# Characterisation of different GLRaV-3 variant infections by determining virus concentration ratios and miRNA expression profiles

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## Summary

Grapevine leafroll disease (GLD) is present in all grape-growing regions of the world and is considered the most significant grapevine viral disease. Grapevine leafroll-associated virus 3 (GLRaV-3) is considered the primary cause of GLD and in South African vineyards five genetic variant groups (I, II, III, VI and VII) have been confirmed. Biological distinctions between GLRaV-3 variants have not been fully validated. By characterising virus concentration and stress-responsive microRNA expression in GLRaV-3 infected plants, this study aimed to glean a better understanding of the possible biological distinctions between GLRaV-3 variants. Quantitative reverse transcription PCR was utilised for virus concentration ratio (VCR) determination and miRNA quantitation in GLRaV-3 positive and negative grapevines grown under greenhouse and field conditions. This study found statistically significant differences in VCRs in plants singly infected with different GLRaV-3 variants. Interestingly, no difference in mean VCRs were observed between data sets, despite notable differences in plant age, duration of GLRaV-3 infection, scion/rootstock combination and growing conditions. Several miRNAs showed statistically significant expression modulation between infected and healthy samples. miRNA expression between data sets varied substantially and a greater overall miRNA response was observed in plants with more established GLRaV-3 infections. The lack of significant differences in mean VCRs between data sets, coupled with the consistent modulation of certain miRNAs in plants that have likely been infected for longer is a promising result. This finding could indicate that successful inhibition of further virus replication by plant defence mechanisms occurred, and that these miRNAs are implicated in this response.

K e y　w o r d s :　virus concentration ratios; grapevine leafroll disease; microRNAs; RT-qPCR; 'Cabernet Sauvignon', grapevine leafroll-associated virus 3.

## Introduction

The grapevine industry has global economic importance, however, its susceptibility to virus infection, and the resulting negative effects of the associated disease complexes, threaten its sustainability (Naidu *et al*. 2014). Internationally, more viruses have been identified in grapevine than any other fruit crop (Martelli 2014). Diseases caused by the various virus infections of grapevine can be divided into five major viral disease complexes, of which Grapevine leafroll disease (GLD) is arguably the most economically important (Atallah *et al*. 2012, Almeida *et al*. 2013, Maree *et al*. 2013 and Naidu *et al*. 2014, 2015). GLD significantly impairs overall plant health, with negative effects such as decline in plant vigour and lifespan, disruption of phloem, reduction of crop yield and quality (Cabaleiro *et al*. 1999, Naidu *et al*. 2014, Alabi *et al*. 2016). The foliar symptoms of most red-fruited cultivars, as reviewed by Maree *et al*. (2013), include a downward rolling of leaf edges towards the later stages of the growing season, as well as reddening of interveinal leaf areas. Less pronounced yellow discolouration of leaves can be seen in some white cultivars. Although some asymptomatic grapevine varieties have been identified, no natural source of GLD resistance has been found in *Vitis vinifera* (Weber *et al*. 1993, Martelli 2000, Naidu *et al*. 2014).

Several virus species in the family *Closteroviridae* contribute to GLD etiology, of which *Grapevine leafroll-associated virus 3* (GLRaV-3) is considered the primary causative agent (Maree *et al*. 2013). Eight genetic variant groups of GLRaV-3 have been identified internationally (Ling *et al*. 2004, Engel *et al*. 2008, Maree *et al*. 2008, Jooste *et al*. 2010, Gouveia *et al*. 2011, Bester *et al*. 2012a, Maree *et al*. 2015, Thompson *et al*. 2018). To date, five of these variant groups (I, II, III, VI and VII) have been identified in South African vineyards (Maree *et al*. 2008, Jooste *et al*. 2011, 2012, Bester *et al*. 2012a, Goszczynski 2013, Jooste *et al*. 2015, Maree *et al*. 2015). A survey by Jooste *et al*. (2012) showed GLRaV-3 to be the most prevalent virus in South African vineyards. Recent findings from around the world indicate that some variants are more prevalent than others in screened vineyards (Sharma *et al*. 2011, Jooste *et al*. 2011, 2015, Farooq *et al*. 2013, Chooi *et al*. 2013). This may point toward differences in the efficiency of virus variants to infect host plants and spread within and between vineyards. The first evidence of GLRaV-3 variants being biologically distinct was produced by Blaisdell *et al*. (2012), who showed significant differences in transmission efficiency between variant groups I and VI, as tested in the Napa Valley, California. In a recent South African report, Bester *et al*.

