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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

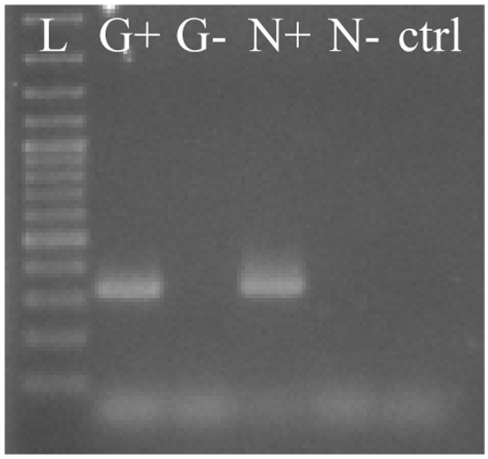

Fig. 1. GLRaV-3 infects *N. benthamiana*. L: Ladder. G+: GLRaV-3 positive *V. vinifera*. G-: GLRaV-3 negative control in grape, *V. vinifera*. T+: GLRaV-3 positive *N. benthamiana*. T-: GLRaV-3 negative control in *N. benthamiana*. Expected fragment length 320 base pairs (bp).

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.