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# Interactions Within Susceptible Hosts Drive Establishment of Genetically Distinct Variants of an Insect-Borne Pathogen

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**ABSTRACT** Coinfections are common, leading to pathogen interactions during transmission and establishment in a host. However, few studies have tested the relative strengths of pathogen interactions in vectors and hosts that determine the outcome of infection. We tested interactions between two genetically distinct variants of the mealybug-transmitted *Grapevine leafroll-associated virus 3*. The transmission efficiency of each variant in single variant inoculations by two vector species was determined. The effects of vector species, a coinfecting source, and simultaneous inoculation from multiple hosts to one host on variant establishment were examined. Within-vector interactions could have a role in transmission from hosts containing mixed infections, but not when vectors were moved from separate singly infected source plants to a single recipient plant. The invasive *Planococcus ficus* (Signoret) was a more efficient vector than *Pseudococcus viburni* (Signoret). Transmission efficiency of the two variants did not differ in single variant inoculations. Overall infections were the same whether from singly or coinfecting source plants. In mixed inoculations, establishment of one variant was reduced. Mixed inoculations from two singly infected source plants resulted in fewer mixed infections than expected by chance. Therefore, the observed outcome was determined subsequent to host inoculation rather than in the vector. The outcome may be due to resource competition between pathogens. Alternatively apparent competition may be responsible; the pathogens' differential ability to overcome host defenses and colonize the host may determine the final outcome of new infections. Detailed knowledge of interactions between pathogens during transmission and establishment could improve understanding and management of disease spread.

**KEY WORDS** apparent competition, Grapevine leafroll-associated virus 3, *Planococcus ficus*, *Pseudococcus viburni*, resource competition

Coinfections with multiple pathogen species or strains that may vary in virulence are common in many disease systems of humans, other animals, and plants (Chandler et al. 1987, Garrett et al. 2004, Bell et al. 2006, Gómez et al. 2009, Lecoq et al. 2011), inevitably leading to interactions between pathogens during transmission and establishment in new hosts. The strength and direction of pathogen interactions can differ within host and vector, potentially exerting opposing influence on overall spread. Competition and complementation have both been found between pathogens in vectors during transmission, as well as in potential hosts (Kassanis and Govier 1971, Power 1996, Pruss et al. 1997). Interactions between pathogens in hosts can lead to resource competition, in which pathogens compete for a limiting resource such as nutrients (Lacroix et al. 2014). Alternatively apparent competition

between two pathogens, in which the defense response of the host is a shared enemy of multiple pathogens (Cobey and Lipsitch 2013), may determine the outcome of mixed infections.

Vectors can affect the differential transmission of plant pathogens and consequent disease spread (Srinivasan et al. 2012, Salvaudon et al. 2013). In some cases, a pathogen already present at low prevalence can become widespread after a new more effective vector is introduced (Purcell and Feil 2001), or after adaptation by a pathogen to a vector that is already present (Tssetsarkin et al. 2011). Vector behavior, such as within-host feeding preference, can mediate the risk of pathogen transmission (Daugherty et al. 2010). One pathogen in a vector can affect the transmission of another pathogen; for example, potato aucuba mosaic virus can be transmitted by aphids only if they have fed on plants infected with potato virus Y (Kassanis and Govier 1971). Furthermore, vector transmission from a coinfecting source can lead to differential transmission between two virus strains (Srinivasan et al. 2012, Péréfarres et al. 2014). For vector-borne plant viruses transmitted both in a circulative and noncirculative manner, insects transmit one to a few virus particles or genomes per inoculation event, even when several

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million virus genomes are present in the insect; thus, vectors can be strong bottlenecks (Moury et al. 2007, Péréfarres et al. 2014). At regional landscape scales, vector populations can drive patterns of host coinfection by closely related pathogens, perhaps even overriding the direct influence of hosts on pathogens (Power 1996, Seabloom et al. 2009).

