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FINAL REPORT 2022

Winetech Number: GenUS 17-03

1. PROGRAMME & PROJECT LEADER INFORMATION

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2. PROJECT INFORMATION

Project title	Application of virus vectors against grapevine leafroll disease
Short title	Virus vectors against GLD

Fruit kind(s)	Wine Grapes		
Start date (mm/yyyy)	01/2018	**End date** (mm/yyyy)	06/2022

Key words	Biotechnology, virus-based expressions systems, RNAi

	CFPA	RAISIN SA	HORTGRO	SATI	WINETECH	ARC	OTHER
TOTALS All years	R 0	R 0	R 0	R 0	R 1720405	R 0	R 0

Total cost of entire project	R 1720405

3. EXECUTIVE SUMMARY

Objectives and Rationale

Grapevine leafroll-associated virus 3 (GLRaV-3) and grapevine proteins that interact with each other can be targeted by control strategies to develop plant immunity. The aim of this study was to identify these proteins, and to evaluate the use of an infectious clone to suppress the replication and spread of GLRaV-3.

Methods

The use of a grapevine virus A (GVA)-based RNAi infectious clone to deliver silencing information to *Nicotiana benthamiana* and *Vitis vinifera* was optimised. Interacting virus and/or plant proteins involved in GLRaV-3 infection were identified using a Yeast-two-hybrid (Y2H) system and Bimolecular fluorescence complementation (BiFC). All interactions were validated in plants.

Key Results

Two different methods were used to screen for and validate the interaction of eight virus protein pairs. A Y2H library for the screening of V. vinifera proteins was constructed. Five grapevine proteins that possibly interact with GLRaV-3 proteins were identified. Two of these could be validated in plants.

Key Conclusion of Discussion

Infiltration of V. vinifera with the GVA infectious clone was unsuccessful.
We identified interacting protein pairs that could be possible targets for a control strategy for GLRaV-3.
This study provides information on the functional role of GLRaV-3 ORFs and increases our understanding of the pathogenic role of GLRaV-3 in GLD.

Take Home message for Industry

Potential targets for GLRaV-3 control were identified that can be exploited by future research.
We propose the use of genome editing technology to investigate the two host genes identified.

4. PROBLEM IDENTIFICATION AND MOTIVATION

Problem Identification

Grapevine Leafroll Disease (GLD) is an economically important viral disease affecting wine grape cultivars. As the uptake and impact of GLD management protocols in South Africa have been limited, it remains a major cause for concern. GLD is linked to infection by grapevine leafroll-associated virus 3 (GLRaV-3). GLRaV-3 infection alters photosynthesis, berry cluster size, fruit ripening, and the levels of anthocyanins and phenolic compounds. The precise underlying mechanisms behind the role of GLRaV-3 in GLD remains unclear.

Transcriptional or post-transcriptional gene silencing (RNAi), is an antiviral defence mechanism utilised by plants. The presence of double stranded RNA (dsRNA) triggers a pathway leading to the degradation of mRNA complementary to the dsRNA. Virus vectors carrying fragments of host genes can be used to suppress the expression of these genes in plants by triggering the RNAi system.

The aim of this project was to study virus-virus and virus-host protein interactions, to identify virus or host genes that play a key role in the proliferation of GLRaV-3 and the onset of GLD. The possibility of using these genes as targets for an RNA interference (RNAi)-based control measure for GLRaV-3, delivered by a Grapevine virus A (GVA) vector, was also investigated.

Motivation

GLRaV-3 has not been successfully eliminated from mature vines and no natural source of resistance has been reported. The functions of viral proteins have largely been inferred by sequence homology to related viruses, and no grapevine genes essential for the replication and spread of GLRaV-3 have been identified.

Although the application of a virus vector to trigger the RNAi system of grapevine and slow down or stop the spread of GLRaV-3 was not successful, we identified several protein-protein interactions that may help to clarify of the role of virus and plant proteins in the replication cycle and spread of GLRaV-3. In addition to understanding which virus or host genes play a key role in the proliferation of GLRaV-3 and the onset of GLD, our understanding of the complex molecular interactions between plant viruses and their hosts was expanded, providing valuable information to assist in the development of disease control or prevention strategies.

