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Dissecting Novel Grapevine-Mealybug-Virus Interactions

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by

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Abstract

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Professor Rodrigo P.P. Almeida, Chair

The biological mechanisms underlying vector transmission of grapevine leafroll-associated virus 3 (GLRaV-3) remain poorly understood due to limitations of a technically challenging host-pathogen system in *Vitis vinifera*. GLRaV-3 was able to infect the model organism *Nicotiana benthamiana* by insect-vector mediated transmission using the vine mealybug, *Planococcus ficus.* Working with GLRaV-3 infected *N. benthamiana* revealed distinct advantages in comparison with its natural host *Vitis vinifera*, yielding both higher viral protein and virion concentrations in western blot and transmission electron microscopy (TEM) observations, respectively. Immunogold labelling of thin sections through *N. benthamiana* petioles revealed filamentous particles in the phloem cells of GLRaV-3 positive plants. Comparison of assembled whole genomes from GLRaV-3 infected *V. vinifera* vs. *N. benthamiana* revealed identical sequences. High throughput sequencing was used to compare host response to GLRaV-3 infection between *V. vinifera* and *N. benthamiana*. General families of differentially expressed genes (DEGs) common in both hosts followed similar expression changes with six upregulated, seven downregulated, and two stably expressed genes in common. Overall, both hosts have many DEGs unique to each host as well as responses in common to GLRaV-3 infection. The vine mealybug, *Planococcus ficus,* fed through a membrane feeding system on GLRaV-3 viral purifications from both *V. vinifera* and *N. benthamiana,* and transmitted the virus to test plants. An immunofluorescence approach was used to localize virions to two retention sites in *P. ficus* mouthparts. Assays testing molecules capable of blocking virus transmission demonstrated that GLRaV-3 transmission by *P. ficus* can be disrupted. Our results indicate that our membrane feeding system and transmission blocking assays are a valid approach and can be used to screen other candidate blocking molecules. GLRaV-3 continues to impact grape-growing regions worldwide and the lack of knowledge surrounding virus-vector interactions remains limiting to the field. Elucidating the transmission biology of this important virus contributes to the eventual goal of blocking of transmission in insect vectors and the development of improved control strategies in vineyards.

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Chapter 1

INTRODUCTION

Plant diseases caused by insect-transmitted pathogens are of great importance to agriculture worldwide. Epidemics of such diseases have led to famine in African countries and severe economic losses in North America, Europe and elsewhere. These diseases are primarily studied by focusing on the pathogen or vector alone, rather than the combined interactions between the pathogen and vector required for disease spread. Consequently, control strategies usually focus on pesticide applications to reduce vector populations or the long-term development of resistant plant material; these approaches are costly, and in the case of widely used pesticides, have negative environmental impacts. The management of these pathogens would greatly benefit from the development of novel disease control strategies.

Grapevine leafroll disease (GLD) is a disease of *Vitis vinifera* and has been described in historical records from mid-nineteenth century Europe and the Mediterranean basin (Naidu, Maree, & Burger, 2015). The disease was first shown to be associated with virus particles in the 1970s (NAMBA et al., 1979). GLD is associated with a complex of viruses from the family *Closteroviridae. Grapevine leafroll-associated virus 3* (GLRaV-3), the main etiological agent of the disease, is a rapidly increasing problem in California and all grape-growing regions of the world. GLRaV-3 has several biologically distinct genetic variants, representing eight major clades (Naidu et al., 2015). GLD symptoms are variable depending on grape cultivar, season, and climate (Maree et al., 2013). In red cultivars, leaves develop red color while interveinal regions remain green. In contrast, white cultivars often remain symptomless or may exhibit subtle chlorotic interveinal regions in leaves making detection and management in vineyards challenging. Infected vines exhibit delayed ripening resulting in lowered brix, which negatively impacts wine quality (Almeida et al., 2013; Over de Linden & Chamberlain, 1970). The total economic impact of GLD remains poorly understood, although it is estimated that yield reductions of up to 40%, increased management costs, and shortened vineyard life spans all can be attributed to GLRaV-3 infection in vineyards (Almeida et al., 2013). Despite the economic importance of this disease little is known about the biology of the virus.

GLRaV-3 is an 18Kb ssRNA virus in the genus *Ampelovirus* transmitted by phloem-sap sucking mealybugs (Hemiptera, Pseudococcidae). GLRaV-3 are transmitted in a semi- persistent manner; the foregut (i.e. mouthparts) is thought to be the site of virus retention in insect vectors for a period of a few days (C. W. Tsai et al., 2008). There appears to be a lack of transmission specificity among mealybugs and other scale insects although the focus of my work is on the vine mealybug *Planococcus ficus* because it has been shown to be an important invasive pest in California vineyards. In addition, fast generation time and efficient virus transmission from first instar nymphs makes them preferable for research purposes (C.-W. Tsai, Rowhani, Golino, Daane, & Almeida, 2010; C. W. Tsai et al., 2008).

For other vector-borne plant viruses transmitted semi-persistently, virus-encoded proteins specifically interact with receptors in the vector, allowing for retention and, consequently, successful transmission between plants (Ng & Falk, 2006). Vector transmission is a complex event in the virus life cycle; however, the molecular determinants of virus-vector interactions of many important plant viruses, including GLRaV-3, remain unknown. Specific proteins, domains, and amino acids required for virus binding to vectors have been identified for the major viral genera transmitted by aphids. Involvement of viral coat proteins and other accessory proteins (helper components) are known to be the two main strategies used by virus

for their transmission (Ng & Falk, 2006). The identity of putative virus receptors in vectors remains a major gap in virus-vector interaction research. However, a recent study identified a protein rich region (called “acrostyle”) at the tip of aphid stylets, which functions as the binding site for *Cauliflower mosaic virus* (CaMV; (Uzest et al., 2007)). This research was the first to identify proteins as putative vector receptors for a non-circulative plant virus. Further advances in this system have allowed the identification of Stylin- 1 the first cuticular protein specifically described in aphid stylets. It has now been shown that stylin-1 interacts with the helper component required for CaMV transmission (Webster et al., 2018). Thanks to these data on virus- insect transmission, it is now feasible and urgent to pursue related questions with GLRaV-3.

Because of the continued impact of GLRaV-3 on grape growing regions worldwide and the lack of knowledge surrounding virus-vector interactions, I studied the development of novel plant model hosts to enable and facilitate the study of the transmission biology of the virus. GLRaV-3 infection in *Vitis vinifera* is a notoriously difficult system to work with because of the length of time required for transmission experiments and low virus yields from purifications. Because of these technical challenges, much of GLRaV-3 research has concentrated on epidemiology or the development of detection assays (Almeida et al., 2013). I was able to infect a herbaceous novel host with GLRaV-3, demonstrate its usefulness as a model organism, and answer initial questions delving into the transmission mechanisms of GLRaV-3 in mealybug vectors. Elucidating the transmission biology of this important virus could contribute to the eventual blocking of transmission in insect vectors and the development of improved control strategies in vineyards.

SUMMARY OF CHAPTERS

Chapter 2 describes the discovery of a novel host for GLRaV-3. I attempted to infect with GLRaV-3 several plant species that are model organisms through insect vector mediated transmission, using the vine mealybug *Planococcus ficus*. I present several lines of evidence demonstrating that GLRaV-3 can infect the herbaceous species *Nicotiana benthamiana*. Infection of *N. benthamiana* allowed me to optimize a protocol for efficient viral purifications, transmission experiments that take half the time needed for detection in the natural *Vitis vinifera* host, and viral protein isolation. I also demonstrated that GLRaV-3 is phloem limited in the novel host and mechanical transmission was not possible. This work lays the groundwork for a less labor-intensive host-pathogen system for future studies and has important implications for GLRaV-3 management in the field.

Because *N. benthamiana* is a novel host for GLRaV-3, it is not known how this organism responds to virus infection. If *N. benthamiana* response to infection is similar to the gene expression profiles observed in *V. vinifera,* it can be a useful model for future genetic studies. Chapter 3 uses next generation sequencing (NGS) to compare the gene expression profiles of GLRaV-3 infected *N. benthamiana* to infected *V. vinifera*. I summarize several differentially expressed genes that were found to be the same between the two respective plant hosts, as well as describe unique up, down, and stably expressed genes in response to GLRaV-3 infection. This is the first look at how *N. benthamiana* responds to GLRaV-3 infection.

The work described in chapters 2 and 3 was all done in the effort to develop a system to be able to study GLRaV-3 transmission biology without the complications that arise from working with *V. vinifera*. In chapter 4 I describe initial efforts to localize a virus retention site in

P. ficus vectors using an artificial diet system with viral purifications from both *N. benthamiana* and *V. vinifera*. To further characterize the interactions between the virus and *P. ficus* I developed an assay to block GLRaV-3 transmission by feeding potential blocking compounds. These results provide an initial idea of the nature of the receptor required for successful transmission events and serve as a "proof of concept" for testing future blocking compounds.

The chapters in this dissertation have been prepared for publication in peer-reviewed scientific journals explaining some of the redundancy in different sections of the text. References corresponding to each chapter have been placed immediately after the respective chapter. Chapter 2 has been accepted for publication in the 'Virology'. Chapter 4 has been submitted for publication. Chapter 3 is being prepared for submission.

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Chapter 2

Infection and Colonization of *Nicotiana benthamiana* by *Grapevine leafroll-associated virus 3*

This chapter is a postprint of a paper submitted to and accepted for publication as:
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Depositories: The GenBank accession number for the nucleotide sequence of Grapevine leafroll-associated virus 3 isolate GLRaV-3-I-LR101 is KY886362

ABSTRACT
Grapevine leafroll disease is an increasing problem in all grape-growing regions of the world. The most widespread agent of the disease, *Grapevine leafroll-associated virus 3* (GLRaV-3), has never been shown to infect species outside of the genus *Vitis*. Virus transmission to several plant species used as model systems was tested using the vine mealybug, *Planococcus ficus*. We show that GLRaV-3 is able to infect the model organism *Nicotiana benthamiana*. Working with GLRaV-3 infected *N. benthamiana* revealed distinct advantages in comparison with its natural host *Vitis vinifera*, yielding both higher viral protein and virion concentrations in western blot and transmission electron microscopy (TEM) observations, respectively. Immunogold labelling of thin sections through *N. benthamiana* petioles revealed filamentous particles in the phloem cells of GLRaV-3 positive plants. Comparison of assembled whole genomes from GLRaV-3 infected *V. vinifera* vs. *N. benthamiana* revealed identical sequences. These results open new avenues and opportunities for GLRaV-3 research.

INTRODUCTION
Research using model systems has been fundamental to the progress of science. Model organisms facilitate scientific progress because they are relatively well studied, and ensure the propagation of knowledge when ethics, costs, and technical difficulties can be an impediment to experiments. In medicine, model systems have been central to important discoveries from the development of vaccines to aid in the eradication of infectious diseases to the implementation of important medical techniques like organ transplantation (Academies, 2004). Plant model systems have also been indispensable to biology; *Arabidopsis thaliana* and *Nicotiana benthamiana* have become widely used for the study of fundamental questions in molecular plant-microbe interactions and other areas of plant biology.

Nicotiana benthamiana is an important experimental host in plant virology because a diverse range of viruses have been shown to successfully infect it (M. M. Goodin, Zaitlin, Naidu, & Lommel, 2008). In addition to this trait, *N. benthamiana* has become an important tool in plant biology to study protein interactions, localization, and plant-based systems for protein expression (M. Goodin, Yelton, Ghosh, Mathews, & Lesnaw, 2005; Ohad, Shichrur, & Yalovsky, 2007). The susceptibility of *N. benthamiana* to a range of plant viruses has been linked to a naturally occurring mutation in an RNA-dependent RNA polymerase gene present in the *N. benthamiana* genome (Yang, Carter, Cole, Cheng, & Nelson, 2004). Plants without the mutation in this gene exhibit enhanced virus resistance. In addition, *N. benthamiana* as an herbaceous plant is relatively easy to work with compared to woody plants because it grows quickly (weeks vs. months) in the greenhouse and can be grown year round. A draft genome has also recently become available making *N. benthamiana* an indispensable tool for plant biology and a highly sought after model organism for pathogen-host systems that are difficult to work with (Bombarely et al., 2012).

One example of a notoriously labor-intensive host-pathogen system is that of Grapevine leafroll disease (GLD) in grapevines. The disease is associated with a complex of viruses in the family *Closteroviridae* with *Grapevine leafroll-associated virus 3* (GLRaV-3) regarded as the most important causative agent (Maree et al., 2013). Because of its narrow host range limited to *Vitis* species and the fact that the virus is limited to the phloem, most GLRaV-3 research has concentrated on epidemiology or the development of detection assays (Almeida et al., 2013). In

addition, studying GLRaV-3 in grapevines in the greenhouse requires several months for the virus to be detectable with current detection assays, and symptom development can require even more time. Viral populations are typically low making virion purifications that could be useful for biological studies arduous. There is also no GLRaV-3 infectious clone available for research. GLRaV-3 research would benefit from infection in a plant model organism that could help overcome these issues. Despite its significance as an important viral disease of grapevine, little is known about viral replication and gene expression, and knowledge of the function of many GLRaV-3 genes is based only on inference from related viruses in the same family.

GLRaV-3 is an 18Kb ssRNA virus transmitted primarily by phloem-sap sucking mealybugs (Hemiptera, Pseudococcidae). GLRaV-3 is transmitted in a semi-persistent manner; the foregut (i.e. mouthparts) is thought to be the site of virus retention in insect vectors for a period of a few days (Tsai et al., 2008). Typically in *Vitis* GLD symptoms vary between cultivars, with red varieties showing reddening of the leaves compared to white varieties exhibiting leaf yellowing between major veins. In both cases the primary veins remain green and leaves become brittle and roll downwards. Substantial economic losses to the wine, table, raisin, and nursery industries have been documented with yield losses of 20–40% (Maree et al., 2013). Contributing to these economic hardships, diseased vines show a reduction in yield and cluster size, delayed and irregular fruit ripening, and changes in berry color hindering premium wine production (Goheen, Harmon, & Weinberger, 1958; Over de Linden & Chamberlain, 1970). Berry quality also is significantly decreased one year after infection under field conditions (Blaisdell et al., 2016).

