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

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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 *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 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 substitutions in the 5′ UTR. These results open new avenues and opportunities for GLRaV-3 research.

1. 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 (Goodin et al., 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 (Goodin et al., 2005; Ohad et al., 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 et al., 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

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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 18 kb 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 et al., 1958; Over de Linden and Chamberlain, 1970). Berry quality also is significantly decreased one year after infection under field conditions (Blaisdell et al., 2016).

Previously, GLRaV-3 had never been shown to infect hosts outside of the genus *Vitis*. 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 a labor-intensive and technically challenging host-pathogen system.

2. Results

2.1. 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 model plants were performed. Host plants tested included *Arabidopsis thaliana, Capsicum annuum, Nicotiana benthamiana, Nicotiana tabacum,* and *Solanum lycopersicum* (Table 1). GLRaV-3 could be detected 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 protein, 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 four months post-inoculation.

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

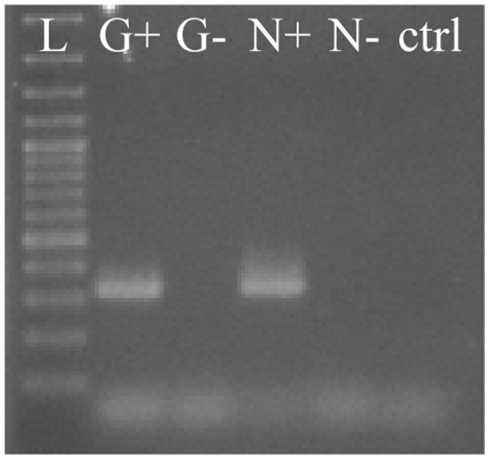

Fig. 1. GLRaV-3 infects *N. benthamiana*. L: Ladder. G+: GLRaV-3 positive *V. vinifera*. G-: GLRaV-3 negative control in grape, *V. vinifera*. T+: GLRaV-3 positive *N. benthamiana*. T-: GLRaV-3 negative control in *N. benthamiana*. Expected fragment length 320 base pairs (bp).

Table 2
Summary of GLRaV-3 vector transmission experiments with *Nicotiana benthamiana* and *Vitis vinifera*. *N. benthamiana* petioles were tested two months post-inoculation while *V. vinifera* was tested four months post-inoculation.

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

[a] HC-Pro N. benthamiana used.
[b] 20 mealybugs were placed in clip cages on a leaf in addition to 50 mealybugs placed freely on the plant. Mealybugs were placed freely on plants without clip cages for trials 2, 5, 6, 7.

2.2. 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.

2.3. 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 petioles taken four months post-inoculation), amounts of GLRaV-3 coat protein (CP) purified from *N. benthamiana* appeared 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.

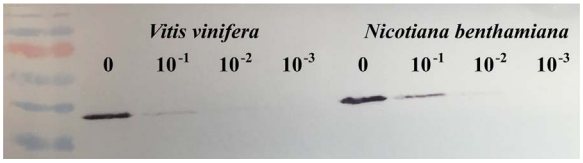

Fig. 2. Western blot analysis of GLRaV-3 virion purifications. Comparisons of serial 10-fold dilutions of *V. vinifera* vs. *N. benthamiana* purifications from 1 g of petioles collected four months post-inoculation. Blots were probed with antiserum to GLRaV-3 coat protein (CP). Darker bands from *N. benthamiana* purifications indicate the presence of higher concentrations of viral CP.

