnostic PCRs

Agrobacterium vitis

In order to detect *A. vitis* by means of PCR the primer set, A (ATG CCC GAT CGA GCT CAA GT) and E (CCT GAC CCA AAC ATC TCG GCT GCC CA) of Haas *et al*. (1995) was used. These primers produce a PCR fragment of 338 bp in samples positive for *A. vitis*. The PCRs were performed with cycling conditions of 1 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, followed by a final elongation step of 5 min at 72°C. In the PCR reaction mix 0.1 mM dNTPs, 0.3 μM of each primer, 0.04 U/μl KapaTaq DNA polymerase, 1X

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KapaTaq buffer A and 1X cresol was used. Diagnostic colony-PCRs were performed by picking one colony, dissolving it in 10 µl dH$_2$O and boiling it for 5 min at 99°C. The suspension (2.5 µl) was used as template in the above-mentioned PCR.

Xylophilus ampelinus

A nested-PCR procedure was used for *X. ampelinus* detection. During the first PCR, a 742 bp fragment was amplified, using the primers A1 (AGT CGT AAC AAG GTA AGC CG) and B1 (CYR YTG CCA AGC ATC CAC) (Barry *et al*., 1991). The cycling conditions for this PCR was 3 min at 94°C, 25 cycles of 30 s at 94°C, 30 s at 52°C and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. A fifty times dilution of the first PCR product served as template for the nested reaction. This nested-PCR made use of the primers S3 (GGT GTT AGG CCG AG TAG TGA G) and S4 (GGT CTT TCA CCT GAC GCG TTA) (Botha *et al*., 2001), and amplified a 277 bp fragment. In this study the cycling conditions were 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, followed by a final elongation step of 10 min at 72°C. In both reactions, the PCR mixture contained 0.1 mM dNTPs, 0.3 µM of each primer, 0.05 U/µl KapaTaq DNA polymerase, 1X KapaTaq buffer A and 1X cresol.

3.2.3 Determination of the tumorigenic nature of *A*. *vitis* strains

Not all *A*. *vitis* strains are tumorigenic. To determine the tumorigenic nature of the *A*. *vitis* strain A39, small cuts were made with a scalpel on the side of an *in vitro* propagated *V*. *vinifera cv.* Sultana plant, and 5 µl of *A*. *vitis* cells suspended in water (OD$_{600}$ of 0.3) were dropped on the damaged tissue. The plant was then incubated for 8 weeks in a growth chamber with a 16 h light and 8 h dark photoperiod at 25°C and 20°C respectively, before the plants were inspected for gall development. Control plants that were inoculated with dH$_2$O instead of bacterial suspension were included.

3.2.4 Bacterial tagging with reporter genes

To aid the visualisation and quantification of bacteria, two methods were applied in an effort to stably tag the two bacteria, *A*. *vitis* and *X*. *ampelinus*, either with GFP or with luciferase. The one method used electroporation for plasmid transfer, while the other made use of bacterial mating. To achieve stable transformation of the bacteria, an intron-based system was used. The plasmids used for tagging are listed in Table 2 and were all kindly supplied by S. Molin (BioCentrum-DTU, Technical University of Denmark). Both pUT plasmids contained the transposase on the plasmid itself and could be used to transform the bacterial cells independently. The two mini-Tn7 plasmids however relied on an additional plasmid (pUX-BF13), which contained the

transposase, for transformation. The plasmid pRK600 was used to mediate plasmid transfer, between bacteria, during transformation via bacterial mating.

Table 2: List of plasmids used for tagging of bacteria, as well as their *E. coli* host strains and the antibiotic selection needed after transformation.

Plasmid	Strain	Description	Selection*
pUTGm-P$_{A1/04/03}$CDABE(lux)[1]	MV1190	Luciferase donor plasmid	Gm
pUTKmGfp[2]	MV1190	GFP donor plasmid	Km
pBK-miniTn7-gfp1 (AKN65)[3]	XL1 Blue	GFP donor plasmid	Gm
miniTn7(Gm)PrmB1-gfp-a [4]	MT102	GFP donor plasmid	Km
pUX-BF13[5]	SM10Δ pir	Helper plasmid Tn7 Transposase	
pRK600[6]	HB101	Helper plasmid for mating	

*Selection of bacteria after successful transformation. Gentamicin (Gm) or Kanamycin (Km); [1]Weitz *et al*., 2001; [2]Tombolini *et al*., 1997; [3]Koch *et al*., 2001; [4]Lambertsen *et al*., 2003; [5]Bao *et al*., 1991; [6]Figurski and Helinski, 1979.

Xylophilus ampelinus cells were made electro-competent using a protocol described by Grall and Manceau (2003). Three-day old shaker cultures were pelleted and the pellet dissolved in dH$_2$O. The OD$_{580}$ was adjusted to 1 and 2 ml of the suspension was added to a clean 2 ml reaction tube. After centrifugation, 13000 *g* for 10 min at 4°C, the supernatant was discarded and the pellet dissolved in 1 ml of dH$_2$O. This step was repeated twice and the final pellet dissolved in 50 µl of dH$_2$O. Two micro litres of plasmid DNA were gently mixed with 40 µl of cell suspension and cells were electroporated as described in section 3.1.5. Electroporated cells were shaken at 125 rpm (25°C) and after 2 h plated onto selective NA plates with antibiotic selection as stated in Table 2. After 5 days of incubation at 25°C, plates were visually inspected for GFP expression using a UV lamp or for luminescence using the Ivis® Lumina Imaging System from Caliper/Xenogen. The Living Image® Software parameters were set to luminescent, auto exposure, field of view D and a subject height of 1 cm.

Electro-competent *A. vitis* cells were prepared using the same protocol as described in section 3.1.5. No antibiotic selection was used. The same electroporation conditions and steps were also used as in section 3.1.5 to transform the *A. vitis* cells. After shaking the electroporated cells for 4 h at 28°C, 100 µl of cell suspension was plated on selective mannitol media plates and incubated at 28°C. After 3 days of incubation, plates were screened for positively transformed cells by visual inspection with an ultra violet (UV) lamp in the case of GFP and by making use of the Ivis system to visualise the luminating bacterial cells.

Plasmid concentrations of the tagging plasmid were very low when it was extracted from host strains listed in Table 2. To overcome these low concentrations used in the electroporation, rolling circle amplification (RCA) was applied. This method makes use of the isothermal DNA polymerase Phi29 to amplify circular DNA and results in linear concatamers of the original DNA molecule. Rolling circle amplification was performed on pUTKmGfp and pUTGm-$P_{A1/04/03}$CDABE(lux). In an RCA reaction, 4.5 µl of plasmid DNA was used as template and added to 5 µl of dNTPs (2.5 mM) and 0.5 µl of Exo-Resistant Random Primers (Fermentas). The mixture was filled up to a final volume of 10 µl with dH_2O and incubated for 3 min at 95°C. The reaction tube was cooled on ice and 0.25 µl of Phi29 DNA polymerase, 2 µl of 10X Phi29 Buffer and dH_2O were added to a final reaction volume of 20 µl. The mixture was then incubated for 18 h at 30°C. This was followed by a 10 min step at 65°C after which the RCA product was purified using Sure Clean (Bioline). The pUTKmGfp RCA product was digested using *Not* I (Fermentas) while the pUTGm-$P_{A1/04/03}$CDABE(lux) RCA product was digested using *Eco* RI (Fermentas). Each digestion generated two fragments. The larger fragment was treated with Shrimp Alkaline Phosphatase (SAP) followed by ligation to the smaller fragment to form the original plasmid again. These ligated products were then introduced into *A. vitis* via electroporation (section 3.1.5). Electroporated cells were incubated on selective media and after 3 days of incubation at 28°C screened for transformed colonies by visual inspection as before.

Agrobacterium vitis chemical competent cells were made and chemically transformed. Briefly, 2 ml of an overnight *A. vitis* culture was used to inoculate 50 ml of mannitol media. After shaking for 4 h at 28°C, until the OD_{600} reached between 0.5 and 0.6, cultures were incubated in 50 ml centrifuge tubes for 10 min on ice. This was followed by centrifugation for 10 min at 3000 *g* (4°C), discarding the supernatant and dissolving the pellet in 1 ml of ice cold $CaCl_2$ (20 mM). Aliquots were made; flash frozen and stored at -80°C. For the transformation, 100-200 ng of plasmid DNA was added to a 100 µl aliquot of cells that were thawed by the heat of one's finger tips. One millilitre of mannitol medium was added and the cell suspension incubated for 4 h at 28°C, shaking at 150 rpm. The cells were then pelleted by centrifugation for 30 s and resuspended in 100 µl of the supernatant, the rest were discarded. The cells were then plated on selective media, as listed in Table 2, and incubated for 3 days at 28°C. Colonies were visually inspected with a handheld UV lamp for GFP expression.

Tagging of bacteria by means of bacterial mating requires antibiotics selecting for the recipient cells but eliminating the donor or helper cells, thus selecting for the desired transformed cells. A literature study did not yield any antibiotic used in selection of natural *A. vitis* or *X. ampelinus* strains. Therefore, readily available antibiotics were screened for their ability to select these

bacteria. All bacteria (donor, helper and recipient) were tested for growth against ampicillin, gentamicin, chloramphenicol and rifampicin. *Agrobacterium vitis* and all the donor and helper bacteria were tested for growth against different concentrations of rifampicin, kanamycin, chloramphenicol, tetracycline and streptomycin. When no suitable antibiotic was found, bacterial mating was performed without any selection specific to the recipient bacteria itself. Bacterial identity was then confirmed with colony-PCRs.

Bacterial mating was performed in order to tag *A. vitis* cells. *Agrobacterium vitis* cells were streaked on mannitol agar media and incubated for 3 days at 28°C while plasmid-containing *E. coli* cells were streaked 2 days later on selective Luria Bertani agar media, and incubated overnight at 37°C. Liquid media were inoculated and shaken overnight, for *A. vitis* at 28°C shaking at 200 rpm and for the different *E. coli* strains at 37°C shaking at 180 rpm. Of each overnight culture, 2 ml were spun down for 2 min at 4500 g. The resulting pellet was resuspended in 1 ml of dH_2O and the centrifugation repeated. This final pellet was then resuspended in 100 µl of dH_2O whereafter 100 µl of *A. vitis* suspension was mixed with 50 µl of each of the respective *E. coli* suspensions as explained above. The mixture was pipetted onto in the middle of a Petri dish containing mannitol media without any antibiotics. After 5 days of incubation at 28°C the cells were dissolved in 200 µl of liquid mannitol medium and plated with antibiotic selection, as indicated in Table 2. The cells were then incubated for 3 days at 28°C after which colonies were visually inspected for GFP expression with a handheld UV lamp and for luminescence using the Ivis system (using the same settings as mentioned before in this section). To further refine the inspection, growth were also visualised under a microscope equipped with an UV light.

3.3 *In vitro* AMP activity screening against *Agrobacterium vitis* and *Xylophilus ampelinus*

3.3.1 Culturing of pathogen strains

Throughout the experiment *A. vitis* (strain A39) was cultured on mannitol media for 3 days at 28°C while *X. ampelinus* (strain VS9) cultures were grown for 5 days at 25°C on NA plates. Liquid cultures were prepared by inoculating 20 ml of the respective liquid media with 5-10 colonies of the bacteria, and incubated it at the appropriate temperatures, at 180 rpm, overnight or for 3 days for *A. vitis* and *X. ampelinus*, respectively.

3.3.2 AMP plate testing

Stock solutions of 1 mg/ml D4E1 (Mr = 2611.87 g/mol) were made fresh just before use and dilutions were made in liquid media of the respective bacteria. The peptide effect was first tested against the bacteria at peptide concentrations of 40, 20, 10, 4, 2, 1, 0.5 and 0 µg/ml. This was achieved by adding peptide solution to bacterial liquid cell cultures in a 1.5 ml reaction tube to a final volume of 1ml and at an OD_{600} (*A. vitis*) or OD_{580} (*X. ampelinus*, Grall and Manceau, 2003) of 0.1. Individual dilutions were performed in triplicate. After shaking the suspension for an hour at 180 rpm at the appropriate temperature, 100 µl of the suspension were plated on solid media. Three or five days after plating, for *A. vitis* and *X. ampelinus* respectively, bacterial cfu were counted and compared to bacterial cell cultures without D4E1 or cultures to which kanamycin (50 µg/ml) was added. To further refine the results the experiment was repeated with D4E1 concentrations between 10-20 µg/ml in 2 µg/ml intervals. These were the two concentrations at which the bacteria showed the highest peptide sensitivity in the first round of experiments.

For statistical analysis, in order to perform t-tests, it was assumed that the number of colonies on the plants showing more than 1000 colonies were only 1000 cfu (a conservative estimation). Poisson distribution was also assumed due to the nature of the data. The standard error for the cfu of 1000 was therefore calculated to be 31.62 (S.E. = $\sqrt{1000/3}$). The Welch two-sample t-test was used to determine statistical differences between numbers of cfu at different D4E1 concentrations.

3.4 Screening the *in planta* activity of AMPs against the pathogens of interest

The disease-free *V. vinifera* cv. Sultana plants were agro-infiltrated with the constructed vectors and used to test the *in planta* effect of the transiently expressed AMPs against *A. vitis* and *X. ampelinus*. In all experiments, 5 plants infiltrated with the AMP expression vector, serving as the treatment group, and 5 control plants infiltrated with Agrobacterium cells containing an empty binary vector, lacking the AMP gene, were used.

3.4.1 Inoculation of *V. vinifera* plants with *A. vitis* and *X. ampelinus*

Plants were inoculated with *X. ampelinus*, 3 days after *A. tumefaciens*-mediated infiltration of the AMP-expressing 35S vector. This was achieved by dipping the plants in a suspension of *X. ampelinus* in sterile water with an OD_{580} of 0.1. These plants were grown for 4 days at 25ºC in light for 18 h and at 20ºC in the dark for 6 h. Plants infiltrated with empty binary vectors served

as controls and were also infected with *X. ampelinus*. Following the 4-day incubation period, DNA extractions were performed on the leaves of all plants after rinsing them in dH_2O, and the total extracted DNA (see section 3.4.2) subjected to qPCR (see section 3.4.6) for bacterial titre determination. Inoculated plantlets were also evaluated over a period of 21 days for symptom development.

In order to determine the *in planta* effect of the AMPs against *A. vitis*, plants were inoculated with the bacteria by vacuum infiltrating a suspension of bacteria with an OD_{600} of 0.01. The bacterial inoculation was performed 3 days after plants were vacuum-infiltrated with *A. tumefaciens* cells containing an AMP vector or empty binary vector. The plants infiltrated with Agrobacterium containing an empty binary vector again served as controls. In an effort to improve this method, the test was repeated but instead of using vacuum infiltration, the plants were dipped in an *A. vitis* suspension with an OD_{600} of 0.1. After 4 days of incubation, plant leaves were rinsed in dH_2O, the DNA extracted and used as template for qPCR in order to determine bacterial titres. Further optimisation of this method of AMP efficacy screening against *A. vitis* was performed by reducing the bacterial concentration to an OD_{600} of 0.05 and elongating the period of incubation between infection of the plant with the bacteria and DNA extraction, to 7 days.

3.4.2 DNA extraction procedures

Total DNA extraction was performed according to a CTAB method described by Malan (2009). The concentration of the DNA was determined by means of a Nanodrop® ND-1000 spectrophotometer and stored at -20°C.

3.4.3 qPCR primers and PCR optimisation

Bacterial titres were determined using qPCR and were compared between AMP-treated and the untreated control plants. Reference grapevine genes were used to normalise the data. The primers used for the relative quantification of the bacteria are listed in Table 3. Primers were first optimised using normal PCR to determine the specificity and efficiency of the primer set by screening them at different annealing temperatures and the reaction compositions, for example primer concentrations. The PCR fragments were cloned into pGem®-T Easy and sequenced to confirm that the amplified sequences were of the expected origin.

Table 3: List of primers used for quantitative real-time PCR.

Primer name	Organism	Sequence	Genomic region	Tm (°C)	Source
Vv_Actin_F	*V. vinifera*	CTTGCATCCCTCAGCACCTT	Actin	57.7	Reid *et al*., 2006
Vv_Actin_R	*V. vinifera*	TCCTGTGGACAATGGATGGA	Actin	55.9	Reid *et al*., 2006
Vv_EF1a_F	*V. vinifera*	GAACTGGGTGCTTGATAGGC	EF1-α	55.9	Reid *et al*., 2006
Vv_EF1a_R	*V. vinifera*	AACCAAAATATCCGGAGTAAAAGA	EF1-α	52.4	Reid *et al*., 2006
VIRD62F23	*A. vitis*	AACCATTCAGCAGGTTAT	VirD2	48.5	Bini *et al*., 2008
VIRD62R135	*A. vitis*	TGGTAATTTGATCAGGCG	VirD2	49.8	Bini *et al*., 2008
Xamp_14F	*X. ampelinus*	CCCGATGATAAATACCGAAAACTC	16S rRNA	53.7	Dreo *et al*., 2007
Xamp_104R	*X. ampelinus*	TGTCTTCTGGTTGTTTTGGTTTTAAT	16S rRNA	53.9	Dreo *et al*., 2007
Xamp1.3A	*X. ampelinus*	GATGTAGCCGGACGTACCG	16S rRNA	57.6	Manceau *et al*., 2000
Xamp1.3B	*X. ampelinus*	CGTTTTCCGTGGCATCGATG	16S rRNA	57.0	Manceau *et al*., 2000
Xamp2.0A	*X. ampelinus*	GTGTGGCGATAATCGTGACG	16S rRNA	56.2	Manceau *et al*., 2000
Xamp2.0B	*X. ampelinus*	TGAAAGAGCGCGGCGGCAGT	16S rRNA	65.1	Manceau *et al*., 2000
S3	*X. ampelinus*	GGTGTTAGGCCGAGTAGTGAG	16S rRNA	56.9	Botha *et al*., 2001
S4	*X. ampelinus*	GGTCTTTCACCTGACGCGTTA	16S rRNA	57.3	Botha *et al*., 2001
AAY_F	Phytoplasma	TTGGGTTAAGTCCCGCAAC	16S rRNA	55.5	Angelini *et al*., 2007
AAY_R	Phytoplasma	CCCACCTTCCTCCAATTTATCA	16S rRNA	54.6	Angelini *et al*., 2007
AY_F	Phytoplasma	AAACCTCACCAGGTCTTG	16S rRNA	51.9	Present study
AY_R	Phytoplasma	AAGTCCCCACCATTACGT	16S rRNA	53.4	Present study

3.4.4 qPCR conditions

After primer optimisation, a Rotor-Gene Q (Qiagen) thermal cycler was used to perform all qPCRs, and the Rotor-Gene Q Series Software 1.7 was used for run setup and analysis. The PCR conditions for all the runs were the same. The reaction volume was 25 µl and consisted of template DNA, 1X SensiMix No-ROX (Quantace), 0.2 µM forward and reverse primer and ddH$_2$O. The SensiMix No-ROX kit is a SYBR Green-based system. Cycling conditions were as follows: 10 min at 95°C followed by 35 cycles of 15 s at 95°C and 60 s at 60°C. After each run melting curve analysis were performed to determine the specificity of the amplified products.

3.4.5 Determination of qPCR efficiencies

Standard curves were set up for each primer pair in order to determine the efficiencies of the respective qPCRs. The standard curve for *X. ampelinus* quantification was established by making a 5-fold dilution series of a DNA sample extracted from a grapevine tissue culture plant infected with the bacteria. The dilution series ranged from 24 ng to 0.0384 ng of total DNA per reaction. Primers S3 and S4 were used and reactions were performed in triplicate for each DNA concentration. The same reaction was performed to set up a standard curve for quantification of the reference gene, actin, using the primers Vv_Actin_F and Vv_Actin_R. The primer pair VirD62F23 and VIRD62R135 was used to establish the standard curve for *A. vitis* quantification. A 5-fold dilution series of a DNA sample extracted from a *V. vinifera* tissue culture plantlet

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infected with the bacteria was made. The amount of DNA in the dilution series ranged from 60 ng to 0.096 ng of total DNA per reaction. In theory the dilution series only has to span the concentration of the DNA which needs to be detected.