Previously, GLRaV-3 has never been shown to infect hosts outside of *Vitis vinifera.* Here, we show that GLRaV-3 is able to infect the model organism *N. benthamiana,* and report several advantages over *V. vinifera* when comparing time from infection to detection, relative ease of virion purifications, as well as visualization of viral particles and structural proteins. These results have implications for future research in a field that has been limited by studies in labor-intensive and technically challenging host-pathogen system.

RESULTS

Vector-mediated infection of *N. benthamiana* with GLRaV-3. To determine if GLRaV-3 could infect a non-grape host, assays using vector transmission of the virus to several species of typical model plants were performed. Host plants tested included *Arabidopsis thaliana, Capsicum annuum, Nicotiana benthamiana, Nicotiana tabacum,* and *Solanum lycopersicum* (Table 1). Weekly sampling of petioles showed detection of GLRaV-3 in *N. benthamiana* at two months post-inoculation but not before (Fig. 1). To ensure that these results could be repeated, additional transmission experiments in *N. benthamiana* were performed (Table 2). After a second trial that resulted in no transmission to *N. benthamiana*, a third transmission experiment using transgenic *N. benthamiana* expressing the Turnip mosaic virus P1/HC-Pro sequence, a silencing suppressor, was conducted to determine if transmission efficiency could be increased. Again, *P. ficus* transmitted GLRaV-3 to 1 out of 41 *N. benthamiana* test plants revealing that transmission efficiency did not appear to change using transgenic plants. All subsequent trials were completed using HC-Pro *N. benthamiana* seedlings. The proportion of *N. benthamiana* plants infected was significantly lower than the proportion of *V. vinifera* infected with GLRaV-3 (Table 2; X-squared = 130.10, df = 1, p= 0.0001). In total, 1 out of 47 *N. benthamiana* and 11 out of 178 HC-

Pro *N. benthamiana* plants tested positive for GLRaV-3 two months post-inoculation. All trials included *V. vinifera* as controls and in comparison, 59 out of 90 *V. vinifera* plants tested positive for the virus.

Mechanical inoculations of GLRaV-3 did not lead to infections. Attempts to mechanically inoculate *N. benthamiana* seedlings using different strategies were not successful. Extracts from GLRaV-3 infected *N. benthamiana*, *V. vinifera*, and crude purifications from *N. benthamiana* were each tested with different buffers routinely used for mechanical inoculations. After two months post-inoculation none of the 408 plants tested were GLRaV-3 positive. Plants were not tested after two months because they grew too large for greenhouse conditions and were discarded.

Virus purifications and virion analysis. Purifications of GLRaV-3 virions from both *V. vinifera* and *N. benthamiana* yielded different viral protein concentrations. When purifying from the same amount of leaf material (1 g), amounts of GLRaV-3 coat protein (CP) purified from *N. benthamiana* were greater than when purified from *V. vinifera* (Fig. 2). SDS-PAGE of viral purifications from *N. benthamiana* suggests the presence of the four structural proteins associated with *Closteroviridae* virions (Fig. 3). Four proteins observed corresponded with the 59 kDa heat shock protein 70 homologue, HSP70; a 55 kDa protein, P55; the 35 kDa major coat protein, CP; and the 53 kDa minor coat protein, CPm. The expected molecular mass of the 35 kDa CP calculated from its amino acid sequence is 34.8 kDa. The band corresponding with CP in Fig. 3 and confirmed by western blot in Fig. 2 indicates this protein migrated less than expected. TEM was used to observe purified virions (Fig. 4); concentrations of particles on grids were also greater from *N. benthamiana* purifications when compared to observations of virions purified from *V. vinifera*. Grids with *N. benthamiana* GLRaV-3 purifications yielded highly concentrated aggregates of virions. When observing GLRaV-3 purifications from *V. vinifera*, few particles were found and they were not seen in concentrated aggregates. Immunogold labelling with polyclonal GLRaV-3 antisera against virions confirmed that the observed particles were GLRaV-3 (Fig. 4B, D). The average length of observed particles was 1811.52 nm ± 459.8 nm based on the measurements of 20 virus particles. The expected length of GLRaV-3 particles is 1800-2200 nm.

Ultrastructural observations confirmed detection results. Filamentous particles could be observed in the phloem cells of *N. benthamiana* petiole sections (Fig. 5). Virions were not observed in the xylem. Immunogold labelling was used to confirm that particles were GLRaV-3. Virus particles were clearly visible in aggregated bundles or scattered through the cytoplasm of cells.

Whole-genome sequencing did not identify substitutions. Alignment of whole genome sequences from GLRaV-3 infected source *V. vinifera* (314,702 reads) and subsequently infected *N. benthamiana* (121,939,749 reads) revealed that sequences were mostly identical (a minimum coverage of 100x). At position 761 in the 5’ UTR of the *V. vinifera* sequence there was a W (A/T) with coverage of 708x. In the *N. benthamiana* sequence at the same position there was an A. Alignment with six other full length GLRaV-3 genomes determined that this position as well as the 5’ UTR are, in general, variable. The sequence of the GLRaV-3 isolate found in *N. benthamiana* was deposited in GenBank under the accession number KY886362.

DISCUSSION

In an effort to find alternatives to study GLRaV-3 biology outside of its technically challenging natural host-pathogen system, we attempted to infect different plant species. We demonstrate that GLRaV-3 infects the model organism *N. benthamiana.* Working with GLRaV-3 infected *N. benthamiana* revealed distinct advantages in comparison with its natural host *V. vinifera*. Working with *V. vinifera* in a greenhouse is time consuming, taking months from initial planting of cuttings to provide plant material suitable for vector transmission experiments. From initial seed planting of *N. benthamiana,* seedlings were ready to use for transmission experiments in a matter of weeks. Typically it takes several months for GLRaV-3 to be detectable in *V. vinifera* by RT-PCR after inoculation using mealybugs. In comparison, RT-PCR confirmation of GLRaV-3 in *N. benthamiana* only took two months. Purifications of virions from *N. benthamiana* revealed greater concentrations of viral CP protein by western blot analysis as well as greater concentrations of virus particles when observed by TEM. SDS-PAGE analysis of *N. benthamiana* purifications suggest that the structural components of GLRaV-3 virions may be similar to the four structural proteins encoded by related viruses in the family *Closteroviridae.* The Crinivirus, *Lettuce infectious yellows virus* encodes proteins found to constitute the long flexuous rod shape virions including a heat shock protein 70 homologue, HSP70h; a 59 kDa protein, P59; major coat protein, CP; and the minor coat protein, CPm (Tian, Rubio, Yeh, Crawford, & Falk, 1999). Four homologous proteins have also been described to compose the structural components of *Citrus tristeza virus* (genus: *Closterovirus*) (Satyanarayana et al., 2000). These proteins were not visible in SDS-PAGE analysis of virion purifications from *V. vinifera* likely due to their low concentration. These results highlight the advantages of being able to obtain GLRaV-3 from an herbaceous host like *N. benthamiana*.

There were some notable differences between GLRaV-3 infection in *N. benthamiana* vs. *V. vinifera* when comparing symptom development and transmission efficiency. At two months post-inoculation clear symptoms of GLRaV-3 infection were not present in either *N. benthamiana* or *V. vinifera* infected plants, even if the plant was positive for GLRaV-3 infection. In grapes, it is expected to observe classical leafroll symptoms many months after infection and symptom development can vary greatly depending on cultivar and season (Maree et al., 2013). In *N. benthamiana* no clear symptoms were observed that could be attributed directly to GLRaV-3 infection. Observations of GLRaV-3 infected *N. benthamiana* showed yellowing and downward curling of leaves while interveinal regions remained green approximately three months post-inoculation although some kind of nutrient deficiency or other factor could not be ruled out. In addition to symptom development, vector transmission efficiency differed between *N. benthamiana* and *V. vinifera*. It should be noted that transmission could be affected by *P. ficus* host plant preference. First instar *P. ficus* had a 4-day inoculation access period on test plants before removal. After 4 days, mealybugs could still be observed on *V. vinifera* test plants while it was difficult to observe any live mealybugs left feeding on *N. benthamiana* plants. The preference of *P. ficus* to feed on *V. vinifera* in relation to *N. benthamiana* likely affected infection rates. It is apparent from our results that developing a system to improve infection rates in *N. benthamiana* would be helpful. Future studies testing transmission with different insect vectors as well as with other commonly used techniques like grafting or transmission by parasitic dodder species might help increase the infection rate of GLRaV-3 in *N. benthamiana.*

When comparing genomes from GLRaV-3 infected *V. vinifera* and *N. benthamiana* plants, the identical sequences obtained indicate that there were no adaptive mutations accumulated within the one passage analyzed. The possible single nucleotide variation in the GLRaV-3 genome from *V. vinifera* is not surprising because variation in the 5' UTR has been described previously as characteristic between GLRaV-3 isolates (Jooste et al., 2010). No functional significance for the 5' UTR has been found. It is not unusual to observe strong selection and rapid evolution in response to "new-host stress" (Ebert, 1998). In our experiment, one serial passage from GLRaV-3 infected *V. vinifera* to *N. benthamiana* might not have been enough time to observe any mutations. Mutations and recombination events are often associated with changes in virulence in many serial passage experiments (Ebert, 1998). Further experiments comparing genomes after many serial passages through *N. benthamiana* are needed to elucidate changes in virulence or other factors that might have contributed to the infection of a novel host.

Although this is the first time GLRaV-3, an *Ampelovirus*, has been shown to infect an herbaceous host by insect-mediated transmission, there have been previous reports of successful transmission of viruses in the family *Closteroviridae* to *Nicotiana* species. Infection by a *Closterovirus* in *N. benthamiana* and possibly even by a GLRaV have been published although the species of GLRaV was not differentiated and the complex organization of the family *Closteroviridae* was unknown at that time (Tanne, Sela, & Harpaz, 1974; Woodham & Krake, 1983). Previously, GLRaV-2 (genus *Closterovirus*) which typically infects woody hosts was the only grapevine leafroll associated virus thought to be capable of infecting an herbaceous host, *N. benthamiana* (Goszczynski, Kasdorf, Pietersen, & Van Tonder, 1996). A vector of GLRaV-2 transmission has yet to be described and infection of *N. benthamiana* was completed by mechanical transmission. A recent report showed the successful transmission of GLRaV-7 (genus *Velarivirus)* by the parasitic dodder *Cuscuta europea* to *Nicotiana occidentalis* (Mikona & Jelkmann, 2010). No insect vector has been described for any virus in the genus *Velarivirus* so it is plausible that its transmission biology differs from other members of the family *Closteroviridae* (Al Rwahnih, Dolja, Daubert, Koonin, & Rowhani, 2012).

Other members of the family *Closteroviridae* have been shown to successfully infect *N. benthamiana.* In contrast to other members of the family *Closteroviridae*, viruses in the genus *Crinivirus* generally are able to infect a wide range of herbaceous hosts. *Lettuce infectious yellows virus*, the type virus from the genus *Crinivirus,* can be successfully transmitted to *N. benthamiana* by both agroinoculation and by whitefly vectors (J. Wang, Turina, Stewart, Lindbo, & Falk, 2009). It was also demonstrated that two other *Criniviruses*, *Tomato chlorosis virus* and *Tomato infectious chlorosis virus*, were able to be transmitted to *N. benthamiana* by whitefly mediated transmission (Wintermantel, Cortez, Anchieta, Gulati-Sakhuja, & Hladky, 2008). Mechanical transmission is not expected for phloem-limited viruses in *Closteroviridae* and our results showing that GLRaV-3 could not be mechanically transmitted to *N. benthamiana* supported this. Mechanical transmission has also not been shown for GLRaV-3 in its original host *V. vinifera*.

Our results have implications for the future of GLRaV-3 research. *N. benthamiana* has already proven to play a crucial role in several seminal discoveries in other host-pathogen systems as already described in recent work (Bisaro, 2006; M. M. Goodin et al., 2008; R. Y.-L. Wang & Nagy, 2008). In one example, research on host factors required for replication of *Tomato bushy stunt virus* (TBSV), a plus-stranded RNA virus, were identified in yeast models.

N. benthamiana was used as a plant model to demonstrate that the same host factors were required for replication in plants. *A. thaliana* could not be used as a model because it is a non-host of TBSV (R. Y.-L. Wang & Nagy, 2008). Other technical advances have made *N. benthamiana* an important tool for plant virology. *N. benthamiana* has become a popular reverse genetics system with the development of virus-induced gene silencing and RNA silencing allowing the systemic down regulation of any gene of interest in plants (Burch-Smith, Anderson, Martin, & Dinesh-Kumar, 2004). The ease and speed of agroinfiltration in *N. benthamiana* is well established for studying specific proteins of interest, often fused to autofluorescent proteins in plant cells proving indispensable for protein localization and interaction studies (Citovsky et al., 2006; Ohad et al., 2007; Tardif et al., 2007). None of these technologies have been available to GLRaV-3 research in *V. vinifera* explaining the current lack of basic knowledge of viral replication and gene expression and function of this virus.

In conclusion, our analyses of GLRaV-3 infection in *N. benthamiana* establish that the host range of GLRaV-3 may not be as narrow as previously thought. This has implications for the development of *N. benthamiana* or other hosts as model plants for future GLRaV-3 research. Further investigations could test the possibility of infection of *N. benthamiana* with an infectious clone and provide a much needed system to study gene function and viral replication and movement. GLRaV-3 is one of the most important viruses of grapevine but despite this, there remain several gaps in our understanding of the biology of this virus. The finding of a plant model system will help drive research in this field forward.

MATERIAL AND METHODS

Mealybug transmission assays. *Planococcus ficus* (Hemiptera, Pseudococcidae) colonies were maintained on butternut squash (*Cucurbita moschata*) at 22 °C, with a 16:8-h photoperiod. First instars were used for all experiments because they were shown to be the most efficient vector of GLRaV-3 (Tsai et al., 2008). To determine if GLRaV-3 could infect non-grape hosts, transmission experiments were carried out on *Arabidopsis thaliana, Capsicum annuum, Nicotiana benthamiana, Nicotiana tabacum,* and *Solanum lycopersicum. Vitis vinifera* cv. Cabernet Sauvignon was also tested as a control. Whatman filter papers were wet and placed on top of mealybug colonies. After 30 min the papers were pinned to GLRaV-3 source vine cuttings (accession LR101; variant I) provided by Foundation Plant Services, University of California Davis, CA. After a 24 h acquisition access period (AAP), first instars were transferred manually with a small paintbrush to healthy test plants. In some trials small groups of insects (~20) were also clipped to leaf blades with clip cages. After 4 days, any visible mealybugs were removed from the test plant and plants were moved to the greenhouse. Petiole samples were collected from plants 2 months post-inoculation and RNA extractions were completed on 100 mg of petiole tissue (Sharma et al., 2011). One step reverse transcription-polymerase chain reaction (RT-PCR) was then performed and PCR products were analyzed using fragment analysis as described previously (Sharma et al., 2011).