Pathogens can interact during the early stages of a new infection of a susceptible host (Bell et al. 2006, Julve et al. 2013, McWhorter et al. 2013). Recent studies have shown that two viral pathogens within one host plant will not colocalize within individual plant cells during establishment of new infections, leading to a mosaic pattern of cells that are singly infected with each of two viruses (Blanc et al. 2011, Julve et al. 2013). Furthermore the newly infected host plant acts as a bottleneck as the virus spreads throughout the plant, resulting in a subset of the originally inoculated viral population diversity spreading throughout the plant (Li and Roossinck 2004, Miyashita and Kishino 2010, Gutiérrez et al. 2012). In plant populations, simultaneous inoculation with multiple pathogens could occur when a source plant is infected with multiple pathogens and one or more vectors move from one source plant to susceptible host plants. Alternatively, multiple host plants in a region may be singly infected with different pathogens, and visited by vectors who then travel from those sources to the same susceptible host. It is common that the life cycle of a vector species is synchronous and pathogen transmission occurs at a vector's particular life stage; predicting the outcome of the resulting simultaneous inoculations from multiple hosts could inform predictions of disease spread. Coinfections of closely related pathogen species or even multiple genetically distinct variants of one pathogen species are common. Examples of plant diseases that commonly occur as coinfections of multiple virus strains or variants are barley yellow dwarf virus, tomato yellow leaf-curl virus, and grapevine leafroll-associated viruses (Garrett et al. 2004, Seabloom et al. 2009, Jooste et al. 2011, Sharma et al. 2011, Péréfarres et al. 2014).

*Grapevine leafroll-associated virus-3* (GLRaV-3), in the family *Closteroviridae*, genus *Ampelovirus*, is only known to affect *Vitis* spp. (grapevines), and is transmitted by several species of mealybugs and soft scale insects (Hemiptera, Pseudococcidae, and Coccidae, respectively; Tsai et al. 2010, Klaassen et al. 2011, Le Maguet et al. 2012, Almeida et al. 2013, Maree et al. 2013). It is transmitted by mealybugs in a semipersistent manner or can be transmitted via grafting, but cannot be transmitted by leaf-rub inoculations. Within the host plant, GLRaV-3 is limited to the phloem. GLRaV-3 is the primary virus species that causes grapevine leafroll disease, which affects crop quality in grape-growing regions worldwide (Almeida et al. 2013). GLRaV-3 is subdivided into several genetically distinct variants, consecutively numbered I-VI (Maree et al. 2013). Coinfections of multiple GLRaV-3 variants within a single host plant are common (Jooste et al. 2011, Sharma et al. 2011). GLRaV-3-I through GLRaV-3-V have relatively high genetic similarity, while

GLRaV-3-VI is distinct from other variants (Maree et al. 2013).

In vineyards of Napa Valley, CA, two mealybug vectors of GLRaVs are *Pseudococcus viburni* (Signoret) (obscure mealybug) and *Planococcus ficus* (Signoret) (vine mealybug). *Ps. viburni* is neotropical in origin (Charles 2010). Because of past taxonomical confusion, it is unknown how long *Ps. viburni* has been present, but it is thought to have been in California for at least 50 years (Daane et al. 2008). *Ps. viburni* has three generations per year, with typical population densities low enough that they do not cause direct damage to the grapevines (Daane et al. 2012). The invasive *Pl. ficus* is thought to be Mediterranean in origin, and was first discovered in southern California in 1994. It has been expanding its range northward since then (Daane et al. 2004). In contrast to *Ps. viburni*, it can produce seven overlapping generations per year with higher population density, often causing direct damage to vines in addition to being a vector of GLRaV-3 (Gutierrez et al. 2008).

Nothing is known about the ecology of the genetically distinct GLRaV-3 variants with respect to variation in transmission efficiency, differential transmission by different vector species, symptom severity, host specificity, or interactions with each other during transmission and establishment of new infections. We used two variants of GLRaV-3 to determine virus interactions during vector transmission and infection establishment in a new host: GLRaV-3-I, which is commonly found and genetically similar to most described GLRaV-3 variants, and GLRaV-3-VI, which is genetically distinct from the other five known variants. We tested the transmission efficiency of each variant and their interactions during vector transmission and establishment in a new host, to address the role of coinfections in the spread of genetically distinct variants of one virus species.

## Materials and Methods

**Plant Material.** Foundation Plant Services at University of California, Davis, provided dormant grape cuttings of virus accession LR101, collected during the dormant season of winter 2011 (*Vitis vinifera* 'Italia 3'), which were used as the source for single infections of GLRaV-3-I. GLRaV-3-VI-infected source plants were obtained from cuttings of known infected mature *V. vinifera* 'Merlot' vines in Oakville, Napa Valley, CA (collected during winter dormancy in 2011, site 43 in Sharma et al. 2011), and used as the source for single infections of GLRaV-3-VI. For source plants with mixed infections of GLRaV-3-I and GLRaV-3-VI, one cutting was collected from each of nine mature symptomatic *V. vinifera* 'Chardonnay' vines in Napa Valley in August 2011 (Site 14 in Sharma et al. 2011), all of which were coinfecting with GLRaV-3-I and GLRaV-3-VI. All source plants were tested for all known variants of GLRaV-3 prior to use in experiments to confirm infection status.