3.4.6 Determination and comparison of bacterial titres

Quantitative analysis of bacterial titres in *V. vinifera* was performed using qPCR in order to determine the *in planta* activity of the AMPs against the pathogens. The concentration of the bacteria in the AMP-treated group was compared relative to the concentration of the bacteria in the untreated control group. For this, qPCR amplifications were performed for each plant. Relative qPCRs were performed for the first experiment in triplicate for both the gene of interest (GOI) and the reference gene. The GOIs are situated within the bacterial genomes, while the reference gene is situated within the grapevine genome. From here on, reactions were only performed in duplicate. When amplification showed inconsistency between two reactions of the same sample, the qPCR was repeated for those samples. In each run one sample, for both the GOI and the reference gene, was included to serve as a calibrator. These samples were used to construct the respective standard curves and were included in the quantification run at a concentration used during the setup of each dilution series.

To determine the Ct values for each sample, the Rotor-Gene Q Series Software 1.7 was used. The software imported the two standard curves, one for the GOI and one for the reference gene, and adjusted the curves according to the calibrator samples included in each run. The Ct values derived were then exported and incorporated into the Relative Expression Software Tool (REST) program. To determine the bacterial titres in the treated plants relative to those of the untreated plants, this programme carries out 10000 mathematical iterations and statistical analysis (Pfaffl *et al*., 2002).

3.5 Aster yellows phytoplasma AMP activity screening

3.5.1 Phytoplasma infected plant samples

Cane material from phytoplasma infected *V. vinifera* cv. Chardonnay plants was collected from an infected vineyard in the Slanghoek area near Rawsonville in the Western Cape. These plants were potted and phytoplasma infection confirmed by means of a nested-PCR reaction (section 3.5.2). The plants were kept in a greenhouse until needed. Infected cane material (*V. vinifera* cv. Shiraz) was also collected from a farm near Vredendal in the Olifants River region of the Western Cape. These canes were stored at 4°C for later used in tissue culture (section 3.5.4).

3.5.2 Aster yellows phytoplasma diagnostic PCR

Aster yellows phytoplasma was detected by a nested-PCR procedure. Universal diagnostic primers, P1 (AAG AGT TTG ATC CTG GCT CAG GAT T) (Deng and Hiruki, 1991) and P7 (CGT CCT TCA TCG GCT CTT) (Smart *et al*., 1996), were used for the first PCR. The reaction mixture contained 0.2 mM dNTPs, 0.5 µM of each primer, 0.05 U/µl KapaTaq DNA polymerase, 1X KapaTaq buffer A and 1X cresol, while the PCR cycling conditions were as follows: 5 min at 94°C, 35 cycles of 20 s at 94°C, 30 s at 59°C and 45 s at 72°C, followed by a final elongation step of 7 min at 72°C. The first PCR products were then, after diluting 30 times, used in nested-PCR reactions using the primers R16F2N (GAA ACG ACT GCT AAG ACT GG) and R16R2 (TGA CGG GCG GTG TGT ACA AAC CCC G) (Lee *et al*., 1993). The reaction mixture was the same as for the first PCR and the cycling conditions were as follows: 2 min at 94°C, 35 cycles of 20 s at 94°C, 30 s at 60°C and 45 s at 72°C, followed by a final elongation step of 10 min at 72°C. Positive samples displayed an amplicon of 1860 bp (although not always visible) in agarose gel electrophoresis after the first PCR reaction and a 1245 bp amplicon after the nested-PCR reaction.

3.5.3 Aster yellows phytoplasma qPCR

Real-time PCR primers were designed for a SYBR Green-based assay from the primer of Hollingsworth *et al*. (2008) and the probe used in the TaqMan® assay of Angelini *et al*. (2007) for aster yellows phytoplasma. The sensitivity of the detection of aster yellows with qPCR using these primers, AY_F (AAA CCT CAC CAG GTC TTG) and AY_R (AA GTC CCC ACC ATT ACG T), were then compared to the sensitivity of detection by means of the nested-PCR described in section 3.5.2. Total DNA extracted from an aster yellows infected grapevine plant, was used to make a 10-fold dilution series ranging over 10 different concentrations (2.5 ng to 2.5 x 10^{-9} ng). Real-time PCR and normal nested-PCR runs were then performed to compare detection of aster yellows phytoplasma at each of the different concentrations. Diagnostic runs were repeated to confirm the results.

3.5.4 Establishing plant cultures infected with phytoplasma

Infected *V. vinifera* cv. Shiraz material from the field was sterilised according to a protocol by Alplanta (Germany). Cane material stored at 4°C, was cut into pieces of 2-3 cm in length each containing one node. Sterilisation was performed by washing the cuttings with a brush in water containing a strong detergent and then rinsing it in water containing a mild detergent. The cuttings were then stirred for 2 min in 70% ethanol and then for 10 min in a solution of 10% CaClO$_2$. After rinsing 4 times alternating in 0.25% CaCl and sterile water, the cuttings were

planted in perlite with sterile water. They were then grown in an incubator with a 16 h light and 8 h dark photoperiod at 25°C and 20°C respectively.

3.5.5 *In vitro* grafting for phytoplasma transmission

In vitro established *V. vinifera* cv. Shiraz scions infected with phytoplasma, were grafted onto uninfected *in vitro* cultured *V. vinifera* cv. Shiraz plants (protocol by Pathirana and McKenzie, 2005). Explants, having one node with a leaf, were obtained from phytoplasma infected plantlets. Using a sharp scalpel blade the apex was cut to form a longitudinal cleft of 2-4 mm and positioned on media. The basal part of a scion stem with similar size also having a single node and leaf, was cut into a wedge and fixed in the recipient plantlet cleft. These grafted plants were used in an effort to propagate sterile tissue culture grapevine plants infected with phytoplasma.

3.5.6 Transient expression in *Catharanthus roseus* (periwinkle)

Recently it was also found that infected *M. fuscovaria* insects will feed on *C. roseus* and transfer the phytoplasma to these plants (Pers. Com. K. Kruger, Department of Zoology and Entomology, University of Pretoria). Phytoplasma are known to accumulate in high concentrations in *C. roseus* (Berges *et al*., 2000), making these plants ideal to be used in the study phytoplasma.

Before testing the efficacy of AMPs against aster yellows phytoplasma in *C. roseus*, a transient expression system for foreign proteins first had to be evaluated. Leaf material of *C. roseus* plants were vacuum-infiltrated with an *A. tumefaciens* (strain C58C1) cell suspension containing the GUSi construct 35S:GUSi, as described in section 3.1.6. After 3 and 6 days a GUS assay (section 3.1.9) was performed to determine GUS expression. *Nicotiana benthamiana* plants served as positive control while uninfiltrated plants were used as negative control for the GUS assay. The assay was repeated adding a different *A. tumefaciens* strain (EHA105) containing the 35S:GUSi construct to compare GUS expression of different *A. tumefaciens* strains.

The *C. roseus* plant used in the first two GUS assays was more than a year old. A 6-week-old *C. roseus* plant was also vacuum-infiltrated with an *A. tumefaciens* (strain C58C1) cell suspension containing with the same constructs and under the same conditions.

Chapter 4

Results

4.1 Construction of transient expression vectors

Viral vectors as well as 35S expression vectors containing the foreign genes were successfully constructed, in order to conduct AMP *in planta* activity screening. These constructs included vectors for the expression of the AMPs, D4E1 and Vv-AMP1, as well as control constructs for GFP and GUS expression. Sequencing results confirmed the integrity of the inserted foreign genes. The 35S expression vectors that were constructed are pBin61S-VvAMP1 and pBin61S-D4E1 (Figure 11). The 35S:GUSi vector was previously designed and available for use. The GVA118-based viral vectors, pBinSN-GVA118-GFP, pBinSN-GVA118-GUS, pBinSN-GVA118-VvAMP1 and pBinSN-GVA118-D4E1 are illustrated in Figure 12, along with the Gr5ΔORF2-based viral vector pBinSNGr5ΔORF2-VvAMP1.
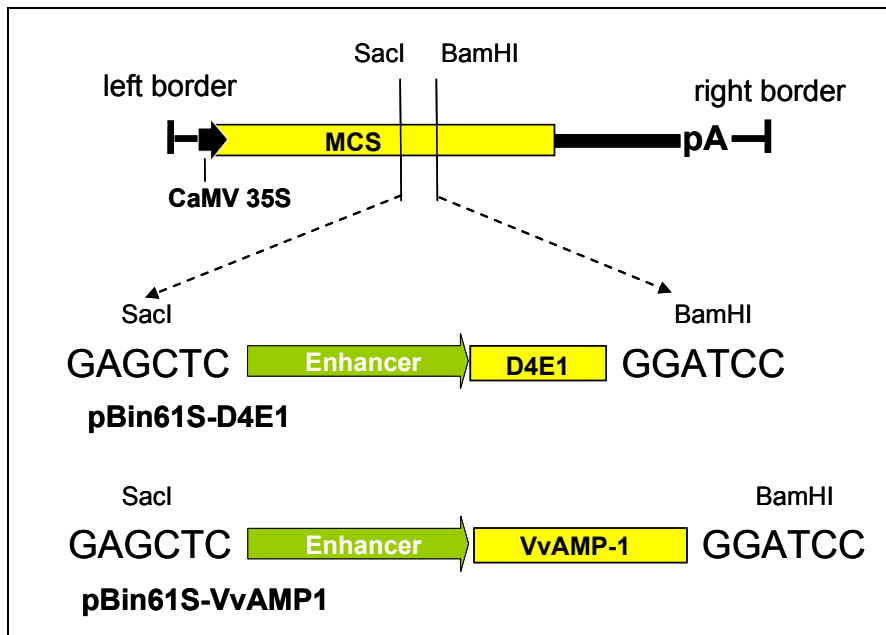
Figure 11: Schematic representation of the region between the right and left border of the 35S expression vectors using the pBin61S binary vector backbone. The vectors pBin61S-VvAMP1 and pBin61S-D4E1 are shown.
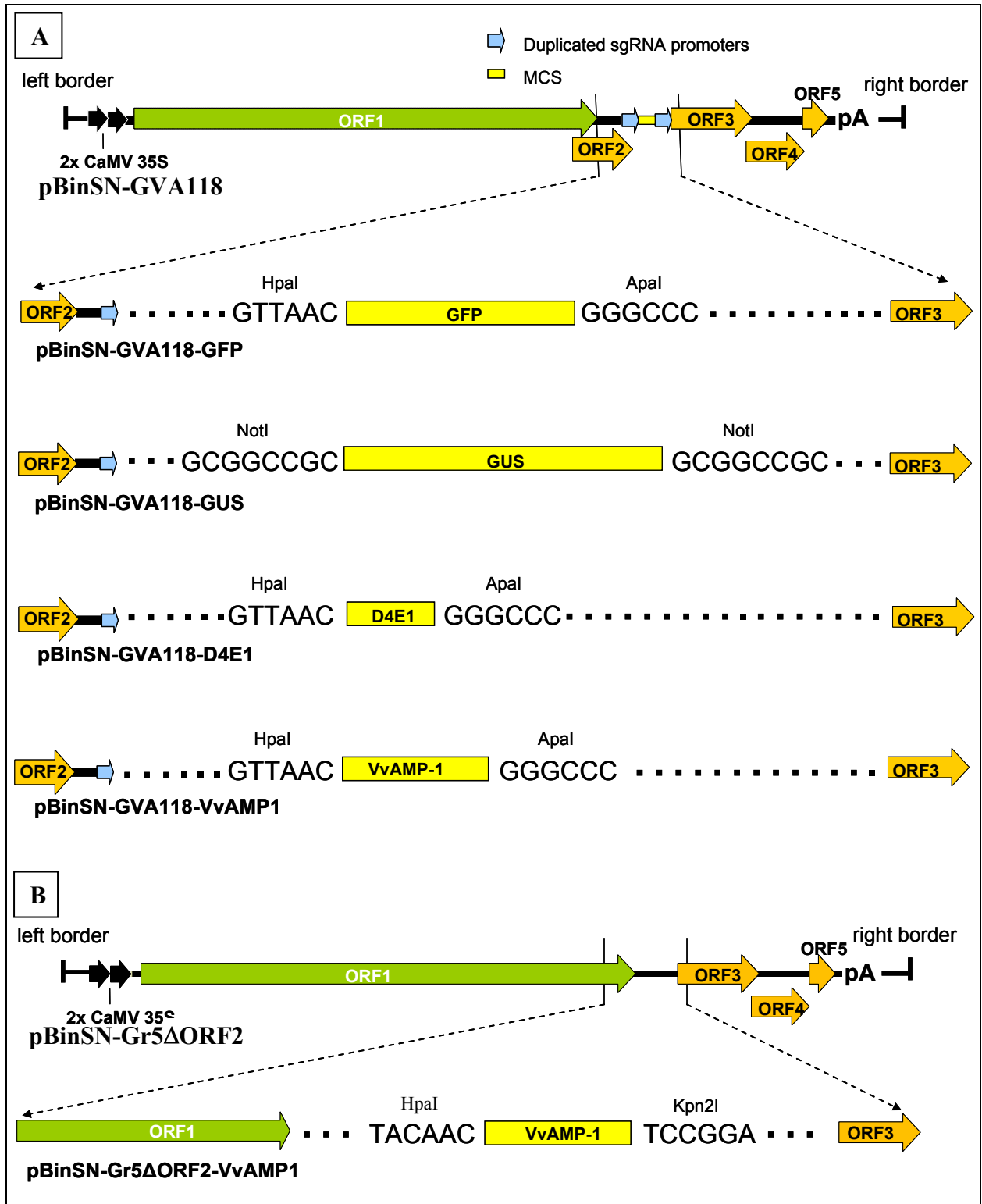
Figure 12: Schematic representation of the viral expression vector regions between the right and left border of the pBinSN binary vector. The vectors pBinSN-GVA118-GFP, pBinSN-GVA118-GUS, pBinSN-GVA118-VvAMP1 and pBinSN-GVA118-D4E1 are shown in (A) and the GR5-ΔORF2 construct pBinSN-Gr5ΔORF2-VvAMP1 is shown in (B).

4.1.1 Testing the infectivity of viral constructs

Tissue print immuno-assays were performed in the model plant *N. benthamiana* to indicate successful viral replication that originated from the viral vectors. Local replication in the leaves inoculated with the viral vectors was determined by using the inoculated leaves in the TPIAs. Positive samples showed dark purple-like spots on the membrane after the BCIP/NPT colouring reaction (Figure 13). The negative control plant showed none of these dark spots, only green spots were observed as background resulting from the leaf tissue. Plants infiltrated with either pBinSN-GVA118-GFP, pBinSNGVA118-VvAMP1 or pBinSN-GVA118-D4E1 tested positive for GVA replication.
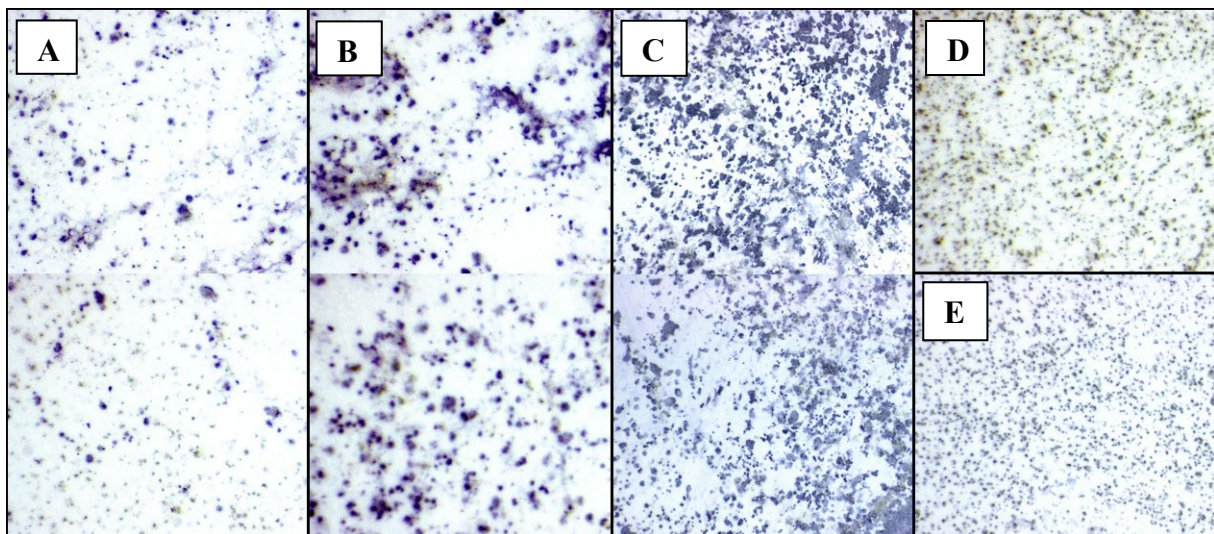
Figure 13: Tissue print immuno-assay of *Nicotiana benthamiana* leaves showing GVA specific antibody binding to the coat protein of recombinant GVA viruses that originated form the viral expression vectors (A) GVA118-GFP; (B) GVA118-VvAMP1 and (C) GVA118-D4E1. Purple spots were indicative for viral replication. The negative controls for GVA118-GFP replication and GVA118-VvAMP1 replication (D) and for GVA118-D4E1 replication (E) were leaves of plants that were not infiltrated with any construct and did not show viral replication only leaf background.

To determine whether the viruses were moving systemically in *N. benthamiana* plants, uninfiltrated leaves, of plants that were infiltrated with the viral constructs, were visually inspected for symptom development. These leaves developed symptoms associated with viral infection (yellowing of the veins), as shown in Figure 14. Viral infection in these leaves was confirmed by TPIA's which showed BCIP/NPT colouring reactions where GVA antibodies bound to areas of petiole prints of the plants infiltrated with pBinSN-GVA118-VvAMP1 and

pBinSN-GVA118-D4E1 (Figure 15). The TPIA of the negative control petiole print showed no BCIP/NPT colouring reactions.

Figure 14: *Nicotiana benthamiana* plant showing systemic GVA associated symptoms on leaves after being infiltrated with (A) pBinSN-GVA118-VvAMP1; (B) pBinSN-GVA118-D4E1; (C) Uninfiltrated leaf showing no symptoms.
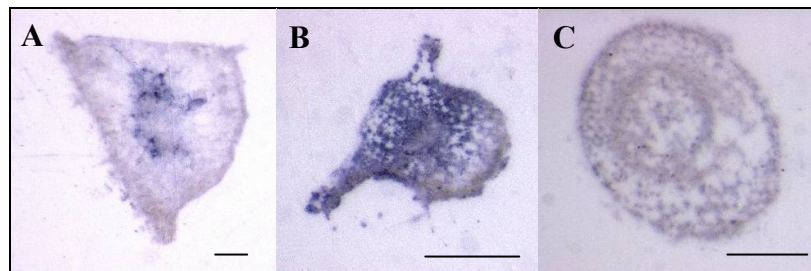
Figure 15: Tissue print immuno-assay of *Nicotiana benthamiana* leaf petioles showing the systemic movement of the viruses in plants infiltrated with (A) pBinSN-GVA118-Vv-AMP1 and (B) pBinSN-GVA118-D4E1 in comparison to an uninfiltrated control plant (C). Bars indicate 1 mm.