Following results from the first experiment, a similar protocol was used to determine if GLRaV-3 transmission in *N. benthamiana* could be reproduced. Six total trials were completed. Starting with trial 3, transgenic *N. benthamiana* expressing the Turnip mosaic virus P1/HC-Pro sequence kindly supplied by B. Falk (University of California, Davis) were used as test plants to determine if transmission efficiency could be increased (J. Wang, Turina, Medina, & Falk,

2009). Eight petiole samples representing *N. benthamiana* across the different trials were tested by PCR to confirm that HC-Pro was indeed expressed in these plants. In all trials, young seedlings of *N. benthamiana* were used as test plants. The Chi-squared test of proportion was calculated using R (Version 3.0.2, R. RStudio, Inc., Boston, MA [http://www.rstudio.com/].

Mechanical inoculations. *N. benthamiana* seedlings were dusted with carborundum and inoculated with different combinations of three different virus sources and three different buffers for a total of nine experimental treatments. The first source of virus tested was from 0.7 g of GLRaV-3 infected *N. benthamiana* leaves ground to a fine powder using a chilled mortar and pestle. The second virus source was from virions purified from *N. benthamiana* leaves as described below. The final virus source was a crude purification from 10 g of GLRaV-3 infected *N. benthamiana* leaves ground in liquid nitrogen with a mortar and pestle. 80 ml of extraction buffer (0.1 M-Tris-HC1, 0.5 % (w/v) Na_2SO_3, 0.5 % (v/v) 2-mercaptoethanol pH 7.4) was added to the ground plant material. TritonX-100 was added to a final concentration of 2 % (v/v) and the solution was stirred for 1 hr over ice. The mixture was centrifuged in a Beckman 50.2 Ti rotor at 7500 rpm for 10 min at 4°C and used immediately for mechanical inoculations. Each of these starting virus sources was inoculated with three different buffer conditions using a ratio 1:5 (plant material:buffer). The first buffer tested was 0.1 M phosphate buffer, pH 7 prepared as described previously (Martelli, 1993). The second buffer tested was 0.01 M potassium phosphate and 0.01 M cysteine HCl (Boscia et al., 1993). The final buffer used was 0.02 M phosphate buffer (pH 7.4) with addition of 2% (w/v) of polyvinylpyrrolidone (PVP). After 2 months petioles were collected from plants and RNA extractions, RT-PCR, and fragment analysis were completed as described above.

Virion purification and analysis. GLRaV-3 virions were purified using a modified protocol as previously described (Klaassen, Boeshore, Dolja, & Falk, 1994). 10 g of GLRaV-3 infected *Vitis vinifera* or *Nicotiana benthamiana* leaves were ground in liquid nitrogen with a mortar and pestle. 80 ml of extraction buffer (0.1 M-Tris-HC1, 0.5 % (w/v) Na_2SO_3, 0.5 % (v/v) 2-mercaptoethanol pH 7.4) was added to the ground plant material. TritonX-100 was added to a final concentration of 2 % (v/v) and the solution was stirred for 1 hr over ice. The mixture was centrifuged in a Beckman 50.2 Ti rotor (Beckman Coulter, Inc., CA, USA) at 7500 rpm for 10 min at 4°C. The supernatant was transferred to a new ultracentrifuge tube, then 1 ml of 20 % sucrose in TE (10 mM TrisHCl, 1 mM EDTA, pH 7.4) was added as a cushion underneath the supernatant followed by centrifugation in a Beckman 70.1 Ti rotor at 35,000 rpm for 2 hrs at 4°C. The supernatant was removed and pellet was soaked in 500 µl of TE overnight at 4°C. The pellet was resuspended with gentle pipetting up and down and centrifuged at 7400 rpm for 2 minutes. The supernatant was removed and the pellet was resuspended in 50 µl of TE and stored at 4°C for further analysis. Purified GLRaV-3 virions were analyzed by SDS–PAGE and western blot. Blots were probed with antiserum to GLRaV-3 coat protein diluted 1:1000 and detected using Immun-Blot® AP Colorimetric Kits for immunodetection according to the manufacturer's directions (Bio-Rad, CA, USA).

Electron microscopy of purified virions. Formvar carbon-coated copper grids were floated on top of drops of GLRaV-3 purifications for 10 min. For negative staining, grids were then moved to drops of 1% aqueous uranyl acetate for 10 min. Grids were viewed with a FEI Tecnai 12 transmission electron microscope (FEI, Massachusetts, USA). Average length ± standard

deviation was calculated from length measurements of 20 virion filaments using ImageJ software (version 1.45s; National Institutes of Health, USA [http://imagej.nih.gov/ij].

Immunogold labelling of purified virions. Formvar carbon-coated copper grids were placed on drops of purified virions for 10 min in a moist chamber. Grids were blocked with blocking buffer (1% BSA, 10 mM Tris HCl pH 7.4, 100 mM NaCl, 0.1% Tween 20) for 15 min and transferred to drops of primary antisera (supplied by Dr. Adib Rowhani) diluted 1:200 in blocking buffer for 1 h. Grids were then rinsed on several drops of TE and placed in blocking buffer for 30 min. Grids were incubated with goat anti-rabbit antiserum conjugated with 10 nm gold (1:30 in blocking buffer) for 1 h. Grids were rinsed in TE and stained with 2% uranyl acetate for 10 min. Grids were viewed with a FEI Tecnai 12 transmission electron microscope. Negative controls were completed by floating grids on purifications from healthy *N. benthamiana* or *V. vinifera* followed by the protocol described above.

Preparation of petioles for ultrastructural analysis. Small pieces of petiole (1-2 mm) were removed from GLRaV-3 infected *N. benthamiana* or healthy non-infected *N. benthamiana* as a negative control and fixed in 2% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer and processed for EM observation by embedding in London Resin White for immunogold labelling. Serial ultrathin (70 nm) sections were cut using a Reichert Ultracut E, RMC MT6000 Microtome (Reichert-Jung, Vienna, Austria) and collected on formvar/carbon coated copper grids for labelling. Grids were floated on blocking buffer (1% BSA, 0.1% cold water fish gelatin) for 15 min. Grids were then floated on primary antisera (supplied by Dr. Adib Rowhani) diluted 1:200 on blocking buffer for 1 hr. Grids were rinsed on 1 drop of PBST (0.02% Tween 20 in PBS) followed by several washes in PBS. Grids were incubated in goat anti-rabbit antiserum conjugated with 10 nm gold (1:30 in blocking buffer) for 1 h followed by a PBST rinse and PBS washes. Grids were fixed in 0.5% glutaraldehyde in PBS for 5 minutes followed by PBS and H_2O washes. Grids were stained in 2% aqueous uranyl acetate and lead citrate, and observed with a FEI Tecnai 12 transmission electron microscope.

Next generation sequencing. 0.1 g of petioles from a known GLRaV-3 infected *N. benthamiana* plant and 0.1 g petioles from the original GLRaV-3 source *V. vinifera* were used for next generation sequencing. For RNA extractions, petioles were ground in liquid nitrogen and added to 5 ml of Guanidine extraction buffer (4 M Guanidine thiocyanate, 0.2 M sodium acetate, 25 mM EDTA, 2.5% polyvinylpyrrolidone-40) and 1% beta-mercaptoethanol. 20% sarcosyl buffer was added followed by vigorous mixing and incubation in a 57 °C water bath for 12 minutes, vortexing every 3 minutes for better lysis efficiency. The extract was then added to QIAshredder columns (Qiagen) and the remainder of the protocol was followed according to Qiagen RNeasy Plant Mini Kit instructions (Santos, 2013). Sequencing libraries were constructed at the Functional Genomics Lab (FGL), a QB3-Berkeley Core Research Facility (UC Berkeley). Quality of RNA was checked on a 2100 Bioanalyzer (Agilent Technologies, CA, USA). The library preparation was done using Apollo 324™ with PrepX™ RNAseq Library Prep Kits (WaferGen Biosystems, Fremont, CA), and 13 cycles of PCR amplification was used for index addition and library fragment enrichment. Genomic sequencing was done on the Miseq v2 platform (Illumina, Inc., CA, USA) using 50PE by Vincent J. Coates Genomics Sequencing Laboratory (UC Berkeley). Quality control check was done using FastQC (Babraham Bioinformatics, UK). Reads were trimmed using library sickle (Bioinformatics Core, UC Davis, USA). Trimmed reads were mapped to a GLRaV-3 complete genome (GenBank Accession:

GQ352633) using default settings in Geneious (Version 9.1.2 [https://www.geneious.com, Kearse et al., 2012]). Although coverage throughout the genome was variable, a minimum of 100x coverage was obtained for all nucleotides; a strict consensus sequence was obtained for the two samples for sequence comparison.

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Table 1. GLRaV-3 is vector transmitted to a non-grape host.
Experiments testing GLRaV-3 infection in different host plants; *N. benthamiana* was found to be GLRaV-3 positive two months post-inoculation.

Host Plant	Plants infected/Plants inoculated
Arabidopsis thaliana	0/12
Capsicum annuum	0/22
Vitis vinifera	4/10
Nicotiana benthamiana	1/17
Nicotiana tabacum	0/13
Solanum lycopersicum	0/7

Table 2. Summary of GLRaV-3 vector transmission experiments with *Nicotiana benthamiana* and *Vitis vinifera*.

Trial	*N. benthamiana*	*V. vinifera*	Mealybugs used/plant
1	1/17	4/10	70[†]
2	0/30	5/10	100
3	1/41[*]	4/10	70[†]
4	2/24	6/10	70[†]
5	4/54	7/10	50
6	2/35	16/20	50
7	2/24	17/20	50

[*]HC-Pro *N. benthamiana* used after Trial 3

[†]20 mealybugs were placed in clip cages on a leaf in addition to 50 mealybugs placed freely on the plant

FIGURES

Fig. 1. GLRaV-3 infects *N. benthamiana* . L: Ladder. G+: GLRaV-3 positive *V. vinifera.* G-: GLRaV-3 negative control in grape, *V. vinifera.* T+: GLRaV-3 positive *N. benthamiana.* T-: GLRaV-3 negative control in *N. benthamiana.* Expected fragment length 320 base pairs (bp).

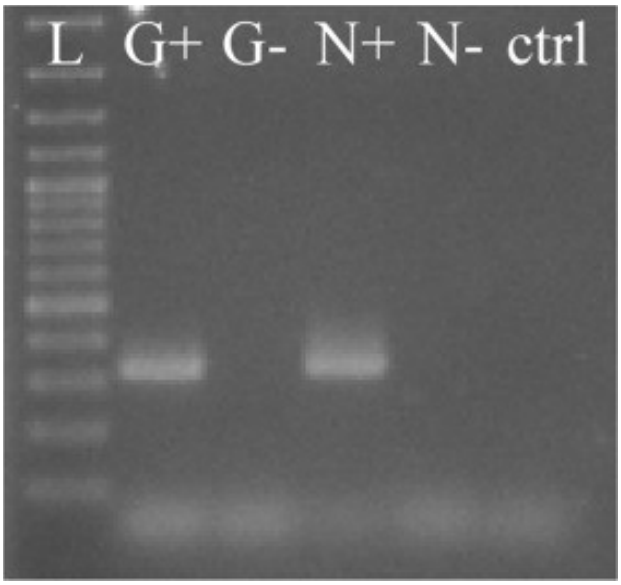


Fig. 2. Western blot analysis of GLRaV-3 virion purifications. Comparisons of serial 10-fold dilutions of *V. vinifera* vs. *N. benthamiana* purifications probed with antiserum to GLRaV-3 coat protein (CP). Darker bands from *N. benthamiana* purifications indicate the presence of higher concentrations of viral CP protein.

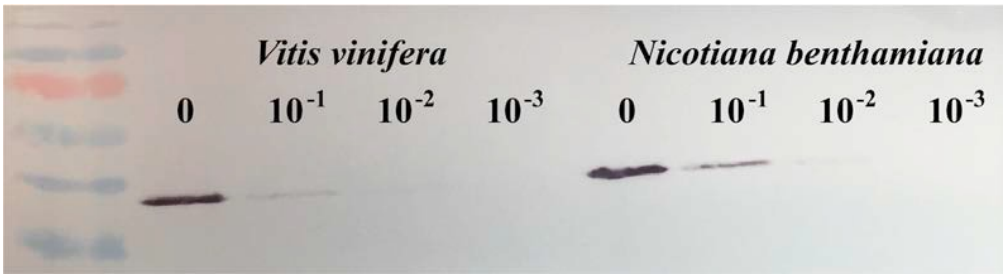


Fig. 3. SDS-PAGE analysis of *N. benthamiana* purifications. Lanes A, B, C represent three purifications from *N. benthamiana*. In lane C, the four proteins visible likely correspond with GLRaV-3 structural components; 59 kDa heat shock protein 70 homologue, HSP70; a 55 kDa protein, p55; the 35 kDa major coat protein, CP; and the 53 kDa minor coat protein, CPm. Gel was revealed with silver staining.