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(2014) found a significant difference in virus concentration ratio (VCR) between plants infected with variant groups II and VI, respectively. Variant group II showed a higher concentration when compared to group VI, indicating possible differences in the efficiency of viral infection and replication within the host, between variants of GLRaV-3. Chooi *et al.* (2016) reported significant differences in GLRaV-3 concentration between rootstocks infected with variants groups I and VI, evaluated under greenhouse conditions in New Zealand. The validation and characterisation of such differences at the molecular level is an essential next step in understanding GLRaV-3 infection, and the contribution of the different variants to GLD etiology.

Limited studies have focussed on characterising the molecular basis of plant-pathogen interactions in GLRaV-3 infection. Grapevine leafroll disease has been recognised as a potential threat to the viticulture industry for several decades, yet our knowledge of the disease remains limited due to the complex nature of its etiology and contrasting symptom expression in red- and white-fruited cultivars (Naidu *et al.* 2014). Gaining knowledge of the molecular mechanisms underlying GLRaV-3 infection therefor remains a high priority. Plant small RNAs, such as microRNAs (miRNAs), play a crucial role in virtually all aspects of plant growth and development (Chuck and O'Connor 2010), as well as to mediate stress responses to environmental factors (Guleria *et al.* 2011, Khraiwesh *et al.* 2012, Sunkar *et al.* 2012). MicroRNAs negatively regulate the expression of target genes through cleaving of target mRNAs (Guleria *et al.* 2011, Khraiwesh *et al.* 2012) or via transcriptional/translational repression (Guleria *et al.* 2011). Investigating biotic stress-responsive miRNA expression in GLRaV-3 infection may yield valuable insights into the molecular mechanisms of the GLRaV-3 stress response. Additionally, the characterisation of miRNA expression profiles could show a correlation with differences observed in virus concentration and prevalence of certain variants observed in screened vineyards. This data could facilitate further host-pathogen interaction studies, with specific reference to the genetic variability of GLRaV-3. This could also ultimately aid in the development of more targeted GLRaV-3 and GLD intervention strategies.

**Material and Methods**

Plant material: A set of own-rooted *Vitis vinifera* 'Cabernet Sauvignon' plants representing GLRaV-3 variant groups I, II, III and VI has been established under greenhouse conditions, from cuttings of highly symptomatic GLRaV-3 infections found in commercial vineyards. These plants have been previously confirmed to be free of the following common grapevine viruses; grapevine leafroll-associated virus 1, -2, -5, -9, grapevine rupestris stem pitting-associated virus, grapevine virus A, grapevine virus B and grapevine fanleaf virus. Phloem material from these plants was sampled to yield the first data set (2014 GH). Additionally, young 'Cabernet Sauvignon' plants, obtained from a certified nursery, were established and graft-inoculated with single infections of the five known variants of GLRaV-3 found in South African vineyards. Plants were maintained in a climate-controlled greenhouse and sampled twice, six months apart, to yield two data sets (2015 1 GH and 2015 2 GH). Greenhouse conditions included natural light, with temperatures ranging between 22 °C and 28 °C. Soil- and potting conditions included the use of five litre bags filled with a mixture of sand (45 %), vermiculite (10 %) and coco peat (45 %). A fourth data set (2016 field) comprised of GLRaV-3 positive and negative plants sampled at one time point, in mid-summer of 2016, from five 'Cabernet Sauvignon' vineyards between two farms (Farm A and B) in Stellenbosch. These vineyards were sampled as part of a larger GLD survey. An overview of all 'Cabernet Sauvignon' rootstock/scion combinations investigated in this study is provided in Tab. 1.

