CFPA	DFPT	DFTS	Winetech
Canning Fruit Producers' Assoc. Submit to: Wiehahn Victor PO Box **426** **Paarl, 7620** **Tel: +27 (0)21 872 1501** **inmaak@mweb.co.za**	Deciduous Fruit Producers' Trust Submit to: Louise Liebenberg Suite 275, Postnet X5061 Stellenbosch, 7599 Tel: +27 (0)21 882 8470/1 **louise@dfptresearch.co.za**	Dried Fruit Technical Services Submit to: Dappie Smit PO Box 426 Paarl, 7620 Tel: +27 (0)21 872 1501 **dappies@dtd.co.za**	Submit to: Jan Booysen PO Box 528 Paarl, 7624 Tel: +27 (0)21 807 3324 **booysenj@kwv.co.za**

			X

Indicate (X) client(s) to whom this final report is submitted.
Replace any of these with other relevant clients if required.

FINAL REPORT
FOR 2015

PROGRAMME & PROJECT LEADER INFORMATION

	Programme leader	**Project leader**
Title, initials, surname	Prof JT Burger	Dr M-J Freeborough
Present position	Associate Professor	Post-Doctoral Fellow
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PROJECT INFORMATION

Project number	GenUS 6/2

Project title	Grapevine transformation with virus resistance constructs and evaluation of resistance (GenUS6/2)

Industry programme	**CFPA**	
	DFPT	
	DFTS	
	Winetech	Grapevine viruses
	Other	

Fruit kind(s)	Grape

Start date (dd/mm/yyyy)	08/06/2007	**End date** (dd/mm/yyyy)	31/12/2014

FINAL SUMMARY OF RESEARCH PROJECT

PROGRAMME & PROJECT LEADER INFORMATION

Title, initials, surname	Prof JT Burger	Dr M-J Freeborough
Institution	Stellenbosch University	Stellenbosch University
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Start date (dd/mm/yyyy)	08/06/2007	**End date** (dd/mm/yyyy)	31/12/2014

(Give a summary of the *total* project in no more than 250 words).

Two virus resistance constructs created in projects GenUS1/2 (ΔRSP-LR-SAScon) and GenUS1/4 (ΔHSP-Mut) were revived from cold storage and validated to contain the correct virus resistance constructs. These two constructs were supplied to the IWBT for transformation in to embryogenic grapevine tissue. *In vitro* plantlets of rootstock Richter110 transformed with the HSP-Mut have been supplied for genetic analysis. These plantlets have been subjected to genetic analysis to determine copy number and transcript.
The Merlot material containing the HSP-Mut construct and the Richter110 material containing the RSP-LR-SAScon construct were not delivered.

Because of several technical difficulties to stock sufficient rootstock material, an extension to the project was requested. The project was revived two years ago to evaluate the remaining transgenic lines in greenhouse conditions for GLRaV-3 resistance. All tested transgenic grapevine lines consistently express a mutated HSP protein from GLRaV-3 (HSP-Mut) in the grapevine rootstock Richter 110. In this milestone, resistance was tested by grafting shoots of six transgenic plant lines transgenic shoots onto healthy Chardonnay and Cabernet Sauvignon plants in the greenhouse using bud-grafting. After approximately five months, GLRaV-3 virus titres of all grafted plants were quantified relative to two reference genes using RT-qPCR. Results showed that resistance levels of plant line #3 was significantly enhanced (>99%) and remarkably, plant line #14, showed to be more susceptible to the virus.

FINAL REPORT
(Relevant publications may replace the final report)

1. Problem identification and objectives

State the problem being addressed and the ultimate aim of the project.

Grapevines are subject to many virus diseases. Some of these diseases are associated with one or more grapevine infecting viruses. The complexity of virus-associated diseases makes control of the diseases difficult, as most molecular strategies designed to control virus diseases generally target specific viruses. This laboratory has designed and developed two antiviral constructs that may have the ability to control one or more viruses associated with important grapevine virus diseases.

The aim of this project is to transform into grapevine callus two antiviral constructs and generate transgenic grapevine plants for further analysis of virus resistance. The constructs have been provided to the IWBT for transformation into Richter110 and Merlot embryogenic callus material. The objectives for this year are to receive plant material from each of the three transgenic events and perform genetic analysis on the transformed plant material to

determine the copy number and transcript levels of the different transgenes via qPCR analysis. We aim to select certain plants to be hardened off and bulked up for virus challenge and resistance experiments.