In order to confirm that the viral vector-derived virus was not only replicating and spreading systemically through the *N. benthamiana* plants, but still contained the inserted foreign gene, RT-PCR reactions were performed to determine the presence of the recombinant viruses in leaf tissue acropetal to the infiltrated leaves. The region of amplification included the inserted foreign gene as well as a part of ORF2. The RT-PCR products were visualised on an agarose gel (Figure 16) and the expected amplicons of about 200 bp for GVA118-D4E1 (lane 1) and 300 bp for GVA118-Vv-AMP1 (lane 3) could be detected. The plants infected with the control viral construct pBinSN-GVA118-GUS did not show the expected amplicon of 2070 bp (lane 5). None of the negative control reactions (even numbered lanes) yielded any products.
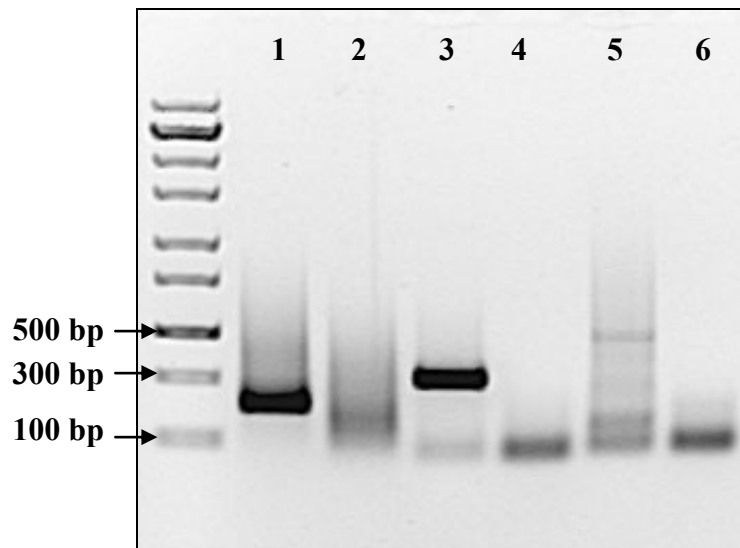
Figure 16: Agarose gel electrophoresis of RT-PCR products used to determine the systemic spread of viral vectors containing foreign gene inserts. Lane 1: GVA118-D4E1, lane 3: GVA118-Vv-AMP1, lane 5: GVA118-GUS, lanes 2, 4, and 6: Water negative controls.

4.1.2 Foreign protein expression by viral expression vectors

Both grapevine and *N. benthamiana* plants were subjected to a GUS expression assay after infiltration with pBinSN-GVA118-GUS, using 35S:GUSi as positive control. This was done to verify that the inserted foreign gene did not disrupt the vector and to evaluate foreign gene expression in the respective leaf tissues. The blue colouration of GUS substrate in the leaves as a result of GUS expression, as it was observed in the plants, are shown in Figure 17. Plants infiltrated with 35S:GUSi showed GUS expression in almost 100% of the infiltrated tissue. The GUS expression was also observed on *N. benthamiana* leaves infiltrated with pBinSN-GVA118-GUS. Only small spots of GUS-staining could be detected in grapevine leaves when infiltrated with pBinSN-GVA118-GUS. These spots were visualised under a microscope (Figure 17) and seemed to be only detectable at leaf veins. Leaves of the negative control plants, not infiltrated with either of the GUS expressing vectors, did not show any blue areas of GUS expression. Two to three leaves were infiltrated per construct and showed similar degrees of expression.
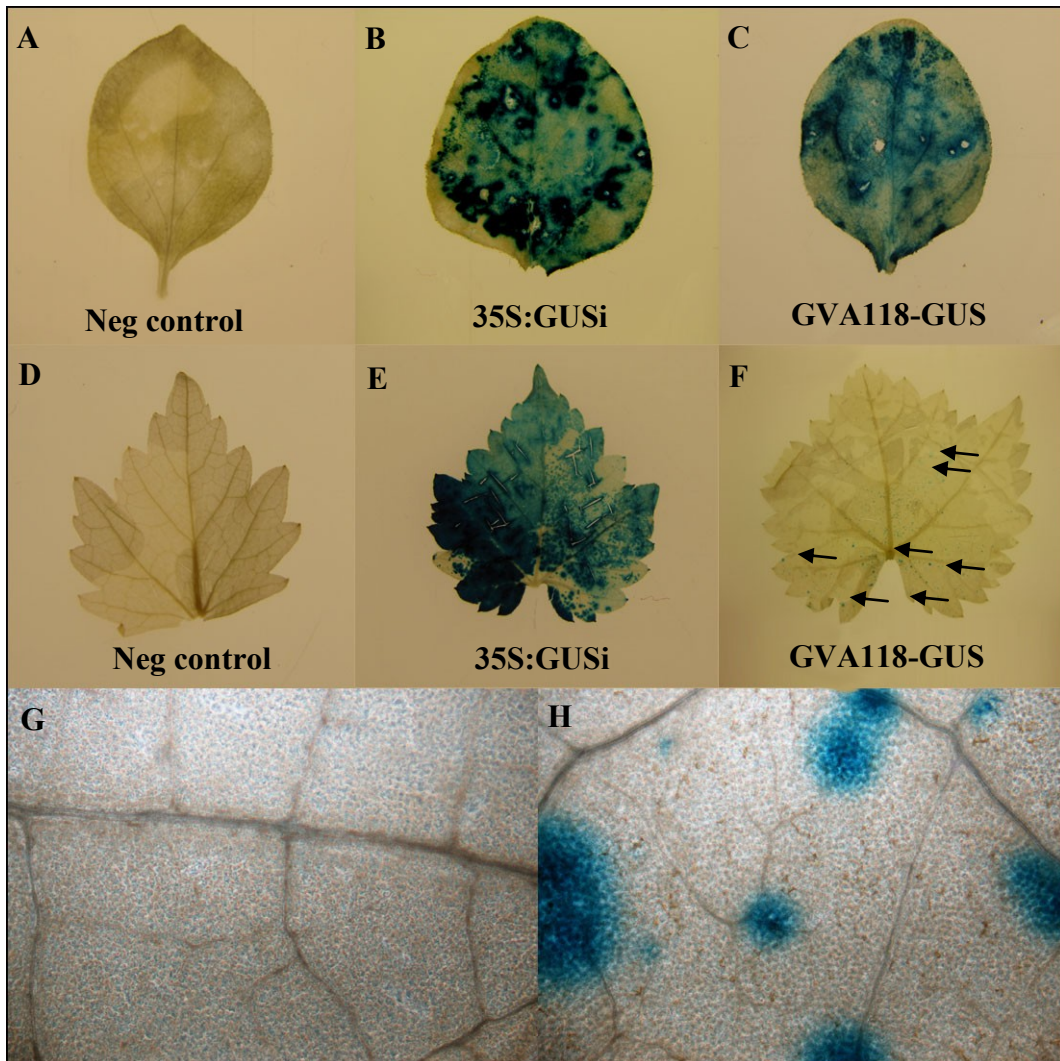
Figure 17: Photographs showing GUS expression in *Nicotiana benthamiana* (A-C) and *Vitis vinifera* (D-F) leaves at 6 dpi. The negative control leaves (A and D) show no GUS expression. All the leaves infiltrated with either the 35S:GUSi (B and E) or pBinSN-GVA118-GUS (C and F) show GUS expression. Visualised under 100 x magnification: (G) Negative control *V. vinifera* leaf; (H) GUS expression in a *V. vinifera* leaf infiltrated with pBinSN-GVA118-GUS.

Vitis vinifera and *N. benthamiana* were used to test the expression of GFP from the recombinant virus GVA118-GFP. Microscopic analysis of *N. benthamiana* leaves infiltrated with pBinSNGVA118-GFP was performed and showed single cells expressing GFP (Figure 18). No GFP expression was observed in leaves of *V. vinifera* plants infiltrated with this viral construct, nor in uninfiltrated plants which served as negative control.
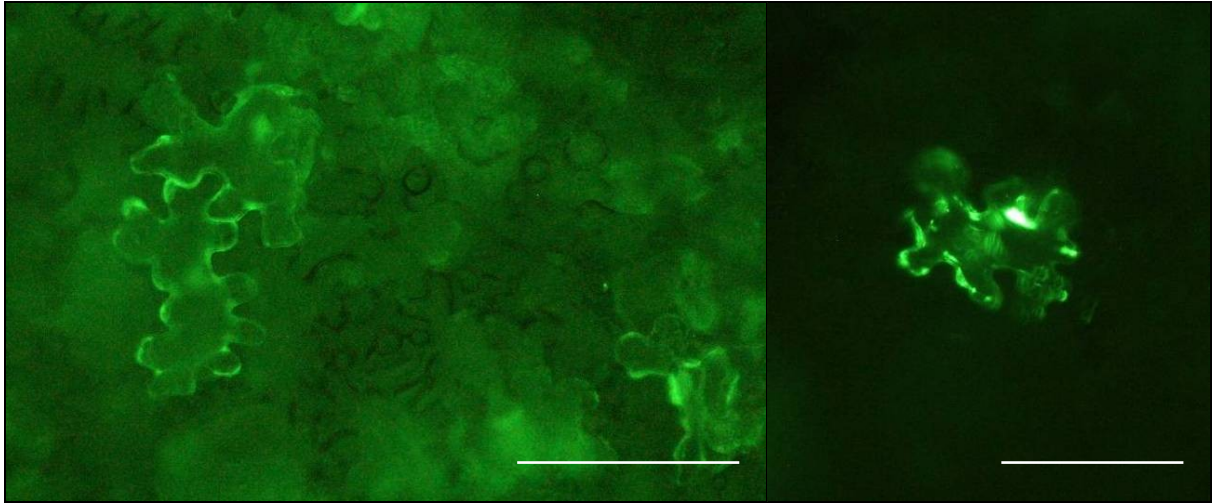
Figure 18: GFP expression in *Nicotiana benthamiana* epidermis cells infiltrated with pBinSNGVA118-GFP as seen under a microscope. Bar indicate 50 μm.

A GUS assay was performed on leaves acropetal to the leaves infiltrated with pBinSN-GVA118-GUS in order to illustrate the systemic movement of the virus, and also whether it was still actively expressing the foreign protein. The presence of the recombinant virus could be detected by GUS expression 17 dpi in leaves acropetal to in infiltrated ones (Figure 19). The GUS expression was associated with the leaf veins, which confirmed systemic spread of GVA, containing a stably integrated and functional GUSi gene in its genome.
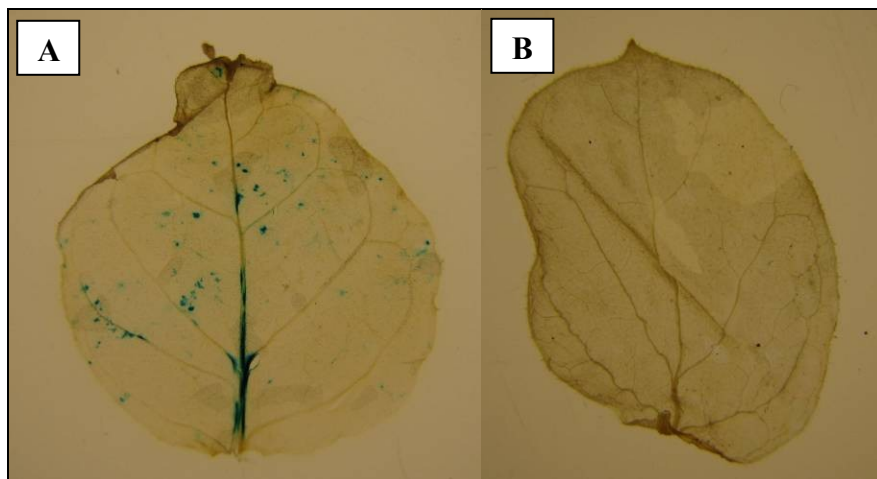
Figure 19: *Nicotiana benthamiana* leaves that were used for determining systemic GUS expression from GVA118-GUS at 17 dpi. A) A leaf acropetal to pBinSN-GVA118-GUS infiltrated leaves, showing GUS expression associated with the vascular tissue. B) A leaf of an uninfiltrated negative control plant showing no GUS expression.

4.1.3 Testing of viral AMP expression

Western blot analysis was performed on crude protein extracts from *N. benthamiana* leaves, infiltrated with pBinSN-GVA118-VvAMP1, to test for the expression of Vv-AMP1 at 6 dpi. Crude protein extracts of *N. benthamiana* leaves infiltrated with pBinSN-GVA118-D4E1, served as negative control. All samples, including the negative control, displayed the same pattern of antibody binding to the membrane (Figure 20). Four attempts were made to detect Vv-AMP1 in these plants, but all failed. Since no antibodies were available for D4E1, no blots to test its expression were performed.
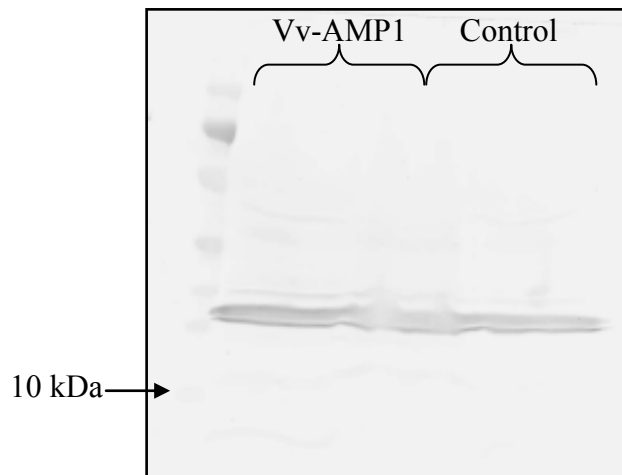
Figure 20: Western blot results for Vv-AMP1 expression in *Nicotiana benthamiana* plants infiltrated with pBinSN-GVA118-VvAMP1 and negative control plant infiltrated with pBinSN-GVA118-D4E1.

4.2 Tagging of pathogens of interest

The pathogens of interest needed to be tagged with a reporter gene in order to quantify them *in planta*. The tagging methods could only be applied to *A. vitis* and *X. ampelinus* as it is not possible to culture phytoplasmas in an artificial environment.

4.2.1 Verification of *A. vitis* and *X. ampelinus* cultures

To verify the identity of the bacteria, PCRs were performed on bacterial colonies growing on solid media. The amplicons were cloned and sequenced. The sequences were analysed with the Basic Local Alignment Search Tool. The *A. vitis* amplicon aligned to the virD2 gene of *A. vitis* (see Addendum). The *X. ampelinus* amplicon aligned to the 16S ribosomal RNA region of *X. ampelinus* (see Addendum).

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4.2.2 Biotest to determine the tumorigenic nature of *A. vitis* strain A39

To determine whether the *A. vitis* strain, later used for AMP activity screening, is tumorigenic and therefore of agricultural importance, its ability to induce tumours in grapevine was investigated. The *V. vinifera* cv. Sultana plantlet inoculated with an *A. vitis* suspension on a bruised stem developed a tumour 8 weeks after infection at the site of inoculation (Figure 21), while the control plant inoculated with dH_2O did not develop any tumours.

Figure 21: *Vitis vinifera* cv. Sultana plant showing crown gall development as a result of *A. vitis* inoculation at 56 dpi.

4.2.3 Bacterial tagging with reporter genes

Agrobacterium vitis and *X. ampelinus* cells were submitted to various transposon-based tagging methods in an attempt to label them with a reporter gene to facilitate quantification by the Ivis system. These results are summarised in Table 4. In most tagging attempts of *A. vitis* and *X. ampelinus* no colonies developed on selective plates after electroporation or chemical transformation. When colonies developed, no luminescence or GFP emission could be observed. The antibiotics tested for selection during bacterial mating were not effective in discriminating between the donor and the recipient bacteria. When bacterial mating was however applied to *A. vitis* cells, without the initial use of antibiotic selection, colonies developed that showed GFP expression when visualised under a microscope (Figure 22). They were confirmed to be *A. vitis* by colony-PCR. The expressed GFP was only visible under a microscope, but not by a hand held UV lamp, and was therefore inadequate for quantification by means of the Ivis system. As a result of the limited expression of GFP in *A. vitis* and the failure to tag *X. ampelinus*, quantification was performed by means of qPCR.

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Table 4: Results from bacterial tagging attempts. The methods used and the plasmids involved are shown for each bacteria.

Bacteria	Method	Plasmids	Result
X. ampelinus	Electroporation	pUTGm-$P_{A1/04/03}$CDABE(lux)	Unsuccessful
		pUTKmGfp	Unsuccessful
A. vitis	Electroporation	pUTGm-$P_{A1/04/03}$CDABE(lux)	Unsuccessful
		pUTKmGfp	Unsuccessful
		pBK-miniTn7-gfp1; pUX-BF13	Unsuccessful
		miniTn7(Gm)PrmB1-gfp-a; pUX-BF13	Unsuccessful
	Electroporation after RCA	pUTGm-$P_{A1/04/03}$CDABE(lux)	Unsuccessful
		pUTKmGfp	Unsuccessful
	Chemical transformation	pUTGm-$P_{A1/04/03}$CDABE(lux)	Unsuccessful
		pUTKmGfp	Unsuccessful
		pBK-miniTn7-gfp1; pUX-BF13	Unsuccessful
		miniTn7(Gm)PrmB1-gfp-a; pUX-BF13	Unsuccessful
	Bacterial mating	pUTGm-$P_{A1/04/03}$CDABE(lux); pRK600	Unsuccessful
		pUTKmGfp; pRK600	Successful*
		pBK-miniTn7-gfp1; pUX-BF13; pRK600	Unsuccessful
		miniTn7(Gm)PrmB1-gfp-a; pUX-BF13; pRK600	Successful*

* GFP expression visible under a microscope
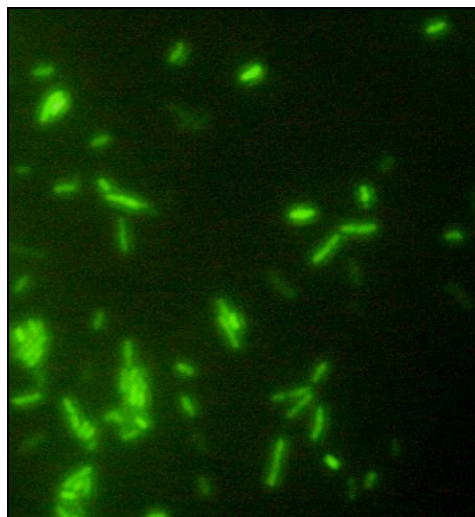
Figure 22: *Agrobacterium vitis* cells showing GFP fluorescence when exposed to a UV light and visualised under a microscope.

4.3 Screening of the *in vitro* activity of D4E1 against the pathogens of interest

To give an indication of the effect of the AMPs against the grapevine pathogens of interest, *A. vitis* and *X. ampelinus* were used to monitor the *in vitro* activity of D4E1. Vv-AMP1 peptide was not available in sufficient amounts, and was therefore excluded from these tests. A D4E1 dilution series was screened in triplicate against the two pathogens, the cfu were counted and the average cfu for each D4E1 concentration calculated. Aster yellows phytoplasma could not be included in this assay due to its inability to be cultured *in vitro*.

After a first round of *in vitro* peptide screening a reduction of cfu was observed for *A. vitis* and *X. ampelinus* when D4E1 concentrations from 10 to 20 μg/ml were used. To refine these results, D4E1 concentrations of 20, 18, 16, 14, 12 and 10 μg/ml were applied following the same procedure. For *A. vitis*, no cfu could be detected on any of the plates after treatment with either D4E1, at concentrations between 20-18 μg/ml, or with kanamycin (50 μg/ml). On the negative control plates, without D4E1 treatment, more than one thousand cfu were observed, while there was a reduction in average number of cfu for D4E1 concentration from 10 μg/ml to 16 μg/ml (Figure 23).

