BIOLOGY AND SPREAD OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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ABSTRACT
Grapevine red blotch-associated virus (GRBaV) was isolated from table and wine grapes, as well as rootstocks, affected by red blotch, a recently recognized viral disease. Phylogenetic analyses revealed the clustering of GRBaV isolates two groups (clades) of genetic variants (Krenz et al., 2014, Al Rwahnih et al., 2015). Producing and using a full-length infectious clone of a representative isolate of each of the two clades showed systemic GRBaV infection of healthy grapevines following agroinoculation and the manifestation of typical disease symptoms, i.e. interveinal reddening on red-berried cultivars and chlorotic and necrotic leaf areas on white-berried cultivars, while infection was latent in rootstocks with the exception of SO4. This work demonstrated that GRBaV isolates of both clades cause red blotch disease. Analysis of the spatio-temporal incidence of GRBaV in a selected vineyard of Cabernet franc in California was consistent with the occurrence of virus spread. GRBaV isolates spreading in California corresponded to phylogenetic clade 2. A survey of alternate hosts in proximity to the diseased Cabernet franc vineyard showed that free-living grapevines in riparian areas are infected with GRBaV (Perry et al., 2016). The GRBaV isolates from free-living grapevines, some of which being fingerprinted as hybrids of *Vitis californica* x *Vitis vinifera* cv. Sauvignon blanc, belonged to phylogenetic clade II, as did most of the GRBaV-infected vines in adjacent Cabernet franc and Merlot vineyards. The presence of GRBaV in free-living grapevines suggests the existence of a hemipteran vector. Insect sticky traps placed in the section of the California vineyard with extensive clustering of diseased vines in 2014 and 2015 showed a diversity of insect families, genera and species that visited the vineyard, among which, the majority of specimens of four species consistently tested positive for GRBaV in PCR. These four species are vector candidates and their potential to transmit GRBaV in controlled conditions in the greenhouse is investigated. Among the four vector candidates, *Spissistilus festinus* - the three cornered alfalfa treehopper - was shown to transmit GRBaV from infected to healthy vines in the greenhouse. This finding revealed the potential of this treehopper as a vector of epidemiological significance.

LAYPERSON SUMMARY
Red blotch is a newly recognized viral disease of grapevines that is widely distributed in U.S. vineyards. Limited information is available on spread of its associated virus called Grapevine red blotch-associated virus (GRBaV). Similarly, limited information is available on the association between virus variability and pathogenicity. We showed that GRBaV isolates cause red blotch disease, regardless of their genetic makeup and variability. Studying changes in virus prevalence over time in selected vineyards of Cabernet franc in California and New York revealed an increased virus incidence in the California but not in the New York vineyard. Free-living grapevines proximal to diseased vines in the California vineyard were found infected with GRBaV, suggesting their potential role as alternate host. Among insects visiting the California vineyard, four species were found to carry the virus, suggesting a potential role as vector. Subsequent work in the greenhouse showed that one of these vector candidates, the three cornered alfalfa treehopper (*Spissistilus festinus*) transmits GRBaV from infected to healthy vines, revealing this treehopper is likely a vector of epidemiological importance in vineyards.

INTRODUCTION
Red blotch is a recently recognized disease of grapevines (Calvi 2011; Sudarshana et al., 2015). It was described for the first time on Cabernet Sauvignon at the UC Oakville Research Field Station in 2007 (Calvi 2011). Leaves of GRBaV-infected vines of red wine grapes show red specks and blotches first on old leaves at the bottom of the canopy in late June or July. Symptoms progressively appear upward in the shoots over time. Veins underneath the leaf blade often turn partly or fully red. For white wine grapes, foliar symptoms are less conspicuous; they correspond to localized and generalized foliar discoloration or chlorosis, sometimes combined with necrotic areas

at the edge of leaf blades (Sudarshana et al., 2015). Diagnosis based on specific symptoms can be challenging because of several confounding factors, including striking similarities between foliar symptoms elicited by red blotch and leafroll. There are also similarities between foliar symptoms of red blotch and abiotic factors such as poor root health, or physical injuries due to trunk or shoot girdling, mite damage, mineral deficiencies, or even the presence of *Xyllela fastidiosa* or *Agrobacterium tumefaciens* in young vines. Because symptom variation makes visual diagnosis of GRBaV-infected vines difficult, only DNA-based assays such as PCR are reliable for accurate diagnosis (Sudarshana et al., 2015).