Uninfected test plant material was provided by Foundation Plant Services at University of California, Davis. *V. vinifera* 'Pinot Noir', a known indicator variety of GLRaV-3 infection, was used for all transmission

experiments. Dormant cuttings were pruned to contain two buds each, treated with RootBoost (GardenTech, Lexington, KY) rooting hormone, and put in flats of 1:1 vermiculite: perlite. Cuttings were then kept on a mist bench for 6 wk, until they produced ~1-inch roots and a few leaves. Cuttings were then transplanted to a potting mix of 2:1:1 Supersoil: sand: perlite in 4-inch pots and kept in a greenhouse with no insecticide treatment until use in insect inoculations. Once inoculated, plants were treated weekly with insecticides.

#### **Vector Inoculations and Greenhouse**

**Conditions.** *Pl. ficus* and *Ps. viburni* were collected from California wine grape vineyards, and maintained in colonies on butternut squash in a growth chamber at 22°C, with a photoperiod of 12:12 (L:D) h. Only first-instar individuals were used in the experimental inoculations, as this life stage is the most efficient vector of GLRaV-3 (Tsai et al. 2008, Charles et al. 2009, Le Maguet et al. 2012). A large number of first-instar insects were collected by placing moistened filter paper on butternut squash in the insect colonies, and allowing mealybugs to crawl onto the filter paper. After 1 h, filter paper containing insects was secured to the leaf underside of source plants, and insects were allowed to crawl onto the source plants. The mealybugs were left on the known infected plants for a set acquisition access period. Infected source plants were then gently shaken so that insects fell onto a piece of paper, and insects were then transferred with a paintbrush from the paper to small leaf cages (previously described in Tsai et al. 2008). Mealybugs from each source plant were transferred to uninoculated test plants, using one cage with five mealybugs transferred from each source plant to a test plant. The mealybugs were confined on the lower surface of one fully expanded leaf of each test plant for a set inoculation access period. After the inoculation access period, the mealybugs were removed from the test plants, and then the test plants were treated with a contact insecticide to kill any remaining mealybugs.

After inoculation, test plants and uninoculated negative control plants were arranged in a fully randomized design and maintained in the greenhouse for 4 mo to allow virus establishment, after which we can reliably detect new infections using molecular diagnostic testing (Tsai et al. 2008). Uninoculated, virus-free plants were included to assure that no infections were the result of other potential sources besides our experimental inoculation treatments. Greenhouse conditions were 25:20°C days:nights and a photoperiod of 16:8 (L:D) h. Because grapevine leafroll disease symptoms cannot be reliably discerned under greenhouse conditions, and there is no known difference in symptoms between variants, we relied on molecular testing for diagnosis of infection. Three petioles were collected from different locations on each plant and pooled for laboratory testing.

**Molecular Testing.** To verify infection status of source plants, RNA extractions were performed on 100 mg of petioles from all infected source plants using Qiagen Plant Mini Kits (Qiagen, Valencia, CA). One step RT-PCR was then performed using Qiagen kits, and PCR products were analyzed using fragment analysis as described in Sharma et al. (2011). To test the

experimentally inoculated plants for infection with GLRaV-3-I and GLRaV-3-VI, total RNA was extracted based on the protocol in Sharma et al. (2011). The fluorescent primer sets included in each multiplexed reaction are listed in Supp Table 1 (online only), and multiplex conditions are in Supp Table 2 (online only).

#### **Experimental Design.**

**Experiment One, Single Infections.** To compare the transmission efficiency of GLRaV-3-I and GLRaV-3-VI by *Pl. ficus* and *Ps. viburni*, five different combinations of acquisition and inoculation access periods were used for each mealybug species-variant combination. To determine the effect of acquisition access period on transmission efficiency, insects were allowed acquisition access periods of 2, 8, or 24 h on known infected source plants followed by an inoculation access period of 24 h on uninoculated recipient test plants. To determine the effect of inoculation access period, five insects per plant were allowed 24-h acquisition access period, followed by inoculation access periods of 2, 8, or 24 h. Three replicate source plants with 10 replicate test plants per source plant were used for each variant, for a total of 30 test plants per mealybug-variant-access time combination.