5. ACCUMULATED PROGRESS TABLE

Objectives	**Milestones (Significant event or stage in a project)**	**Date Achieved**
O1. Optimise the application of infectious clones	M1.1 Optimise the infiltration of wild type (wt) GVA and/or GLRaV-3 infectious clones to N. benthamiana	2019-01-30
	M1.2 Optimise the infiltration of wt GVA and/or GLRaV-3 infectious clones to V. vinifera. Not Achieved	
O2. Construct infectious clones (GVA and GLRaV-3)	M2.1 Construct recombinant GVA and/or GLRaV-3 infectious clones (GFP and PDS)	2019-01-30
	M2.2 Construct recombinant GVA and/or GLRaV-3 infectious clones (RNAi targets) Not Achieved	
O3. Evaluate RNAi virus vectors to control the systemic spread of GLRaV-3	M3.1 Infiltrate V. vinifera with RNAi constructs Not Achieved	
	M3.2 Evaluate GLRaV-3 concentrations in infiltrated V. vinifera Not Achieved	

O4. Identify potential RNAi targets by using Yeast-two-Hybrid assays	M4.1 Clone GLRaV-3 ORFs in bait and prey constructs	2019-05-30
	M4.2 Perform one-on-one mating experiments to identify virus-virus interacting proteins	2019-08-30
	M4.3 Construct V. vinifera Yeast-two-Hybrid library	2019-10-30
	M4.4 Perform library scale mating experiments to identify virus-host interacting proteins	2021-03-30
	M4.5 Validate interacting protein pairs in planta	2022-06-30

6. WORKPLAN (MATERIALS AND METHODS)

W1. Optimise the application of infectious clones

Since no GLRaV-3 infectious clone could be obtained, we focused on the GVA infectious clone.

1.1. *N. benthamiana* was infiltrated with a wild type GVA infectious clone (GVA118) and success was evaluated using a diagnostic RT-PCR.
1.2. *V. vinifera* plants were not infiltrated with the wild type GVA clone, but instead were subjected to infiltration with the recombinant clones (W2).

*The recombinant GVA infectious clone was used (see W 2.1) to optimise the infiltration of *V. vinifera*, as this would allow us to assess the success of RNAi induced by the clone.

W2. Construct recombinant infectious clones (GVA and GLRaV-3)

A GVA infectious clone was used since GLRaV-3 full length clone was determined to be non-infectious.

2.1. GVA infectious clones with RNAi targets towards *N. benthamiana* (Nb) and *V. vinifera* (Vv) phytoene desaturase (PDS) in sense (F) and antisense (R) orientation (NbPDSF, NbPDSR, VvPDSF and VvPDSR) to serve as silencing vectors, and clones carrying a green fluorescent protein (GFP) or β-glucoronidase (GUS) gene to serve as expression vectors, were constructed. Each of the clones was used to infiltrate *N. benthamiana* using *A. tumefaciens*. Leaves from infected plants were evaluated visually 3-4 weeks post infiltration. Leaf material from new growth (not directly infiltrated) was also sampled for RNA extraction on the same day, and extracted RNA was kept at -80 °C. An RT-PCR and nested RT-PCR was performed with primers targeting the GVA coat protein gene (CP), as well as the foreign gene insert site, to determine whether GVA spread throughout the plant. Following these experiments, *V. vinifera* plants maintained *in vitro* were infiltrated with GVA infectious clones (VvPDSF and VvPDSR) using an *Agrobacterium tumefaciens* root-drench method. After 30 days, plants were transferred from the *in vitro* incubator to harden off in the greenhouse. Leaf and petiole material was sampled 5 months post-infiltration and subjected to the same treatment as described for *N. benthamiana*. Plants that screened positive for the presence of GVA were repotted to allow them to grow for another 9 months. Leaf, petiole and cane material was sampled and screened using the same method as before.

2.2. Construct GVA infectious clones with RNAi targets towards GLRaV-3 or the grapevine host genome.*

*Milestone 2.2 was never carried out, as infectivity and silencing/expression capabilities of the GVA RNAi infectious clone in *V. vinifera* could not be proven.

W3. Evaluate RNAi virus vectors to control the systemic spread of GLRaV-3 (track GLRaV-3 titre with RT-qPCR).

Source plants infected with various GVA RNAi infectious clones targeting GLRaV-3/*V.vinifera* (from W2.2) would have been prepared and evaluated for their ability to prevent the systemic spread or suppress GLD symptom expression of a wild type GLRaV-3 challenge (bud graft).*

*Objective 3 was never carried out, as infectivity and silencing/expression capabilities of the GVA RNAi infectious clone in *V. vinifera* could not be proven.

W4. Investigate protein-protein interactions between GLRaV-3 proteins, and proteins from either GLRaV-3 or *V. vinifera*, using the yeast two-hybrid system

As no GLRaV-3 clone was available, the workplan was amended. We elected to use a Yeast-two-Hybrid system instead of a mutant GLRaV-3 infectious clone to identify protein-protein interactions between the virus and itself, and the virus and the host, to elucidate the function of various ORFs.