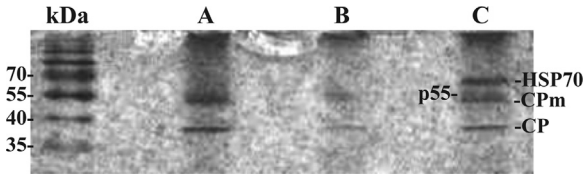

Fig. 3. SDS-PAGE analysis of *N. benthamiana* purifications. Lanes A, B, C represent three purifications from *N. benthamiana*. In lane C, the four proteins visible likely correspond with GLRaV-3 structural components; 59 kDa heat shock protein 70 homologue, HSP70; a 55 kDa protein, p55; the 35 kDa major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

The band corresponding with CP in Fig. 3 and confirmed by western blot in Fig. 2 indicates this protein migrated slower 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 (from *N. benthamiana*). The expected length of GLRaV-3 particles is 1800–2200 nm.

2.4. 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.

2.5. Whole-genome sequencing identified substitutions in the 5′ UTR

Alignment of whole genome sequences from GLRaV-3 infected

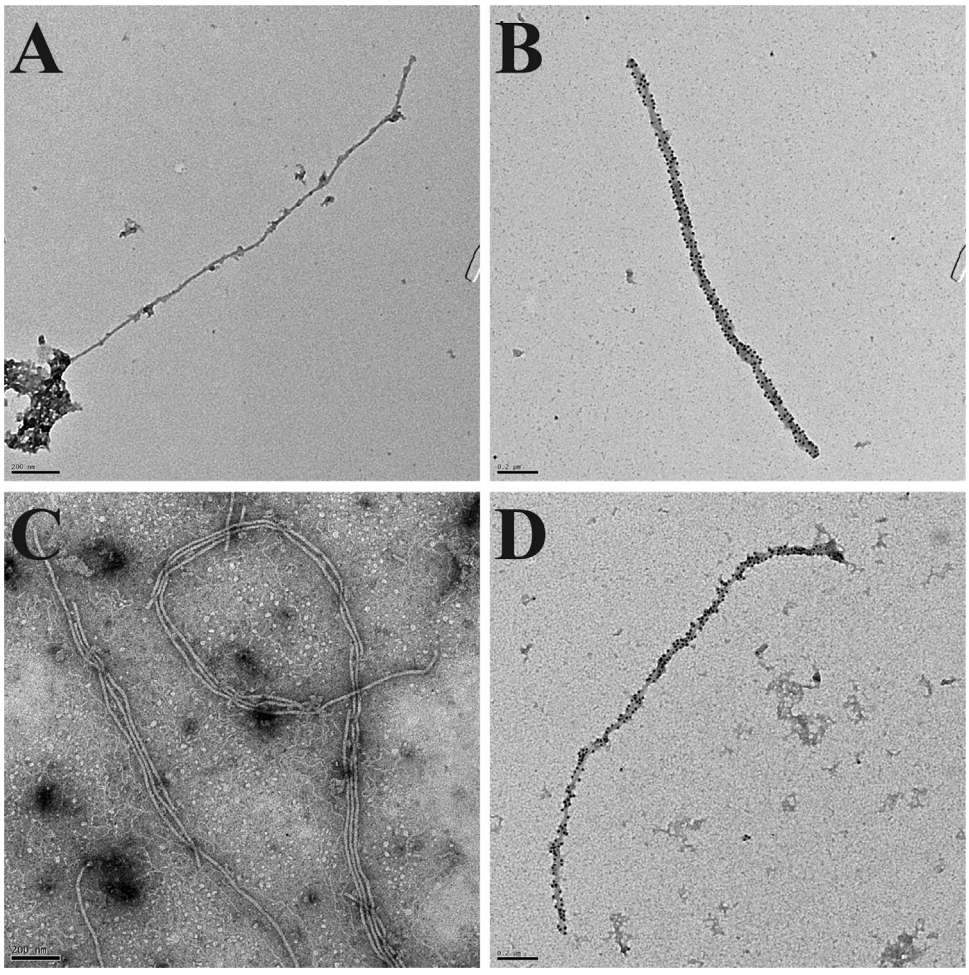

Fig. 4. Transmission Electron Microscopy and immunogold labelling analysis of purified GLRaV-3 virions. (A) Virion purified from GLRaV-3 infected *V. vinifera* petioles. (B) Virion purified from *V. vinifera* petioles labelled using antiserum to the GLRaV-3 CP. (C) Virions purified from GLRaV-3 infected *N. benthamiana* petioles 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.