**Experiment One, Mixed Infections From Two Sources.** An additional treatment for each mealybug species was included as part of this experiment to discern possible interactions between the two variants, using 24-h acquisition and inoculation access periods. For this treatment, five mealybugs were moved from each of two singly infected source plants, one infected with GLRaV-3-I and one with GLRaV-3-VI. These 10 mealybugs were placed together in one cage and confined on the underside of one leaf of a test plant. The same three replicate source plants of each variant that were used in single variant inoculations were used for each of 10 inoculated test plants, for a total of 30 inoculated recipient test plants. The single variant inoculations served as a reference for comparison. Thirty replicate uninoculated negative control plants were included in the experiment.

**Experiment Two, Mixed Infections From Coinfected Source.** A second experiment testing possible interactions between the variants during transmission from coinfecting hosts was performed. Nine coinfecting cuttings were placed in flasks with water immediately after collection and used as infected sources. *Pl. ficus* were allowed an acquisition access period of 24 h on the source cuttings, followed by an inoculation access period of 24 h on 20 test plants per source cutting, for a total of 180 test plants. Five mealybugs were confined on each recipient test plant for the inoculations. Twenty replicate uninoculated negative control plants were included in the experiment.

**Data Analyses.** For each experiment, resulting successful inoculations from replicate source plants of each infection status (GLRaV-3-I, GLRaV-3-VI, or coinfecting with GLRaV-3-I and GLRaV-3-VI) were compared using a Pearson chi-square test. Proportion of successful infections did not differ among replicate source plants within each variant or variant combination; therefore, data from the replicate source plants of each infection status were

pooled for further data analyses, which are described in the next two paragraphs.

For Experiment One, to determine the effect of acquisition access period, variant, and vector species, we performed a logistic binary regression using a generalized linear model, to test the proportion of plants infected depending on variant, vector species, their interaction, and acquisition access period (Crawley 2012). The same procedure was repeated to test the effect of inoculation access period, variant, and vector species.

Pearson chi-square tests were performed to test for differences in transmission of each variant in single versus mixed infection inoculations. For two by two chi-square tests, reported *P*-values are based on Yates correction. Post hoc pairwise proportion tests were used to determine differences among the three possible combinations of infections in mixed inoculation trials. To determine whether mixed infections were more common than expected, a Pearson chi-square test was used to compare single versus mixed infections. In instances where observed values were less than five, a Fisher's exact test was used in place of a chi square test. We report *P*-values that are not corrected for multiple tests, but we note that the statistical significance ( $P < 0.05$ ) is unchanged when a Bonferroni–Holm correction for multiple tests is applied (Abdi 2010). For each experimental treatment, we calculated the estimated probability of transmission by a single insect (*Ps*) and 95% confidence intervals based on Swallow (1985). All analyses were performed using R version 3.0.1.

## Results

**Vector Species, But Not Virus Variant, Affect Transmission Efficiency.** All treatments and transmission efficiencies obtained are reported in Supp Table 3 (online only). No negative control plants became infected with GLRaV-3. In Experiment One, transmission efficiency increased from shorter to longer acquisition access periods ( $z = 5.71$ ,  $P < 0.0001$ ,  $df = 349$ ), but was not significantly affected by inoculation access periods ( $z = 1.19$ ,  $P = 0.23$ ,  $df = 348$ ; Fig. 1; Supp Tables 4 and 5 [online only]). The invasive *Pl. ficus* transmitted GLRaV-3 more efficiently than *Ps. viburni* ( $z = 3.78$ ,  $P = 0.0002$ ,  $df = 349$ ). Transmission efficiency of GLRaV-3-I and GLRaV-3-VI were equivalent in single variant transmission tests ( $z = 0.46$ ,  $P = 0.64$ ,  $df = 349$ ). There was no interaction between variant and vector species ( $z = -1.35$ ,  $P = 0.17$ ,  $df = 349$ ).