4.1. A set of bait and prey constructs, each construct containing one GLRaV-3 ORF, was constructed.

4.2. One-on-one mating experiments were performed to identify virus-virus interacting proteins. Some interacting protein pairs were expected, but not detected. We hypothesised that some interactions may require the presence of a third protein. Therefore, another set of experiments were conducted to screen all possible three-way combinations of genes in the quintuple gene block (ORF 4-7; ORF3 is not included as it is a non-structural protein. This involves expression of a third protein in the system, which can act as a 'bridge' between the bait and prey proteins. Twenty additional bait plasmids were constructed, and 90 additional one-on-one mating experiments performed. Furthermore, since the Yeast-two-Hybrid assay relies on the protein of interest being fused to either an activation or DNA-binding domain, the N-terminal of the protein of interest may be blocked. Some protein-protein interactions might require this terminal to be available. The orientation in which the proteins bind or interact may also play a role in the success of the activation of reporter genes. The bimolecular fluorescence complementation (BiFC) system circumvents the limitation of blocking terminal ends with the DNA-binding domain by allowing us to screen sets of possible interacting partners in various orientations (Fig 1). Therefore, a second set BiFC experiments were conducted on all the different combinations of proteins produced by GLRaV-3 ORFs 3-

7 (the quintuple gene block, encoding proteins involved in virion assembly and intercellular movement) for interactions that could have been missed due to the limitations of Y2H screening.

4.3. A prey library was constructed from mRNA extracted from GLRaV-3 infected *V. vinifera*. Plant material used were pooled petiole, leaf and phloem tissues.

4.4. Using bait constructs constructed in 4.1., GLRaV-3 ORFs 4, 5, 6, 7, 8, 9 and 10 were screened against the *V. vinifera* library to identify virus-host interacting proteins. These GLRaV-3 ORFs are involved in cell-to-cell movement, virion assembly, and suppression of host silencing.

4.5. Interacting protein pairs were validated *in planta* using bimolecular fluorescence complementation (BiFC) in *N. benthamiana*.

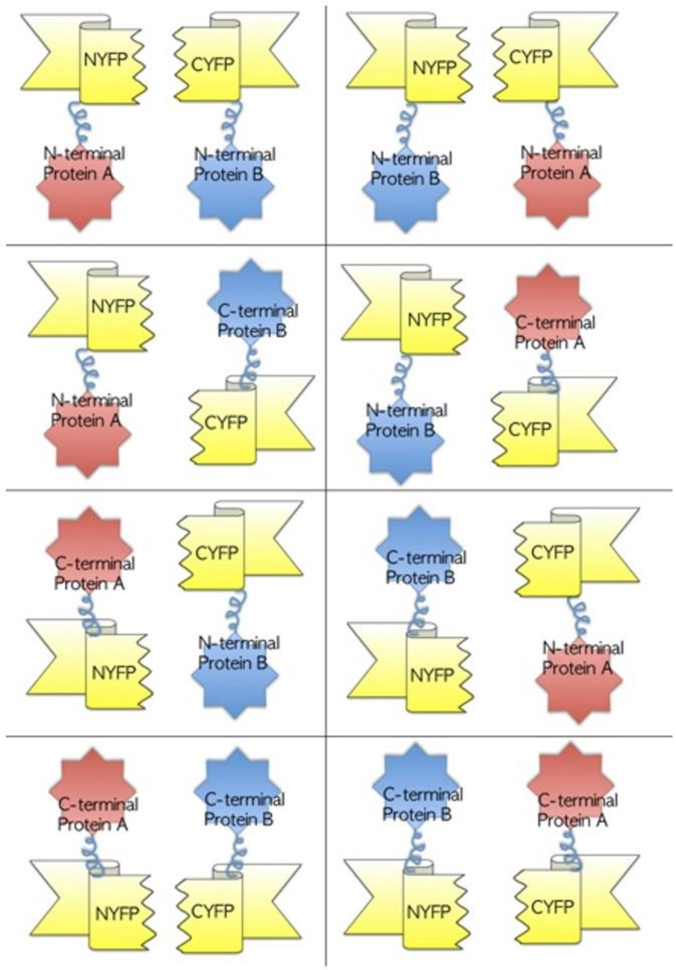

Fig 1: A diagram illustrating the effect of different orientations of interacting partners on the success of YFP reconstitution in the BiFC system. The Y2H system does not make allowances for different orientations and availability of active domains when screening for protein-protein interactions.