Table 1

Summary of scion and rootstock clones and rootstock cultivars of all plants

| | Vineyard block | Scion clone | Rootstock clone | Rootstock cultivar |
|---|---|---|---|---|
| 2015 Greenhouse plants | | | | |
| All | N/A | CS 338 C | RQ 28 C | Richter 110 |
| Field plants (Survey) | | | | |
| Farm A | 1 | CS 163 I | AA 219 F | 101-14 |
| | 2 | CS 163 O | AA 219 F | 101-14 |
| Farm B | 3 | CS 34 B | AA 219 F/662 | 101-14 |
| | 4 | CS 169 A | AA 26 B /25 A | 101-14 |
| | 5 | CS 169 B | AA 219 F | 101-14 |

RNA extraction: A CTAB buffer extraction protocol (Carra *et al.* 2007) as modified in Bester *et al.* (2014) was used for total RNA extraction from two grams of phloem scrapings from lignified canes in all instances. Phloem material was exposed by removing the bark layer in each case, which allowed it to be scraped and collected using a sharp blade. DNase treatment was performed using RQ1 RNase-free DNase (Promega). Five µg of total RNA were treated in 50 µL reactions, following instructions provided by the manufacturer. The quality of RNA was assessed by spectrophotometry (Nanodrop 1000 or 2000) and gel electrophoresis (2 % Tris-acetate-EDTA (TAE) agarose gel).

Virus detection

GLRaV-3 infection status and variant group screening: The GLRaV-3 infection status of all plant samples was confirmed using an end-point RT-PCR assay (Bester *et al.* 2014). Initial virus screening of the 2015 greenhouse plants was performed using a rapid one-step RT-PCR method (MacKenzie 1997) with primers targeting ORF1a of GLRaV-3 (Bester *et al.* 2014) to confirm the infection status of the newly graft-inoculated 'Cabernet Sauvignon' plants. A real-time PCR high-resolution melting curve RT-PCR assay (Bester *et al.* 2012b) for variant groups I, II, III and VI, in combination with an end-point RT-PCR assay (Jooste *et al.* 2015) for variant group VII were used to verify the GLRaV-3 variant statuses of all plants.

Virus concentration ratio determination: To determine the relative abundance of GLRaV-3 within plants, virus concentrations were normalised with three stably expressed reference genes to produce virus concentration ratios (VCRs). Virus concentration ratios of all samples were determined using a SYBR Green RT-qPCR assay (BESTER *et al*. 2014) on the Rotor-Gene Q thermal cycler (Qiagen). Virus concentrations were quantified by comparing the expression of the ORF1a gene of GLRaV-3 to the geometric mean of three reference genes used, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH), actin and alpha-tubulin. All reactions were performed in triplicate in Rotor-Gene Q 0.1 mL tube-and-cap strips.

RT-qPCR miRNA expression profiling: MicroRNAs investigated in this study were selected from various sources. Six miRNAs that showed significant expression differences were selected from a study by ALABI *et al*. (2012), in which differential miRNA expression between GLD and healthy samples was assessed using next-generation sequencing (NGS). Four additional miRNAs were selected based on their high expression levels in GLD samples, as determined by a miRNA microarray study (BESTER *et al*. 2017a). Two highly expressed miRNAs, miR167a and miR159c, were selected as reference miRNAs based on their expression stability in healthy and GLD samples (VARKONYI-GASIC *et al*. 2007, ALABI *et al*. 2012). MicroRNA expression levels were measured with a probe-based RT-qPCR assay (CHEN *et al*. 2005, VARKONYI-GASIC *et al*. 2007) using the Rotor-Gene Q thermal cycler (Qiagen). Standard curve samples and controls (NTCs and no-RT) were visualised by 4 % TAE agarose gel electrophoresis to validate primer specificity.

Data analysis: Polymerase chain reaction efficiency, Cq values and quantitation values for all qPCR runs were calculated using the Rotor-gene Q software version 2.3.1 (Qiagen). For the purpose of quantitation, all runs performed included the second dilution point (25X) of the dilution series prepared per gene/miRNA to compensate for possible inter-assay variability. A web-browser application, Harbin (BESTER *et al*. 2017b) was used for all concentration ratio (CR) calculations, by comparing the expression of targets to that of the references/reference gene index. The geometric means of the triplicate reactions were used for all relative quantitation calculations. The geometric mean of the concentration of the appropriate references was used for normalisation of gene/miRNA expression levels. Differential expression analysis between experimental groups was performed using the Wilcoxon rank sum test; with a p-value significance threshold of 0.05 selected in all instances. An Excel-based application, BestKeeper (PFAFFL *et al*. 2004) was used to confirm the stability of all reference genes/miRNAs utilised in this study.

## Results and Discussion

Virus detection
GLRaV-3 infection status and variant group screening in greenhouse plants: The GLRaV-3 infection status of the sixteen plants of the 2014 GH data set was confirmed as described previously.