**Mixed Inoculations from Two Sources.** Mixed inoculations by *Pl. ficus* from two singly infected source plants (one source plant infected with GLRaV-3-I, and the other infected with GLRaV-3-VI) resulted in more plants becoming infected with GLRaV-3-VI than with GLRaV-3-I ( $\chi^2 = 11.47$ ,  $P = 0.0007$ ,  $df = 1$ ). Overall GLRaV-3 infections resulting from inoculations from two separate source plants by *Pl. ficus* were higher than from a single source plant ( $\chi^2 = 5.63$ ,  $P = 0.018$ ,  $df = 1$ ), because 10 instead of 5 mealybugs were used to inoculate each test plant. The calculated

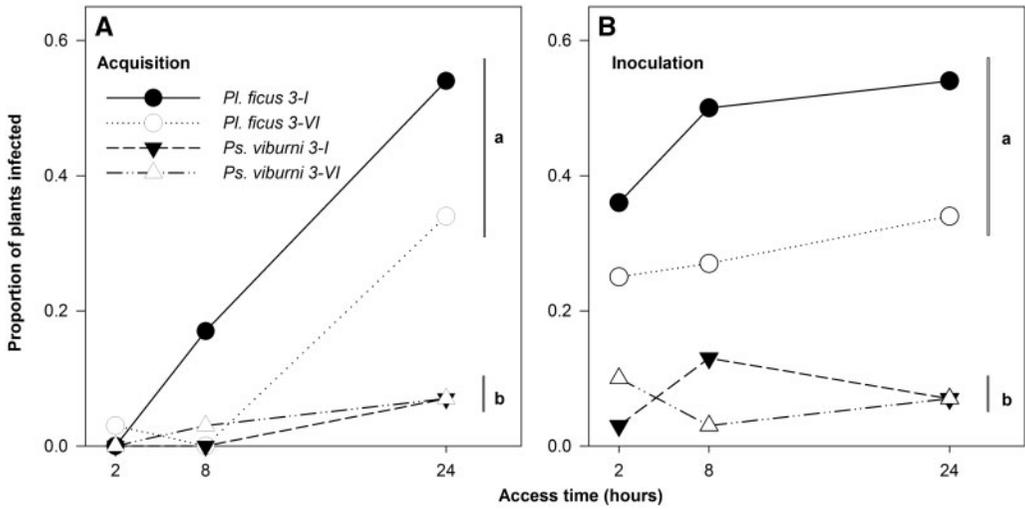
probability of a single insect transmitting virus (*Ps*) (Swallow 1985) was similar between the two experiments (Supp Table 3 [online only]). A pairwise proportion test showed that single infections with GLRaV-3-I and mixed variant infections did not differ from each other, but single infections with GLRaV-3-VI were most common ( $P = 0.0011$ ; Fig. 2a). Single infections were more common than expected by chance ( $\chi^2 = 18.05$ ,  $P < 0.0001$ ,  $df = 1$ ), indicating exclusion of GLRaV-3-I by GLRaV-3-VI.

In mixed inoculations from two source plants, overall GLRaV-3 transmission by *Ps. viburni* was less efficient than by *Pl. ficus* ( $\chi^2 = 18.05$ ,  $P < 0.0001$ ,  $df = 1$ ). Resulting infections from inoculations with *Ps. viburni* did not differ significantly whether inoculations were from one or two sources ( $\chi^2 = 1.25$ ,  $P = 0.26$ ,  $df = 1$ ); it is likely that at such low transmission rates a potential difference could not be detected. We did not find a significant difference in resulting infections among possible combinations of variants by *Ps. viburni*, nor did we find that resulting single infections were significantly more or less common than expected ( $P = 0.06$ ; Fig. 2b). However, the relationship among variants appears to follow a similar trend to that resulting from inoculations by *Pl. ficus*.

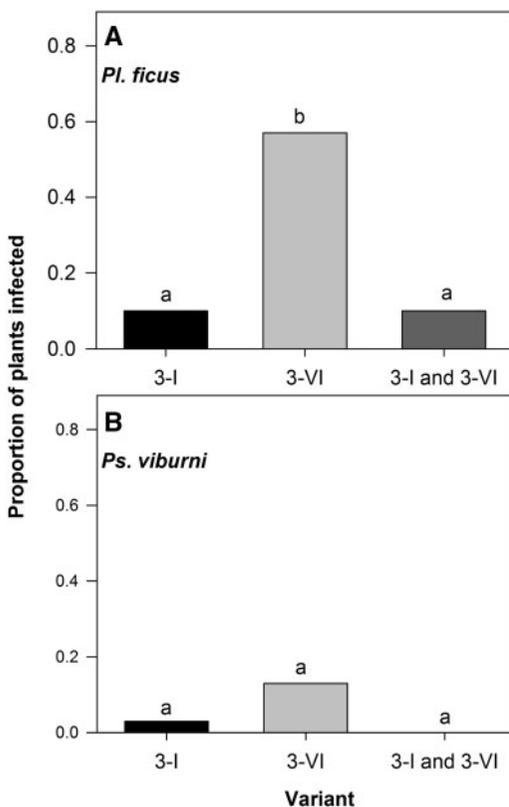
**Mixed Inoculations from Coinfected Source.** In Experiment Two, overall GLRaV-3 transmission efficiency by *Pl. ficus* from a host co-infected with GLRaV-3-I and GLRaV-3-VI was equivalent to transmission efficiency from singly infected sources ( $\chi^2 = 4.02$ ,  $P = 0.13$ ,  $df = 2$ ; Fig. 3). From a coinfecting source, there were fewer resulting infections of GLRaV-3-I compared to single-source inoculations ( $\chi^2 = 6.48$ ,  $P = 0.0109$ ,  $df = 1$ ), but resulting GLRaV-3-VI infections were unaffected by the coinfection of the source ( $\chi^2 = 2.81$ ,  $P = 0.09$ ,  $df = 1$ ). A pairwise proportion test showed that established infections with only GLRaV-3-I differed from mixed infections and infections with only GLRaV-3-VI ( $P < 0.0001$ ), which did not differ from each other ( $P = 0.53$ ; Fig. 4). Mixed infections were not more or less common than expected by chance ( $\chi^2 = 1.24$ ,  $P = 0.27$ ,  $df = 1$ ).