To optimise the Bimolecular Fluorescence Complementation (BiFC) system, two *A. thaliana* genes previously shown to interact: CBL10 (a member of the calcineurin B-like calcium sensor gene family) and CIPK24 (a serine-threonine protein kinase), were cloned into vectors expressing these proteins fused to half of a yellow fluorescent protein (YFP). When CBL10 and CIPK24 interact, the two halves of the YFP are brought together and reconstituted, and a signal can be observed using fluorescent microscopy. Initially, “empty” vectors (vectors encoding half of the YFP not fused to anything) were used as negative controls. However, this led to a very high background signal. Subsequently, for the virus-virus interactions, the CIPK24 plasmid was used as a negative control, as it was assumed no GLRaV-3 protein interacts with *A. thaliana* CIPK24.

A preliminary experiment was conducted to identify the combination of orientations that produces the strongest YFP signal for each set of proteins to be screened.

Following that, three leaves on each of three *N. benthamiana* plants were infiltrated with *A. tumefaciens* bacteria carrying one of the following combinations of plasmids, and imaged using fluorescent microscopy:
1) Protein A + Protein B
2) Protein A + CIPK24
3) Protein B + CIPK24

To normalise YFP signal across samples, an additional BiFC plasmid was modified to express mCherry, a red fluorescent protein. In all experiments, the mCherry vector is co-infiltrated with the YFP constructs. The strength of the protein interaction and subsequent YFP signal of each of the nine images was then quantified as (*mean intensity YFP – mean intensity mCherry*).

7. RESULTS AND DISCUSSIONS

O1 M1.1 and O2 M2.1: Construction of infectious GVA clones and optimisation of application in *Nicotiana benthamiana*

Previous reports described the successful application of GVA as an RNAi vector in *N. benthamiana*, with infected plants showing chlorophyll photobleaching (white veins on leaves) as a result of PDS silencing in areas where GVA is present. An attempt was made to engineer an expression vector based on the same GVA clone. However, even though GVA could be detected using RT-PCR, levels of foreign gene expression were too low for practical application and observations could only be made under a confocal microscope.

Several attempts were made to optimise the use of the GVA clone as an expression vector, including the use of different foreign genes (two types of green fluorescent protein (GFP) and the β-glucoronidase (GUS) reporter gene), different controller elements, altering the position of the foreign gene within the GVA genome, and co-infiltration with different silencing suppressors.

Although differences in GVA titer could be observed, no expression of foreign genes could be observed with the naked eye. We concluded that, although the clone is successful at inducing RNAi, it is not a successful expression vector.

O2 M2.2: Application of recombinant GVA infectious clones in *Vitis*

To optimize infiltration of *Vitis* plants, GVA-118 VvPDSF (containing a sense-fragment of the *V. vinifera* PDS gene) and GVA-118 VvPDSR (containing an antisense-fragment of the *V. vinifera* PDS gene) silencing clones were used. *In vitro* lines were established of grapevine plants from our greenhouse and subjected to root-drench experiments. Four cultivars were used: Chenin Blanc, Cabernet Sauvignon, Chardonnay, and Sultana. We also investigated the effect of the presence of GLRaV-3 in the plants on the success of infiltration.

The transformation of *Vitis* is a rare event and the first two rounds of infiltrations delivered only one plant possibly containing the clone. Since the GVA titer in this plant was so low, a nested RT-

PCR detection assay specific to this GVA clone was developed.

Infection in the plant did not persist. Another large-scale infiltration of 118 plants, 58 GLRaV-3 positive and 60 GLRaV-3 negative, was performed. After 5 months, no PDS silencing symptoms could be observed. Nine plants screened positive for the presence of GVA. All of these plants were infected with GLRaV-3, indicating that the presence of GLRaV-3 could improve the chances of a successful infiltration. Four of the nine plants contained GVA-118 VvPDSF, and five contained GVA-118 VvPDSR. The plants were then repotted, allowed to grow bigger, and screened after 9 months to see if infection persisted.

None of the plants screened positive for the presence of GVA. We concluded that the use of the GVA clone for silencing or expression in *V. vinifera* is not feasible.

O4 M4.2: Perform one-on-one mating experiments to identify virus-virus interacting proteins

To identify suitable targets for RNAi, we used a Yeast-two-Hybrid assay to look for interacting protein pairs between GLRaV-3 and itself, and between GLRaV-3 and its host.