GRBaV was isolated from grapevines affected by red blotch disease (Sudarshana et al., 2015). This virus is a putative member of a new genus in the family *Geminiviridae* (Varsani et al., 2014; Sudarshana et al., 2015). The new genus is tentatively named *Grablovirus* (Zerbini, personal communication). GRBaV has a single-stranded DNA genome that codes for seven open reading frames (Al Rwahnih et al., 2013; Krenz et al., 2012; Perry, unpublished; Poojary et al., 2013; Seguin et al., 2014). Efforts to investigate the role of GRBaV in the etiology of red blotch disease showed that GRBaV is the causal agent of red blotch disease (Fuchs and Perry, unpublished).

GRBaV was documented in major grape-growing US States (Krenz et al., 2014). The virus was also reported in British Columbia and Ontario (Poojari et al., 2016) in Canada, and in a *Vitis* germplasm collection (Al Rwahnih et al., 2015a), indicating its widespread presence in North America. GRBaV was found in table grapes, wine grapes, French-American interspecific hybrids, and rootstocks (Al Rwahnih et al., 2015a; Sudarshana et al., 2015). The widespread occurrence of GRBaV and its wide geographic distribution in North America suggest that propagation material has played a significant role in its dissemination. The virus was also found in an archival sample (Al Rwahnih et al., 2015b). Analysis of the genetic diversity among isolates of GRBaV indicated the existence of two groups (clades) of genetic variants (Krenz et al., 2014). The majority of isolates belong to the predominant clade II and recombination is underlying some of the variation seen among GRBaV genomes within clade I.

Most vineyard managers and vintners report ripening issues with GRBaV-infected wine grapes. Reductions of 1-6°Brix have been consistently documented in fruits of infected vines, as well as lower berry anthocyanin and skin tannins, particularly in red wine grapes such as Cabernet franc and Cabernet Sauvignon (Calvi 2011; Sudarshana et al., 2015). Based on the effect of GRBaV on fruit quality and ripening, several growers are culling infected vines and replacing them with clean, virus-tested ones.

Free-living grapevines proximal to vineyards were found infected with GRBaV (Bahder et al, 2016a; Perry et al., 2016). The GRBaV isolates in free-living grapevines was genetically related to clade II isolates in proximal Cabernet franc and Merlot vineyards (Perry et al., 2016). The presence of the virus in a potential alternative host that is at least 150 ft away from the natural host suggested the existence of a hemipteran vector. The ziczac leafhopper (Virginia creeper; *Erythroneura ziczac)* was claimed to transmit GRBaV from vine to vine in the greenhouse (Poojari et al., 2013) but a vector of GRaBV of epidemiological significance in vineyards remains to be identified.

OBJECTIVES

The overarching goal of this project is to advance our understanding of red blotch disease and its causal agent, GRBaV, with a major emphasis on horizontal spread in vineyards and optimized detection methodologies. Our specific objectives are to:

1. Investigate spread of GRBaV in selected vineyards in California and New York
2. Improve diagnostics for GRBaV
3. Determine if either of the two groups of GRBaV isolates show greater virulence and pose an increased threat to vineyard production
4. Disseminate research results to farm advisors and the industry

RESULTS AND DISCUSSION

To address objective #1 and study spread of GRBaV, two vineyards of Cabernet franc were selected, one in California and one in New York. The California and New York vineyards were planted in 2008. In 2013 and 2014, virus prevalence was determined in the two selected vineyards. This information served as a baseline to determine the spatio-temporal incidence of GRBaV. A comparative analysis of the infection rate of GRBaV as measured by the number of symptomatic vines in the selected vineyard in California between 2014 and 2015 indicated a 1.5% increase, suggesting the possibility of virus spread (Figure 1). In addition, an investigation of the spatial distribution of symptomatic vines through an ordinary runs analysis, a statistical test for randomness of infected plants, revealed disease clustering in the majority of rows within the selected vineyard (–Z > 1.64 in 32/44 rows). These data confirmed the occurrence of GRBaV spread in the California vineyard as a result of either vine-to-vine transmission within the selected vineyard or of an influx from adjacent vineyards. Characterizing 10 randomly selected GRBaV isolates in the selected Cabernet franc vineyard in California by PCR followed by sequencing indicated that they all correspond to the phylogenetic clade 2 that was previously reported (Krenz et al., 2014).