Similar to *A. vitis*, a reduction of *X. ampelinus* cfu after D4E1 treatment was found. An average number of below 30 cfu per plate at D4E1 concentrations between 14 and 20 μg/ml were found. At D4E1 concentrations of 12 and 10 μg/ml, respectively, the average number of cfu observed was more than a thousand colonies per plate (Figure 23). The untreated controls showed more than a thousand cfu while on positive controls no cfu could be observed. D4E1 had no effect on *X. ampelinus* at peptide concentrations of 12 μg/ml and lower.
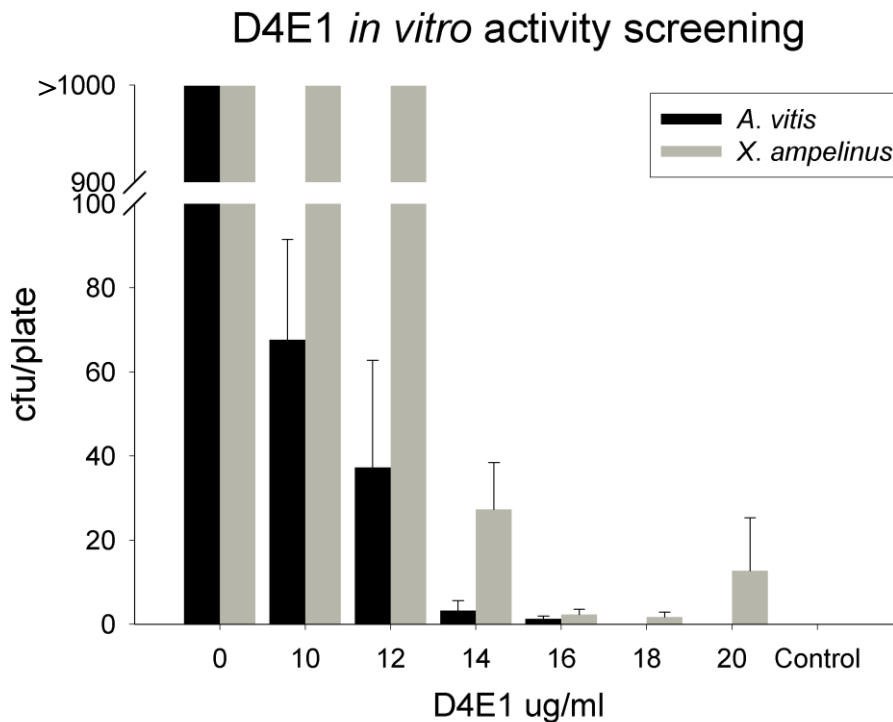
Figure 23: Bar graph showing the average number of cfu per 100 µl of *Agrobacterium vitis* or *Xylophilus ampelinus* liquid culture on solid media after treatment with D4E1, with concentrations from 10 µg/ml to 20 µg/ml. Kanamycin treated liquid cultures served as positive control and untreated cultures (0 µg/ml) as negative control. Error bars indicate the standard error of means.

The lowest concentration at which a complete inhibitory effect of D4E1 against *A. vitis* could be observed after one hour of exposure was 18 µg/ml (6.89 µM). A decrease in the average number, from 68 to 3 cfu, was observed for D4E1 treatment concentrations from 10 µg/ml to 16 µg/ml. This decrease in cfu was not statistically significant (p = 0.05414). Although a complete inhibition of *X. ampelinus* growth was not detected, the low number of cfu at D4E1 concentrations between 20 and 14 µg/ml is indicative of an inhibitory effect, by visual inspection, at these concentrations. The decrease in cfu, observed at D4E1 concentrations between 12 and 14 µg/ml, points toward an inhibitory effect of D4E1 against *X. ampelinus* from concentrations of 14 µg/ml (5.36 µM) and higher. This decrease in *X. ampelinus* cfu between 12 and 14 µg/ml was statistically significant (p = $5.237e^{-6}$). The same applied for the decrease in *A. vitis* cfu between 0 and 10 µg/ml (p = $5.659e^{-6}$).

4.4 Screening of the *in planta* activity of AMPs against the pathogens of interest

A 35S transient expression system was used to express the AMPs D4E1 and Vv-AMP1 in *in vitro* cultured *V. vinifera* plantlets to evaluate the efficiency of a transient expression system for *in planta* AMP activity screening. Bacterial titres in plants inoculated with either *A. vitis* or *X. ampelinus* were determined by qPCR to examine the inhibitory effect of the AMPs on these bacteria. The AMP expressing viral vectors were not used for these purposes as their foreign gene expression was found to be limited in *V. vinifera* to the leaf phloem tissue (see section 4.1.2). The *in planta* effect of the AMPs was also not screened against aster yellows phytoplasma in this study as no suitable phytoplasma infected *in vitro* plant material could be established.

4.4.1 Establishment of qPCR protocols for pathogen quantification

Real-time PCR protocols were optimised for the detection and quantification of *A. vitis*, *X. ampelinus* and aster yellows phytoplasma. The optimisation involved the identification or design of primers specific to the pathogens or the host plant, optimisation of qPCR conditions and the construction of standard curves to determine the efficiency of the qPCR reaction. Since no phytoplasma infected plants were available for the transient AMP expression procedure, only the reaction efficiencies of the qPCR protocols for *A. vitis*, *X. ampelinus* and *V. vinifera* quantification could be determined.

qPCR optimisation and efficiency determination for Vitis vinifera quantification

Two candidate reference genes for *V. vinifera* DNA quantification were used, actin and EF1-α. The actin-specific primers amplified a DNA fragment of 166 bp, while the EF1-α specific primers amplified a DNA fragment of 163 bp. These amplicons were slightly larger than the original amplicons obtained by Reid *et al.* (2006), who used mRNA as template instead of DNA as used in this study. Sequence analysis indicated the presence of an intron in the region amplified by the actin-specific primers. During the optimisation of these primers no non-specific amplification was found, and the actin primer set was chosen for use as reference gene in the subsequent qPCRs. The PCR product could clearly be distinguished from a product resulting from RNA contamination.

The efficiency of qPCR for quantification of grapevine DNA in a sample was determined by a standard curve established for the actin reference gene from a dilution series of total extracted DNA (Figure 24). The series ranged from 24 ng to 0.0384 ng of total DNA. When the Ct values

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(obtained from the amplification profiles) were plotted against the logarithm of their relative concentrations, the efficiency (E) of the curve was 1.04 ($E = 10^{1/-M}$-1). With a slope (M) of -3.2372 and a relative regression correlation coefficient (R^2) value of 0.9975, the reactions proved to be sufficient to be used in further quantifications as it was highly reproducible and the amount of DNA doubled with each subsequent cycle.
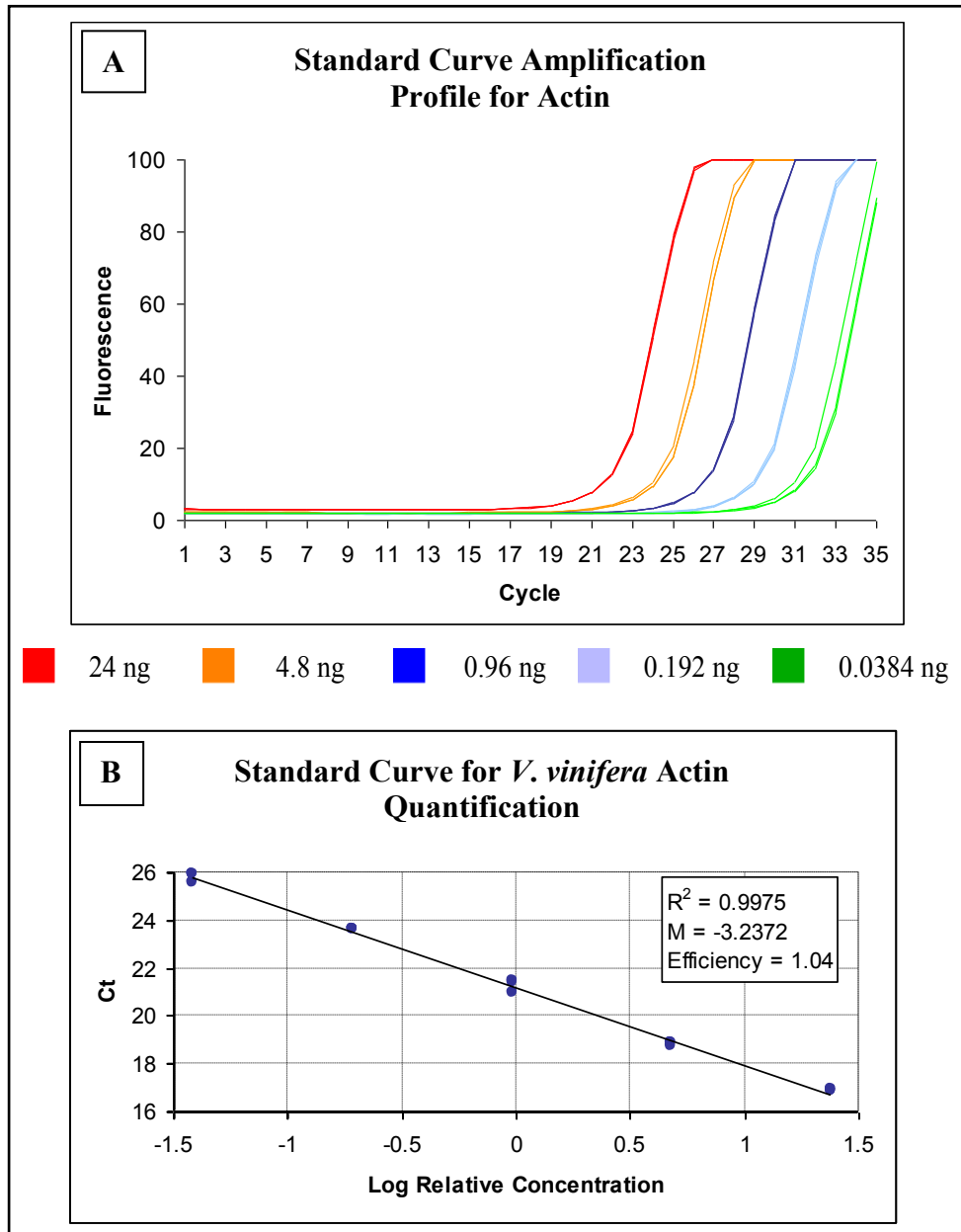
Figure 24: Standard curve for the relative quantification of the *Vitis vinifera* actin gene using the primers Vv_Actin_F and Vv_Actin_R. A) Amplification profile of the qPCR reactions of a dilution series of total DNA. B) The standard curve resulting from the threshold (Ct) values of each triplicate plotted against the logarithm of the relative concentration of the sample.

qPCR optimisation and efficiency determination for Xylophilus ampelinus quantification

Different primers were analysed for their efficiency in a *X. ampelinus* qPCR-based quantification procedure. The pairs Xamp_14F/Xamp_104R, Xamp1.3A/Xamp1.3B and Xamp2.0/Xamp2.0B, all amplifying a region of the 16S rRNA gene of *X. ampelinus*, were first tested in conventional PCR. All three primer sets showed non-specific amplification when used in a SYBR Green-based qPCR system. The amplification reactions could not be optimised by changes in annealing temperatures and reaction compositions and therefore these primer sets were excluded from further experiments.

The primer pair S3 and S4 was optimised for *X. ampelinus* quantification. These primers did not show any non-specific amplification after optimisation. They amplified a 277 bp DNA product in positive samples, and because of their specificity they were used in further reactions for *X. ampelinus* quantification by means of qPCR.

To determine the efficiency of the primers S3 and S4 in qPCR, a standard curve was established for *X. ampelinus* DNA amplification using a dilution series from 24 ng to 0.0384 ng of total extracted DNA, containing both grapevine and bacterial DNA. The Ct values were plotted on a graph against the logarithm of their relative concentrations (Figure 25). The PCR efficiency of the standard curve was 0.97, since the slope of the trendline was -3.387. The reaction efficiency of nearly 100% indicated a near doubling of DNA product with every cycle, as expected for a highly efficient PCR. The R^2 value for the curve was 0.9984.
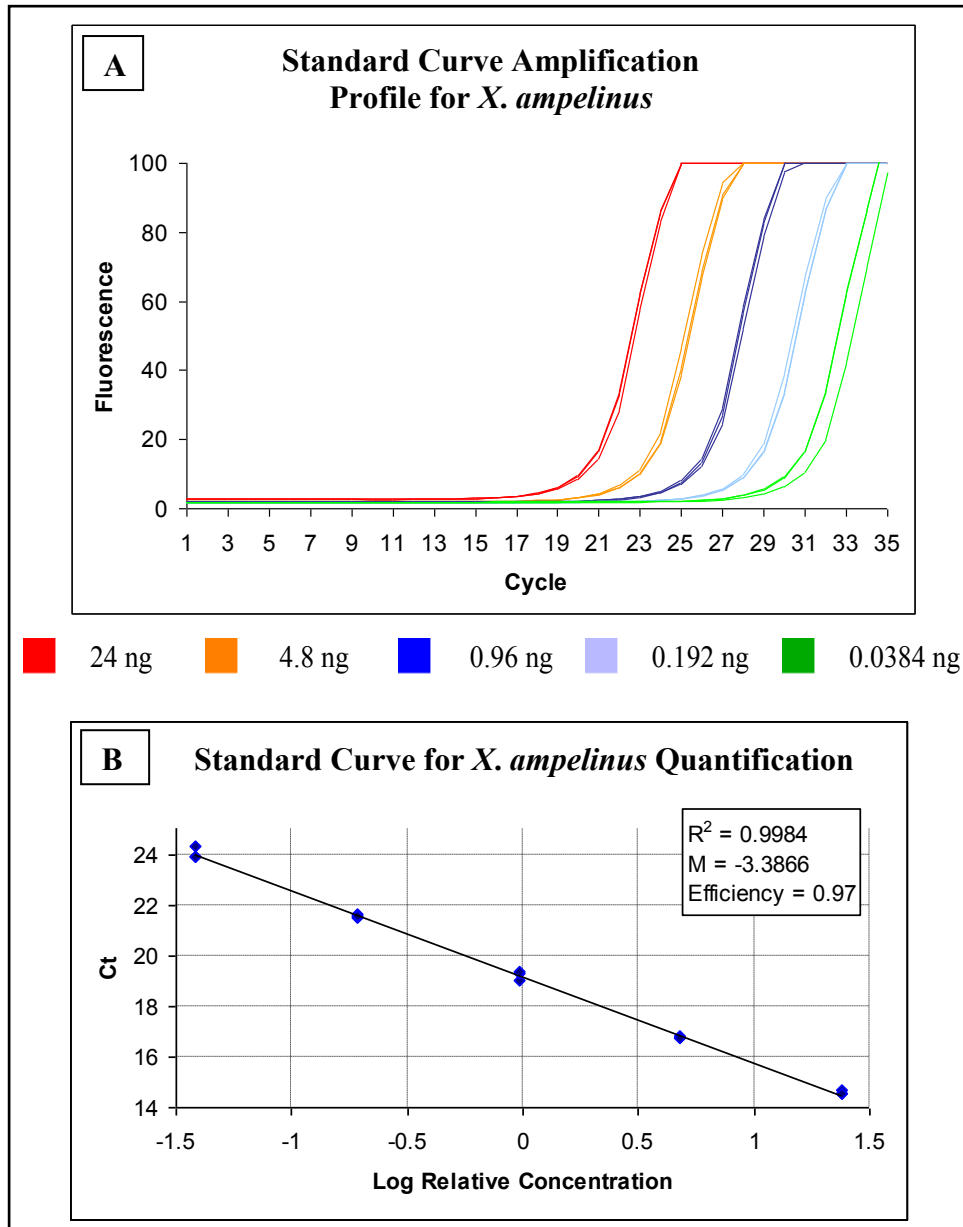
Figure 25: Standard curve for the real-time quantification of *Xylophilus ampelinus* titres in *Vitis vinifera* using the primers S3 and S4. A) Amplification profile of the qPCR reactions of a dilution series of total DNA. B) The standard curve resulting from the threshold (Ct) values of each triplicate plotted against the logarithm of the relative concentration of the sample.

qPCR optimisation and efficiency determination for Agrobacterium vitis quantification

For *A. vitis* DNA amplification, the primer pair VIRD62F23 and VIRD62R135 was optimised. These primers amplified a 113 bp product and showed no amplification product in any of the negative control samples. In order to determine the qPCR efficiency, a DNA dilution series from 60 ng to 0.096 ng of total extracted DNA was used to set up a standard curve for *A. vitis* amplification. The standard curve had a slope of -3.18 which describes an approximate doubling of DNA after every amplification cycle (Figure 26). The efficiency was 1.06 and the R^2 value for

the curve was 0.9956. Since these reactions were almost 100% reproducible, they were sufficient to use in *A. vitis* quantification.
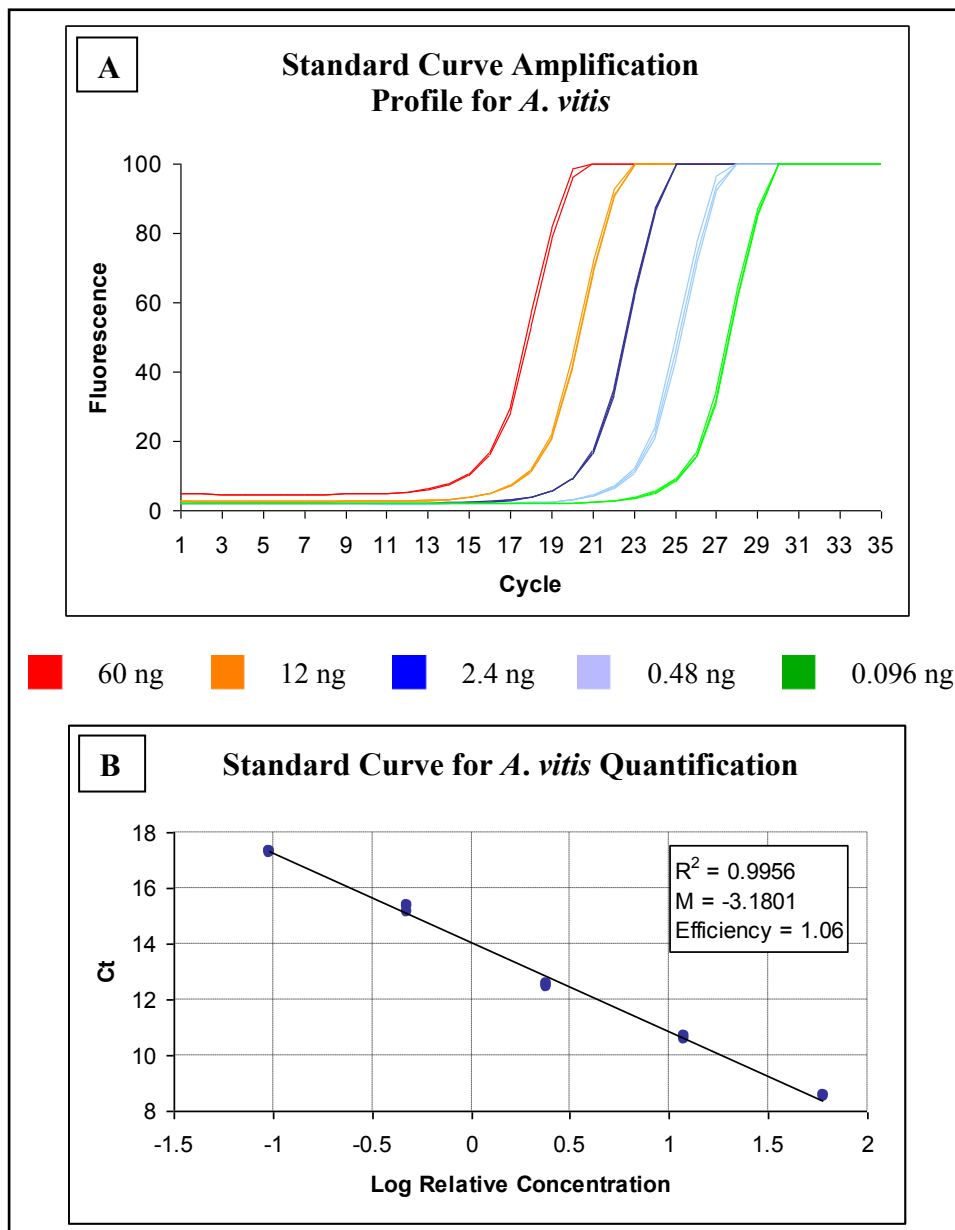
Figure 26: Standard curve for the real-time quantification of *Agrobacterium vitis* titres in *Vitis vinifera* using the primers VIRD62F23 and VIRD62R135. A) Amplification profile of the qPCR reactions of a dilution series of total DNA. B) The standard curve resulting from the threshold (Ct) values of each triplicate plotted against the logarithm of the relative concentration of the sample.