Virus-virus screenings using the standard Yeast-two-Hybrid assay identified four interacting pairs (Table 1). These results correlate with protein-protein interaction studies from pineapple mealybug wilt-associated virus 2 (PMWaV-2), and with our knowledge of the roles of these proteins based on sequence homology with other members of the family, most notably beet yellows virus (BYV). Although no further interactions were detected in the three-way interaction experiments, the supplementary BiFC experiments revealed five additional interactions (Table 1). All of the results are discussed under O4 W5.4.

O4 M4.3: Construct *Vitis vinifera* Yeast-two-Hybrid library

A Yeast-two-Hybrid library was constructed using RNA from a GLRaV-3-infected Cabernet Sauvignon plant maintained in the greenhouse at Stellenbosch University. The number of independent clones in the library was calculated at 2.1×10^6. Any number above 1 million is considered adequate to proceed to screening. The cell density of the library was 2.2×10^8 cells/mL, which is above the acceptable threshold of 2×10^7. The average insert size after removal of plasmid sequences was 784 bp. This corresponds to the modal gene size of *Vitis vinifera* (501-1000 bp).

O4 M4.4: Perform library scale mating experiments to identify virus-host interacting proteins

Several experiments had to be conducted to optimise yeast library mating efficiency. Ultimately, mating efficiencies of more than 10% were achieved for all screened proteins (a minimum of 2% is required to screen an adequate amount of diploids).

The bait plasmid containing ORF 8 caused autoactivation of the reporter genes in yeast and could not be used for library screening. Six GLRaV-3 proteins (encoded by ORFs 4, 5, 6, 7, 9 and 10) were screened against a library of *V. vinifera* protein-coding genes. Potential interacting partners were subjected to further screening in yeast: Plasmids were isolated and transformed into haploid yeast cells, which were then mated with yeast containing an ‘empty’ bait plasmid (encoding the DNA binding domain not fused to any protein) to eliminate false positives.

No potential partners were detected for ORFs 4 and 6. Two potential partners for ORF 5 (encoding a HSP90 homolog), were found, however, further screening revealed that these were false positives.

ORF 7, a protein coating the head of the virion, putatively interacts with *V. vinifera* 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) 02. DAHPS is the first enzyme in the Shikimic acid pathway, a plant-specific metabolic pathway necessary for the production of many secondary compounds, including auxin and aromatic amino acids.

Five potential interacting partners, one of which was a duplicate, were found for ORF 9. Although the exact role of p20A, the protein encoded by ORF 9, has not been described, genes similarly located in other closteroviruses are involved in the suppression of the host's RNAi defence system, and long distance transport. The *V. vinifera* proteins that potentially interact with p20A are:

- A 18.1 kDa class I heat shock protein involved in stress response
- A chlorophyll a-b binding protein CP26, functioning as a light receptor during photosynthesis
- A SMAX1-LIKE 6 protein, the homolog of which, in *Arabidopsis thaliana*, is proposed to be involved in branching control
- MAP3K epsilon protein kinase 1, the homolog of which, in *A. thaliana*, is involved in the regulation of cell expansion, cell elongation and embryo development.

O4 M4.5: Validate interacting protein pairs *in planta*

Bimolecular fluorescence complementation assays were conducted to validate the interaction of the proteins *in planta*. Microscopic images of the screening of an interacting set of proteins (ORF 3 x ORF 3) and a non-interacting set (ORF 9 x chlorophyll a-b binding protein CP26) are shown as an example (Fig 2). The remainder of the results are summarised in Table 1.