Twelve plants tested positive for GLRaV-3, and the four healthy control plants were verified to be free of GLRaV-3. The four variant groups of GLRaV-3 (I, II, III and VI) included in this data set were confirmed using a real-time PCR high-resolution melting curve RT-PCR assay (BESTER *et al*. 2012b). Each variant group infection was represented by three biological replicates.

Initial virus screening of the 2015 greenhouse plants was performed to confirm the GLRaV-3 infection status of the newly graft-inoculated *V. vinifera* 'Cabernet Sauvignon' plants. Plants were screened at three time-points, 50, 77 and 105 d post-inoculation. GLRaV-3 variant groups I and II graft-inoculated plants showed the most rapid infection rate, and by 50 d post-inoculation had yielded the most GLRaV-3 positive plants per group, indicating that they might be transmitted more efficiently. Due to the range in transmission efficiency, not all variant group infections were represented by the desired eight biological replicates. A minimum of five GLRaV-3 positive plants per group was deemed sufficient for comparison between variant group infections. Plants infected with variant groups VI and VII had to undergo multiple rounds of grafting to yield at least five positive plants per group. The infection success rates of the different variants after the first round of graft-inoculations were 71.4 %, 62.5 %, 50 %, 12.5 % and 25 % for groups I, II, III, VI and VII, respectively. These findings could imply biological differences between variant groups in terms of pathogenicity, though the contribution of possible virus concentrations differences in the grafting sources may be a contributing factor. The VCRs of the grafting sources were not specifically tested for prior to graft-inoculations. The source material with lower VCRs would likely produce grafting material with a lower abundance of those specific GLRaV-3 variants, thereby reducing the probability of virus transfer and replication in host plants.

Grapevine leafroll disease survey: A GLD survey was conducted early in 2016 to collect symptomatic and asymptomatic material in commercial vineyards. One hundred and seventy-five 'Chardonnay', 'Mourvedre', 'Shiraz', 'Pinot Noir' and 'Cabernet Sauvignon' symptomatic and asymptomatic plants were sampled based on phenotypic (visual) assessment, of which 113 tested positive for GLRaV-3. Due to possible variability of symptom expression, five symptomatic and five asymptomatic plants were sampled per vineyard to compensate for potential false assessments. Thirty plants (26.5 %) tested positive for GLRaV-3 single-variant infections, with the remaining plants showing multiple variant infections of several different combinations. The most prevalent virus variant found was GLRaV-3 variant group II in either single- or mixed infections. The most abundant mixed variant infection was GLRaV-3 II/VI. These findings are in agreement with what was found by JOOSTE *et al*. (2015), in surveys of viruses affecting Western Cape vineyards. This study showed variant groups II and VI to be the most abundant as single-variant infections and in combination with other variants in vineyards screened. The number of single-variant infections found in this study relative to that of Jooste *et al*. (2015) was also comparable at 26.5 % and 37.8 %, respectively. A summary of the GLRaV-3 variant groups detected in all the vineyards sampled is provided in Fig. 1.
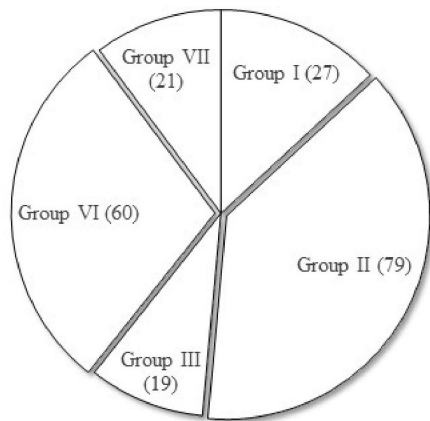
Fig. 1: GLRaV-3 variant group presence as single and mixed infections in all survey plants screened.
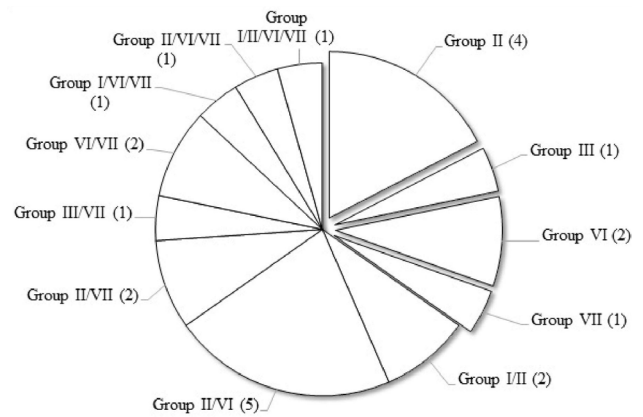


Fig. 2: GLRaV-3 variant group infection status of the 23 GLRaV-3 positive 'Cabernet Sauvignon' plants sampled between Farms A and B. Single-variant infections made up 28.6 % of total infections detected and are shown as elevated segments in the chart. No single-variant infections were detected for GLRaV-3 group I.