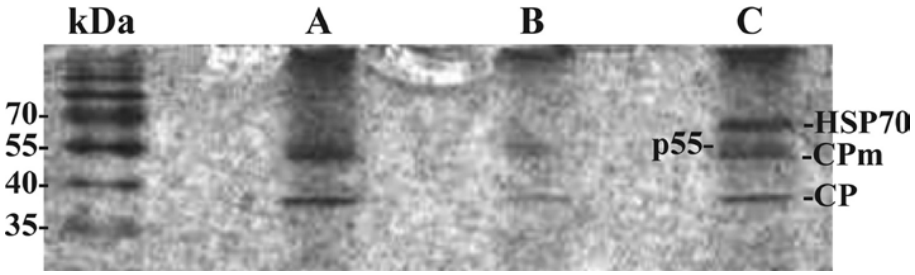


Fig. 4. TEM and immunogold labelling analysis of purified GLRaV-3 virions. (A) Virion purified from GLRaV-3 infected *V. vinifera*. (B) Virion purified from *V. vinifera* labelled using antiserum to the GLRaV-3 CP. (C) Virions purified from GLRaV-3 infected *N. benthamiana* were commonly found in aggregates. (D) Virion purified from *N. benthamiana* labelled using antiserum to the GLRaV-3 CP. Average particle length of virions purified from *N. benthamiana* was 1811.52 nm ± 459.8 nm based on 20 virus particle measurements. Expected size: 1800-2200 nm. Bars represent 200 nm.

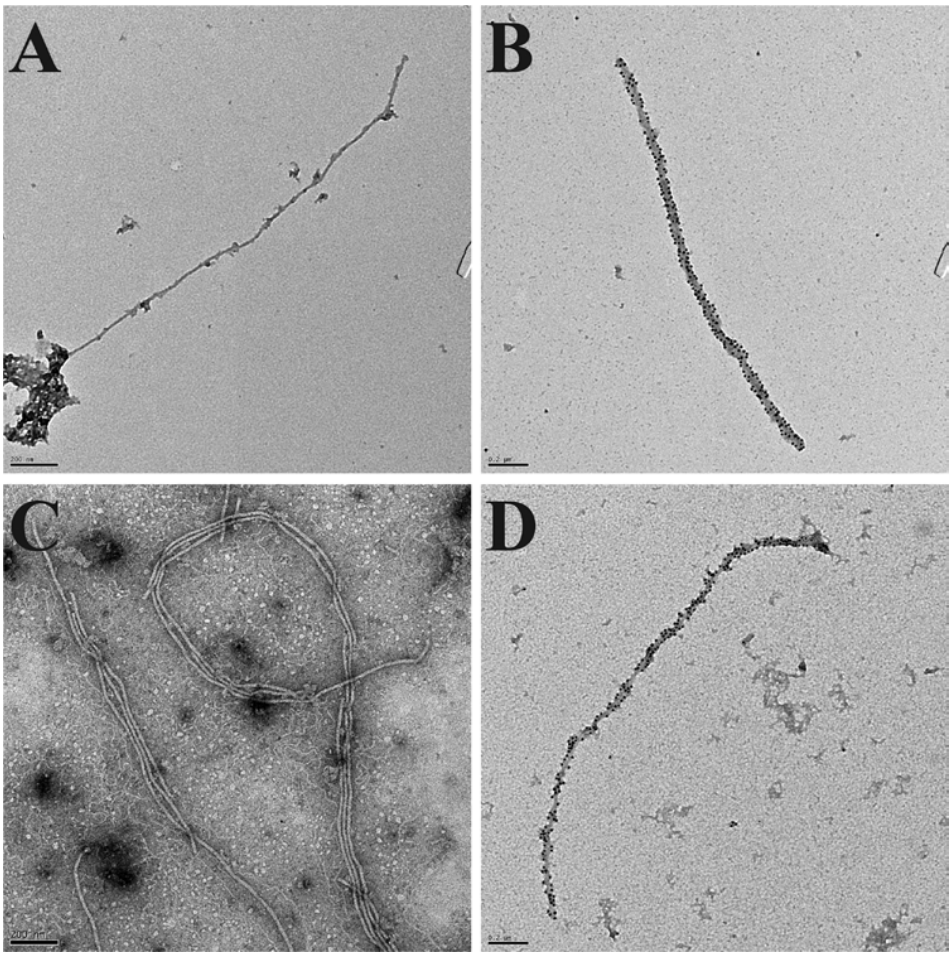
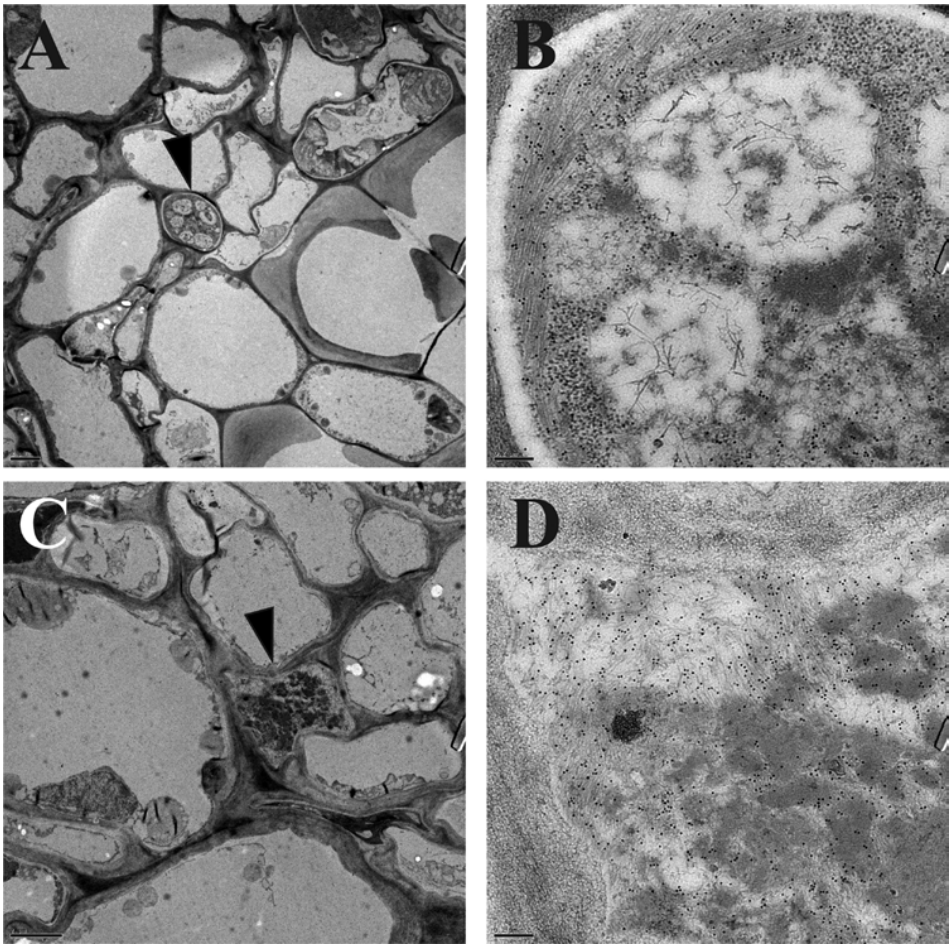


Fig. 5. TEM and immunogold labelling analysis of GLRaV-3 virions in infected *N. benthamiana* petioles. (A, C) Low magnification view of area surrounding GLRaV-3 infected cells. Arrow points to phloem cells where virions could be observed. (B, D) Higher magnification view of phloem cells corresponding with (A) and (C) respectively, showing immunogold labelled GLRaV-3 virions in infected *N. benthamiana* cells. Labelled virions could be observed dispersed throughout the cytoplasm of phloem cells. Bars represent 200 nm.



Chapter 3

Comparison of two different host plant responses to grapevine leafroll-associated virus 3 infection

This chapter is currently being prepared for submission by:

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ABSTRACT
Grapevine leafroll-associated virus 3 (GLRaV-3), the main causative agent of grapevine leafroll disease, is one of the most important viruses of grapevine but despite this, there remain several gaps in our understanding of the biology of this virus. Because of its narrow host range limited to *Vitis* species and the fact that the virus is restricted to the phloem, most GLRaV-3 research has concentrated on epidemiology and the development of detection assays. The recent discovery that GLRaV-3 could infect *Nicotiana benthamiana,* a plant model organism, makes new opportunities available for future research in this field. In this work, we use RNA-seq to compare both *V. vinifera* and *N. benthamiana* host responses to GLRaV-3 infection. This is the first analysis of gene expression profiles beyond *Vitis* to mealybug-transmitted GLRaV-3.

INTRODUCTION

One of the gaps in our knowledge of plant virology remains our understanding of how virus infection impacts whole plants physiologically and biochemically, especially at cellular resolution. Viruses are biotrophs and plants respond with a highly polymorphic innate immune response to infection (Dangl & Jones, 2001). Determining how the plant adapts to virus infection and which sets of genes are differentially expressed represents a first step to better understand the mechanisms behind the regulatory pathways involved (Ekblom & Galindo, 2010). High throughput sequencing (HTS) technologies have been increasingly carried out in model as well as in non-model plants for gene expression studies to aid in understanding host and virus responses during infection cycles (Pervaiz et al., 2016). Model organisms are indispensable to scientific progress because they are well studied and provide a biological setting to undertake experiments when ethics, costs, and technical difficulties can impair research. In the context of understanding virus infection in plants and in determining the usefulness of a model organism for future work, a critical comparison is of host response to virus infection between its original non-model host versus a novel potential model organism.

Grapevine leafroll-associated virus 3 (GLRaV-3) was previously thought to be limited to *Vitis* species, but was recently demonstrated that it could also infect *Nicotiana benthamiana* (Prator, Kashiwagi, Voncina, & Almeida, 2017). GLRaV-3, a ssRNA virus within the family *Closteroviridae*, is regarded as the most important agent of grapevine leafroll disease (GLD) that results in substantial economic losses (20-40%) to the wine, table, raisin, and nursery industries (Bester, Burger, & Maree, 2017a). The virus is transmitted primarily by phloem-sap sucking mealybugs (Hemiptera, Pseudococcidae) in a semi-persistent manner and infects the phloem tissue of both *V. vinifera* and *N. benthamiana* hosts (Prator et al., 2017; Tsai et al., 2008). The finding that GLRaV-3 infects *N. benthamiana* provides advantages of working with this model organism when comparing time from infection to detection, relative ease of virion purification, as well as visualization of viral particles and structural proteins. Importantly for *N. benthamiana* to be used as a model host for GLRaV-3 research the two different hosts must share key responses to GLRaV-3 infection.

Plant viruses cause significant changes in host gene expression in response to infection (Barba, Czosnek, & Hadidi, 2014). In recent years, RNA-sequencing technology has progressed rapidly providing a more sensitive method to detect low-abundance host gene expression changes due to stresses induced by viral infection than previously observed using microarray technologies (Garber, Grabherr, Guttman, & Trapnell, 2011; Z. Wang, Gerstein, & Snyder, 2009). RNA-seq has quickly become the preferred tool for gene expression analyses in important model hosts like *N. benthamiana*. This popular experimental host has become an indispensable tool in plant virology because of its susceptibility to infection by a

large number of diverse plant viruses, perhaps due to a naturally occurring mutation in an RNA-dependent RNA polymerase gene (Goodin, Zaitlin, Naidu, & Lommel, 2008). This, combined with the recent release of the draft genome sequence for *N. benthamiana*, has made it a particularly useful for host–pathogen studies focused on innate immunity and defence signalling, protein localization and interactions, and a system for protein expression and purification research (Bally et al., 2015; Nakasugi et al., 2013).

In contrast, gene expression studies in *V. vinifera* in response to viral infection are relatively limited. Most RNA-sequencing work on *V. vinifera* as a host has focused on differential gene expression analysis during berry development and other developmental stages of plant growth (Pervaiz et al., 2016; Sweetman, Wong, Ford, & Drew, 2012). HTS has also been adapted for detection of virus infection in *V. vinifera* (Coetzee et al., 2010; Visser, Bester, Burger, & Maree, 2016). Recently, one group has described transcriptome analyses and differential expression profiles of small RNAs associated with GLRaV-3 infection in grapes (Bester et al., 2017a; Bester, Burger, & Maree, 2017b). It is our hope that together with these studies, the gene expression profiles in response to GLRaV-3 infection will help lead to a better understanding of host-pathogen interactions of grapevine leafroll disease.

We used RNA-seq to analyse the gene expression profiles of *N. benthamiana* and *V. vinifera* responses to GLRaV-3 infection. This is a first assessment of how a novel host, outside of *V. vinifera,* responds to GLRaV-3 infection. Responses between the two hosts are show several shared gene expression results. This, together with the small number of shared gene expression differences demonstrates that *N. benthamiana* could serve as a useful tool for future studies.

RESULTS

RNA sequencing of *N. benthamiana* and *V. vinifera.* RNAseq data were mapped to the respective *N. benthamiana* or *V. vinifera* genome and the results of that mapping assessed (Table 1). In all cases, the majority of sequenced reads mapped to the appropriate genome, although a proportion of reads mapped to multiple loci probably due to repeats or gene families. A substantial proportion of reads did not map to the genome. To determine whether RNA from other species had been included in the RNAseq data it was assessed by analysis of ribosomal RNA which demonstrated that cross-contamination between other organisms is not a major source of unmapped reads; poor-quality sequence was removed as part of pre-processing. Some sequencing libraries produced substantially less sequence than others, due to the difficulty of obtaining quality RNA, furthermore, some conditions did not have replicates. However, differential gene expression was performed using edgeR, with appropriate normalisation, allowing for statistically valid gene expression comparisons.

***V. vinifera* response to GLRaV-3 infection.** In our analyses, 494 genes were differentially expressed in response to GRLaV-3 infection in *V. vinifera* (Figure 1a; Table S1). Of these differentially expressed genes (DEGs), 222 were downregulated while 272 were upregulated. Additionally, 44 genes were shown to be stably expressed when comparing infected versus healthy plants. Kinases were the most commonly observed DEGs (Figure 2a) of which serine/threonine protein kinases were the most common kinases observed. Other notable genes were associated with transcription regulation and transporter genes.

***N. benthamiana* response to GLRaV-3 infection.** In the novel host, 157 genes were shown to be differentially expressed (Figure 1b; Table S1). Of these, 107 were downregulated and 49 were upregulated while 28 genes were stably expressed. In contrast to the trends observed in *V. vinifera*, heat shock and chaperone associated genes were the most common DEGs (Figure 2b). Similarly to trends observed in *V. vinifera,* expression of transcription and transporter associated genes were also a prominent response to virus infection.