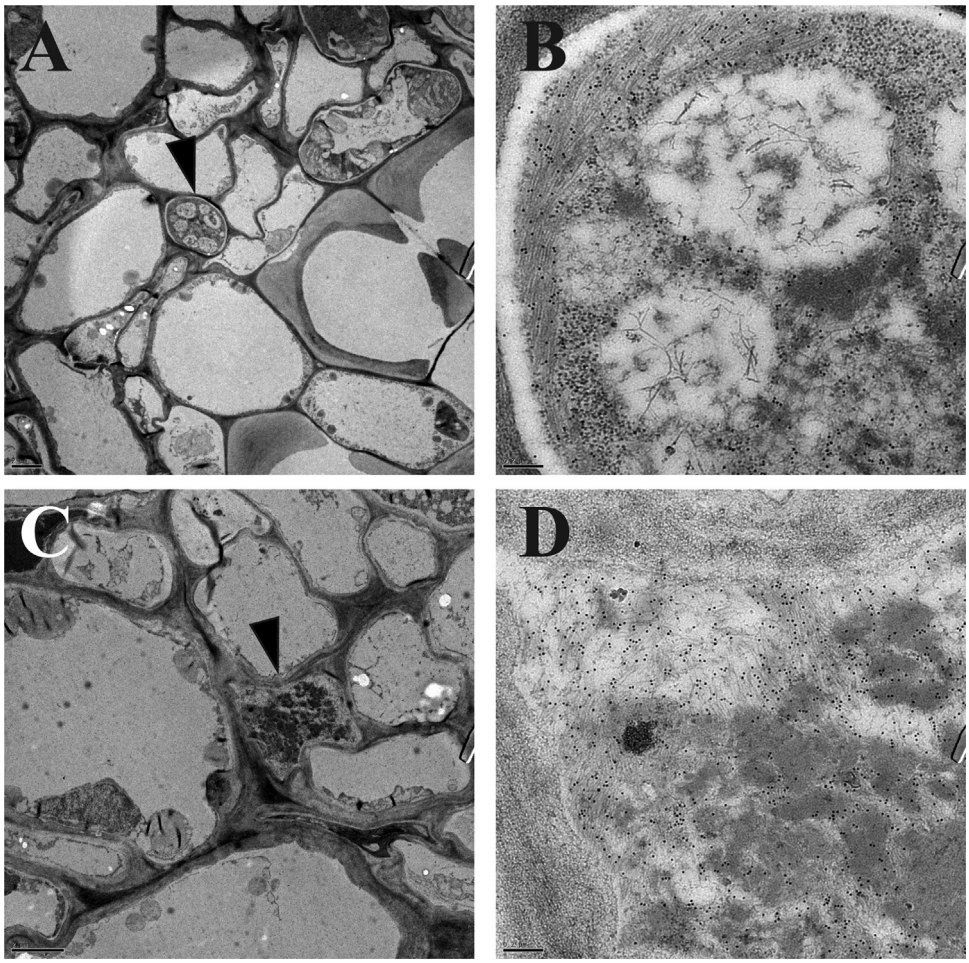

Fig. 5. TEM and immunogold labelling analysis of GLRaV-3 virions in infected *N. benthamiana* petioles. (A, C) Low magnification view of area surrounding GLRaV-3 infected cells. Arrow points to phloem cells where virions could be observed. (B, D) Higher magnification view of phloem cells corresponding 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.