For the VCRs and miRNA expression comparisons between variant groups, only the field plants that were of the cultivar 'Cabernet Sauvignon' were used, as this was the same cultivar that was used in the greenhouse data sets. Five vineyard blocks were sampled between two farms. The trend of single-variant infections as a percentage of total infections was upheld in the 'Cabernet Sauvignon' plants (28.6 %). Thirty-eight plants, of which 23 tested positive for GLRaV-3 and 15 negative controls, were used for RT-qPCR profiling of VCRs and miRNA expression levels. The virus-variant infection status of the GLRaV-3 positive plants is summarised in Fig. 2. No single-variant infections for GLRaV-3 group I were found in the 'Cabernet Sauvignon' plants sampled, confirming the low prevalence of variant group I observed in previous South African studies (Jooste *et al.* 2012; 2015). These findings are in contrast to the high prevalence of variant group I found in other grape-growing regions around the world, including the United States of America (Napa Valley), China and New Zealand (Sharma *et al.* 2011, Farooq *et al.* 2013, Chooi *et al.* 2013).

Virus concentration ratios
Virus concentration ratios per data set: A summary of the mean VCRs of all samples in each data set is provided in Fig. 3. The 2016 field data set showed the highest average VCR (1.37) calculated for all GLRaV-3

positive plants. The 2015 2 GH data set had the lowest average VCR (1.05) of the four data sets. No statistically significant differences in mean VCRs were observed between data sets. This finding is of interest when considering the substantial differences between plants comprising these data sets. Plants differed in terms of origin, duration of infection, rootstock, growing conditions and the number of GLRaV-3 variants co-infecting the same plant (Fig. 2). For plants sampled as part of the GLD survey, possible co-infection with other frequently-occurring grapevine viruses could also potentially influence GLD etiology, though these viruses were not specifically tested for. For the sake of brevity we will also refer to field samples that tested negative for GLRaV-3 as "healthy". The lack of statistically significant differences in mean VCRs between data sets, despite likely differences in the duration of GLRaV-3 infection between these plants, is intriguing. This finding suggests that the detrimental effect of GLRaV-3 over time is not directly proportional to the abundance of the virus within the host plant.

Virus concentration ratios per variant group in greenhouse plants: Comparisons were made between VCRs calculated for plants singly infected with different variants of GLRaV-3 to identify possible biological distinctions between variants. Variant groups I
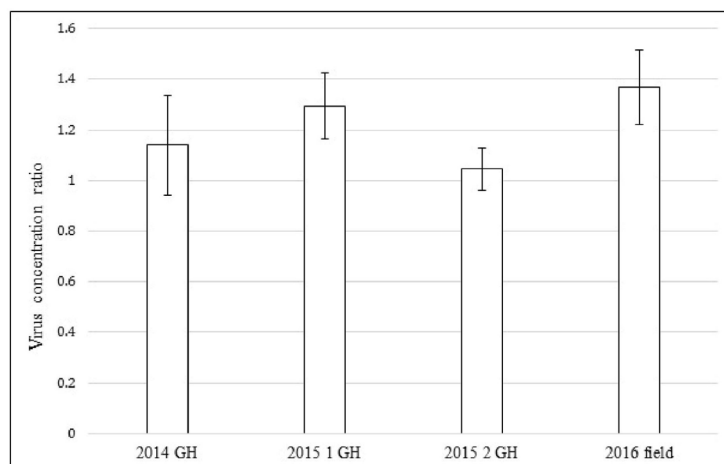