Comparison of differential gene expression between *N. benthamiana* and *V. vinifera.* To determine if *N. benthamiana* could serve as a suitable model organism for this host-pathogen system, we determined how similar it responded to GLRaV-3 infection when compared to *V. vinifera*. Generally, heat shock, chaperone, and transporter gene groups appeared the most common between the different plant host data sets (Figure 2c).
General families of DEGs common in both hosts also followed similar expression changes with six upregulated, seven downregulated, and two stably expressed genes in common (summarized in Table 2 with specific gene ID, transcript accumulation summarized in Table S2). Only 1% of the shared DEGs observed in both hosts showed up or down regulation patterns that were different from each other (Table 3). Shared genes were categorized into general groups and annotated with functions associated with expression based on literature searches. Several DEGs were shared but present in up (n = 10), down (n = 13), and/or stable (n = 3) categories concurrently (Table S3).
RT-qPCR validation of selected genes. Validation of differentially expressed genes was performed by RT-qPCR. In total, twelve DEGs (seven grapevine and four *N. benthamiana*) were verified. Based on the transcriptome data, selection of genes whose expression did not vary among healthy or GLRaV-3-infected plants, were used as references in RT-qPCR. Results showed that all gene expression levels were consistent with the results of the transcriptome analysis for both hosts (Figure S1). Four of the grapevine genes (V8856.2, V8031, V6014, V9000) were consistently downregulated while three grapevine genes (V2636, V1578, V9187) showed upregulation consistent with the transcriptome results. Of the four *N. benthamiana* genes verified, two genes (B1018, B2016) showed downregulation while the other two genes (B0002, B0006) showed upregulation consistent with the transcriptome analysis.

DISCUSSION

Until recently, GLRaV-3 infection was thought to be limited to infection in *V. vinifera*. The discovery that GLRaV-3 could infect the novel model host *N. benthamiana* is an important finding for a research field where studies have been limited by a difficult host-pathogen system. In this work, we compared the host responses between GLRaV-3-infected *V. vinifera* and *N. benthamiana* to determine if gene expression profiles were similar. These are also the first gene expression data for GLRaV-3 infection in a novel host.

Reads that did not map to either the *V. vinifera* or *N. benthamiana* genomes could be due to the presence of RNA from other sources, e.g. RNA from GLRaV-3, other viruses, bacteria or fungi, or may be a consequence of poor quality sequence, or sequences that are correct but not present in the genome. The rRNA analysis to detect contamination from other organisms showed this as only a small contributor to unmatched reads. Therefore, it is likely that most unmapped reads do not map to the respective genome because that part of the genome is missing or is significantly different.

Data showed transcriptional changes in both GLRaV-3-infected *vs* healthy *V. vinifera* and infected *vs* healthy *N. benthamiana.* We observed 494 DEGs in infected *V. vinifera* when compared to healthy plants. In contrast, only 157 DEGs were observed in GLRaV-3 infected *N. benthamiana*. Several variables could explain the increase in the number of DEGs expressed in *V. vinifera* when compared to *N. benthamiana*. One limitation is the lack of a well-annotated genome for *N. benthamiana* relative to the available *V. vinifera* genome. As future research continues to improve available annotations for the *N. benthamiana* genome more DEGs could be uncovered that were missed in our analysis. In other gene expression studies on *N. benthamiana* response to virus infection, smaller numbers of DEGs have been associated with infection of virus resistant plant varieties as well as infected hosts with less severe observable symptoms (Dardick, 2007; Fan et al., 2014; Senthil et al., 2005). In

GLRaV-3-infected *N. benthamiana*, no clear symptoms were observed that could be attributed directly to GLRaV-3 infection when petioles were collected for analysis at two months post-inoculation. The smaller number of DEGs in *N. benthamiana* could be attributed to a lack of observable symptoms as has been previously described in other virus infections in this host. In addition, our results provide an arbitrary snapshot of plant host response to GLRaV-3 infection taken during one timepoint. Future studies looking at multiple timepoints of virus infection as well as an increase in replicates used for sequencing could help decipher this trend.

To determine if *N. benthamiana* could serve as a suitable model host for future studies, we wanted to determine how similar it responded to GLRaV-3 infection when compared to *V. vinifera*. Alternatively, this also meant determining if there were any major differences in expression profiles between the two hosts. The majority of DEGs observed between the two hosts were unique and not found in the other. It should be noted that the comparison between hosts is limited by replication number available for sequencing. Among the shared DEGs between the two hosts, only 1% in *N. benthamiana* showed up or down regulation that was different than those observed in *V. vinifera*. Only one of these, thaumatin-like protein, which was downregulated in *V. vinifera* and upregulated in *N. benthamiana,* is found to be associated with response to biotic stress and in this case is thought to be antifungal (Vigers et al., 1992). The other three gene families detected, glutaredoxin, RHOMBOID-like protein, and UDP-glycosyltransferase are involved in other plant cellular processes not known to be associated with pathogen infection (Keegstra & Raikhel, 2001; Knopf & Adam, 2012; Rouhier et al., 2005). This small number of genes observed to have differing gene expression, and the fact that only one appears to be related to biotic stress is encouraging for future work to determine the use of *N. benthamiana* as a model host for this system.

Response to GLRaV-3 infection resulted in a common set of genes being differentially expressed in both hosts. When comparing gene expression patterns that are shared between the two hosts, two interesting outcomes are observed: (i) the down regulation of genes associated with stress chaperones; (ii) the induction of gene families involved in primary plant physiological processes. We observed a shared pattern of down regulatied genes associated with drought stress. Among these downregulated genes, are molecular chaperones and specifically HSP70, which is part of a larger group of heat shock proteins. Typically chaperone synthesis is a common aspect of plant virus infection and the induction of biotic and abiotic stress response genes can be associated with exposure to other various stressors including, thermal changes, heavy metal accumulation, pH variation, and hypoxia (Dardick, 2007; Lindquist & Craig, 1988; Pockley, 2003; Qian & Patterson, 2007). Our samples used for sequencing were collected at two months post-inoculation when virus infection would have been well established throughout the plant. It is possible that downregulation of genes associated with a stress response could be the result of a successful long-term GLRaV-3 infection as a strategy to escape plant defences. The downregulation of stress-related genes has also been described before in *Prunus necrotic ringspot ilarvirus* infection of *N. benthamiana* where mild symptoms were observed in comparison to other viruses infecting this host (Dardick, 2007). It is possible that the lack of observable symptoms associated with GLRaV-3 infection at two months post-inoculation in both hosts could be attributed to the escape of plant stress responses.

The shared downregulation of HSP70s observed in both hosts is another example of a molecular chaperone commonly associated with host response to stress and viral infection being repressed. Recently, it has been shown that heat shock proteins are associated with regulation of the plant immune response and HSP70 as well as other chaperones have been shown to be transiently up or down regulated in a dynamic manner through different stages of

viral infection (Park & Seo, 2015; Phillips, Abravaya, & Morimoto, 1991; Wainberg, Oliveira, Lerner, Tao, & Brenner, 1997). Many plant and animal viruses recruit host cellular HSP70s for replication and cytoplasmic HSP70 has also been shown to enhance infection of *N. benthamiana* by tobacco mosaic virus, potato virus X, and watermelon mosaic virus (Park & Seo, 2015). Although HSP70s appear to be an important factor for plant-virus infections, viruses do not typically encode their own HSP70s and must rely on availability in an infected host (Peremyslov, Hagiwara, & Dolja, 1999). In contrast, viruses from the family *Closteroviridae* encode their own homologs of HSP70s (HSP70h) (Peremyslov et al., 1999). For GLRaV-3, HSP70h is thought to be associated with cell-to-cell movement and virion assembly (Maree et al., 2013). Because GLRaV-3 encodes its own customised HSP70h, it does not have to rely on the host to provide this protein, thus explaining a possible model for the downregulation of these genes in *N. benthamiana* and *V. vinifera.* This could also be another strategy used by the virus to avoid host stress responses.

Both *V. vinifera* and *N. benthamiana* share upregulation of a few genes associated with general plant or cellular processes such as sugar transport and ubiquitination proteins. The induction of U-box domain-containing proteins in both hosts could be interpreted as a mechanism to cope with GLRaV-3 infection. The initiation of plant immune responses requires ubiquitination for positive and negative regulation and is also involved in hormone signalling required for cellular integration of biotic stress cues (Trujillo & Shirasu, 2010). Recent studies have also demonstrated that ubiquitination associated proteins are targeted by pathogen virulence effectors, emphasizing its importance in immunity (Trujillo & Shirasu, 2010).

Another commonly upregulated family of proteins was associated with sugar transport. This group of proteins is associated with drought stress as well as cellular sugar transport (Yıldırım, Yağcı, Sucu, & Tunç, 2018). Accumulation of soluble sugars, decreased photosynthesis and increased respiration have been linked to virus infection in plants (Lemoine et al., 2013). Previous work has also demonstrated that GLRaV-3 infection induces genes related to sugar metabolism, such as sugar transporters and glycosyl transferases (Espinoza et al., 2007). In GLRaV-3 infection of grapevines, it is common to observe symptoms like leaf curling that are associated with decreased photosynthesis. This is thought to be due to the movement and accumulation of sugar to the roots of the plant (Blaisdell et al., 2016). Since GLRaV-3 affects sugar accumulation, it is therefore not surprising to see an upregulation of sugar transport proteins in hosts infected by GLRaV-3. The upregulation of genes involved in this pathway in *N. benthamiana* demonstrates the virus has the same effect on sugar transport in the novel host. Future experiments should test the sugar content of roots in GLRaV-3 infected *N. benthamiana.*

N. benthamiana has already shown several promising advantages over *V. vinifera* to serve as a model organism including a shortened time from infection to detection, relative ease of virion purifications, as well as visualization of viral particles and structural proteins (Prator et al., 2017). Model systems are essential to the propagation of knowledge when ethics, costs, and technical difficulties can be an impediment to experiments (Li, Zanin, Xia, & Yang, 2018; Shaw et al., 2012). In addition, *N. benthamiana* is an herbaceous plant, capable of being grown in greenhouse conditions year around compared to *V. vinifera*, a deciduous, woody host, where transmission experiments are limited by growing season. Ease of genetic transformations methods and use for virus induced gene silencing or transient protein expression, make *N. benthamiana* a popular choice as a tool in plant biology (Goodin et al., 2008).

In conclusion, our results indicate that *N. benthamiana* and *V. vinifera* show a promising amount of similar gene expression patterns in response to GLRaV-3 infection, although many of the DEGs observed were unique to each respective host. As research on

genome annotation of both organisms progresses, we expect further interesting insights into host response to GLRaV-3 infection in the future. To help continue our understanding of this disease, future work could isolate proteins of interest for reverse genetic experiments to test for roles in viral pathogenesis and provide insights into signalling pathways that are affected by GLRaV-3 infection.

MATERIALS AND METHODS

Plants used for the analysis

Planococcus ficus (Hemiptera, Pseudococcidae) colonies were maintained on butternut squash (*Cucurbita moschata*) at 22 °C, with a 16:8-h photoperiod. First instars were used for all experiments because they were shown to be the most efficient life stage to transmit GLRaV-3 (Tsai et al., 2008). Whatman filter papers were wet and placed on top of mealybug colonies. After 30 min the papers were pinned to GLRaV-3 source vine cuttings (accession LR101 (cv. Italia-3); group I) provided by Foundation Plant Services, University of California Davis, CA. After a 24 h acquisition access period (AAP), approximately 20 first instars were transferred manually with a small paintbrush to either healthy *V. vinifera* (cv. Cabernet Sauvignon) or *N. benthamiana* expressing the turnip mosaic virus P1/HC-Pro, kindly supplied by B. Falk (University of California, Davis). After 4 days, any visible mealybugs were removed and plants were sprayed with insecticide before being moved to the greenhouse. To test for GLRaV-3 infection, petiole samples were collected from plants at two months post-inoculation and RNA extractions were completed on 100 mg of petiole tissue (Sharma et al., 2011). One step reverse transcription-polymerase chain reaction (RT-PCR) was then performed and PCR products were analyzed using fragment analysis as described previously (Sharma et al., 2011). At two months post inoculation for this experiment, petioles from four non-infected *V. vinifera*, four GLRaV-3 infected *V. vinifera*, three non-infected *N. benthamiana*, and four GLRaV-3 infected *N. benthamiana* were collected for RNA extractions and HTS submission.

RNA extractions

0.1 g of petioles from a known GLRaV-3 infected *N. benthamiana* plant and 0.1 g petioles from the original GLRaV-3 source *V. vinifera* were used for next generation sequencing. For RNA extractions, petioles were ground in liquid nitrogen and added to 5 ml of Guanidine extraction buffer (4 M Guanidine thiocyanate, 0.2 M sodium acetate, 25 mM EDTA, 2.5% polyvinylpyrrolidone-40) and 1% beta-mercaptoethanol. 20% sarcosyl buffer was added followed by vigorous mixing and incubation in a 57 °C water bath for 12 minutes, vortexing every 3 minutes for better lysis efficiency. The extract was then added to QIAshredder columns (Qiagen) and the remainder of the protocol was followed according to Qiagen RNeasy Plant Mini Kit instructions as previously described (Santos, 2013). RNA concentration and quality were evaluated by measuring the absorbance at 260 nm and the absorbance ratio 260/280 with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at -80°C until further analysis.

RNASeq library preparation and sequencing

Sequencing libraries were constructed at the Functional Genomics Lab (FGL), a QB3-Berkeley Core Research Facility (University of California, Berkeley). Quality of RNA was checked on a 2100 Bioanalyzer (Agilent Technologies, CA, USA). The library preparation was done using RiboZero and Apollo 324™ with PrepX™ RNAseq Library Prep Kits (WaferGen Biosystems, Fremont, CA) and 15 cycles of PCR amplification was used for index addition and library fragment enrichment. RNA samples with >RIN 8 were selected for sequencing. From this selection, one healthy *V. vinifera*, two GRLaV-3 infected *V. vinifera*, two healthy *N. benthamiana*, and one GLRaV-3 infected samples were sequenced.

Genomic sequencing (150 bp paired-end) was done using the Illumina platform (Illumina, Inc., CA, USA) at Vincent J. Coates Genomics Sequencing Laboratory at University of California, Berkeley.