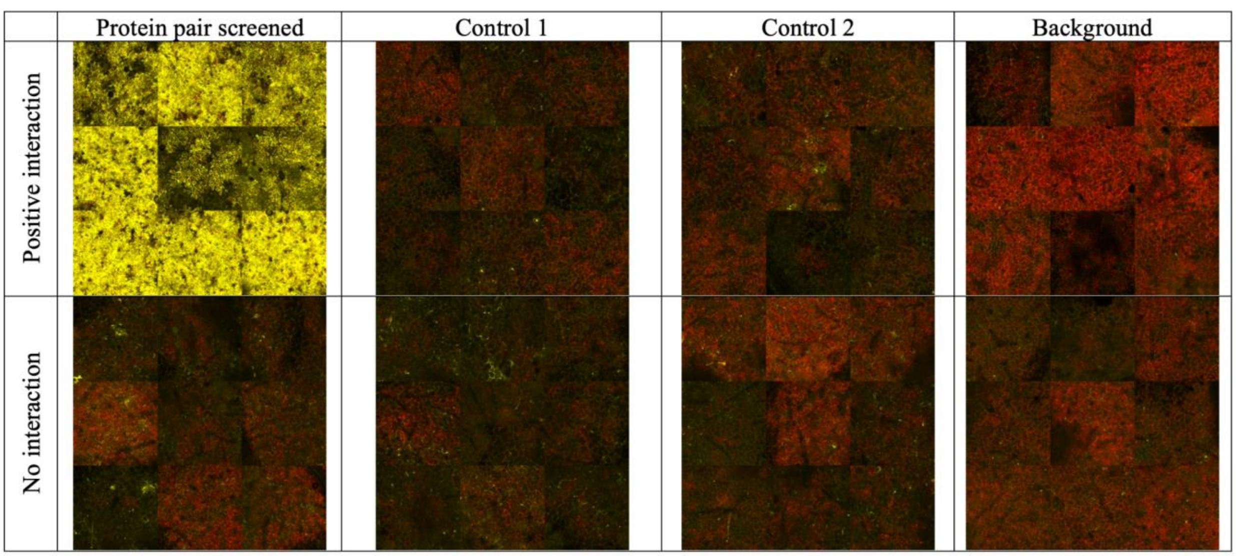

Fig 2: . Superimposed confocal microscopy images of YFP and mCherry expression in infiltrated leaves. The two rows are representations of how interacting proteins (ORF3 self-interaction, top panel) and non-interacting proteins (ORF9 with chlorophyll a-b binding protein CP26, bottom panel) can be discerned in the BiFC system. The image of each sample is made up of nine individual photos taken from the leaves of three infiltrated plants (three leaves per plant). The first column shows the protein pair screened. In the positive interaction row, the YFP can clearly be seen, whereas very little YFP is visible in the image of the non-interacting pair. The second and third columns show the two controls, one for each respective protein in the pair with *A. thaliana* CIPK24 (an unrelated protein that does not bind to either of the proteins of interest). The final column is plants infiltrated only with mCherry, to serve as a background control (no YFP expression).

Table 1: A summary of all protein-protein interactions screened for and found using the Yeast-two-Hybrid (Y2H) or Bimolecular fluorescence complementation (BiFC) assays.

Virus-virus interactions			
Protein A	**Protein B**	**Y2H**	**BiFC**
ORF2 (Unknown)	ORF6 (CP)	yes	no
ORF4 (HSP70h)	ORF4 (HSP70h)	yes	yes
ORF4 (HSP70h)	ORF6 (CP)	yes	yes
ORF10 (Silencing suppressor)	ORF10 (Silencing suppressor)	yes	yes
ORF6 (CP)	ORF6 (CP)	no	yes
ORF3 (Transmembrane protein)	ORF3 (Transmembrane protein)	no	yes
ORF4 (HSP70h)	ORF10 (Silencing suppressor)	no	yes
ORF6 (CP)	ORF10 (Silencing suppressor)	no	yes
ORF7 (CPm)	ORF10 (Silencing suppressor)	no	yes
Virus-host interactions			
Protein A	**Protein B**	**Y2H**	**BiFC**
ORF7 (CPm)	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) 02	yes	yes
ORF9 (p20A)	18.1 kDa class I heat shock protein	yes	yes
ORF9 (p20A)	Chlorophyll a-b binding protein CP26	yes	no
ORF9 (p20A)	SMAX1-LIKE 6 protein	yes	no
ORF9 (p20A)	MAP3K epsilon protein kinase 1	yes	no

The following virus-virus protein interactions were confirmed:

ORF4 (HSP70h) x ORF4 (HSP70h)

It is known that HSP70h of members of the family *Closteroviridae* forms a homomultimeric structure during assembly of virion head.

ORF4 (HSP70h) x ORF6 (CP - body)

While it has been found that HSP70h is not necessary for assembly of virion body in BYV, the protein has been speculated to act as chaperones to carry stable virions to plasmodesmatal necks.

ORF10 x ORF10 (silencing suppressor)

BYV silencing suppressor forms an RNA-binding octameric ring structure. Although there is no BYV homolog of ORF10, it is possible that the silencing mechanism of this protein also requires polymerization.

No significant interaction could be observed between ORF2 (a potential ORF of unknown function) and ORF6 (CP - body). As ORF2 is not present in several GLRaV-3 variants, it is unlikely to be a true reaction.

Several additional virus-virus protein interactions were found in the second set BiFC experiments conducted on proteins from the quintuple gene block.

ORF6 (CP-body) x ORF6 (CP-body)

In a previous study using the standard yeast-two-hybrid system, no self-interaction was found between major coat proteins of PMWaV-2. Initially, we proposed that the presence of RNA or a third protein is necessary for the CPs to interact. However, the results of this BiFC experiment show a clear interaction between the coat proteins. It is possible that the proteins require a plant cell environment to interact, or, more likely, that the reporter gene activation domains fused to the proteins in the Yeast-two-Hybrid system interfere with the CP-CP interaction sites.