Aster yellows phytoplasma qPCR optimisation

The first set of primers used for aster yellows phytoplasma qPCR optimisation was AAY_F and AAY_R. These primers were designed to produce an amplicon of 102 bp by means of a TaqMan®-based reaction, which relies on the additional use of a probe. The primers were used

in a SYBR Green-based system in this study. In the absence of the probe, PCRs using these primers produced non-specific amplicons of about of the same size in negative samples, and resulted in the elimination of these primers from aster yellows phytoplasma quantification in this study.

The primers AY_F and AY_R that were subsequently designed for a SYBR Green-based quantification system, amplified a 172 bp region of the 16S rRNA gene sequence of AY phytoplasma. No non-specific amplification was observed and positive samples were confirmed by nested-PCR. To determine the detection threshold for these primers, in comparison to conventional nested-PCR, both methods were used to screen a dilution series of a phytoplasma positive sample. Results of this comparison are shown in Figure 27.
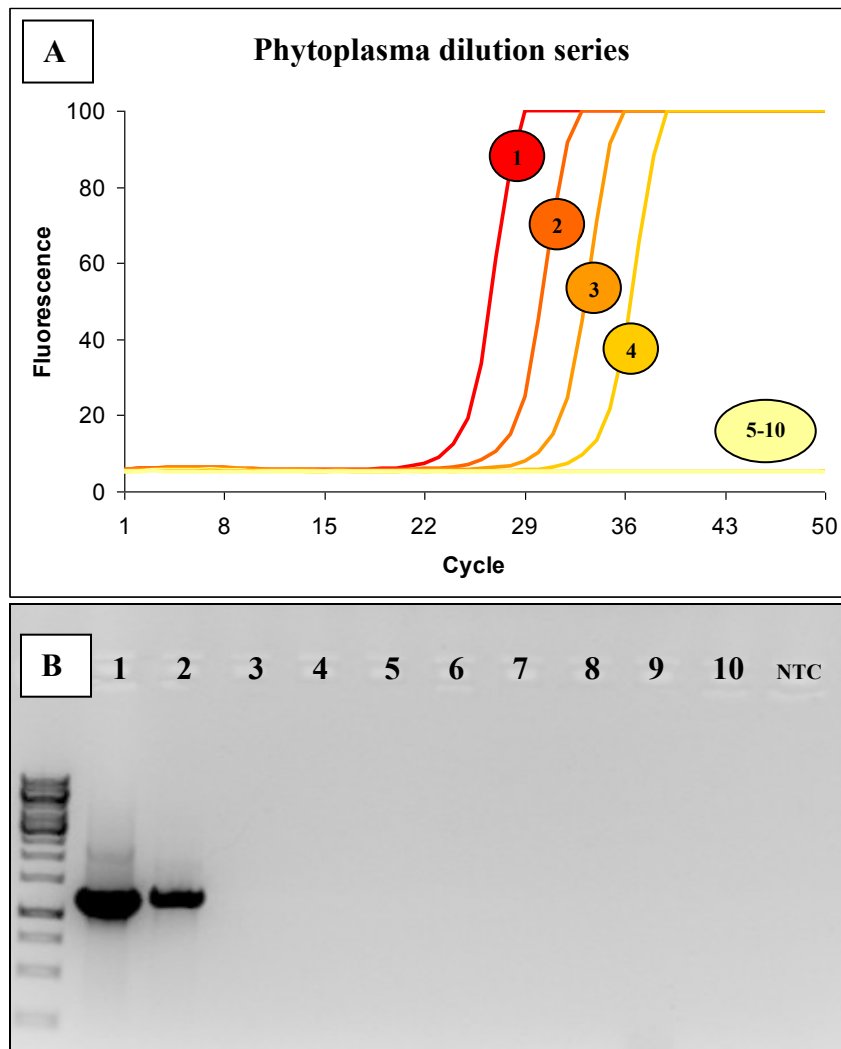
Figure 27: Comparison of the sensitivity of qPCR vs. standard nested-PCR to detect aster yellows phytoplasma in a dilution series. A) Real-time PCR amplification profiles and (B) agarose gel electrophoresis of nested-PCR products. Reaction 1) 10^{0} dilution; Reaction 2) 10^{-1} dilution; Reaction 3) 10^{-2} dilution; Reaction 4) 10^{-3} dilution; Reactions 5-10) 10^{-4}-10^{-9} dilutions and no template negative control.

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The qPCRs were able to detect the aster yellows phytoplasma up to a dilution of 10^{-3} (reaction 4). The nested-PCR reaction only detected the aster yellows phytoplasma in reactions 1 and 2, up to a dilution of 10^{-1}.

4.4.2 Screening of the *in planta* efficacy of D4E1 and Vv-AMP1 against *X. ampelinus* and *A. vitis*

The *in planta* effect of the AMPs D4E1 and Vv-AMP1 was evaluated by comparing the bacterial titres in one treatment group relative to that of another. Each treatment group consisted of 5 grapevine plants either infiltrated with an AMP 35S expression vector (pBin61S-D4E1 or pBin61S-VvAMP1) or by the empty binary vector, pBin61S (negative control). The bacterial titres were normalised to the amount of plant DNA in an individual sample and a relative group-wise comparison was performed by REST. The bacterial DNA served as the gene of interest (GOI) and the *V. vinifera* actin gene as the reference gene. Since the AMP Vv-AMP1 was not available in a soluble peptide form to be used in the *in vitro* plate tests, the transient expression assay allowed for the first time during this study a screening of the effect of Vv-AMP1 against any of the grapevine pathogens. The results for the qPCR as well as the REST analysis are presented below.

Screening of the efficacy of D4E1 against X. ampelinus

Real-time PCR analysis for relative bacterial quantification was performed in 2 separate runs to determine the effect of D4E1 on *X. ampelinus* in grapevine. Sample 3 of the D4E1-treated samples was included in both runs to verify the reproducibility of the reactions. Figure 28A shows the amplification profiles (fluorescence against the cycle numbers) of samples 1-3 of the D4E1 treatment group (pBin61S-D4E1-infiltrated) and samples 4 and 5 of the control group (empty pBin61S infiltrated), amplified using the *X. ampelinus*-specific primers. The amplification profiles of samples 3-5 of the D4E1 treatment group and 1-3 of the control group are shown in Figure 28B. The control group is indicated in red and the D4E1-treated group in blue.
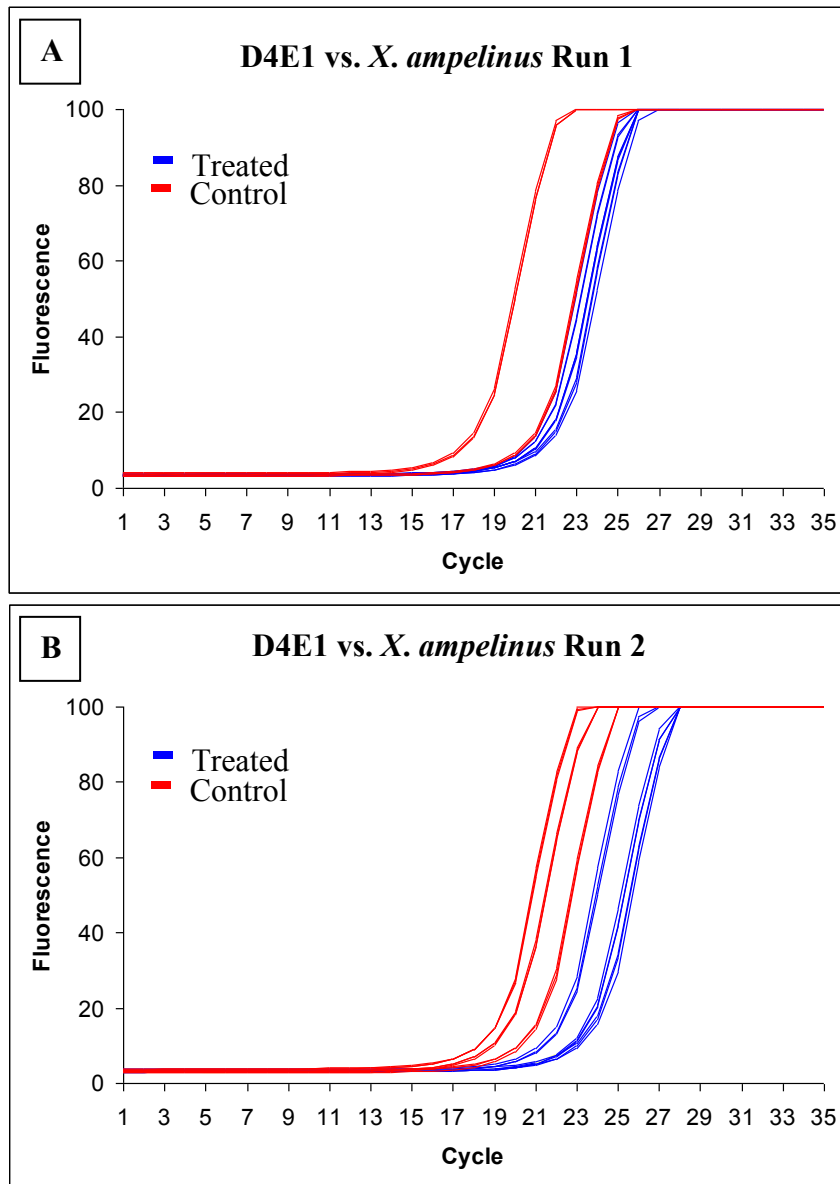
Figure 28: Amplification profiles, using the *X. ampelinus*-specific primers S3 and S4, on *V. vinifera* samples inoculated with *X. ampelinus*. Samples exposed to D4E1 after infiltration with pBin61S-D4E1 and control samples infiltrated with the empty pBin61S vector are shown. A) First run which included plants 1-3 of the D4E1 treatment group and plants 4 and 5 of the control group. B) Second run which include plants 3-5 of the D4E1 treatment group and plants 1-3 of the control group.

The amplification profiles for the *X. ampelinus* DNA (GOI) showed a clear distinction between the cycles at which the fluorescence of the control samples rose above the threshold (background fluorescence) and that of the treated samples. The data was normalised with the reference gene (actin) amplification to eliminate differences in total DNA in the reactions. Table 5 lists the Ct values of each of the samples for their GOI and their reference gene. These Ct values were then analysed by the REST programme, along with the efficiencies of their respective standard curves, to determine the *X. ampelinus* concentration in the D4E1 treated plants relative to those

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in the untreated plants. For sample 3 of the treatment group the Ct values obtained in the second run was chosen because the ratio of the GOI to the reference gene Ct value of the two runs were almost the same.

Table 5: Ct values obtained from qPCR profiles of *Vitis vinifera* plants infected with *Xylophilus ampelinus* that were treated with D4E1 (pBin61S-D4E1 infiltrated) and untreated (empty pBin61S infiltrated) control plants. The bacterial DNA is represented by the gene of interest and the DNA of the plant internal control by the reference gene.

Sample	GOI	Reference Gene
1	15.62	18.08
2	15.06	18.00
3*	15.86	18.79
4	17.02	18.37
5	17.34	18.81
Control 1	13.02	18.51
Control 2	13.29	19.31
Control 3	14.60	18.39
Control 4	14.90	18.37
Control 5	12.23	17.25

*Ct value from the second run used

When the Ct values were processed with the REST programme the average concentration of the GOI from *X. ampelinus* in the treatment group was found to be 82% lower than the average concentration of the control group. The reduction of detectable GOI indicated a 5.5 times reduction [p(H$_1$) = 0.004] in *X. ampelinus* concentrations as a result of D4E1 exposure. This indicates an effect of D4E1 expressed by the 35S vector against *X. ampelinus* in grapevine tissue. No symptoms developed on any of the *V. vinifera* plantlets inoculated with *X. ampelinus* even at 21 dpi.

Screening of the efficacy of Vv-AMP1 against X. ampelinus

The *X. ampelinus* qPCR amplification profiles of samples exposed to Vv-AMP1, after pBin61S-VvAMP1 infiltration (indicated in blue), and control samples, infiltrated with an empty pBin61S vector (indicated in red) are shown in Figure 29. The profiles were consistent for the duplicates of each sample and all the amplifications were performed in a single run.
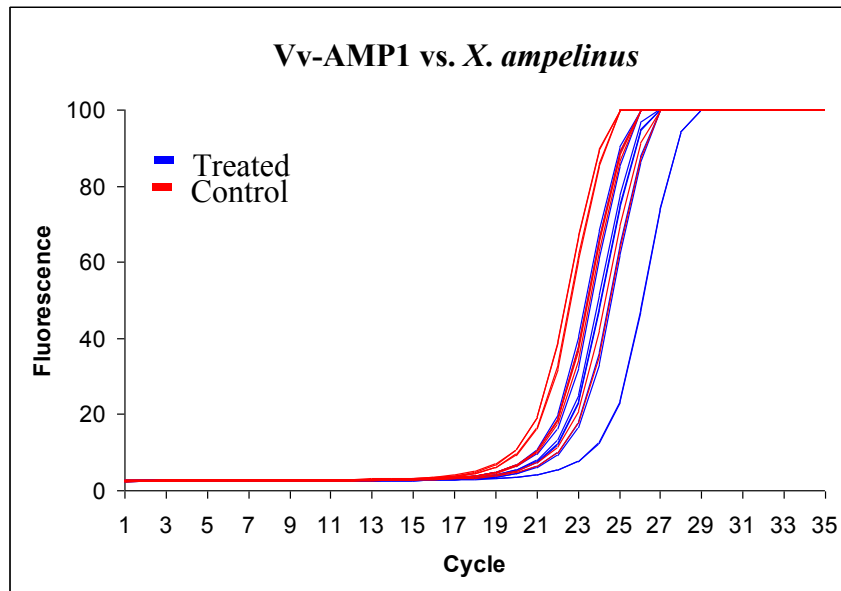
Figure 29: Amplification profiles, using the *X. ampelinus*-specific primers S3 and S4, on *V. vinifera* samples infected with *X. ampelinus*. Samples exposed to Vv-AMP1 after infiltration with pBin61S-VvAMP1 and control samples infiltrated with the empty pBin61S vector are shown.

The cycles in the *X. ampelinus* GOI amplification at which the control samples' fluorescence increased above the threshold could not be clearly distinguished from those of samples exposed to Vv-AMP1. The GOI Ct values were normalised with those of their respective reference genes when incorporated into the REST programme along with the efficiencies of the standard curves for *X. ampelinus* and actin. The Ct values obtained from the amplification run by means of the Rotor Gene software for each sample's GOI and reference gene are listed in Table 6. REST results showed that there was no significant difference between the average concentration of *X. ampelinus* in *V. vinifera* plants that were infiltrated with the Vv-AMP1 35S expression vector and that of the plants that were infiltrated with an empty 35S expression vector. Again, no symptom development was observed on any of the *V. vinifera* plantlets.

Table 6: Ct values obtained from qPCR profiles of *Vitis vinifera* plants infected with *Xylophilus ampelinus* that were treated with Vv-AMP1 (pBin61S-VvAMP1 infiltrated) and untreated (empty pBin61S infiltrated) control plants. The bacterial DNA is represented by the gene of interest and the DNA of the plant internal control by the reference gene.

Sample	GOI	Reference Gene
1	15.55	18.18
2	15.88	18.79
3	17.57	18.84
4	14.89	17.95
5	15.60	18.16
Control 1	15.74	18.09
Control 2	13.94	18.25
Control 3	14.11	17.91
Control 4	14.85	19.29
Control 5	14.90	18.00

Screening of the efficacy of D4E1 against A. vitis

Part of optimising the AMP screening procedure was testing different methods of plant inoculation with *A. vitis.* This was achieved by comparing inoculation with *A. vitis* suspensions of different concentrations and varying the period after inoculation and before DNA extraction. Vacuum-infiltration of grapevine tissue culture plants with an *A. vitis* suspension with an OD_{600} as low as 0.01 had such a damaging effect on the plant that they died 5 days after inoculation. Real-time PCR data showed no pattern of distinction between the D4E1 treated (pBin61S-D4E1 infiltrated) and untreated (empty pBin61S infiltrated) samples at 5 dpi using an inoculum concentration of $OD_{600} = 0.01$.

The effect of D4E1 treatment against *A. vitis* was screened *in planta* with two different *A. vitis* suspension concentrations and at two different time points. It was first screened in plants dipped in an *A. vitis* suspension with an OD_{600} of 0.1 and measuring the bacterial titres *in planta* after 4 days. Three of the five untreated control plants developed dark necrotic lesions at the sites where leaves were previously cut with a scalpel (Figure 30). No difference in bacterial concentrations was observed using this method.

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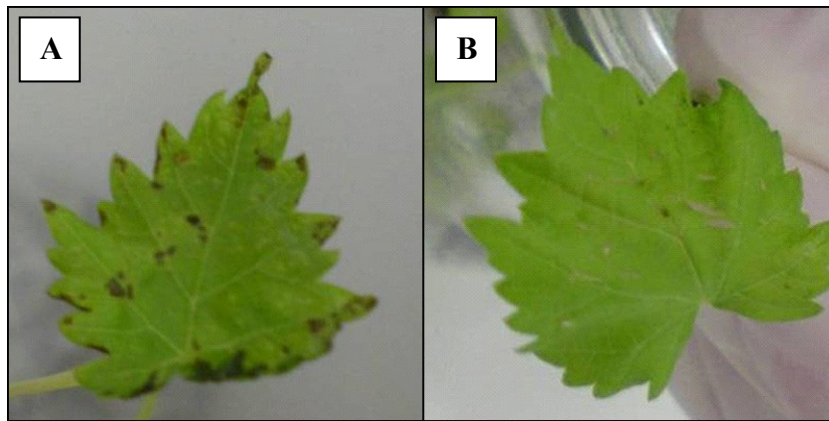
Figure 30: *In vitro* cultured *Vitis vinifera* cv. Sultana plants (4 dpi) inoculated with *Agrobacterium vitis* by dipping in an *A. vitis* suspension. A) Leaf showing dark necrotic lesions. B) Symptomless leaves.