2. Workplan (materials & methods)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage and examination stages and parameters.

Agrobacterium cultures containing two constructs created during previously funded Winetech projects were revived and analysed to determine the construct for transformation. These cultures were grown and delivered to the IWBT for transformation into Richter110 and Merlot cultivars of grapevine. Transgenic Richter110 plantlets were delivered to our laboratory for genetic analysis to determine copy number and relative transcript expression levels. These tests were performed using quantitative PCR technologies using Reletive expression software test (REST).

In the earlier part of the project stable transformed grapevine rootstock was generated in collaboration with the IWBT and was evaluated in a contained environment. The transgenic grapevine lines consistently express a mutated HSP protein from GLRaV-3 (HSP-Mut) in the grapevine rootstock Richter 110. It is thought that the excess of mutated HSP proteins will block cell-to-cell movement of GLRaV-3 and therefore confer resistance. Five transgenic and two non-transgenic in Richter 110 lines will be evaluated for their resistance to GLRaV-3 in this milestone. For that, transgenic lines have been already transferred from in vitro conditions into the greenhouse environment. Shoot material is sterilized and multiplied back into in vitro conditions. This procedure was chosen as contained space for the evaluation of fully-grown plants is limited at the moment. It is planned to infect in vitro plantlets with GLRaV-3 by using the natural mealy bug vector. In collaboration with Dr Kruger from the University of Pretoria a mealybug culture was established in the Stellenbosch laboratory. GLRaV-3 infected plants have also been stocked up in vitro. To test the resistance of the transgenic Richter 110 lines, healthy mealybugs will be allowed to feed on GLRaV-3 infected plants for 24h before they will be transferred to transgenic and non-transgenic Richter 110 plantlets. Mealybugs will be allowed to feed on the transgenic plants and non-transgenic plants for 5 days before plantlets are treated with insecticide. Treated plantlets will be adopted to greenhouse conditions and tested for their GLRaV-3 titre after 4 weeks. GLRaV-3 titres from transgenic and non-transgenic plants will be compared.

3. Results and discussion

State results obtained and list any benefits to the industry. Include a short discussion if applicable to your results.
This final discussion must cover ALL accumulated results from the start of the project, but please limit it to *essential* information.

Milestone	**Achievement**
1. Culture *Agrobacterium* strains and deliver to IWBT	Achieved
2. Transform constructs into grapevine callus	Achieved
3. Receive plants for genetic analysis	Genetic analysis of the Richter110 ΔHSP-Mut in vitro plantlets has been completed. Genetic analysis of the transgenic plantlets delivered have been completed by qRT-PCR

4. Subject plants to virus infection and assay for resistance	Leafroll infected canes have been cultivated, Virus free plants have been obtained and grown in the greenhouse, grafting of transgenic buds on Chardonnay and Cabernet Sauvignon plants were done. Quantitative virus detection assays on these plants have been conducted.

Molecular characterisation of genetically transformed grapevine with resistance to GLRaV-3, was also performed in this study. In addition to detecting and identifying infected grapevine and the removal of these plants, plants can also be genetically engineered to provide resistance to pathogens as an additional strategy for eradicating disease. qPCR with REST analysis was used to estimate the transgene (ΔHSP-Mut) copy numbers relative to three reference genes (β-tubulin, cyclophilin and GAPDH). Southern blot analysis was also performed and the results of the two techniques were compared. The results for both techniques showed some discrepancies, nonetheless 76.5% of samples correlated for the copy number estimations by both techniques.

Details for the qPCR to determine the copy number are listed below, but full details of the REST analsis are listed in Miss Malan's Dissertation.