ORF3 (transmembrane protein) x ORF3 (transmembrane protein)

It has been shown that dimerization of the movement protein of BYV, the homolog of ORF3 in GLRaV-3, is essential to its function in cell-to-cell movement of the virus.

ORF4 (HSP70h) x ORF10 (Silencing suppressor)
ORF6 (CP - body) x ORF10 (Silencing suppressor)
ORF7 (CPm - head) x ORF10 (Silencing suppressor)

It is interesting to note that, in addition to self-interaction, ORF10 also interacts with three of the four structural proteins of GLRaV-3. This has not been reported previously and could provide valuable insights into the mechanism behind the silencing suppressor activity of this protein.

Only two of the virus-host interactions were shown to be statistically significant compared to the controls *in planta*.

ORF7 (CPm) x *V. vinifera* 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS) 02

ORF7 forms the head structure of the virion. In plants, DAHPS-2 is involved in primary carbohydrate metabolism and the biosynthesis of aromatic amino acids. In grapevine, a higher expression of DAHPS-2 relates to the accumulation of hydroxycinnamic acids (phenolic phytochemicals) and tannins. Molecular studies on berries of GLD-infected vines have shown a correlation between decreased sugar and total anthocyanin content with the repression of genes in relation pathways. However, another study suggests that anthocyanin biosynthesis is not directly impaired by GLD infections and that the previously reported effect of virus infection on sugar and secondary metabolites is merely a consequence of the virus induced delay in the phenology of berries.

Long-distance movement of plant virus particles is known to occur via the phloem, in the direction of sugar transport. A previous study theorised that the change in sucrose concentrations in plants affected by cucumber mosaic virus may be due to altered cellular control elements that regulate phloem loading. It has been suggested that the change in expression of genes related to carbohydrate metabolism is simply a defence response by the plant to counteract this manipulation, however, the observation of a direct interaction of a GLRaV-3 protein with an enzyme involved in carbohydrate metabolism may provide clues to understand how viruses manipulate, access, and utilise the phloem transport network of its host for long-distance transport.

ORF9 (p20A; possibly implicated in long distance transport and suppression of host silencing) x A *V. vinifera* 18.1 kDa class I heat shock protein

Heat shock proteins are synthesised by plants in response to a variety of biotic and abiotic stresses. Heat shock proteins can be divided into several classes based on their size: HSP70, HSP60, HSP101 and small HSPs (sHSPs). sHSPs bind to partially folded or denatured proteins to prevent irreversible unfolding or aggregation. The production of these proteins is increased during some stages of growth and development of plants, such as fruit ripening. sHSPs belonging to Class I, such as the one identified in our study, are generally located in the cytoplasm, and have been proposed to play a role in the maintenance of membrane integrity under stress conditions.

Although many studies have focused on the roles of plant HSP70s and HSP90s during viral infection, comparatively few have been conducted on the role of sHSPs. However, an increase in the production of sHSPs in response to infection by RNA viruses, including GLRaV-3, has been shown. It has also been proposed that an interaction between rice stripe virus (RSV) and a rice sHSP may be necessary for transport through plasmodesmata. Systemic acquired resistance (SAR) is a plant defence response which depends on the long-distance movement of stress signals through the phloem. In *A. thaliana*, a sHSP has been found to be enriched in the phloem during SAR. Some potyvirus resistance genes in *A. thaliana* limit long-distance transport. The mechanisms behind this resistance involve transmembrane domain-containing small heat-shock-like proteins. Homologs of these genes are found in several plant families. Finally, the product of the polerovirus gene P0 is a strong silencing suppressor that has been found to target ARGONAUTE1, a key role player in the silencing mechanism of plants, for degradation. Yeast-two-Hybrid assays have identified sHSPs as cellular partners for P0.

It is not clear whether the interaction of GLRaV-3 p20A with the sHSP of *V. vinifera* is a result of the host attempting to protect itself against the virus, the virus manipulating the defence response of the plant for long-distance transport, or a mechanism employed by the virus to counteract RNA-induced silencing of virus genes. In a study conducted on transgenic *N. benthamiana* plants infected by cucumber green mottle mosaic virus (CGMMV), overexpression of sHSPs reduced viral RNA accumulation and slowed down disease development, whereas silencing of the sHSP genes did not affect CGMMV infection.