Decreasing the concentration of the *A. vitis* inoculum, to an OD_{600} of 0.05, increased the period of survival of the plants (up to 7 days). A qPCR run of samples of different infection periods showed an increase in *A. vitis* concentrations from 7 to 10 days, after plants were infected with the bacteria. The titres did not reach a plateau before day 7 of incubation. From day 7 onwards the health of the plants started decreasing rapidly. Using the optimised conditions qPCRs were performed, for *A. vitis* relative quantification, similar to that of *X. ampelinus*. The *A. vitis* qPCR amplification profiles of samples exposed to D4E1 (indicated in blue) and control samples that were not exposed to D4E1 (indicated in red) are shown in Figure 31. The amplification profiles were consistent for the duplicates of each sample and all the amplifications were performed in the same run.
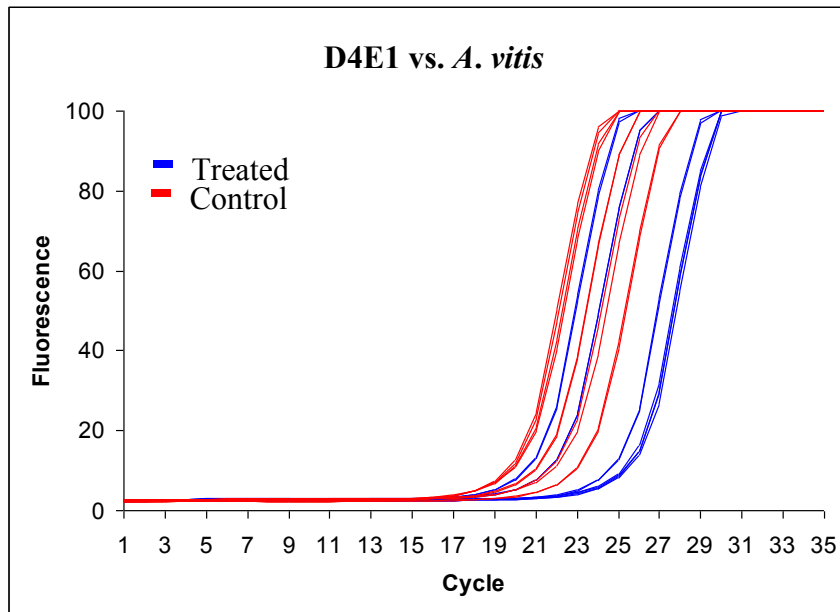
Figure 31: Amplification profiles using the *A. vitis* specific primers, VIRD62F23 and VIRD62R135, on *V. vinifera* samples infected with *A. vitis*. Samples exposed to D4E1 after infiltration with pBin61S-D4E1 and control samples infiltrated with an empty pBin61S vector are shown.

Agrobacterium vitis amplification profiles showed a clear distinction between the cycles at which the control samples' fluorescence increased above the threshold and those of 3 out of the 5 samples exposed to D4E1. The Ct values obtained from the amplification run by means of the Rotor Gene software, for each sample's GOI and reference gene, are listed in Table 7. The Ct values of all the samples were normalised with their respective reference gene Ct values by means of the REST programme. Between the D4E1 treatment group and control group, REST analysis indicated that there was no statistical difference in the average *A. vitis* concentrations, despite the fact that 3 of the 5 treated plants (samples 2, 4 and 5) showed definite lower *A. vitis* concentrations. REST indicated that the average bacterial concentration of these 3 plants were significantly lower than concentration of *A. vitis* in the control group.

Table 7: Ct values obtained from qPCR profiles of *Vitis vinifera* plants infected with *Agrobacterium vitis* that were treated with D4E1 (pBin61S-D4E1 infiltrated) and untreated (empty pBin61S infiltrated) control plants. The bacterial DNA is represented by the gene of interest and the DNA of the plant internal control by the reference gene.

Sample	GOI	Reference Gene
1	13.22	19.22
2	16.76	17.82
3	14.24	18.91
4	16.85	18.98
5	17.53	17.88
Control 1	12.22	18.33
Control 2	14.83	18.94
Control 3	12.84	18.02
Control 4	11.83	17.93
Control 5	13.94	19.51

Screening of the efficacy of Vv-AMP1 against A. vitis

The *in planta* activity of Vv-AMP1 was screened against *A. vitis* in the same way and at the same time D4E1 was screened against it, making the activity of the two peptides comparable using the data set. The same plants infiltrated with the empty pBin61S binary vector were used as negative controls, while plants infiltrated with pBin61S-VvAMP1 served as the experimental plants. The *A. vitis* qPCR amplification profiles of samples exposed to Vv-AMP1 (indicated in blue) and control samples that were not exposed to Vv-AMP1 (indicated in red) were consistent for the duplicates of each sample and all the amplifications could be perform in a single run. Figure 32 shows the *A. vitis* amplification profiles using the primers VIRD62F23 and VIRD62R135.
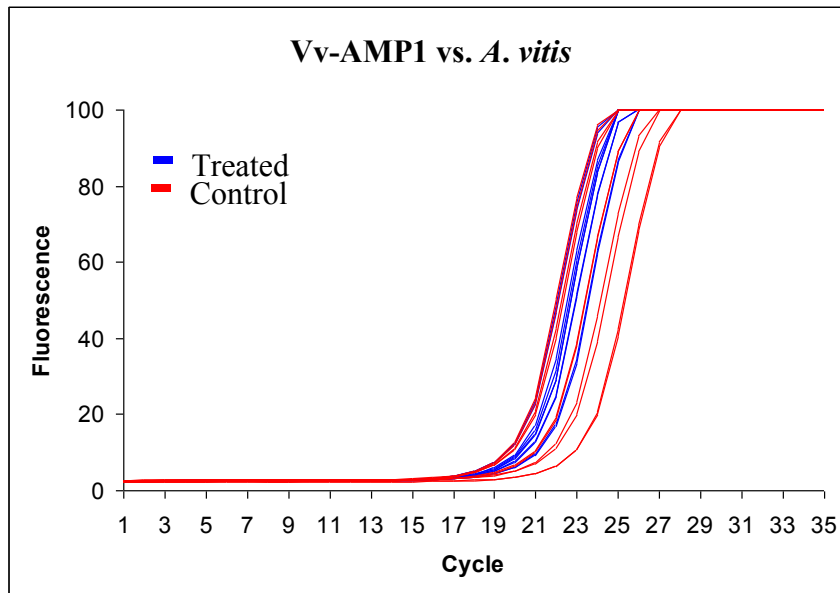
Figure 32: Amplification profiles using the *A. vitis* specific primers, VIRD62F23 and VIRD62R135, on *V. vinifera* samples infected with *A. vitis*. Samples exposed to Vv-AMP1 after infiltration with pBin61S-VvAMP1 and control samples infiltrated with an empty pBin61S vector are shown.

The amplification profiles showed no distinction between the Vv-AMP1 treatment group and control group regarding the bacterial titres. The Ct values obtained from both the *A. vitis* and the reference gene amplification profiles (listed in Table 8) were, along with the efficiencies of the two respective standard curves, incorporated into REST. REST analysis indicated that there was no difference between the concentration of *A. vitis* in the *V. vinifera* plants that were infiltrated with pBin61S-VvAMP1 and the *A. vitis* concentration in the untreated plants.

Table 8: Ct values obtained from qPCR profiles of *Vitis vinifera* plants infected with *Agrobacterium vitis* that were treated with Vv-AMP1 (infiltrated with pBin61S-VvAMP1) and untreated (empty pBin61S infiltrated) control plants. The bacterial DNA is represented by the gene of interest and the DNA of the plant internal control by the reference gene.

Sample	GOI	Reference Gene
1	13.19	18.18
2	13.13	19.14
3	12.18	19.08
4	12.13	18.13
5	12.12	18.31
Control 1	12.22	18.33
Control 2	14.83	18.94
Control 3	12.84	18.02
Control 4	11.83	17.93
Control 5	13.94	19.51

Comparison between the efficacy of D4E1 and Vv-AMP1 against A. vitis

On account of the simultaneously activity screening of D4E1 along with the Vv-AMP1 activity screening against *A. vitis in planta* the difference in effect of the two peptides on *A. vitis* titres could also be compared. The qPCR amplification runs, for the two different AMP treated groups, are shown in Figure 33. The Vv-AMP1 treatment group is represented by the red curves while the D4E1 treatment group is represented by the blue curves.
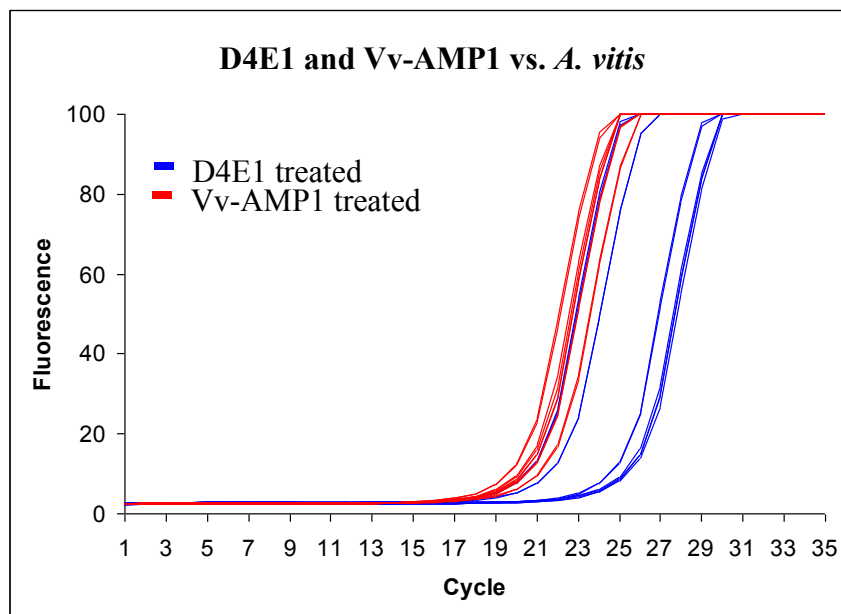
Figure 33: Amplification profiles using the *A. vitis* specific primers, VIRD62F23 and VIRD62R135, on *V. vinifera* samples infected with *A. vitis*. Samples exposed to D4E1, after pBin61S-D4E1 infiltration, and samples that were exposed to Vv-AMP1, after pBin61S-VvAMP1 infiltration, are shown.

A distinction could be made between the cycles in which the profiles of the D4E1 treated samples rose above the threshold and the cycle at which the Vv-AMP1 samples rose above the threshold for the GOI amplification run. These profiles could, however, only be compared after they were normalised by their respective reference gene amplification profiles to determine whether the actual bacterial titres in the plants differed and not only the amount of the bacterial DNA that was inserted into the qPCR reactions. The Ct values obtained for the different groups from their GOI and reference gene amplification profiles by the Rotor Gene software are listed in Table 9. These Ct values were analysed in the REST programme, incorporating the *A. vitis* and actin standard curve reaction efficiencies.

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Table 9: Ct values obtained from qPCR profiles of *Vitis vinifera* plants infected with *Agrobacterium vitis* that were treated with D4E1 (infiltrated with pBin61S-D4E1) and that has been treated with Vv-AMP1 (infiltrated with pBin61S-VvAMP1). The bacterial DNA is represented by the gene of interest and the DNA of the plant internal control by the reference gene.

Sample	GOI	Reference Gene
D4E1 1	13.22	19.22
D4E1 2	16.76	17.82
D4E1 3	14.24	18.91
D4E1 4	16.85	18.98
D4E1 5	17.53	17.88
Vv-AMP1 1	13.19	18.18
Vv-AMP1 2	13.13	19.14
Vv-AMP1 3	12.18	19.08
Vv-AMP1 4	12.13	18.13
Vv-AMP1 5	12.12	18.31

REST analysis indicated that the average concentration of the *A. vitis* bacteria in the plants treated with D4E1 was found to be 9 times less [$p(H_1) = 0.01$] than the average concentration of the *A. vitis* bacteria in the plants treated with Vv-AMP1. This showed the definite reduction in *A. vitis* concentrations as a result of D4E1 with comparison to the titres in plants exposed to Vv-AMP1, further indicating that D4E1 but not VvAMP1 exhibits a reducing effect on *A. vitis* titres in grapevine.

4.4.3 Screening of the *in planta* efficacy of D4E1 and Vv-AMP1 against aster yellows phytoplasma

As phytoplasma can't be cultured *in vitro*, the only means of evaluating the activity of AMPs against phytoplasma would be to perform the testing in the host plant. Phytoplasma infected *in vitro* grapevine plantlets were intended for use in D4E1 and Vv-AMP1 activity screening. The transient expression system was also tested on another aster yellows phytoplasma host, periwinkle (*C. roseus*).

Establishing plant cultures infected with phytoplasma from field material and in vitro grafting

In vitro cultured *V. vinifera* plantlets are easily agro-infiltrated and has proven to be suitable for foreign gene expression by means of a transient expression vector system. For *in planta* AMP activity screening against aster yellows phytoplasma in grapevine it was necessary to establish such phytoplasma infected plantlets from infected field material. Sterilisation procedures were

therefore performed on cane material collected from phytoplasma infected vines. The sterilised canes (70) started to bud after two weeks of incubation. Shoot development was slow (Figure 34) and less than half of all incubated canes developed shoots. More than half of these canes developed fungal contamination, possible as a result of endophytic fungal infection of the grapevine material. When 15 of the uncontaminated shoots were transferred to low sugar MS media, fungal contamination developed after 3 days, subsequently leading to death of the shoot.
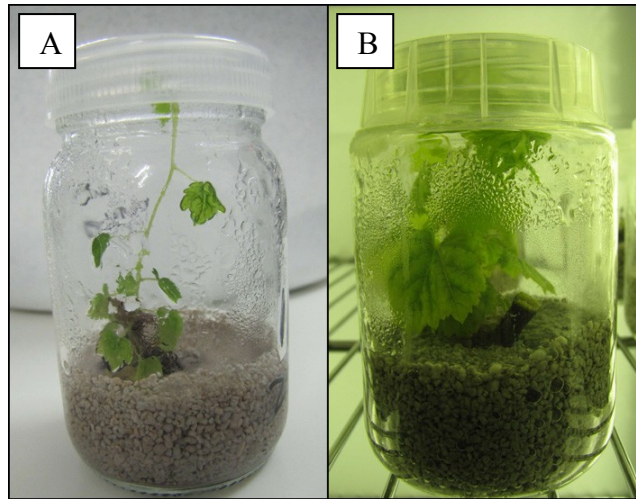
Figure 34: Surface sterilised *Vitis vinifera* canes that has budded and are growing in perlite containing sterile water. A) Phytoplasma infected cane. B) Non-infected cane.

Cuttings from the shoots on the above mentioned sterilised canes were micro-grafted onto sterile *in vitro* cultured *V. vinifera* plants. Fungal growth developed on these plants at the grafting site (Figure 35). Shortly after grafting, the plants died. Too little sterile material was available for further grafting experiments before the end of this study.

Figure 35: A phytoplasma infected shoot, grafted onto a sterile *in vitro* cultured cane. Fungal contamination developed around the site of grafting.

Transient expression in Catharanthus roseus

In an effort to develop an alternative AMP screening method against aster yellows phytoplasma, that did not require *in vitro* cultured plants infected with the pathogen, *C. roseus* was evaluated for its use to facilitate AMP expression. For that, *C. roseus* leaves were infiltrated with the 35S:GUSi construct and evaluated for GUS expression at two time periods. The results for the first GUS expression assay performed on *C. roseus* at 3 (A-C) and 6 dpi (D-F) are shown in Figure 36. Uninfiltrated *C. roseus* leaves showing no GUS expression served as negative control. The 35S:GUSi infiltrated *N. benthamiana* leaves served as positive control and showed either an limited level of GUS expression (3 dpi), seen only in about 20% of the infiltrated area, or an elevated level of expression (6 dpi), covering the whole infiltrated area. GUS expression was only observed in *C. roseus* leaves at 6 dpi, and only at wounding sites made by a scalpel to allow infiltration in the vacuum-infiltration procedure.
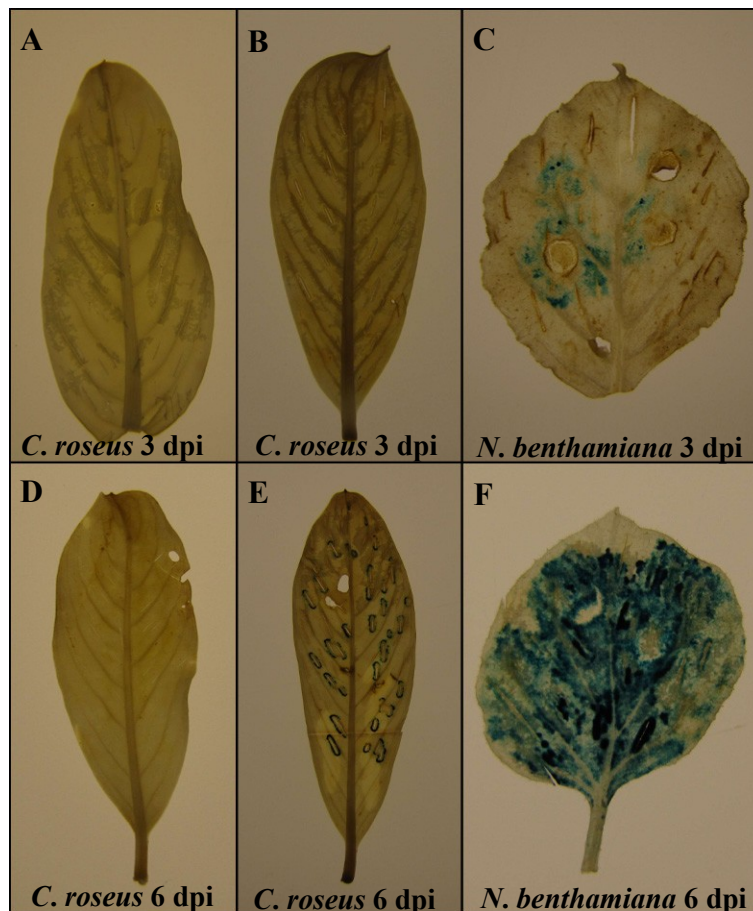
Figure 36: Results of the first GUS assay on *C. roseus* and *N. benthamiana*, 3 (A-C) and 6 dpi (D-F), to determine foreign gene expression in *C. roseus*. A) and D) *C. roseus* negative control, uninfiltrated leaves showing no GUS expression. B) and E) *C. roseus* leaves infiltrated with 35S:GUSi showing GUS expression (6 dpi). C) and F) *N. benthamiana* leaves infiltrated with 35S:GUSi, showing GUS expression.

The limited level of GUS expression, observed during the first GUS assay, was insufficient for AMP screening in *C. roseus* and further tests had to be performed to determine if another *A. tumefaciens* strain besides C58C1 (pCH32) will be more efficient in *C. roseus*. For this reason a second GUS expression assay was perform with *A. tumefaciens* strain EHA105. The results of the second GUS assay are illustrated in Figure 37. No GUS expression was observed in the negative uninfiltrated leaves. *Catharanthus roseus* leaves infiltrated with either C58C1 (pCH32 + 35S:GUSi) or EHA105 (35S:GUSi) showed only a limited level of GUS expression, seen only as small spots of expression mainly confined to areas cut by a scalpel to improve whole leaf inoculation.
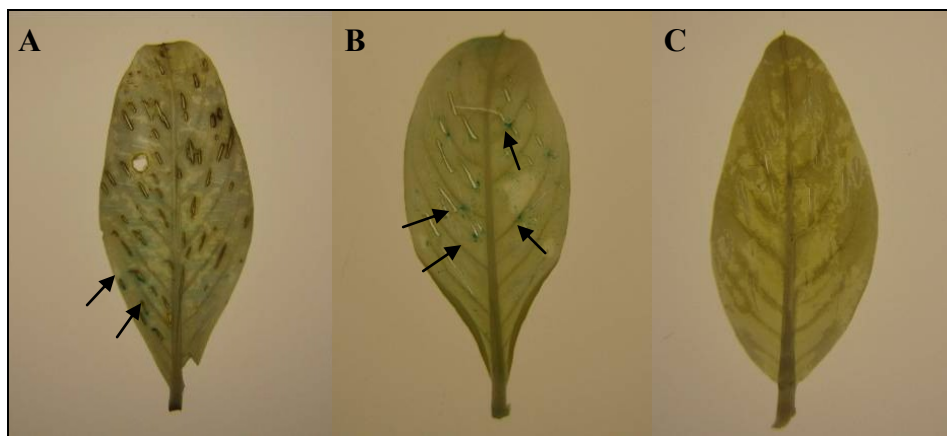
Figure 37: Results of the second GUS assay, to determine foreign gene expression in *C. roseus*. A) Leaves infiltrated with the 35S:GUSi containing *A. tumefaciens* strain C58C1 (pCH32). B) Leaves infiltrated with the 35S:GUSi containing *A. tumefaciens* strain EHA105. C) Negative control leaf, infiltrated with neither of the *A. tumefaciens* strains.