Fig. 3: Mean virus concentration ratios across all GLRaV-3 variant group infections calculated for each data set. Bars indicate standard error.
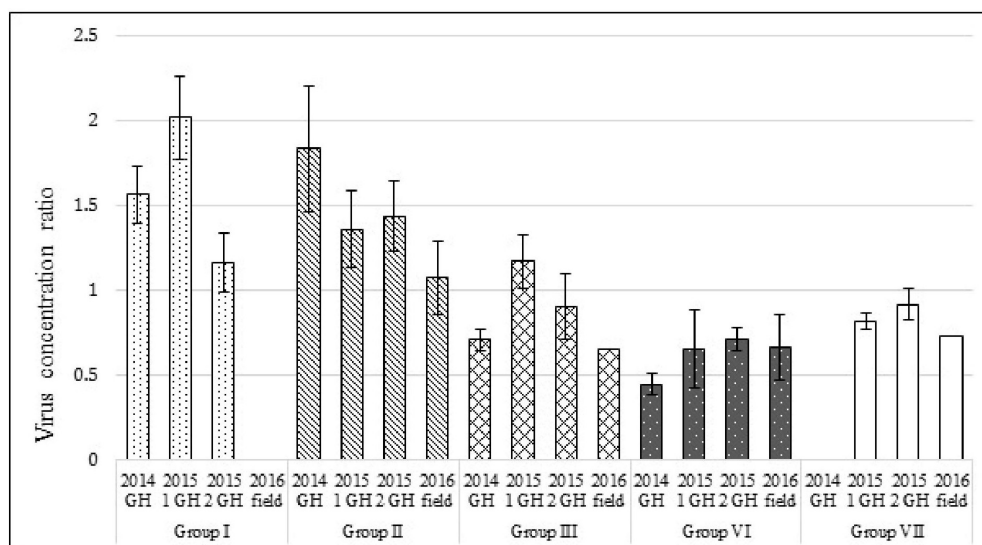
Fig. 4: Mean virus concentration ratios calculated for each GLRaV-3 variant group infection in the four data sets. Bars indicate standard error.

Table 2

Summary of VCR comparisons between variant groups of GLRaV-3 in the greenhouse data sets

| | 2014 GH | | 2015 1 GH | | 2015 2 GH | |
|---|---|---|---|---|---|---|
| | *p*-value | log$_2$ (fold change)[a] | *p*-value | log$_2$ (fold change) | *p*-value | log$_2$ (fold change) |
| Group I *vs* II | 0.7 | 0.2278264 | 0.06494 | -0.5669464 | 0.4634 | 0.3073683 |
| Group I *vs* III | 0.1 | -1.137243 | 0.01515 * | -0.7816216 | 0.2593 | -0.361043 |
| Group I *vs* VI | 0.1 | -1.81377 | 0.009524 * | -1.620869 | 0.01748 * | -0.7092419 |
| Group I *vs* VII | -- | -- | 0.02381 * | -1.305722 | 0.7551 | -0.3391403 |
| Group II *vs* III | 0.1 | -1.365069 | 0.8182 | -0.2146753 | 0.07211 | -0.6684113 |
| Group II *vs* VI | 0.1 | -2.041597 | 0.06667 | -1.053923 | 0.01399 * | -1.01661 |
| Group II *vs* VII | -- | -- | 0.09524 | -0.7387756 | 0.09324 | -0.6465086 |
| Group III *vs* VI | 0.1 | -0.6765274 | 0.1143 | -0.8392476 | 0.8048 | -0.3481989 |
| Group III *vs* VII | -- | -- | 0.1667 | -0.5241003 | 0.7551 | 0.02190273 |
| Group VI *vs* VII | -- | -- | 0.8571 | 0.3151473 | 0.1061 | 0.3701016 |

* Statistically significant differences as determined with the Wilcoxon rank sum test. A *p*-value significance threshold of 0.05 was selected.

[a] The log$_2$ (fold change) values indicate the expression of the last variant group mentioned per line versus the first group (*i.e.* last group/first group).

and II showed consistently higher VCRs when compared to groups III, VI and VII (Fig. 4). This trend was upheld in all greenhouse data sets, possibly indicating variability in the efficiency of virus replication within host grapevine plants, between GLRaV-3 variants. Statistically significant differences in VCRs were observed in five instances (Tab. 2).

The VCRs of plants infected with GLRaV-3 variant groups I and II were significantly higher when compared to variant groups VI and VII in both 2015 GH data sets. The 2014 GH data set yielded no statistically significant VCR differences between variant groups. This was due to the limited number of biological replicates (three) compared per group. Comparisons with variant group VII was also not possible for the 2014 GH data set, as it only consisted of variant groups I, II, III and VI.