## Discussion

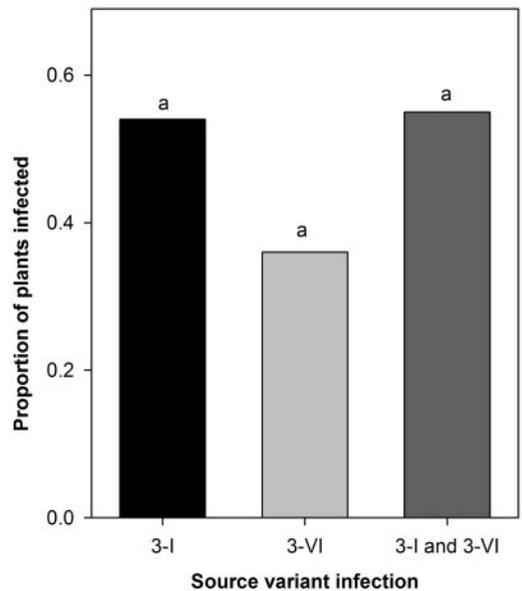
Our experiments revealed that two pathogen variants with similar vector transmission efficiency in single-variant inoculations interacted after introduction to a new uninfected host plant, resulting in reduced establishment of one of the variants. In single variant inoculations we found no difference in transmission efficiency between the two variants, and no indication of variant-vector specificity. Inoculations from a coinfecting host plant did not affect overall virus transmission, but number of plants successfully infected with one variant was reduced, indicating limitation due to the other variant during vector transmission and/or establishment in the susceptible host. Interestingly, the effect was stronger with mixed inoculations from two individually infected source plants and vectors that were exposed to only one variant than from a coinfecting source, as shown by the exclusion of resulting



**Fig. 1.** Transmission of GLRaV-3-I and GLRaV-3-VI by *Pl. ficus* and *Ps. viburni* (Experiment One). (A) Acquisition access time, (B) Inoculation access time. Different lowercase letters indicate statistically significant differences in proportion of recipient test plants that became infected with GLRaV-3 among vector and variant combinations.



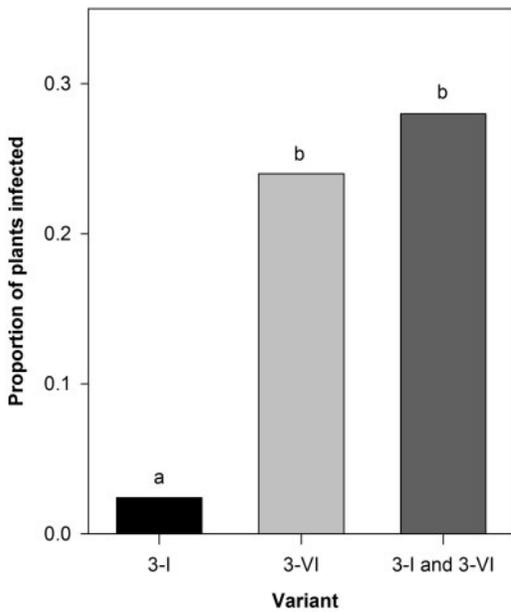
**Fig. 2.** Overall transmission of two GLRaV-3 variants resulting from mixed-variant inoculations from two singly infected source plants per recipient test plant (A) by *Pl. ficus*, and (B) by *Ps. viburni*. Different lowercase letters indicate statistically significant differences in proportion of recipient test plants that became infected with GLRaV-3-I, GLRaV-3-VI, or mixed infections of both virus variants.