The *C. roseus* leaves used in the first two assays were not young leaves. In order to determine whether the age of the leaves has a limiting influence, a third GUS assay was performed on leaves from 6 weeks old young plants (Figure 38). The area of detectable GUS expression in these younger leaves, which were not cut by a scalpel to facilitate easy infiltration, was as high as an estimated 20-30% of the whole leaf area (Figure 38A). Leaves that were cut before agro-infiltration showed dark areas of GUS expression located around the wounding sites and also in unwounded tissue (Figure 38B). The leaf area showing GUS-expression on the plants was estimated 30-40% of the whole leaf area. The negative control showed no GUS expression (Figure 38C).
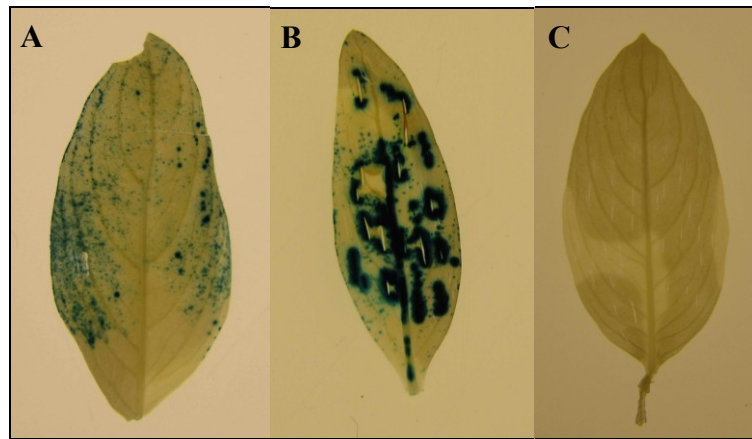
Figure 38: Results of the third GUS assay, to determine foreign gene expression in *C. roseus*. A) Leaves infiltrated with the 35S:GUSi containing *A. tumefaciens* strain C58C1 (pCH32) (uncut). B) Leaves infiltrated with the 35S:GUSi containing *A. tumefaciens* strain C58C1 (pCH32) (cut before agro-infiltration). C) Negative control leaf, not infiltrated with 35S:GUSi expressing *A. tumefaciens* strains.

Chapter 5

Discussion

5.1 Construction of transient expression vectors

The use of transient expression systems are a fast and effective way of expressing foreign genes in plants. These systems, both viral and non-viral, have been implemented in the past for, amongst others, RNA silencing in plants (Liu *et al*., 2002; Hellens *et al*., 2005; Naylor *et al*., 2005; Bhaskar *et al*., 2009), the expression of proteins for disease resistance studies (Saitoh *et al*., 2001; Donini *et al*., 2005; Guan *et al*., 2010), gene functional analysis (Sawers *et al*., 2006) and vaccine production (Awram *et al*., 2002; Gleba *et al*., 2005). In this study transient expression systems were used for determining the *in planta* efficacy of AMPs against grapevine pathogens. To validate the systems, the effects of two AMPs, Vv-AMP1 and D4E1, were tested against *A. vitis* and *X. ampelinus*. For this purpose, four GVA118-based viral vectors, expressing GUS, GFP, Vv-AMP1 and D4E1 respectively, as well as two 35S expression vectors, expressing the peptides Vv-AMP1 and D4E1, were constructed.

5.1.1 Testing the infectivity of viral constructs

The infectivity of the viral vectors was confirmed by TPIAs of *N. benthamiana* leaf material infiltrated with the vectors. Symptom development in the leaves acropetal of the area of infiltration along with their leaf petiole-prints confirmed that the viruses were replicating and moving systematic. The systemic movement of the recombinant viruses is essential for studying the *in planta* effect of virus-expressed AMPs against grapevine phytoplasma. In grapevine, both GVA and phytoplasma are phloem-limited (Rosciglione *et al*., 1983; Minafra *et al*., 1997; Doi *et al*., 1967; Lee and Davis, 1992), and will therefore be in close proximity to each other leading to pathogen exposure to the viral expressed AMP. Phytoplasmas are mostly confined to the phloem sieve elements of their plant hosts (Christensen *et al*., 2004; Hogenhout *et al*., 2008), but have been found on rare occasions in the phloem parenchyma cells that are associated with the phloem sieve cells and vascular system (Sears and Klomparens, 1989; Sillers *et al*., 1987). GVA expresses its proteins in cells associated with the phloem (Minafra and Hadidi, 1994). The

bacterial pathogens *A. vitis* and *X. ampelinus* are however confined to the xylem cells of plants (Burr *et al*., 1998; Grall and Manceau, 2003) and are therefore spatially separated from AMPs expressed via a GVA-based vector.

The fact that the viruses were replicating and spreading systemically through the plants did not imply that the foreign gene was still integrated in the virus genome. Some viral vectors become unstable and loose the inserted foreign gene (Gleba *et al*., 2007). The instability may result from insert size or the position of insertion (Dawson, 1989). The stable integration of the AMP genes into the recombinant virus genomes, in this study, was confirmed by RT-PCR. Although the RT-PCR for the GVA-GUS recombinant virus failed, possibly due to the use of an inadequate primer, systemic GUS expression (see section 5.1.2) confirmed the stable integration of the GUS gene. The RT-PCR results along with the TPIA results illustrated that the inserted peptide gene sequences were stably integrated and did not disrupt the viral replication system at the integration sites used in this study. Infectious recombinant viruses therefore originated form the viral vectors. These results also correlated with previous studies that showed that the stable integration of the GUS gene into infectious GVA clones still allowed viral replication (Galiakparov *et al*., 2003; Haviv *et al*., 2006).

5.1.2 Protein expression from control transient expression vectors

Nicotiana benthamiana and *V. vinifera* plants were infiltrated with the viral vectors pBinSN-GVA118-GUS and pBinSN-GVA118-GFP to confirm that expression of foreign genes by means of the GVA118 viral vector was possible. Successful expression was found for both control proteins in *N. benthamiana*. The expression levels and the sites of expression in *V. vinifera*, however, were limited. No GFP expression was observed and the GUS expression was only restricted to the veins of the infiltrated *V. vinifera* leaves. Although GVA naturally occurs in the grapevine phloem tissue, it was expected that all of the infiltrated tissue including the mesophyll cells would show GUS expression resulting from the recombinant virus, as was the case for the *N. benthamiana* leaves in this study as well as in previous studies (Galiakparov *et al*., 2003; Haviv *et al*., 2006). In a study on the phloem-limited beet mild yellowing virus infectious full-length cDNA clone ($BMYV_{fl}$), different plants that supported the local $BMYV_{fl}$ agro-infection, indicated the presence of the viral coat protein in the complete infiltrated mesophyll tissue of leaves (Stephan and Maiss, 2006). Various plants infiltrated with a viral expression vector, based on the phloem-limited tomato yellow leaf curl virus, also showed the expression of foreign genes in the mesophyll cells (Peretz *et al*., 2007). Taliansky *et al*. (2003) discussed the cell-to-cell movement properties of another phloem-limited virus, namely potato leafroll virus (PLRV). These viruses spread through the sieve elements and are able to occupy both the phloem

parenchyma and companion cells. They stated that the movement protein for this virus could only mediate viral movement between phloem cells. Although PLRV can replicate in the mesophyll cells of the plants (Taliansky *et al*., 2003), the systemic movement of movement protein mutated PLRV could not be accommodated in tomato plants but rather in the model plant *N. benthamiana* (Lee *et al*., 2002). This indicated the host-dependent method of viral movement between cells. It was suggested that these viruses not only lose their movement function in mesophyll cells, but also their ability to overcome RNA silencing. The host-dependent nature of viral vectors may therefore be responsible for the phloem-limited GUS expression observed in grapevine relative to that in *N. benthamiana*. The limited GUS expression in *V. vinifera* indicates that GVA does not replicate to detectable amounts outside the phloem tissue and therefore might be only of limited value for expression studies in grapevine when whole plant infiltrations are performed.

Only single cells showed GFP fluorescence under a microscope in *N. benthamiana* leaves infiltrated with pBinSN-GVA118-GFP. These results correlated with the comparison between GUS and GFP expression in grapevine from 35S vectors in a previous study (Santos-Rosa *et al*., 2008). The observation that GFP expression seems to be more limited than GUS expression may be deceptive. Santos-Rosa *et al*. (2008) stated that GUS assays are very sensitive, but could however give a misleading indication of the expression, as the substrate can diffuse within the tissue giving the impression of a wider distribution of GUS expression. The nature of GFP to be expressed only in single cells is therefore a better indication of the true expression of the foreign gene. They also stated that expression of the same low level as the GFP expression in their study by another transient expression system has shown to be sufficient for viral resistance studies. To overcome possible silencing effects of the foreign gene it can also be useful to co-infiltrate the plants with a virus silencing suppressor (Vionnet *et al*., 2003; Chiba *et al*., 2006). No silencing suppressors were used in this study as the testing of suppressors for grapevine is still on its way. Additionally, green plant material contains large amount of molecules that prevent UV light from reaching and exiting GFP molecules, while photosynthetic pigments have been shown to emit high levels of autofluorescence (Lang *et al*., 1991). These factors may reduce the amount of emitted GFP observed, and could explain the limited amount of GFP expression observed in this study. A recent study by Zhang *et al*. (2010) describes a method of enhancing the detection of GFP fluorescence in plants by eliminating chlorophyll and xanthophyll from transgenic tobacco leaves by means of virus-induced gene silencing of phytoene desaturase expression. This method circumvents the use of confocal laser scanning microscopy by making GFP visualisation possible by means of a stereomicroscope with GFP-specific filters. This method can in the future

also be applied to optimise the detection of GFP expression in plants through transient expression systems.

The GUS expression in the veins of *N. benthamiana* leaves acropetal to the leaves infiltrated with pBinSN-GVA118-GUS, confirmed the systemic spread of the recombinant GVA118-GUS virus containing a stably integrated and functional GUSi gene in its genome. The viral association with the veins of uninfiltrated leaves was expected since the recombinant virus would spread through the vascular system of a plant. This was shown for another phloem-limited viral infectious clone, BMYV$_{fl}$, which also showed the systemic movement of the virus to be associated with the vascular tissue of the plants (Stephan and Maiss, 2006).

In *N. benthamiana* and *V. vinifera* plants infiltrated with the 35S:GUSi vector, GUS expression occurred across the complete infiltrated area of the leaves including the mesophyll cells. This was expected as it was previously shown using the same vector (Santos-Rosa *et al*., 2008). It was also shown that the degree of GUS expression differed with different *V. vinifera* cultivars and that it is dependant on the age of the infiltrated leaves. These factors are important to keep in consideration when performing AMP activity assays in grapevine as they may influence the outcome. The results obtained from the GUS expression assays via the 35S:GUSi vector confirmed that a 35S transient expression vector can be used to obtain sufficient amounts of foreign gene expression throughout the entire infiltrated leaf of *V. vinifera* plantlets. These vectors were consequently used for AMPs expression in order to determine their *in planta* activity against *A. vitis* and *X. ampelinus*.

5.1.3 Testing of viral AMP expression

In order to confirm the expression of Vv-AMP1 by the recombinant virus GVA118-VvAMP1, western blots were performed on crude protein extracts from *N. benthamiana* leaves. Expression was not tested in *V. vinifera* since the results from the viral vector-based expression showed insufficient GUS expression, excluding the viral vectors for further applications during this study. The antibody used was raised in mice against a GST-Vv-AMP1 fusion protein, and was shown to be specific to Vv-AMP1 by western blot analysis, using 2 μg of purified peptide (De Beer, 2008). The peptide was produced in and extracted from *E. coli*. Western blot analysis using the described antiserum failed to detect viral expressed Vv-AMP1 in the present study. A factor that complicated western blot analysis was the non-specific binding of the antibody against a *N. benthamiana* protein background (De Beer, 2008). For this reason, De Beer (2008) could not tie western blot results to the expression of Vv-AMP1 by Vv-AMP1 transgenic *N. benthamiana,* even after enriching for cationic peptides from crude leaf extracts. Since no enrichment was

performed for peptides in this study, future studies should focus on more effective peptide enrichment and purification and maybe consider another model plant for determining peptide expression. Another problem discussed by De Beer (2008) is the possibility that the abundance of Vv-AMP1 may have increased the peptide's degradation in *N. benthamiana*. These plants express a peptide highly homologous to Vv-AMP1, resulting in the down regulation of both these peptides due to their combined over-expression. The concentration of Vv-AMP1 in the plants may therefore also have been too low for western blot detection. Due to these problems with the Vv-AMP1 western blots no attempts were made on *N. benthamiana* leaves infiltrated with pBin61S-VvAMP1.

The small size of the peptide (~5.5 kDa) may also be problematic. Over the years studies have developed improved methods of addressing these problems. Peptides less than 10 kDa in size often bind to SDS as they are of a related size and charge (Lu *et al*., 2007). Schagger and Von Jagow (1987) adapted the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method and developed a protocol using Tricine-SDS-PAGE to separate peptides between 1 and 100 kDa. Later, a glycine-SDS-PAGE (Sarfo *et al*., 2003) system was also developed, which could separate peptides between 1 and 3 kDa. Even more recently, Lu *et al*. (2007) developed a bis(2-ethylhexyl) sulfosuccinate-PAGE system that is able to separated peptides between 0.8 and 17 kDa. In this system the smaller peptides migrate through the gel slower than the larger peptides. This method did not only improve the resolution of previous systems but is also simpler and more cost effective to implement and could be included in future studies to detect *in planta* AMP expression. These systems can therefore be used to optimise the confirmation of Vv-AMP1 expression in the future.

The amphipathic nature of D4E1 leads to the accumulation of the peptide and the inability to migrate through a SDS-PAGE gel. Furthermore, polyclonal antibodies raised against D4E1 were shown to generate a high intensity background (Rajasekaran *et al*., 2005). These reasons resulted in the exclusion of D4E1 from western blot analysis. Despite the lack of a sufficient assay to confirm the expression of the AMPs, the visual detection of expressed control genes confirmed the expression of foreign genes from the vector systems also used for AMP expression.

5.2 Tagging of pathogens of interest

5.2.1 Verification of *A. vitis* and *X. ampelinus* cultures

Sequencing analysis that was performed confirmed the identity of the two bacterial cultures and found them to be *A. vitis* and *X. ampelinus*. The primers for *A. vitis* detection were used by Haas

et al. (1995) to detect different pathogenic Agrobacterium strains. These primers were designed based on the virD2 gene sequences of *A. tumefaciens* and *A. rhizogenes*. The sequence aligned best to the *A. vitis* sequence available on GenBank and the possibility of the bacteria being either *A. tumefaciens* or *A. rhizogenes* could be excluded. Little sequence data was available on Genbank for *X. ampelinus*. The sequence of the potential *X. ampelinus* strain did however align to that of a *X. ampelinus* sequence available, and the bacterial identity was therefore confirmed. These two bacterial cultures could then be used for further analysis.

5.2.2 Determination of *A. vitis* strain A39 tumorigenic nature

Tumour development in a mechanically inoculated *V. vinifera* plantlet confirmed the pathogenic nature of the strain. Different strains of *A. vitis* have shown varying susceptibility to AMPs (Rosenfield *et al.*, 2010). Antimicrobial peptide activity screening is therefore important to be performed on the same or closely related strain that will be targeted in the field. Since bacteria endangering the plant would be the primary target, tests were performed to determine the tumorigenic nature of the *A. vitis* strain A39. Virulent *A. vitis* strains differ from avirulent strains in that they hold a Ti-plasmid (Van Larebecke *et al.*, 1974). The T-DNA region of the *A. vitis* Ti-plasmid is inserted into the host genome, causing the host cells to grow undifferentiated, due to the over-expression of phytohormones, and develop tumours (Burr *et al.*, 1998). The tumour development induced by *A. vitis* strain A39 in this study, is indicative of its pathogenicity in grapevine and as a result was used for *in planta* AMP activity screening.

5.2.3 Bacterial tagging with reporter genes

Agrobacterium vitis cells were successfully tagged with GFP during this study. The fluorescent emission from these cells could, however, only be detected under a microscope. This expression was insufficient for *in planta* determination of bacterial titres by the Ivis system, available for fluorescence quantification. The decreased fluorescence of GFP tagged *A. vitis* cells was also observed by other research groups (Pers. Com. Prof. T. Burr, Cornell University, Department of Plant Pathology and Plant-microbe Biology). It is possible that the cell structure of *A. vitis* may by obstructive for GFP or luciferase detection. In this study, tagging of *X. ampelinus* with either GFP or luciferase was not successful. A previous study was, however successful in tagging *X. ampelinus* with GFP and made use of a Tn5 transposon system, introduced by means of electroporation (Grall and Manceau, 2003). The fluorescence emitted by these cells could be implemented to characterise the bacteria in an *in planta* environment. Transposon systems have also been used in the past to tag various other plant pathogenic bacteria with a reporter gene for monitoring their *in planta* activity, using both electroporation (Tombolini *et al.*, 1999; Gau *et al.*,

2002; Tanaka *et al.*, 2006) or tri-parental mating (Kobayashi *et al.*, 2009) to introduce the intron-containing plasmid to the recipient cell. Tagging of *X. ampelinus* by means bacterial mating is complicated by their slow growth on artificial media. Other bacteria will out compete their growth, especially since no antibiotic selection is available for *X. ampelinus* cells. The inability of this study to tag *X. ampelinus* by means of electroporation suggests that future studies should look into the development of a protocol to obtain chemical competent *X. ampelinus* cells for transformation. This method might be less damaging to the bacterial cell than electroporation and result in a higher rate of plasmid uptake.

5.3 Screening of the *in vitro* activity of D4E1 against *A. vitis* and *X. ampelinus*

The *in vitro* activity of D4E1 against *A. vitis* and *X. ampelinus* was investigated. A general trend of a decrease in cfu by increasing the D4E1 concentration could be observed for both pathogens. Nevertheless, experimental variation as measured by differences in counted cfu between repetitions did not allow for the assumption that specific peptide concentrations lead to a statistically significant reduction in cfu. For a confirmation of the clear trend in cfu reduction by increasing the peptide concentration the *in vitro* screening has to be repeated in the future to determine the D4E1 concentration that has the most significant effect on *A. vitis* and *X. ampelinus*.

Previous studies found that bacteria were generally more sensitive to the *in vitro* antimicrobial activity of D4E1 than fungi (Rajasekaran *et al.*, 2001). The minimum inhibition concentrations (MIC) of D4E1 at which the growth of two gram-negative bacteria, *P. syringae* pv. *tabaci* and *X. campestris* pv. *malvacearum* race 18, were completely inhibited were 2.25 µM and 1.25 µM, respectively, while the MICs for the fungi ranged from 4.67 to 25 µM. Another study has also tested D4E1 *in vitro* against fungi and showed MICs ranging from 12.5 µM to 25 µM (De Lucca *et al.*, 1998). The results of the present study showed that D4E1 inhibits the growth of *A. vitis* and *X. ampelinus* at concentrations of 6.89 and 5.36 µM, respectively, suggesting that *X. ampelinus* is less sensitive to D4E1 than *A. vitis*. Differences in media composition could however have affected the activity of the peptide (Yeaman and Yount, 2003). Therefore, *in planta* tests first had to be performed to eliminate the potential effect of the artificial environment before further conclusions could be drawn.