source *V. vinifera* (387,402 reads) and subsequently infected *N. benthamiana* (168,764,540 reads) revealed that sequences were mostly identical (a minimum coverage of 100x throughout the genome). We found three nucleotide deletions and ten nucleotide substitutions in the 5′ UTR of the *N. benthamiana* sequence. Alignment with six other full length GLRaV-3 genomes determined that the 5′ UTR is, in general, variable. The sequence of the GLRaV-3 isolate found in *N. benthamiana* was deposited in GenBank under the accession number MF186605. The sequence of the GLRaV-3 isolate found in the source *V. vinifera* was deposited under the accession KY886362. Mapping of reads from the original GLRaV-3 source *V. vinifera* sequence against a custom library of 57 grapevine-infecting virus species and 4 viroids revealed the presence of GLRaV-3 (647,236-fold coverage), GLRaV-2 (5-fold coverage), and Hop stunt viroid (1.5-fold coverage) at a threshold 1-fold coverage. In comparison, GLRaV-3 (1,481-fold coverage) was the only virus found when mapping reads from infected *N. benthamiana*. This indicates that only GLRaV-3 was transmitted by *P. ficus* from source *V. vinifera* to the recipient *N. benthamiana*.

3. 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 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 et al., 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 similar sequences obtained indicate that there were no adaptive mutations accumulated within the one passage analyzed. The nucleotide variation in the GLRaV-3 genome from *N. benthamiana* 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 et al., 1974; Woodham and 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 et al., 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 and 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 et al., 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 (Wang et al., 2009b). 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 et al., 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 proven to play a crucial role in several seminal discoveries in other host-pathogen systems as already described in recent work (Bisaro, 2006; Goodin et al., 2008; Wang and 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 (Wang and 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 et al., 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.

4. Material and methods

4.1. 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 life stage to transmit 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 weekly until two months post-inoculation when a positive result was obtained 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 (Wang et al., 2009a). 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/].

4.2. Mechanical inoculations

N. benthamiana seedlings were dusted with carborundum and inoculated with different combinations of three different virus sources and three different extraction 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. The purification was completed as described below but omitted the sucrose cushion and final centrifugation steps. Each of these starting virus sources was inoculated with three different extraction buffers 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.

4.3. Virion purification and analysis

GLRaV-3 virions were purified using a modified protocol as previously described (Klaassen et al., 1994). 10 g of GLRaV-3 infected *V. vinifera* or *N. benthamiana* leaf petioles 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 h 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 h 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 min. 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. PageRuler™ Prestained Protein Ladder, 10–180 kDa (Thermo Fisher, MA, USA) was used. Blots were probed with antiserum to GLRaV-3 coat protein (supplied by Dr. Adib Rowhani) diluted 1:1000 and detected using Immun-Blot® AP Colorimetric Kits for immunodetection according to the manufacturer's directions (Bio-Rad, CA, USA).

4.4. Electron microscopy of purified virions

For immunogold labelling 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 1.45 s; National Institutes of Health, USA [http://imagej.nih.gov/ij].

4.5. 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.

4.6. 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 h. 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 min 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.

4.7. 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 min, vortexing every 3 min 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 (50PE) was done using the Illumina platform (Illumina, Inc., CA, USA) at Vincent J. Coates Genomics Sequencing Laboratory (at U.C. Berkeley). Quality control check was done using FastQC (Babraham Bioinformatics, UK). Reads were trimmed using 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 comparison. To determine if viruses other than GLRaV-3 were present in our samples, we mapped data against a custom library of 57 grapevine virus species and four viroids found in grapevine. Briefly, reads were trimmed with Sickle and aligned to a grapevine or *N. benthamiana* genome. Unaligned reads were mapped against the virus and viroid library using Bowtie2 ver. 2.2.9 (Langmead and Salzberg, 2012). Results were analyzed using BAMStats 1.25 program (Kevin E. Ashelford, [http://bamstats.sourceforge.net]).

Depositories

The GenBank accession numbers for the nucleotide sequences of Grapevine leafroll-associated virus 3 isolate GLRaV-3-I-LR101 are KY886362 from *V. vinifera* and MF186605 from *N. benthamiana*.

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