Quality assessment and pre-processing

Sequence data was assessed for overall quality using FastQC (0.11.7) and MultiQC (1.5), before and after every pre-processing step. Ribosomal RNA was removed using SortMeRNA (2.1), and the samples checked for the presence of contaminant ribosomal RNA. Sequences were trimmed by quality and Illumina adaptors removed using Trimmomatic (0.36). This workflow (including MultiQC reports) is described in full in the github repository (Refer to Jupyter notebook and MultiQC report files at https://github.com/PlantandFoodResearch/bioinf_Vitis_Nicotiana_RNAseq).

Reference-based gene expression

Gene expression in both hosts was assessed in reference to existing genomes and annotations. In *N. benthamiana*, the Niben 1.0.1 genome and annotations were obtained from solgenomics.net (https://solgenomics.net/organism/Nicotiana_benthamiana/genome) (Bombarely et al., 2012). For *V. vinifera*, the Genoscope 12X genome and annotations were obtained from Genoscope (www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/),
For both species, pre-processed RNAseq reads were mapped to the appropriate genome using STAR (2.6.1a), deriving raw read counts for each annotated gene in each sample. These workflows are described in full (Refer to Jupyter notebook).

Differential gene expression

Differential gene expression between healthy and infected samples was assessed for both species using edgeR (Robinson, McCarthy, & Smyth, 2010). Briefly, read counts are normalised for library size using the trimmed mean of m-values (Robinson et al., 2010), genes that showed very low abundance were removed, and differential expression assessed using both the likelihood ratio and quasi-likelihood F-test methods. These workflows are described in full (Refer to R markdown notebooks).

Quantitative RT-PCR

Total of 11 DEGs (seven *V. vinifera* and four *N. benthamiana* genes), and four stable expressed genes (two per plant host) identified by RNA-Seq were selected for validating differential expression analysis by RT-qPCR (Table S4). Primers were designed using GenScript Real-time PCR (TaqMan) Primer Design online tool (https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool) requiring one primer pair to cross an exon-exon junction. The OligoAnalyzer 3.1 program (Integrated DNA Technologies, Coralville, USA) was used to analyse the likelihood of prospective primers generating secondary structures.
Total RNAs from petioles from four non-infected *V. vinifera*, four GLRaV-3 infected *V. vinifera*, three non-infected *N. benthamiana*, and four GLRaV-3 infected *N. benthamiana* were collected for RNA extractions and isolated using a modified Qiagen Kit protocol described above. Amplicons were synthesised using two-step qualitative RT-PCR (RT-qPCR), with Superscript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA) used to synthesise the first-strand cDNA, following treatment with Invitrogen™ RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA). qPCR was carried out using PerfeCTa SYBR® Green SuperMix (Quanta Biosciences, Inc., Gaithersburg, MD) on a LightCycler 480 System (Roche Diagnostics, Penzberg, Germany) under the following conditions: 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s followed by a dissociation step. The Ct values and amplification curve data for each reaction generated by the Lightcycler480 software were exported. Relative quantification (RQ) values for each sample were calculated using the method described by Pfaffl (2001) and the geometric average of the plant genes showing stable expression by HTS was used to

normalize the data, instead of using a single reference gene (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007; Vandesompele et al., 2002). Calculated RQ values were log2 transformed and averaged for each of the technical replications of each sample and primer pair combination.

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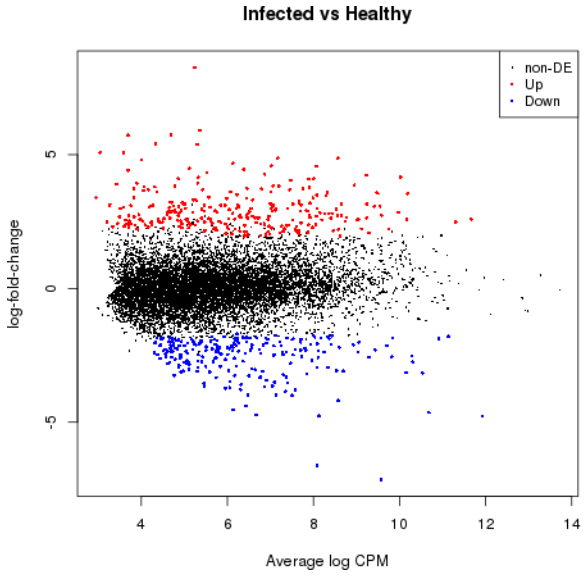
Figure 1. MA-plot of log-fold change (M-values, i.e. the log of the ratio of level counts for each gene between two samples) against the log-average (A-values, i.e. the average level counts for each gene across the two samples) for *V. vinifera* and *N. benthamiana.*

(a) MA-plot comparing GLRaV-3 infected *V. vinifera* to healthy *V. vinifera.*



(b) MA-plot comparing GLRaV-3 infected *N. benthamiana* to healthy *N. benthamiana.*

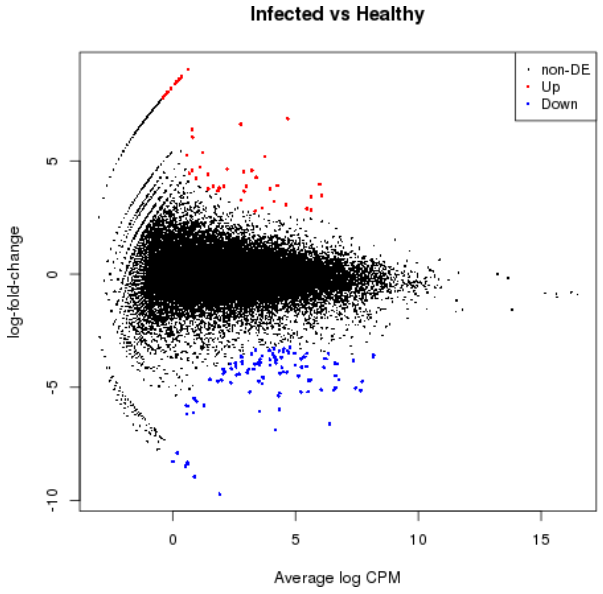


Figure 2. Word cloud analysis shows trends in major gene families observed during transcriptome analyses. The size of words is correlated with the number of times they were observed in the results. Larger words are associated with genes appearing the most in our datasets, while smaller words are associated with genes observed less.

(a) Most common differentially expressed genes in *Vitis vinifera* in response to GLRaV-3 infection.

(b) Most common differentially expressed genes in *Nicotiana benthamiana* in response to GLRaV-3 infection.

(c) Most common differentially expressed genes in both *V. vinifera* and *Nicotiana benthamiana* in response to GLRaV-3 infection.

Table 1. Mapping of RNA sequences to *Nicotiana benthamiana* and *Vitis vinifera*

Species	Sample	Total reads	% mapped (uniquely)	% unmapped
V. vinifera	Healthy	17.5 Mbp	71 (60)	29
	Infected-1	43.3 Mbp	74 (67)	26
	Infected-2	6.5 Mbp	54 (46)	46
N. benthamiana	Healthy-1	52.7 Mbp	78 (66)	22
	Healthy-2	49.4 Mbp	76 (64)	24
	Infected	6.6 Mbp	63 (53)	37

Table 2. Summary of differentially expressed genes in common between *Nicotiana benthamiana and Vitis vinifera*.

Gene Name	Gene Function	Reference
Upregulated		
NAD(P)H-quinone oxidoreductase	Protect organisms from oxidative stress	Heyno et al., 2013
Subtilisin-like serine protease	Immune response	Figueiredo et al., 2014
Sugar transporter protein	Sugar transport, drought response	Yıldırım et al., 2018
U-box domain-containing protein	Degradation of aberrant proteins induced by stress	Azevedo et al., 2001
Beta-glucosidase	Initiate cell division	Brzobohaty et al., 2003
MLP-like protein	Drought tolerance	Wang et al., 2015
Downregulated		
BAG family molecular chaperone regulator	Regulate apoptosis-like processes	Doukhanina et al., 2006
Chaperone protein	Thermotolerance, protein disaggregation	Lee et al., 2004
Copper chaperone	Intracellular delivery of copper to target proteins	Harrison et al., 2000
Ethylene-responsive transcription factor	Transcription regulation	Fujimoto et al., 2000
Heat shock cognate 70 kDa	molecular chaperone, non-covalent folding/unfolding	Chen et al., 2008
Homeobox-leucine zipper protein	Drought tolerance	Lee & Chun, 1998
Peptidyl-prolyl cis-trans isomerase	Thermotolerance, pH stabilization, biotic stress	Pogorelko et al., 2014
Stable		
60S ribosomal protein	Defense against viral infection	Carvalho et al., 2008
Eukaryotic translation initiation factor	Abiotic stress tolerance	Gallino et al., 2018

Table 3. Summary of shared differentially expressed genes with different expression levels between *Nicotiana benthamiana and Vitis vinifera*.

Transcript ID	Gene Name	log2 FC	Gene Description	Reference
GSVIVG01007719001	Glutaredoxin	2.925504	Regulate cellular processes through dithiol–disulfide exchanges with many target proteins	Rouhier et al., 2005
GSVIVG01020614001	Glutaredoxin-C9	2.931628		
Niben101Scf06504g01026	Glutaredoxin family protein	-4.23343		
GSVIVG01019824001	RHOMBOID-like protein 2	3.539202	Catalyzes intramembrane proteolysis. May function in pollen elongation, signaling, development, apoptosis, and mitochondrial integrity	Knopf & Adam, 2012
Niben101Scf18513g00008	RHOMBOID-like protein 14	-3.99537		
Niben101Scf03770g02001	RHOMBOID-like protein 14	-3.39187		
GSVIVG01009928001	Thaumatin-like protein 1b	-2.069703	Involved in anti-fungal response	Vigers et al., 1992
Niben101Scf01400g00014	Thaumatin-like protein	3.664741		
GSVIVG01026054001	UDP-glycosyltransferase 88F3	5.085967	Biosyntheses of cell-wall polysaccharides, the addition of N-linked glycans to glycoproteins, attachment of sugar moieties to various small molecules	Keegstra & Raikhel, 2001
Niben101Scf02751g02006	UDP-Glycosyltransferase superfamily	-8.9506		
Niben101Scf04875g02008	UDP-Glycosyltransferase superfamily	-5.80191		
Niben101Scf06112g01008	UDP-Glycosyltransferase superfamily	-4.73772		

Table S3. Summary of shared differentially expressed genes found in more than one expression category to be up, down, or stably expressed.

Upregulated
Auxin transporter-like protein
Cytochrome P450
NAC domain-containing protein
NAD(P)H-quinone oxidoreductase
Protein NRT1/ PTR
Subtilisin-like serine protease
Sugar transporter protein
Sulfate transporter
U-box domain-containing protein
Zinc finger protein

Downregulated
17.4 kDa class III heat shock protein
Auxin efflux carrier component
BAG family molecular chaperone regulator
Chaperone protein
Copper chaperone
Cytochrome P450
Ethylene-responsive transcription factor
Heat shock cognate 70 kDa
Homeobox-leucine zipper protein
Peptidyl-prolyl cis-trans isomerase
Protein NRT1/ PTR
Sulfate transporter
Zinc finger protein

Stable
60S ribosomal protein
Eukaryotic translation initiation factor
NAC domain-containing protein

Figure S1. RT-qPCR validation of differentially expressed genes. Log2 fold expression values of (a) seven *Vitis vinifera* and (b) four *Nicotiana benthamiana* RNA-seq-selected genes.

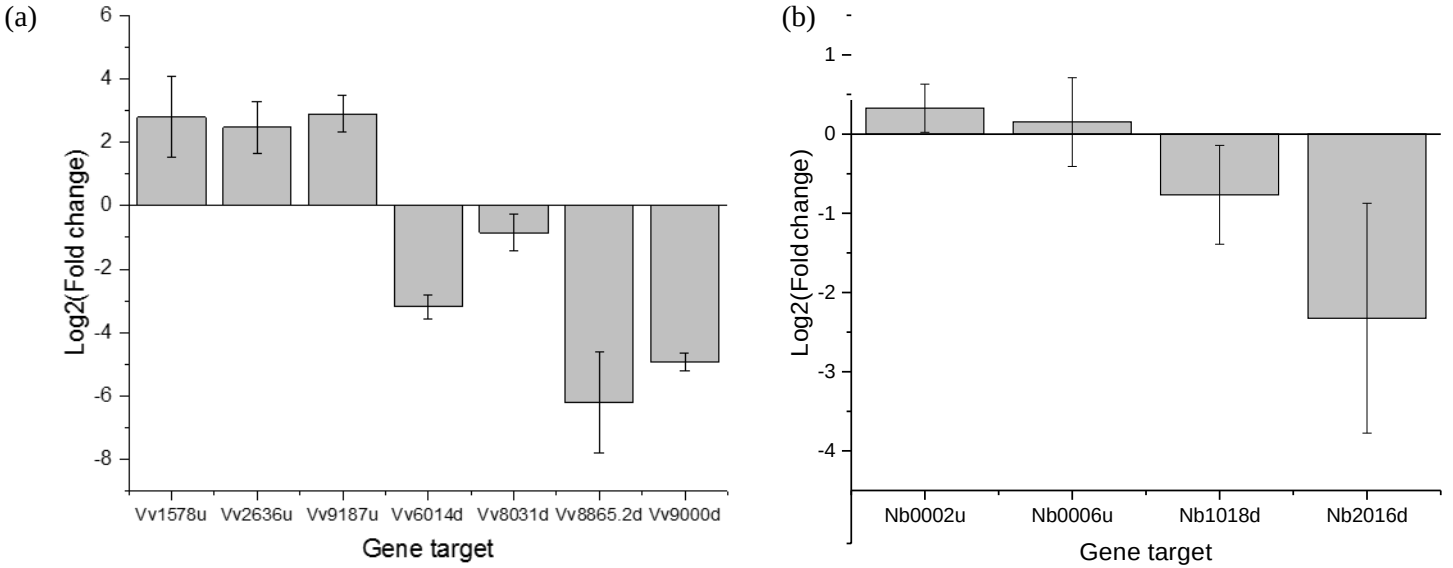


Table S4. Real-time polymerase chain reaction primers and gene targets.