5.4 Screening of the *in planta* activity of D4E1 and Vv-AMP1 against the pathogens of interest

One objective of this study was to illustrate the use of a transient expression system in grapevine to determine the *in planta* effect of AMPs against grapevine pathogens. This goal was achieved by using qPCR to determine the difference in bacterial concentrations in *V. vinifera* plants between two treatment groups, the one expressing an AMP via a 35S expression vector and the other infiltrated only with an empty 35S expression vector. As described earlier, the GVA viral vector-based expression was limited to the vascular tissue in *V. vinifera* after whole leaf infiltrations (see section 5.1.2). The method of plant inoculation applied for these two bacteria will have resulted in them being present also in the mesophyll tissue of the plants and expression by the GVA-based system at few spots in the vascular system was expected to be insufficient. Therefore, only the 35S AMP expression vectors were used for the *in planta* AMP activity screening against *A. vitis* and *X. ampelinus*. The viral vector system will be used in future studies for AMP activity screening against phytoplasma. The phytoplasma will only be present in the vascular tissue of the plant as it would either have been infected with insect transmission or grafting. Additionally, the viral-based system can still be used in further studies when *N. benthamiana* is used as a host plant in a transient expression assay.

5.4.1 Establishment of qPCR protocols for pathogen quantification

The use of qPCR offers a highly accurate method for the determination of bacterial titres in plants (Lie and Petropoulos, 1998; Martin *et al*., 2000 Schmittgen, 2001). This approach is more sensitive and provides more consistent results than previous methods of titre determination through cfu counting after *in vitro* culturing of bacterial extracts (Li *et al*., 2008). For the quantification of the bacteria in the present study, qPCR protocols were not only optimised for the pathogens itself, but also for the quantification of the *V. vinifera* DNA. The amount of pathogen DNA could be normalised against an internal control (reference gene), representative of the amount of plant DNA in the sample (Winton *et al*., 2002; Ruppel *et al*., 2006; Brunner *et al*., 2009; Llorente *et al*., 2010). The quantification of qPCR protocols requires the primers to be highly specific as any non-specific amplification would result in an increased fluorescence, influencing the results negatively. The primer should support high linearity and amplification efficient reactions (Heid *et al*., 1996; Van Guilder *et al*., 2008).

During the qPCR optimisation for aster yellows phytoplasma and *X. ampelinus* quantification, one and three primer sets, respectively, were excluded from further use. The primers described

by Angelini *et al*., (2007) and Dreo *et al*. (2007) were designed to be used in a TaqMan®-based system which requires the additional use of a probe. In a SYBR Green-based system without a probe, as used in this study, these primer sets seemed to lose their specificity. The two primer sets of Manceau *et al*. (2000) were designed to serve as diagnostic primers in a traditional PCR protocol and showed non-specific amplification in the study presented here. The results show that primers are not always directly transferable between different PCR systems. SYBR Green binds to all the double-stranded DNA molecules in a reaction, including primer-dimers and any non-specific amplification (Zipper *et al*., 2003). The binding of the dye to these amplicons contributes to the total amount of fluorescence detected, giving a false indication of the amount of template. Primers therefore have to be carefully designed for SYBR Green assays to avoid any non-specific amplification. This problem does not apply to TaqMan® assays since the dye-containing probe will only bind to the specific DNA. Previous studies have compared SYBR Green-based systems with TaqMan®-based systems, and have found them to be equally sensitive (Papin *et al*., 2004; Andersen *et al*., 2006; Gomes-Ruiz, 2006).

Primers were newly designed for qPCR detection and quantification of phytoplasma. These primers amplified a region of the 16S rRNA gene of these pathogens. In order to test the sensitivity of the primers, the detection of aster yellows phytoplasma was compared to an already establish nested-PCR protocol (Engelbrecht *et al*., 2010). It was shown that the qPCR method for aster yellows phytoplasma detection was at least 100 times more sensitive than the nested-PCR reaction. According to Angelini *et al*., (2007) the TaqMan® assay described by them was as sensitive in the detection of aster yellows phytoplasma as the different nested-PCRs which they used for the detection of different phytoplasmas in grapevine and periwinkle. It could, for this reason, also be possible that the SYBR Green assay designed in this study is as sensitive or even more than the TaqMan® assay designed by Angelini *et al*. (2007). To confirm this proposal, these two qPCR screening methods have to be evaluated on the same samples, in future. The qPCR protocol designed during the current study proved to be highly sensitive and can in the future be applied in standard testing protocols.

Once all the qPCR protocols were optimised for the different reactions, standard curves were set up for each reaction to determine the efficiency thereof as well as the linearity of the standard curves. This was performed for the qPCR reactions for *A. vitis*, *X. ampelinus* and *V. vinifera* DNA quantifications. All the standard curves showed high reaction efficiencies (E >0.97) and linearity (R^2 >0.99). These reactions were sufficiently reproducible and could therefore be used for bacterial quantification.

5.4.2 Screening of the D4E1 and Vv-AMP1 *in planta* activity against *X. ampelinus* and *A. vitis*

Four transient expression assays were performed to determine the *in planta* effect of D4E1 and Vv-AMP1 against *X. ampelinus* and *A. vitis*. The results for the transient expression assay of D4E1 against *X. ampelinus* clearly showed a reduction in *X. ampelinus* concentration in *V. vinifera* plants as a result of D4E1 activity. These results confirm the trends obtained in the *in vitro* screening of the effect of D4E1 against *X. ampelinus*. The mechanism of action of D4E1 still has to be elucidated, but previous results have shown its *in planta* effect against Gram-negative bacteria such as *A. tumefaciens*, *Xanthomonas populi* pv. *populi* (Mentag *et al*., 2003) and fungi such as *Colletotrichum destructivum* (Cary *et al*., 2000). These effects were however only shown in the form of a reduction in symptom development rather than as a reduction in pathogen concentration. Many studies on determining the *in planta* effect of AMPs against plant pathogens based their findings on symptom development only (Ali and Reddy, 2000; Ponti *et al*., 2003; Jones *et al*., 2004; Vidal *et al*., 2006). A recent study stated however, that recording the development of symptoms was not a direct approach of determining pathogen resistance, and rather implemented qPCR to determine fungal resistance in wheat (Brunner *et al*., 2009). For this reason, and because no symptom development could be observed for *X. ampelinus* inoculated plants, even at 21 dpi, the use of qPCR was preferred in the current study.

Different inoculation procedures of *V. vinifera* with *A. vitis* were investigated. It was assumed that the bacterial challenge by vacuum infiltration was too high and as a result the bacteria could overcome the effect of D4E1. This form of inoculation was also damaging to the plant cells which have already been exposed to vacuum infiltration when infiltrating either the pBin61S-D4E1 or the empty pBin61S binary vector. *Agrobacterium vitis* infection does not only cause tumour development in grapevine (Burr *et al*., 1998), but also necrosis on the roots of the vines within 24 to 48 h after inoculation (Burr *et al*., 1988). The necrotic effect is a form of hypersensitive response in non-host plants such as *N. tabacum*, causing rapid cell death in inoculated leaves (Herlache *et al*., 2001). Extreme high concentrations of bacteria might therefore also increase the plant's hypersensitive response in grapevine. The approach of inoculation was shifted to dipping the plants into the bacterial suspension, the same procedure which was used for plant inoculation with *X. ampelinus*. This led to the survival of *A. vitis* inoculated plantlets for more than 4 days. Extending the period of incubation before titre determination and lowering the *A. vitis* inoculum concentration was even more effective. An *in planta* effect of D4E1 against *A. vitis* was shown in 3 out of the 5 treated plants. Repetition of experiments did not lead to an increase in plant numbers in which the effect was significant but

still indicated an effect of D4E1. Increasing the number of experimental plants might give a clear picture of significant differences.

No effect was shown for the *in planta* activity of Vv-AMP1 against *X. ampelinus* or *A. vitis*. Certain AMP groups are selective in their action and therefore only show an effect against a restricted group of microorganisms (García-Olmedo *et al.*, 1998; Yeaman and Yount, 2003; Montesinos, 2007). Since Vv-AMP1 has been classified as an antifungal defensin peptide by De Beer and Vivier (2008), the result obtained in this study could be explained by its possible inability to target bacterial membranes or other bacterial components. Vv-AMP1 was previously found to be active against the fungi *Fusarium oxysporum*, *Fusarium solani*, *Verticillium dahliae* and *Botrytis cinerea*, but not against *Alternaria longipes* (De Beer and Vivier, 2008). The complete target range of this peptide still has to be determined, and may still include bacteria since it is part of the subgroup B1 of plant defensins which shows activity both against bacteria and fungi (De Beer, 2008). Antimicrobial peptides that have shown only antifungal and no antibacterial activity are the sugarcane defensins Sd1, Sd3 and Sd5 (De-Paula *et al.*, 2008) and synthetic peptides developed by De Samblanx *et al.* (1996). The AMP AFP isolated from *Aspergillus giganteus* has also been found to be selectively active against fungi but not bacteria (Lacadena *et al.* 1995; Vila *et al.*, 2001; Moreno *et al.*, 2003; Moreno *et al.* 2005). In the present study, Vv-AMP1 did neither decrease nor increase the bacterial concentration in the plants. Any beneficial effect of Vv-AMP1 on *X. ampelinus* or *A. vitis* growth *in planta* could therefore be excluded. Vv-AMP1 was not available in peptide form and these results could consequently not be confirmed by *in vitro* plate tests.

When the *in planta* effect of D4E1 against *A. vitis* was compared to that of Vv-AMP1, the bacterial concentration was reduced significantly in the D4E1 treatment group in contrast to the Vv-AMP1 treatment group. Vv-AMP1 showed no beneficial effect on *A. vitis* growth and it can therefore be argued that the difference in bacterial titres between these two treatment groups was not due to Vv-AMP1 increasing the growth rate of *A. vitis in planta*, but rather as a result of D4E1 inhibiting the bacterial growth. D4E1 is therefore significantly reducing the titre of *A. vitis* in an *in planta* grapevine environment.

Results indicated that *A. vitis* was less sensitive to D4E1 treatment than *X. ampelinus* and also more pathogenic on *in vitro* cultured grapevine plantlets, as measured by plant survival after infiltration. They have a more destructive effect on the *V. vinifera* plantlets which complicated the optimisation of the *in planta* assay. Future studies should consequently focus on optimising the inoculation system of these bacteria for such assays to be performed with even greater

success. With regards to the two AMPs, D4E1 and Vv-AMP1, it appears that transgenic *V. vinifera* plants expressing Vv-AMP1 will offer protection against neither *X. ampelinus* nor *A. vitis*. The synthetic peptide, D4E1, is a good candidate for transgenic plant development against both bacteria. This assumption is not only corroborated by the *in vitro* activity testing, but also by the *in planta* effect of D4E1 as demonstrated by qPCR analysis.

5.4.3 Screening of the D4E1 and Vv-AMP1 *in planta* activity against aster yellows phytoplasma

The application of *in planta* AMP activity screening is the only way of facilitating AMP activity screening against phytoplasmas. Plant transient AMP expression systems therefore offer an ideal approach of pre-screening AMP activity before the development of transgenic lines. A crucial part of establishing such a system to be implemented on phytoplasma is obtaining infected plant material that will support the expression of AMPs through a transient expression system. *Vitis vinifera* field-grown and potted material are not easy to successfully infiltrate via agro-infiltration (Santos-Rosa *et al*., 2008; Muruganantham *et al*., 2009). Phytoplasma infected *in vitro* culture plants have to be established, which can be easily infiltrated, or an alternative host has to be found that would allow the transient expression approach.

This study was not successful in establishing a sufficient number of phytoplasma infected *in vitro* cultured *V. vinifera* plants from infected field material. A common symptom of phytoplasma infection of grapevine is the effect of delayed budding (Pers. Com. R. Carstens, Agricultural Research Council, Infruitec-Nietvoorbij, Stellenbosch). This could result in sterilised cane material not developing any shoots, as was seen in this study. The sterilisation technique applied in this study seems to be appropriate for removing the epiphytic pathogens but not the endophytic pathogens. A recent study has described the wide diverse range of endophytes that were identified in an entire single vine (West *et al*., 2010). Future studies should implement better sterilisation techniques or make use of different plant material. Younger canes may contain less fungal endophytes as well as canes collected from infected greenhouse plants. The technique of *in vitro* grafting was shown to facilitate the transmission of phytoplasma (Jarausch *et al*., 1999) and future studies may be able to implement grafting as a way to successfully transmit phytoplasma to *in vitro* cultured *V. vinifera* plants.

The implementation of an alternative aster yellows phytoplasma host other than grapevine was additionally investigated during this study. *Catharanthus roseus* was identified as a possible candidate, firstly, because the aster yellows phytoplasma insect vector, in South African vineyards, has recently been identified and is able to transfer the pathogen to *C. roseus* (Pers.

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Com. K. Kruger, Department of Zoology and Entomology, University of Pretoria). Secondly, *C. roseus* plants have been shown to allow foreign gene expression by way of a transient expression system (Di Fiore *et al.*, 2004). Moreover, it was reported that phytoplasma could be successfully transferred from infected *C. roseus* plants to the model plant *N. tabacum* (Kamińska *et al.*, 2010). *Nicotiana benthamiana* plants infected with phytoplasma will assist the screening of AMP activity, as these plants are well supportive of transient expression systems, both by means of 35S and viral expression vectors. Ultimately, AMP activity still has to be screened in *V. vinifera* to show the *in planta* effect against aster yellows phytoplasma in the natural host environment.

GUS expression assays using the 35S:GUSi construct were performed in *C. roseus* to establish a procedure of expressing foreign genes after Agrobacterium vacuum-infiltration. GUS expression was observed at 6 dpi around the cutting sites made for infiltration. In uncut leaves small patches of GUS expression could also be observed. Visual inspection of the leaves showed that younger leaves facilitate better GUS expression. The virulence factor that the *A. tumefaciens* strain contains, was shown to have an influence on transformation and foreign gene expression efficiency (Santos-Rosa *et al.*, 2008). A different *A. tumefaciens* strain (EHA105) was tested, in order to increase the GUS expression, but did not seem to offer any improvement to the system. Di Fiore *et al.* (2004) succeeded in using Agrobacterium vacuum-infiltration to transiently transform the leaves of *C. roseus* plants and in doing so to express terpenoid indole alkaloid enzymes in these plants. This research also stated that the infection efficiency of the Agrobacterium strain along with the physiological condition of the plants and the stress as a result of infiltration may have an effect on the success of the transient assay. These are all factors that have to be considered when optimising transient expression systems for AMP activity screening in *C. roseus*.

Chapter 6

Conclusion

The high incidence of grapevine pathogens threatens the South African grapevine industry. New diseases are constantly emerging in local vineyards with little or no controlling strategies in place, except for preventing the spread thereof. The industry is therefore in need of disease resistant plants that can decrease the economical loss due to vector control and yield loss. Transgenic grapevine development is, however, a tedious and time consuming process which still may lead to plants not being able to combat specific pathogen attacks. Therefore, this study aimed to validate the use of transient expression systems which express antimicrobial peptides to study the *in planta* effect of these peptides against selected grapevine pathogens and compare these results to *in vitro* screenings. It focused on two AMPs, D4E1 and Vv-AMP1, and three grapevine pathogens namely *Xylophilus ampelinus*, *Agrobacterium vitis* and aster yellows phytoplasma.

The results of the *in vitro* D4E1 activity assays against *X. ampelinus* and *A. vitis* pointed towards a total inhibition of bacterial growth at concentrations below 8 μM for both the bacteria. These results were correlated with *in planta* assays to allow conclusions about the potential of the peptide to provide disease resistance to transgenic plants. Transient expression vectors were constructed based on viral- as well as 35S vectors. The viral vectors showed successful replicating recombinant viruses that could spread systemically in *N. benthamiana*. Control vectors expressing GUS, for both systems, showed significant GUS expression in *N. benthamiana*. However, the expression in grapevine by means of the viral expression vectors was limited to small areas associated with leaf veins. The 35S transient expression system showed expression in the whole infiltrated area of the grapevine leaves and was therefore used for determining *in planta* AMP activity. Quantitative real-time PCR assays were established and used for the relative quantification of bacterial titres in the inoculated plants. When the *in planta* effect of the AMPs were screened, Vv-AMP1 showed an effect against neither *X. ampelinus* nor *A. vitis*, while there was also only a significant difference in *A. vitis* titres between 3 of the 5 D4E1 treated plants relative to the untreated control group. D4E1 *in planta* expression resulted in the reduction of *X. ampelinus* titres as well as a reduction in *A. vitis* titres in the D4E1 treatment

group relative to the Vv-AMP1 treatment group. Both bacteria are therefore sensitive to D4E1 in a plant environment. These results illustrated the value of transient expression systems as a pre-screening method of AMP activity in an environment that is more closely related to that in transgenic plants compared to *in vitro* plate assays. Future studies can implement this system to determine the *in planta* activity of a variety of different AMPs against grapevine pathogens in a relatively short period of time. These systems overcome the limitation of *in vitro* assays and can be applied also to unculturable pathogens.

Although no phytoplasma infected material could be established *in vitro* during this study, the constructed viral vectors will be used in future for *in planta* AMP activity screening against aster yellows phytoplasma, once plant material is available. The lack of an outer membrane and cell wall makes phytoplasmas ideal targets for AMPs which do not specifically target these cellular components. Antimicrobial peptides can also be screened against grapevine pathogens that were not included in this study, for example, the fungi responsible for causing powdery and downy mildew. The system can even be implemented to screen the potential *in planta* AMP activity against grapevine infecting viruses or transferred to crop plants other than grapevine. Transient expression systems are flexible and vectors can easily be altered to express different foreign genes. The use of transient expression systems has the potential to play an important role in future disease resistance studies and improvement of economically important crops such as grapevine.

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Internet resources:

http://www.ars.usda.gov/ pandp/people/people.htm?personid =1289

http://www.eppo.org/QUARANTINE/bacteria/Xylophilus_ampelinus/ XANTAM_images.htm

http://www.nysipm.cornell.edu/factsheets/grapes/diseases/crown_gall.pdf

http://www.satgi.co.za

http://www.sawis.co.za

http://www.wynboer.co.za/recentarticles/200808aster.php3

Addendum

Confirmation of pathogen identity

Agrobacterium vitis (strain A39)

Sequencing results:

cctgacccaaacatctcggctgcccattcgcggcttgctgcatgagccgacgtaggatccgtacctgggggggaagcttacgataatgt

gcgtggttaagtcctgttgcctttgctcgtccggcaggctctcgtcataatttccggtctccagaacccagctccgagcaaacctggtaat

ttgatcaggcggtagcggaatatcgaggtgccgggctgagagccggagctccaacttgcccttcctggagagatattcccactgatta

ataacctgctgaatggttttagttccgcccctaggcacaattcgaatgacaacttgagctcg

Blast results:

Aligned to GenBank Accession AM490795.1

99% identity

Agrobacterium vitis Ti-plasmid partial virD2 gene

Xylophilus ampelinus (strain VS9)

Sequencing results:

gcgggktattmcaccaaagtggctttgcagaggcttctttgttgttgatcgatattgttcgatcaatcggctgttctttaaaaattcatagag

tcgaatcagcgttgctggtggaaagagggtcatacctcaccgtgccaccggcaatgtgaatttttgattgcgtcaaaacgaatattcaga

ctaggtctggaattcaagtatcgaagcgagagcttcatacggcataacgcgtcaggtgaaagacc

Blast results:

Aligned to GenBank Accession U76357.1

99% identity

Xylophilus ampelinus 16S ribosomal RNA gene