**Fig. 3.** Overall GLRaV-3 transmission by *Pl. ficus* from a single host source plant containing a mixed infection of GLRaV-3-I and GLRaV-3-VI (Experiment Two) and transmission from a singly infected source plant (Experiment One). Different lowercase letters would indicate differences in overall transmission of GLRaV-3 (I, VI, or both) from source plants infected with different combinations of genetically distinct variants of GLRaV-3.

mixed infections when mixed inoculations were from two singly infected source plants; this provides further evidence for a lack of variant-vector specificity.

Insect vectors are often credited as the primary factor determining the relative prevalence of plant pathogens, even overpowering host defenses (Seabloom et al. 2009). Indeed, recent studies with vector inoculations from a



**Fig. 4.** Infections resulting from source plants with mixed infections of GLRaV-3-I and GLRaV-3-VI, using *Pl. ficus* (Experiment Two). Different lowercase letters indicate differences in proportion of plants that became infected with GLRaV-3-I, GLRaV-3-VI, or mixed infections of both variants.

single source plant with a mixed infection have either concluded that the outcome was due to virus interactions within the vector, or did not discern whether the outcome was determined within vector or susceptible host. Leaf-rub inoculations introduce a much larger quantity of virus than vector-mediated inoculations (Srinivasan et al. 2012, Salvaudon et al. 2013, Péréfarres et al. 2014), and may consequently overpower possible effects of host defenses; therefore, comparing leaf-rub and vector inoculations might lead to erroneous conclusions about the effect of insect vectors on virus establishment in a new host plant (Moury et al. 2007, Péréfarres et al. 2014). In our study, the signature of superiority of one variant over another was actually stronger when competition within the vector was eliminated, by transferring vectors from each of two singly infected source plants to one uninfected test plant, suggesting that virus-virus interactions during establishment in a new host plant may be more important than interactions within the vector. Another possible interpretation of this result is that there may be a synergistic interaction in the vector during transmission that results in establishment of more mixed infections, but this seems unlikely given that neither variant successfully established more infections in mixed compared to single variant inoculations.

There are multiple possible explanations for our observation of exclusion of one virus variant by another in mixed inoculations from two singly infected hosts, but not from one coinfecting host. With 10 vectors instead of five per inoculated plant, the larger number of virus particles introduced to the susceptible host plant may have intensified competition between the

two variants, enabling detection of competitive exclusion for a limiting host resource between the variants in the susceptible host. Alternatively, it may be that one or both variants in the source plants were adapted to their current infection status, i.e., in a singly or coinfecting host, and this resulted in a higher incidence of single infections resulting from singly infected source plants. Another possibility is that each variant may differentially affect vector feeding behavior (Gutiérrez et al. 2013), effectively leading to differences in overall access times and resulting transmission efficiency; however, this seems unlikely based on the results from our single-source inoculations.

Based on our study, it is difficult to rule out apparent competition versus resource competition between the variants in the new host. Plants can have highly specific defenses that could lead to apparent competition, effective against only one of two closely related pathogens (Chung et al. 2012). Recent studies have demonstrated patterns of exclusion of new infections by existing viral infections, even when time of inoculation between the two variants was as short as 6 h (e.g., Julve et al. 2013). These studies show that variants tend to infect in a mosaic pattern, with doubly infected cells being rare. Such evidence indicates that viruses may not directly interact with each other, and therefore apparent competition could be the leading determining factor; in other words variants may differ in their ability to overcome the host plant's defenses and colonize uninfected cells. Unfortunately such studies have not included phloem-limited viruses, and information is lacking. However, quantitative PCR estimates of individual GLRaVs in hosts with single and mixed infections found no significant effect of coinfection on the population of each virus, which supports the notion that the two viruses are not interacting with each other (Velasco et al. 2013). Gouveia and Nolasco (2012) found differences among GLRaV-3 variants in the suppressing activity of the p19.7 silencing suppressor, suggesting that variants may differ in their ability to overcome host plant defenses. The two variants tested in this study share <70% nucleotide sequence similarity, and their predicted protein amino acid sequences are >10% different from each other (Bester et al. 2012). One lacks an open reading frame present in the other that codes for the p6 protein (Seah et al. 2012), and they differ in two other open reading frames also of unknown function. These differences may have a role in their relative abilities to establish new infections in a host. Understanding the functions of these open reading frames may shed light on within-host interactions.