Primer name	**Sequence**	**Fragment size**	**Ta***
HSP-70h F	GGGGGTCAAGTGCTCTAGTT	470 bp	56°C
HSP-70h R	TGTCCCGGGTACCAGATTAT		
Cyclophilin F	TGTGACCTGAACCACTTGA	451 bp	56°C
Cyclophilin R	CCGGTAGGATTGTGATGGAG		
ß-Tubulin F	TGGTGACCTGAACCACTTGA	479 bp	56°C ß-
Tubulin R	TCACCCTCCTGAACATCTCC		
GAPDH F	AGGGAGGAGTCAGAGGGAAA	455 bp	56°C
GAPDH R	GTGTGGCTGTGGCAGAGTTA		
ClosF1	CCATGGAAGTAGGTATAGATTTGG	1500 bp	55°C
ClosR2	TTATCCATTCAAAATCGTGTC		

* Annealing temperatures for DNA template

Detection of the transgene was further optimised with qPCR. For quantification of the transgene, the DNA extracted from transgenic plants was amplified with primers for the reference gene as well as the transgene. For the construction of a standard curve, serial 5-fold dilutions of the DNA of a specific sample were made (from 250ng to 0.4ng) and amplified, in duplicate, with the primers for the gene of interest (HSP- 70h) and reference genes (ß-Tubulin, Cyclophilin and GAPDH). The standard 25µl qPCR reaction was prepared (Appendix A) and the optimised qPCR cycles for the gene of interest and reference genes were followed as described in Appendix A with annealing temperature as indicated in Table 2.

For the amplification of the remainder of the samples, 50ng of DNA from each sample was also amplified in duplicate with both sets of primers (gene of interest and reference genes). One sample had to be defined as a calibrator sample. Preferably this would be a sample of which the copy number is known to which the data of the other samples can be normalised (we chose sample 9 and later verified the copy number by Southern blot). This sample is also included in every run in order to compensate for different efficiencies for the same type of reactions performed in different runs in order to make them comparable.
Different methods were compared for the determination of transgene copy number, namely the two standard curve method, delta delta Ct method and the relative expression software tool (REST). Since the efficiency of the two reactions (transgene and reference gene amplification) differed, the delta delta Ct method could not be used. Thus a model where different efficiencies for reference and transgene are taken
into account, were rather used. This model, REST, was introduced for the determination of mRNA expression levels by Pfaffl et al. (2001), but have since also been employed in copy number determination (Škulj et al. 2008). The Ct values of the sample in question and the

calibrator sample amplified with the two different primer sets (gene of interest and reference gene) are imported into the program, as well as the different efficiency values for these reactions (see equation 1). The program performs 50 000 mathematical iterations to generate an estimated copy number value.

$$\text{Ratio} = \frac{(E\ \text{GOI})^{\Delta Ct\ \text{GOI (Control – Sample)}}}{(E\ \text{Ref})^{\Delta Ct\ \text{Ref (Control – Sample)}}}$$

This indicates that both techniques were able to produce accurate results; however techniques that are less time consuming and labour intensive are more suitable to high through-put analysis. qPCR is able to deliver results within one day, whereas a Southern blot requires several days without any apparent advantage in accuracy. Thus qPCR can effectively be utilized for copy number estimation in transformed plants. qRT-PCR have been utilized in various studies for the estimation of RNA expression levels in transformed plants. In this study, qRT-PCR was used without validation by Northern analysis. The transformed grapevines were subjected to qRT- PCR analysis to determine transgenic mRNA expression levels relative to the GAPDH reference gene. Hereby suitable candidates for further GLRaV-3 resistance studies were identified. Several plant lines showed high levels of ΔHSP-Mut expression (9, 10, 11, 13 14 18 and 19) and would be utilized in further GLRaV-3 resistance trials. Future studies for this aspect of the project would entail exposing these transformed plants to insect vectors carrying GLRaV-3. The qRT-PCR detection system developed in this study can then be used to detect these low titres of GLRaV-3 and confirm resistance of the plant to the virus.

Table 3: ΔHSP-Mut copy numbers for the transgenic samples relatively quantified to each of the reference genes as well as determined by the Southern blot analysis. ΔHSP-Mut expression levels for the transgenic samples relatively quantified to GAPDH

	Copy numbers				***Expression levels***
Sample	**B-tub**	**Cyclophilin**	**GAPDH**	**Southern**	**GAPDH**
1	>4	>4	>4	>4	3
2	1	1	1	1	1
3	1	1	2	-	1
4	1	1	2	>4	7
5	1	1	1	-	4
6	0	0	0	2	0
7	0	0	0	0	0
8	4	4	4	2	1
9	2	2	2	2	>30
10	1	2	2	3	>30
11	>4	>4	>4	>4	>30
12	0	0	0	0	0
13	>4	>4	>4	>4	>30
14	1	1	1	1	>20
15	2	3	3	4	12
16	4	>4	2	3	0
17	0	0	0	0	0
18	2	2	2	2	>20
19	1	1	1	-	>50
20	2	2	2	2	1