Virus concentration ratios of field plants: A statistically significant difference (p=0.0007669) in VCRs was calculated for plants showing single-variant infections relative to plants infected with multiple GLRaV-3 variants. The VCRs of single-variant infected plants were significantly lower [log$_2$ (fold change) value of -0.8947] compared to multiple-variant infected plants. This finding suggests that the GLRaV-3 variants did not have an antagonistic effect on the proliferation of the other variant groups present in the same plant. This could also be an indication that two or more virus variants act in synergy by co-expressing suppressors of silencing for example, as indicated by the higher average VCR calculated for plants showing multiple variant infections. No statistically significant differences in VCRs were measured between plants from the two different

farms sampled for the 2016 field data set. The comparison of VCRs between single-variant infected field plants was not possible due to the limited number of plants per variant group infection (Fig. 2).

R T - q P C R   m i R N A   e x p r e s s i o n   p r o f i l i n g : Several miRNAs showed statistically significant expression modulation between GLRaV-3 infected and healthy samples in the different data sets. The differentially expressed miRNAs are summarised in Tab. 3.

Table 3

Differentially expressed miRNAs across all data sets

| Data set | $\log_2$ (fold change)[a] | *p*-value |
|---|---|---|
| 2014 GH | | |
| miR408 | 2.830734 | 0.01319 |
| miR398b | 2.393181 | 0.01978 |
| miR397a | 1.376623 | 0.05824* |
| miR164a | -0.5376954 | 0.02967 |
| miR162 | -0.3850385 | 0.02967 |
| 2015 1 GH | | |
| miR397a | -0.9089252 | 0.01909 |
| miR162 | -0.7476287 | 0.0312 |
| 2015 2 GH | | |
| miR166h | -0.746873 | 0.007346 |
| 2016 field | | |
| miR408 | -0.9884125 | 0.005518 |
| miR398b | -1.5173534 | 0.0001185 |
| miR397a | -1.733065 | 1.461E-06 |

\* This value is above the p-value threshold of 0.05; however, the significant $\log_2$ (fold change) value warrants inclusion of this miRNA.

[a] $\log_2$ (fold change) was calculated as diseased/healthy.

C o m p a r i s o n   o f   m i R N A   e x p r e s s i o n   b e - t w e e n   d a t a   s e t s : The miRNA expression of plants from the different data sets varied considerably. There were several distinctions to be made between the properties of the plants utilised in each data set. MicroRNA expression observed per data set was therefor compared, to relate the differences observed to these plant differences. Plants differed in terms of growing conditions, rootstock, age and duration of infection.

The 2014 GH data set showed the highest number (five) of differentially expressed miRNAs between diseased and healthy samples. The 2015 1 GH and 2015 2 GH data sets yielded limited statistically significant results. Both the 2014 GH and 2015 GH data sets are of the same cultivar, grown under the same greenhouse conditions. The main differences between these plants are that the 2014 GH data set consists of own-rooted plants established before the 2015 GH data sets, which are grafted plants (Table 1) that were established at the end of 2014. The lower degree of significant miRNA expression modulation seen in grafted Cabernet Sauvignon plants relative to own-rooted plants is consistent with results recently obtained (BESTER *et al.* 2017c).

Differential miRNA expression in the 2016 field data set was also more pronounced than the 2015 GH data sets. The 2015 GH and 2016 field sets all consist of plants that were grafted onto different rootstocks (Tab. 1). Apart from growing conditions differing between the greenhouse and field plants, these data sets differed substantially in terms of age. Vineyards from the 2016 field data set were established in 2003 and 2011 and had lost their mother block status, due to a GLD incidence of more than 3 % per vineyard, in 2011 and 2015 (farms B and A, respectively). Therefore, the GLRaV-3 infection in these plants could be more established compared to the 2015 GH data sets. The data suggests an association between miRNA expression in GLRaV-3 infection and the age of the plant/duration of infection.

M i c r o R N A   e x p r e s s i o n   i n   f i e l d   p l a n t s : The differential miRNAs in the 2016 field data set, miR408, miR398b and miR397a showed similar expression trends between diseased and healthy samples across the two farms. In all three cases the down-regulation observed for the specific miRNAs was the most pronounced for plants sampled from farm B. A summary of mean concentration ratios of these three miRNAs between the different vineyards sampled is provided in Fig. 5. No statistically significant differences in miRNA CRs were measured between single- and multiple-variant group infected plants in the 2016 field data set. Both farms utilised the same rootstock for all plants sampled (101-14), however, the scion clones used in the five vineyards differed (Tab. 1). Given the prominent differences in miRNA regulation observed between the two farms, it was of interest to investigate what distinctions could be made between plants from these farms.