Recently under greenhouse conditions, Lacroix et al. (2014) found evidence of direct resource competition between two closely related phloem-limited virus species, as demonstrated by increased coinfections in hosts fertilized with higher nitrogen. Interestingly, host plant fertilization with phosphorus may lead to the opposite trend under greenhouse conditions (Rúa et al. 2013, Lacroix et al. 2014). In contrast, field studies have found varying effects of host fertilization with nitrogen and phosphorus on virus competition, as well as interactive effects of the two nutrients (Borer et al. 2010,

2014; Seabloom et al. 2013). At this time more studies are needed for definitive conclusions about the conditions under which apparent competition or resource competition is the dominant driver of viral infections.

Evolutionary theory predicts that at higher pathogen prevalence, less virulent pathogen strains will be more common, and this has been supported empirically (Berngruber et al. 2013). In systems where the supply of susceptible host plants is regularly renewed, however, more virulent pathogens may continue to prevail (Smart and Fry 2001). At low pathogen incidence, the two GLRaV-3 variants would be predicted to have similar prevalence. At higher pathogen incidence when coinfecting sources or vectors carrying both variants are more common, we suggest that GLRaV-3-VI would become more common relative to GLRaV-3-I. Because new uninfected hosts are introduced regularly in the wine grape industry in order to replace diseased plants, both variants will probably persist despite the superiority of GLRaV-3-VI.

Because GLRaV-3 is transmitted in a noncirculative, nonpropagative manner, vector transmission is assumed to be limited by putative receptor sites in the vector foregut, rather than by virus replication (Tsai et al. 2008). Viruses may compete for such receptors, or there may be complementation via virus transcapsidation, in which the viral genome of one virus is transmitted inside the capsid protein of another virus (Ng and Falk 2006). Another possible mode of complementation during vector transmission is via a helper protein, where a viral protein forms a bridge between virus particle and receptor on the insect vector (Ng and Falk 2006). The helper protein of one virus may be exploited by a different virus (Froissart et al. 2002). However, we did not find evidence that within-vector competition or complementation affected the relative success of each variant's transmission. The invasive *Pl. ficus* was a more efficient vector than *Ps. viburni*. Transmission efficiency increased significantly with acquisition access period but not with inoculation access period, although there appeared to be a slight upward trend in transmission as inoculation access period increased.

Several mealybug species have been shown to transmit GLRaV-3, including both species included in our current study (Cabaleiro and Segura 1997; Golino et al. 2002; Douglas and Krüger 2008; Tsai et al. 2008, 2010; Charles et al. 2009; Le Maguet et al. 2012). Estimates of transmission efficiency by each species were compiled by Almeida et al. (2013). A wide range of variation in transmission efficiencies among mealybug species was found, as well as variation within mealybug species among different studies. As we found no evidence of vector-variant specificity, existing estimates of vector transmission efficiency may be representative across genetically distinct GLRaV-3 variants. More studies are needed to confirm this. The transmission efficiency by *Pl. ficus* that we found after 24-h acquisition and inoculation access periods, for which transmission reaches its maximum (Tsai et al. 2008), ranges from 0.08 to 0.15 (Supp Table 3 [online only]), and is within the range found by previous studies. To our

knowledge, transmission efficiency by *Ps. viburni* has not previously been estimated. Based on our results, *Ps. viburni* is one of the least efficient mealybug vectors of GLRaV-3 tested to date. Geographic expansion and increased population sizes in California of the newly invasive *Pl. ficus* could lead to drastic increases of virus spread, as well as a change in relative prevalence of genetically distinct variants.

In conclusion, we found that overall pathogen transmission efficiency differed substantially between vector species, but within-host pathogen interactions determined the outcome of vector-mediated infections. A better understanding of apparent competition and resource competition between pathogens could lead to improved prediction of pathogen spread. While new infections can be reliably detected within 4 mo after inoculation (Tsai et al. 2008), temporal dynamics within the host plant exist and may differ among variants (Tsai et al. 2012, Velasco et al. 2013). Therefore the timing of inoculation with different pathogen variants or prior infection status of the experimentally inoculated host may affect the outcome of pathogen interactions, and exploration of these dynamics is needed. Furthermore, more information is needed regarding the specific molecular interactions within vector and host that lead to the superiority of one pathogen variant over another.

### Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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