In the final part of the project, a number of transgenic grapevine lines were generated that were transformed to express a GLRaV-3 HSP-70-like protein from which the ATPase domain was mutated. Early screens of these lines demonstrated a potential resistance phenotype against GLRaV-3. In this milestone resistance was tested by grafting a total of 236 transgenic #HSP Mut plants onto 175 healthy Chardonnay and 61 healthy Cabernet Sauvignon plants in the greenhouse using bud-grafting. The grafting was performed over 10 months of time and the plants were growing for approximately 4-5 months in a standard GMO greenhouse facility. Out of the 236 grafts, 48 plants were already sampled for viral titre analysis, 121 plants died and 65 plants are still growing in the greenhouse. Second and third rounds of sampling on the surviving plants were done during the second half of 2014. A high motality rate of the second batch of plants was caused by fungal infections. The third batch of plants was successful and analyses were performed on all three batches. The six transgenic plant lines (plant lines #1, #3, #9, #14, #15 and #17) as well as a non-modified plant line, were all inoculated with GLRaV-3 by grafting buds of each onto GLRaV-3 infected plant material. After approximately five months, GLRaV-3 virus titres of all grafted plants were quantified relative to two reference genes using RT-qPCR. Results were evaluated by comparing the relative virus titre of each transgenic plant line to that of the non-modified control plant line. Results showed that resistance levels of plant line #3 was significantly enhanced (>99%) and remarkably, plant line #14, showed to be more susceptible to the virus. These results should prompt future studies, in which it will be interesting to graft scion material onto the most promising transgenic plant lines and challenge them with viruliferous mealybugs, which would be a more natural simulation of the field. Subsequently, quantitative analyses of the entire plant are needed to determine the resistance levels to GLRaV-3 as well as the spatial distribution of the virus in the plant.

This study suggested that the use of PDR based on transgenic plant lines containing a dysfunctional viral movement protein can be a potential strategy to control GLRaV-3. Plant line #3 showed significantly enhanced resistance levels to GLRaV-3 when compared to the negative control plant lines. However, not all plant lines in this study showed enhanced resistance levels. More specifically, an increased susceptibility to the virus was observed for plant line #14. Further research on these plant lines, with adequate sample numbers, should provide a better understanding of the mechanism behind the resistance/susceptibility levels.

See MSc thesis, S. Malan:
Real time PCR as a versatile tool for virus detection and transgenic plant analysis.
http://sun.worldcat.org/title/real-time-pcr-as-a-versatile-tool-for-virus-detection-and-transgenic-plant-analysis/oclc/693836455&referer=brief_results

See MSc thesis, F. Suidgeest:
Evaluation of two pathogen-derived resistance strategies for Grapevine leafroll-associated virus 3.
http://hdl.handle.net/10019.1/80066

4. Accumulated outputs

List ALL the outputs from the start of the project.
The year of each output must also be indicated.

Technology developed

The use of qPCR to determine gene copy and transcript levels has been validated for transgenic plants.

At least two transgenic grapevine lines with demonstrated enhanced resistance against GLRaV-3 infection are available for further evaluation.

Human resources developed/trained

Two MSc students (white female) were being trained and graduated with Masters degrees in this project.

Patents

No patents have been applied for at present.

Publications (popular, press releases, semi-scientific, scientific)

One publication will be completed when remaining transgenic plants are subjected to testing.

Two MSc theses were completed during the study.

Presentations/papers delivered

A poster was presented at the SASEV conference 2008 and at the ICVG conference in Dijon France 20009.

4. Total cost summary of project

	Year	CFPA	DFPT	DFTS	Winetech	THRIP	Other	TOTAL
Total cost in real terms for year 1	2007				107 200	107 200		**214000**
Total cost in real terms for year 2	2008				180 000	60 000		**240000**
Total cost in real terms for year 3	2009				225 000	75 000		**300000**
Total cost in real terms for year 4								
Total cost in real terms for year 5								
TOTAL					**512200**	**242200**		**754400**