Primer name	Nucleotide sequence (5'-3')	Transcript ID	Description
Vitis vinifera			
V8856.2F	AGCTGGTGGGTCATTCACTG	GSVIVG01028856001	heat shock protein 83
V8856.2R	GTACTCCACCTGGTCGTCCT		
V8031F	ACGTAGACAAGAAGCTCGCTGA	GSVIVG01038031001	probable glutathione S-transferase parC
V8031R	AGAGAAGGGCACCAGAGCCA		
V2636F	CAATGTGATGGAGGCGAAGG	GSVIVG01012636001	thiamine thiazole synthase 2, chloroplastic
V2636R	GGTAGGACCCATCCTTGGAG		
V1578F	AGCAGGGTATCCTCCACAAC	GSVIVG01015780001	cysteine-rich and transmembrane domain-containing protein A
V1578R	CAAGGCAGCACAACATCCTT		
V9187F	TGGGATTGTCAACAGGCTCT	GSVIVG01009187001	VIN3-like protein 2 isoform X1
V9187R	TCGTATCTGCATCCCTGCTT		
V6014F	TGCGGTTCAGGCTGGTGTTT	GSVIVG01026014001	stromal 70 kDa heat shock-related protein, chloroplastic
V6014R	TTTGCCCATCTGCTGCCGTA		
V9000F	ACAACCCAAGGTGGTAGGTG	GSVIVG01035900001	chaperone protein ClpB4, mitochondrial
V9000R	AATAGTTGCCGCCCAAACCT		
V3149F*	ACTGCGTGTTGGAGTAAGGA	GSVIVG01023149001	auxin response factor 1 isoform X1
V3149R*	CGACCGACTTGTTCTTGGTT		
V1341F*	TGGGAAGAGATCCAGGCAGCA	GSVIVG01013410001	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase
V1341R*	GCTCCTCCACCTTGGGCAAT		
Nicotiana benthamiana			
B1018F	TTGCCTTTGGTGTCCTTGGT	Niben101Scf01182g01018	Bidirectional sugartransporter N3
B1018R	TGAGGAGTGCAGTGTTGGTC		
B2016F	TGCGAGTCGAACGAACTGCT	Niben101Scf00739g02016	Zinc finger Ran-binding domain-containing protein 2
B2016R	CATACCACATCCAATTCTGCTGC		
B0002F	ACCACCAGGATTTAGGTTCTTTCCT	Niben101Scf03366g00002	NAC domain-containing protein 21/2
B0002R	GCCACCTCAGGAAGTTGCCA		
B0006F	TGTCTCTCAGGCTCACCACGA	Niben101Scf03243g00006	Tubulin alpha chain
B0006R	TTCACGTCCTTGGGCACCAC		
B0009F*	GCTTGGAGAAGCGGTTTGGTC	Niben101Scf07824g00009	cellulose synthase 1
B0009R*	GCCCACCGAAGCACCTCTTT		
B0007F*	TGTCCAGGAGAGATTCGGGTCTG	Niben101Scf06579g00007	Cytochrome b-c1 complex subunit 7
B0007R*	ACACGCACGCTCTGTGTCTC		

* genes showing stable expression by HTS used to normalize data

Chapter 4

Mealybug-*Ampelovirus* interactions mediate virus transmission

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ABSTRACT
Grapevine leafroll disease is one of the most important virus diseases of grapevine and occurs in every major grape-growing region of the world. The vector transmission mechanisms of the causative agent, *Grapevine leafroll-associated virus 3* (GLRaV-3) remain poorly understood. We show that the vine mealybug, *Planococcus ficus,* will feed through a membrane feeding system on GLRaV-3 viral purifications from both *V. vinifera* and *N. benthamiana,* and transmit the virus to test plants. Building on this strategy, we used an immunofluorescence approach to localize virions to two retention sites in *P. ficus* mouthparts. Assays testing molecules capable of blocking virus transmission demonstrated that GLRaV-3 transmission by *P. ficus* can be disrupted. Our results indicate that our membrane feeding system and transmission blocking assays are a valid approach and can be used to screen other candidate blocking molecules.

INTRODUCTION
Insect vectors play an essential role in the dissemination of many viruses that cause disease in humans, animals, and plants. Vector transmission is a complex event in the virus life cycle and often can be divided into virus acquisition, retention, and inoculation processes. The molecular determinants of virus-vector interactions of many important viruses remain poorly understood. The majority of plant viruses rely on vectors for efficient transmission such as fungi, nematodes, and arthropods (Ng & Falk, 2006). Insects remain the most common vectors of plant pathogens, and specific relationships as well as specific transmission mechanisms exist between a given virus genus and vector species.

Plant virus-insect vector relationships are characterized by properties associated with virus acquisition, retention and transmission. There are four basic types of virus-vector relationships; nonpersistent and semipersistent, both of which are noncirculative, as well as circulative nonpropagative and propagative, where virus enters via the gut and circulates or replicates within the insect body, respectively (Anna E Whitfield, Falk, & Rotenberg, 2015). Specific viral proteins, molecular domains, and amino acids required for virus binding to vectors have been identified for the major viral genera transmitted by insect vectors. Viral coat proteins and other accessory proteins (helper components) mediate interactions required for virus transmission (Ng & Falk, 2006). The identity of putative virus receptors in vectors remains elusive. However, a recent study identified a protein rich region (called “acrostyle”) at the tip of aphid stylets, which functions as the binding site for *Cauliflower mosaic virus* (CaMV) (Uzest et al., 2007). This research was the first to identify proteins as putative vector receptors for a non-circulative plant virus as well as provided definitive proof of a noncirculative virus retention site located at the stylet tips of hemipteran insects (Blanc, Drucker, & Uzest, 2014). For other vector-borne plant viruses transmitted non-persistently, virus-encoded proteins specifically interact with receptors in the vector, allowing for retention and, consequently, successful transmission between plants (Ng & Falk, 2006). In contrast to CaMV, the viral retention site for a semipersistent virus, *Lettuce infectious yellows virus* (LIYV) has been localized to the cibarium of whitefly vectors instead of stylet tips (Chen, Walker, Carter, & Ng, 2011). *Citrus tristeza virus* (CTV), another related semipersistent virus, has also been shown to be localized to the cibarium of an aphid vector (Nabil Killiny, Harper, Alfaress, El Mohtar, & Dawson, 2016).

Grapevine leafroll-associated virus 3 (GLRaV-3) is associated with grapevine leafroll disease (GLD), an important problem in California and all grape-growing regions of the world (Maree et al., 2013). GLRaV-3 is an 18Kb ssRNA *Ampelovirus* in the family *Closteroviridae* transmitted by phloem-sap sucking mealybugs (Hemiptera, Pseudococcidae) and soft scales

(Coccidae). GLRaV-3 is transmitted in a semipersistent manner; insect vectors can retain the virus for a period of a few days (Tsai et al., 2008). The specific retention site and transmission mechanisms for any ampelovirus has not yet been described.

The vine mealybug (*Planococcus ficus*) is an invasive pest in most grape-growing regions worldwide, as well as an efficient vector of GLRaV-3 (Daane et al., 2006). Mealybug nymphs and adult females have specialized piercing-sucking mouthparts that play an important role in virus transmission. Adult males have modified non-functional mouthparts and therefore are unable to spread the virus. The stylet bundle is composed of two maxillary stylets and two mandibular stylets contained in the labium (Herrbach et al., 2017). When the mealybug does not feed, the stylet bundle is retracted as a loop in the body cavity of the insect inside a sheath called the crumena (Heriot, 1936).There is no evidence that the maxillary stylet tips in mealybugs contain the acrostyle region found to be associated with virus retention and transmission in aphids (Uzest et al., 2010). The food canal is connected to the foregut, comprising the precibarium, the cibarium, equipped with a muscular pump, and the esophagus (Herrbach et al., 2017). During feeding, mealybugs extend the stylets into plant vascular tissues where ingestion of primarily phloem begins, although xylem-sap feeding has also been documented through electropenetrography (EPG) (Cid & Fereres, 2010). GLRaV-3 is phloem-restricted in both *Nicotiana benthamiana* and *Vitis vinifera* thus mealybug vectors must ingest phloem-sap to acquire virions for subsequent transmission (Prator, Kashiwagi, Voncina, & Almeida, 2017).

Previously, the mechanisms of virus-vector interactions of GLRaV-3 in mealybugs were poorly understood. In this work, we used a membrane feeding system to show that GLRaV-3 can be transmitted from purified preparations of *V. vinifera* and *N. benthamiana*. Using an immunofluorescence approach, virions appeared to be localized to two retention sites in *P. ficus* mouthparts. We also tested molecules capable of disrupting virus transmission to gain a general idea of virus-vector interactions. We were successfully able to block GLRaV-3 transmission in *P. ficus* demonstrating this approach is valid and can be used to screen other candidate blocking molecules.

RESULTS

Mealybug transmission of GLRaV-3 through an artificial diet membrane system. To determine if *P. ficus* could transmit GLRaV-3 from artificial diets to test plants, experiments with virus purified from both *V. vinifera* and *N. benthamiana* were conducted (Table 1). Our results indicate that *P. ficus* feeds on solutions from both purified GLRaV-3 plant sources through parafilm and transmits the virus to *V. vinifera* and *N. benthamiana*. *P. ficus* transmitted GLRaV-3 from *V. vinifera* virus purifications to 12 out of 94 *V. vinifera* and only 1 out of 187 *N. benthamiana* tested four months post-inoculation. *P. ficus* specimens fed on diets containing *N. benthamiana* virus-purification transmitted GLRaV-3 to 1 out of 84 *V. vinifera* and 2 out of 125 *N. benthamiana* recipient plants. The main effects of source ($p= 0.368$, $z= -0.900$) or recipient ($p= 0.808$, $z= -0.243$) plants did not have a significant effect on transmission rates. The interaction within plant species transmission was significant when compared to interactions between the two different species ($p= 0.026$, $z= 2.226$). All trials included *P. ficus* feeding on artificial diet without purified virus as controls with no transmission results observed.

GLRaV-3 virions are retained in the mouthparts of vectors. *P. ficus* were sequentially fed on separate artificial liquid diets containing first, purified GLRaV-3 virions, second, anti-GLRaV-3 IgG, and third, a diet with goat anti-rabbit IgG conjugated with Alexa Fluor 488. A final short wash step on artificial diet alone was included to reduce any nonspecific binding. The same

experiment was repeated placing *P. ficus* on GLRaV-3 infected grapevines instead of viral purifications during the first step followed by subsequent diets as described above. All trials included *P. ficus* feeding on artificial diet without purified virus or on healthy plants as controls with no localization observed. Our results show that GLRaV-3 was retained in two binding sites in insect mouthparts (Fig. 1). When feeding on purified virions, 19 out of the 794 specimens of *P. ficus* showed a fluorescent signal in the tip of stylets retracted in the labium (Fig. S1). Fluorescent signal was observed in the cibarium of 11 individuals; in one insect signal was observed in both the cibarium and stylet (Table 2). One individual showed a much stronger signal in the cibarium than any other observed (Fig. 1c). No labeling was observed in any of the 140 insects fed on artificial diet without virions. When *P. ficus* was provided with GLRaV-3 infected plants instead of purified virions, 4 insects were observed with a fluorescent signal in the tip of stylets and 4 insects showed labeling in the cibarium out of 333 individuals observed. No labeling was observed in any of the 70 mealybugs fed on healthy plants. There was no significant effect of the diet (artificial or cuttings) ($p= 0.808$, $z= -0.242$) on the location of signal observed and there was no significant difference in whether the signal was located in the stylet or cibarium ($p=0.190$, $z=1.311$) of insects observed.

GLRaV-3 transmission is blocked by a lectin. Because GLRaV-3 appears to be retained in one or two binding sites, further questions remain regarding the nature of the receptors involved in the mouthparts. Virus transmission experiments conducted with a blocking compound provided to *P. ficus* in artificial diets before feeding on purified virus, and subsequent inoculation on *V. vinifera* were performed (three biological replicates) to determine if transmission could be disrupted. The competitor molecules tested were the lectin wheat germ agglutinin (WGA) with expected affinity to substrates on the cuticle of insect vectors and casein, a molecule commonly used to block nonspecific binding of proteins in immunoassays. Our results show that WGA significantly blocked GLRaV-3 vector transmission to plants (0/45 tested) in comparison to the sucrose controls (Table 3; $X^2 = 10.492$, $p=0.001$). Four out of 45 plants were GLRaV-3 positive when blocked with casein, with no significant difference when compared to sucrose controls ($X^2 = 1.963$, $p=0.161$). As a control, experiments with *P. ficus* fed on sucrose instead of a blocking molecule resulted in 9 out of 46 plants infected with GLRaV-3. This provides evidence that the virus binds to *P. ficus* mouthparts and suggests that there is a receptor implicated in virus retention. This assay also provides proof of concept that this approach is valid for testing other compounds capable of blocking transmission.

DISCUSSION

GLRaV-3 is one of the most important viruses of grapevine but there are significant gaps in our understanding of its transmission biology. GLRaV-3 research has been limited by studies in a labor-intensive and technically challenging host-pathogen system until the recent discovery that GLRaV-3 is capable of infecting an alternative model host, *N. benthamiana* (Prator et al., 2017). Building off that study, we determined that GLRaV-3 purifications from both *N. benthamiana* and *V. vinifera* could be transmitted through an artificial diet membrane system to healthy plants (Table 1). The purification protocol used was based on a protocol previously described for LIYV, a related long filamentous virus (V. A. Klaassen, M. Boeshore, V. V. Dolja, & B. W. Falk, 1994). We eliminated subsequent steps associated with ultra-pure purifications to preserve the integrity of the long flexuous virions as well as maintain virus yields.

GLRaV-3 transmission was highest from *V. vinifera* purifications to *V. vinifera* plants compared to transmission to *N. benthamiana*, or from *N. benthamiana* purifications to *V. vinifera*

or *N. benthamiana* plants. It is possible that transmission could be affected by *P. ficus* host plant preference. Observations of *P. ficus* behavior on *V. vinifera* versus *N. benthamiana* have been previously described and demonstrated that *P. ficus* prefers *V. vinifera* (Prator et al., 2017). In these experiments, it was thought that the membrane feeding would eliminate the plant preference component affecting transmission results; but that was not observed given the low transmission rate from *N. benthamiana* observed. It is possible that *P. ficus* rejects *N. benthamiana* as a recipient host or there is some component purified from *N. benthamiana* that deters *P. ficus* feeding on diets. It also may be that *P. ficus* prefers probing plants instead of parafilm, as the transmission rates published in earlier work from *N. benthamiana* plants to both *N. benthamiana* and *V. vinifera* plants were higher (Prator et al., 2017). Future studies should compare GLRaV-3 transmission from purifications with other mealybug species that may feed on *N. benthamiana*.