8. CONCLUSIONS AND RECOMMENDATIONS

The use of the GVA infection clone was a crucial part in the development of an RNAi-based control strategy for GLRaV-3, as it would have been used to deliver silencing constructs targeted towards GLRaV-3. Unfortunately, we were unable to succeed in establishing stable infections of grapevine using this clone.

This project has, however, delivered interesting insights into the protein-protein interactions involved in onset of GLD by GLRaV-3 infection. Virus-virus protein interactions that have previously been inferred by homology, have been demonstrated experimentally. Additional interactions, most notably those between the structural proteins of the virus and its silencing suppressor, have been identified using BiFC. This could lead to an increased understanding of how the virus overcomes host defence responses.

Two previously unreported virus-host interactions have also been found. The interaction of the GLRaV-3 CP with a key enzyme involved in carbohydrate metabolism may shed light on the underlying mechanisms behind long distance viral transport and the effect of GLRaV-3 on sugar content, secondary metabolites and phenology of infected plants. The interaction of GLRaV-3 ORF9 with a host sHSP could assist in determining the function of this gene. Future studies could focus on the effect of increased or decreased expression of the sHSP on virus replication and disease progression, which can lead to the development of disease-resistant crops.

Recently, advances have been made in the use of CRISPR to confer GLRaV-3 resistance in grapevine by targeting the GLRaV-3 CP and HSP70h genes for degradation. Combining these techniques with knowledge of the role of the proteins identified in this study in the replication and spread of GLRaV-3 and the mechanisms behind viral silencing suppression, could lead to the development of grapevines resistant to a broader spectrum of viruses.

9. PLANNED OUTPUTS

a) TECHNOLOGY DEVELOPMENT, PRODUCTS AND PATENTS

b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

c) HUMAN RESOURCES DEVELOPMENT / TRAINING (STUDENTS)

Student Name and Surname	Student Nationality	Degree (eg Hons, MSc)	Level of studies in final year of project	Total Bursary Cost for Industry for entire project
Honours				
Masters				
PhD				
Ilani Mostert	RSA	PhD		R 0
Postdocs				
Rachelle Bester	RSA	PD		R 0

d) LIKELY PUBLICATIONS (POPULAR, PRESS RELEASES, SCIENTIFIC)

Scientific: Identification of protein-protein interactions between Grapevine leafroll-associated virus 3 and Vitis vinifera

Scientific: Identification of interactions between the proteins of Grapevine leafroll-associated virus 3

e) PRESENTATIONS/PAPERS THAT COULD BE DELIVERED

Previously delivered:
Mostert I, Maree HJ, Burger JT (2019) Optimising the use of grapevine virus A infectious clones for transient gene expression and silencing. 51st Congress of the Southern African Society for Plant Pathology (SASPP), Langebaan, South Africa. (Oral)

Future presentations:
Virus-virus and virus-host protein-protein interactions of Grapevine leafroll-associated virus 3 And Vitis vinifera

10. PROJECT OUTCOME AND IMPACT

New Knowledge	Benefits Supply Chain	Direct Grower Application	Direct Packhouse/Winery/	Other

			Cellar Application	
X				

Other is:

The Value of the project to industry

This project yielded new knowledge on how grapevine leafroll-associated virus 3 (GLRaV-3) interacts with its host at a protein level to induce grapevine leafroll disease (GLD). This new knowledge can be exploited to potentially develop new strategies to combat GLD.

11. PERSONS PARTICIPATING IN THE PROJECT:

INITIALS AND SURNAME	HIGHEST QUALIFICATION	RACE (M,W)	GENDER (M,F)	INSTITUTE DEPARTM	POSITION	TOTAL COST TO PROJECT
RESEARCH PERSONNEL						R 0
SUPPORT PERSONNEL						R 0

POSITION: Co = Co-worker (other researcher at your institution)
Coll = Collaborator (participating researcher that does not receive funding for this project from industry)
PF = Post-doctoral fellow
PL = Project leader
RA = Research assistant
TA = Technical assistant/ technician

12. TOTAL COST OF PROJECT

TOTAL ANNUAL COSTS (ALL YEARS)	CFPA	RAISIN SA	HORTGRO	SATI	WINETECH	ARC	OTHER	TOTAL
2017	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0
2018	R 0	R 0	R 0	R 0	R 75000	R 0	R 0	R 75000
2019	R 0	R 0	R 0	R 0	R 843000	R 0	R 0	R 843000
2020	R 0	R 0	R 0	R 0	R 802405	R 0	R 0	R 802405

2021	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0
2022	R 0	R 0	R 0	R 0	R 0	R 0	R 0	R 0
TOTAL	R 0	R 0	R 0	R 0	R 30	R 0	R 0	R1720405