The most apparent difference between the vineyards from the two farms is the time at which they were established. Vineyard blocks from farm B had already lost their mother block status by the time the vineyard blocks from farm A were first established (2011) and therefor likely had a more established infection status than that of farm A. This finding correlates with what was found in the greenhouse data sets, and data recently generated in our research group by BESTER *et al.* (2017c). These trends suggest a greater miRNA response in plants with a longer and more established infection of GLRaV-3, and is not necessarily correlated with virus concentration.

**Conclusions**

The addition of biological data to support the argument of GLRaV-3 variants being biologically distinct is a useful contribution to GLD research. This data could ultimately aid in further understanding the plant-pathogen interactions in GLRaV-3 infection and the contribution of different virus variants to GLD etiology. The fact that the same miRNAs (miR398b, miR397a and miR408) were differentially expressed in the two data sets from plants that were likely infected with GLRaV-3 for longer (2014 GH and 2016 field) suggests that these miRNAs may be directly involved in defence mechanisms inhibiting GLRaV-3 replication. These miRNAs do not necessarily form part of a universal response to GLRaV-3 infection as the directionality of expression
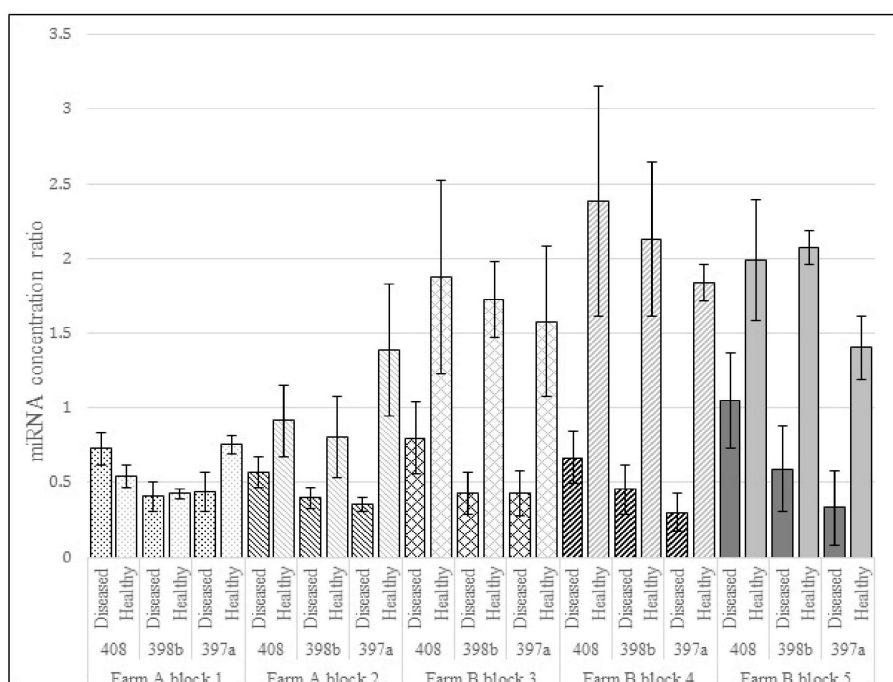
Fig. 5: Comparison of miRNA concentration of miR408, miR398b and miR397a per vineyard sampled in the 2016 field data set. Vineyard blocks are pattern-labelled. Bars indicate standard error.

differed between data sets. By utilising next-generation sequencing (NGS) technology, coupled with qPCR validation, this study can be expanded to investigate miRNAs beyond the small panel evaluated here. Grapevine leafroll disease is unique in that symptom expression, or the lack thereof, corresponds in broad terms to two distinct phenological stages, namely pre-véraison and post-véraison. MicroRNA expression has also been shown to differ in various tissue types and in different physiological growth stages. Thus, sampling at different physiological/phenological stages to enable comparisons between these, could yield valuable insights. It would be of interest to evaluate virus concentration and miRNA expression in GLD at pre-véraison and post-véraison, to see if any expression changes occur with symptom development. This will aid in elucidating the mechanisms underlying GLD symptom development, and may shed light on why some cultivars remain asymptomatic.

## Acknowledgements

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