Because *P. ficus* transmitted GLRaV-3 from artificial diets through membrane feeding, a unique immunofluorescent localization system previously used to investigate LIYV transmission in whitefly vectors was adapted for this study. This approach proved to have low efficiency in this host-pathogen system compared to the results observed in whiteflies, with 31 out of 794 first-instar *P. ficus* observed with a fluorescent signal (Table 2). A similar pattern was observed with 8 out of 333 *P. ficus* fed on GLRaV-3 infected source grapevine cuttings instead of viral purifications (Table 2). In order to observe fluorescence, *P. ficus* was required to feed on four different subsequent diets. If the mealybug did not feed on any one of the diets specific labeling would not be observed, which could explain the low numbers of insects with any labeling. We observed fluorescent signals in the anterior foregut region (cibarium) of *P. ficus* as well as on the retracted stylet tips, regardless of the initial source diet (viral purifications or live plant cuttings). In one insect, signal in both of these regions was observed at the same time. It is possible that the virus is retained in both sites, but further studies must determine if transmission of the virus is associated with one or both of the sites. Without associated transmission data it cannot be concluded whether the retention sites observed are implicated in transmission of GLRaV-3.

Although it cannot be confirmed which site is associated with transmission from this work, it is encouraging that these results follow trends observed in other virus-vector systems. Both the stylet tips and cibarium regions have been implicated in nonpersistent or semipersistent virus transmission. The retention site of the related noncirculative LIYV has recently been identified in the cibarium of the whitefly vector (Chen et al., 2011). Retention sites for other semipersistently transmitted viruses including the leafhopper-transmitted *Maize chlorotic dwarf virus*, aphid-transmitted *Anthriscus yellows virus* and *Parsnip yellow fleck virus* have also been localized to the tips of stylets or foreguts of insect vectors (Ammar & Nault, 1991; Childress & Harris, 1989; Murant, Roberts, & Elnagar, 1976). The stylet tips of aphid vectors were observed to be the retention site for CaMV, another semipersistent virus, as well as *Cucumber mosaic virus* and other potyviruses (Brault, Uzest, Monsion, Jacquot, & Blanc, 2010; Ng & Falk, 2006).

In an effort to further characterize the nature of virus-vector interactions we tested if the transmission of GLRaV-3 could be disrupted by either casein or the lectin wheat germ agglutinin (WGA). Our results showed that WGA, a lectin with affinity to substrates on the cuticular surface of insect vectors, resulted in significantly lower transmission rates than casein, a molecule used to block nonspecific binding of proteins, or the sucrose control. These results demonstrate that our approach to feed mealybugs blocking molecules and determine transmission rates is valid, and that it is feasible to test other molecules for this purpose. Previous work showed that lectins, carbohydrates, antibodies, and peptides affected the transmission rate

of *Xylella fastidiosa*, a noncirculative bacterial pathogen that colonizes the foregut of leafhopper vectors (N. Killiny, Rashed, & Almeida, 2012; Labroussaa, Zeilinger, & Almeida, 2016). This approach provides only a general idea of the type of vector-pathogen interactions. For vector-borne plant viruses, a specific viral protein is required for virus transmission and these have been recently described for related viruses in the *Closteroviridae*. The minor coat protein (CPm) for the *Crinivirus* LIYV and both the CPm and heat shock proteins in *Citrus tristeza virus*, an aphid-transmitted *Closterovirus*, are viral proteins required for successful retention and transmission by insect vectors (Chen et al., 2011; Nabil Killiny et al., 2016). Further research investigating interruption of transmission processes is required to develop novel control strategies as well as develop a basic understanding of transmission mechanisms (Anna E. Whitfield & Rotenberg, 2015).

In conclusion, our analyses of GLRaV-3 transmission mechanisms suggest that viral retention can be narrowed down to one or two binding sites in *P. ficus* mouthparts. Although the viral proteins required for binding remain unknown, vector transmission is blocked after binding of WGA suggesting that the virus interacts with the cuticular surface of the mouthparts. This provides first insights into the transmission biology of this economically important host-pathogen system and demonstrates that mealybug feeding through artificial diet systems works for future studies. Further investigations are needed to elucidate the specific viral proteins required for transmission. The lack of an infectious clone hampers GLRaV-3 research but the creation of recombinant viral proteins and specific antibodies could help confirm the viral retention site and transmission strategies of this system.

MATERIALS AND METHODS

Virion purification

GLRaV-3 virions were purified using a modified protocol as previously described (V. Klaassen, M. Boeshore, V. V. Dolja, & B. W. Falk, 1994). 10 g of GLRaV-3 infected *V. vinifera* or *N. benthamiana* leaves were ground in liquid nitrogen with a mortar and pestle. GLRaV-3 source vine cuttings (accession LR101; variant I) were provided by Foundation Plant Services, University of California Davis, CA. *N. benthamiana* source material was collected from plants infected as described previously (Prator et al., 2017). 80 ml of extraction buffer (0.1 M-Tris-HC1, 0.5 % (w/v) Na_2SO_3, 0.5 % (v/v) 2-mercaptoethanol pH 7.4) was added to the ground plant material. TritonX-100 was added to a final concentration of 2 % (v/v) and the solution was stirred for 1 hr over ice. The mixture was centrifuged in a Beckman 50.2 Ti rotor (Beckman Coulter, Inc., CA, USA) at 7500 rpm for 10 min at 4°C. The supernatant was transferred to a new ultracentrifuge tube, then 1 ml of 20 % sucrose in TE (10 mM TrisHCl, 1 mM EDTA, pH 7.4) was added as a cushion underneath the supernatant followed by centrifugation in a Beckman 70.1 Ti rotor at 35,000 rpm for 2 hrs at 4°C. The supernatant was removed and pellet was soaked in 500 µl of TE overnight at 4°C. The pellet was resuspended with gentle pipetting up and down and centrifuged at 7400 rpm for 2 minutes. The supernatant was removed and the pellet was resuspended in 50 µl of TE.

Membrane feeding transmission assays

P. ficus colonies were maintained on butternut squash (*Cucurbita moschata*) at 22 °C, with a 16:8-h photoperiod. First instars were used for all experiments because they were shown to be the most efficient vector of GLRaV-3 (Tsai et al., 2008). Three of the resuspended virion purifications from either *N. benthamiana* and *V. vinifera* were immediately pooled and added to 1.8 ml of an artificial diet composed of 15% sucrose and 1% BSA in TE as described previously

(Chen et al., 2011). Artificial diet without the addition of purified virions was used as a control. Diets were placed in small glass dishes. Approximately 20 mealybugs were placed in 14- by 20-mm (diameter by height) plexiglass feeding chambers and the opening was covered by a layer of thinly stretched parafilm. The feeding chamber was then placed, parafilm side down into a small dish containing either artificial diet with GLRaV-3 virions or just artificial diet and covered to prevent light disruption. Mealybugs were given an acquisition access period (AAP) of 24 hours on each respective diet before being manually removed from the feeding chamber with a small paintbrush and moved to healthy test *N. benthamiana* or *V. vinifera* (n=10 mealybugs per plant). After a 4-day inoculation access period (IAP), mealybugs were manually removed from the test plant and plants were treated with pesticides and moved to the greenhouse. Petiole samples were collected from all plants 4 months post-inoculation and RNA extractions were completed on 100 mg of petiole tissue (Sharma et al., 2011). One step reverse transcription-polymerase chain reaction (RT-PCR) was then performed and PCR products were analyzed using fragment analysis as described previously (Sharma et al., 2011). Logistic regression analysis was performed using R (Version 3.0.2, R. RStudio, Inc., Boston, MA [http://www.rstudio.com/]).

Virion localization assays

Potential virion retention sites in mealybug vectors were localized following the protocol described by (Chen et al., 2011). For artificial diet experiments, approximately 100 first instar mealybugs at a time were placed in feeding chambers as described above and placed on artificial diets with or without GLRaV-3 virions for a 12-hour AAP. In a second experiment, mealybugs were placed on GLRaV-3 source or healthy vine cuttings (accession LR101; variant I) for a 12-hour AAP. Mealybugs were then placed on a second diet containing a 1/800 dilution of rabbit anti-GLRV-3 polyclonal antisera (kindly supplied by Dr. Adib Rowhani, UC Davis) for 12 hours followed by a third artificial diet containing a 1/200 dilution of goat anti-rabbit antisera conjugated with Alexa Fluor 488 (Invitrogen) for 12 more hours. A final artificial diet was presented to the mealybugs for 4 hours as a wash to remove any nonspecifically bound virions or leftover antibodies present in the mouthparts. Logistic regression analysis was performed using R.

Blocking virus transmission

Five to ten mealybugs were placed in feeding chambers and the opening was covered by a layer of thinly stretched parafilm as described above. The feeding chambers were then placed, parafilm side down into a small dish containing either artificial diet with a competitor molecule or just artificial diet for a 12-hour AAP. The competitor molecules chosen were the lectin wheat germ agglutinin (0.1% (vol/vol)) with expected affinity to substrates on the cuticle of insect vectors (N. Killiny et al., 2012) and casein (0.1% (vol/vol)), a molecule commonly used to block nonspecific binding of proteins. Mealybugs were then moved to an artificial diet containing viral purifications from grapevines as described above for another 12 hours followed by manual placement onto test *V. vinifera* for a 4-day IAP. Petiole samples were collected from plants 4 months post-inoculation and RNA extractions and RT-PCR were completed as described above. A logistic regression with Firth's bias correction was used because no acquisition or inoculation occurred in the sucrose treatment resulting in all zeroes (quasi-complete separation of factor levels) (Heinze & Schemper, 2002). Analyses were performed using R and the logistf package for Firth's logistic regression (Heinze, Ploner, Dunkler, & Southworth, 2013).

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Table 1. *Planococcus ficus* transmits GLRaV-3 from purified virus fed through a membrane system. GLRaV-3 was able to be purified from both *Vitis vinifera* and *Nicotiana benthamiana* source plants and successfully transmitted by insect vectors feeding on artificial diets with an acquisition access period of 24 hours followed by a four day inoculation access period on test plants. All trials included *P. ficus* feeding on artificial diet without purified virus as controls.

Source	Recipient	Plants infected/Plants inoculated	Control
V. vinifera diet	*V. vinifera*	12/94	0/18
	N. benthamiana	1/187	0/37
N. benthamiana diet	*V. vinifera*	1/84	0/17
	N. benthamiana	2/125	0/25

Table 2. Summary of GLRaV-3 retention site observations. *Planococcus ficus* fed on either artificial diet augmented with GLRaV-3 virions or vine cuttings for 12 hours followed by 12 hour acquisition access times on diets containing anti-GLRV-3 polyclonal antisera and antisera conjugated with Alexa Fluor 488, respectively. GLRaV-3 is retained in the stylet and cibarium of *Planococcus ficus.*

Source		Stylet	Cibarium	Both	Total
Artificial diet	Number of *P. ficus* positive	19	11	1	31
	Total *P. ficus* viewed				794
GLRaV-3 vine cuttings	Number of *P. ficus* positive	4	4	0	8
	Total *P. ficus* viewed				333

Table 3. GLRaV-3 transmission is reduced by a lectin. a) Results from three biological replicates GLRaV-3 blocking transmission tests showing number of plants infected/plants inoculated. b) Statistical results from bias-corrected logistic regression testing differences between WGA and casein transmission results from sucrose controls.

a)

Treatment	1	2	3	Total positive
WGA	0/15	0/15	0/15	0/45
Casein	1/15	1/15	2/15	4/45
Sucrose	4/16	3/15	2/15	9/46

b)

Treatment	Estimate	SE	χ^2 statistic	*P* value
Intercept	-1.204	0.517	6.331	0.011
Casein	-0.828	0.608	1.963	0.161
WGA	-3.103	1.429	10.492	0.001

Fig. 1. GLRaV-3 virions are retained in the mouthparts of *P. ficus* vectors. a) First instar head of *P. ficus* control after feeding on membrane diet containing sucrose only followed by subsequent antibody labelled diets showing no labeling. Chitin in insects can be autofluorescent accounting for the small signal observed. b) Retention of GLRaV-3 virions in the retracted stylet tips (near white arrow), or c) cibarium of *P. ficus* after sequential membrane feeding immunolocalization assay. Bars represent 20 µm.

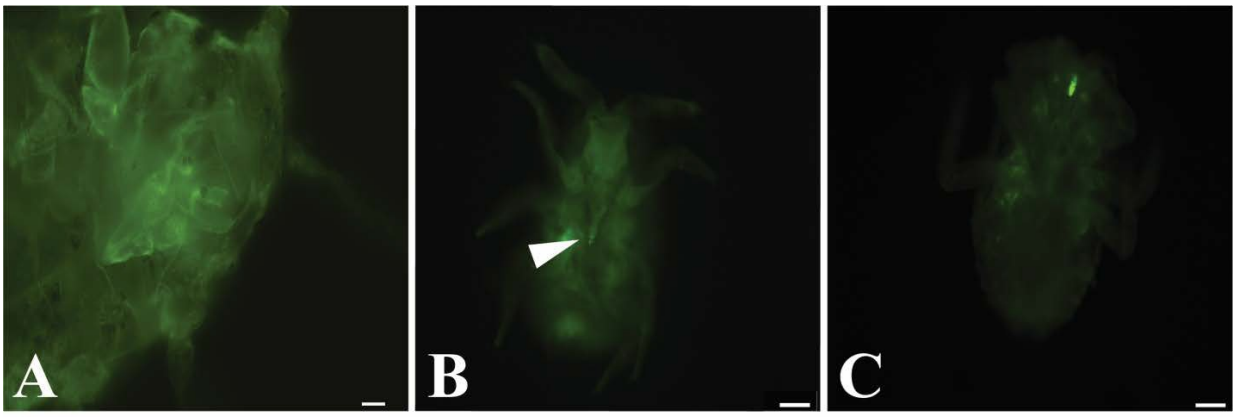

Fig. S1. Evidence of GLRaV-3 virion retention in stylet tip of *P. ficus* vectors. a) Retention of GLRaV-3 virions in the retracted stylet tips with magnified view documented in b). Bars represent 20 µm.