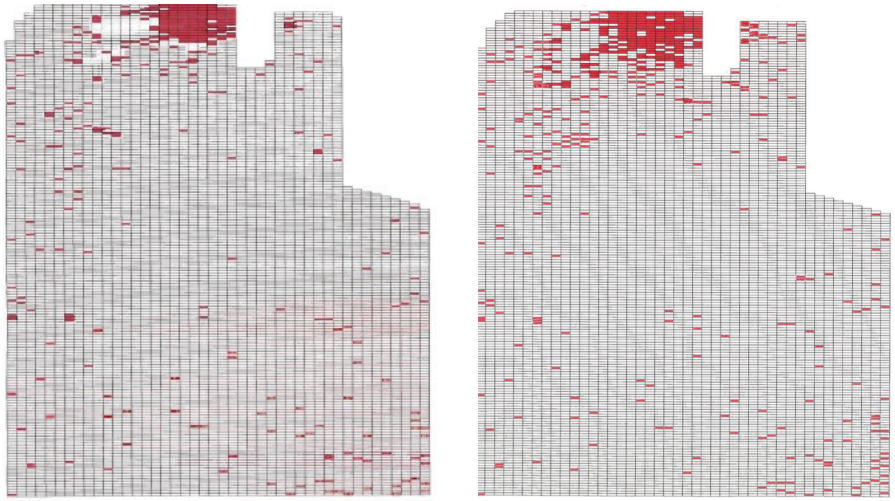

Fig. 1. Spatial distribution of vines showing red blotch symptoms (in red) in a Cabernet franc vineyard in California in 2014 (left) and 2015 (right).

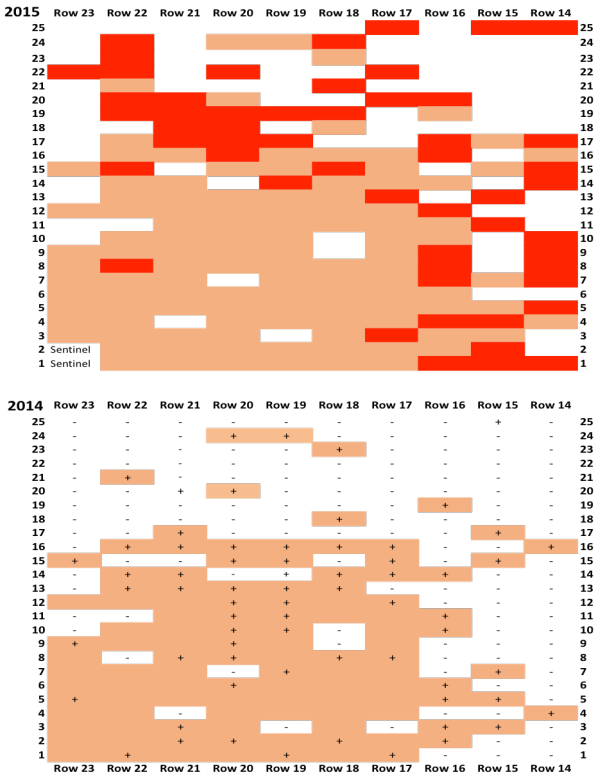

Fig. 2. Clustering of symptomatic vines in 2014 (bottom map) and 2015 (top map) in a California vineyard. Symptomatic vines in 2014 and 2015 are shown in salmon and red, respectively. Vines that tested negative and positive for GRBaV in PCR in 2014 are indicated with (-) and (+), respectively.

Spread of GRBaV was further studied in the vineyard area with extensive clustering of symptomatic vines (top middle area of the maps in Figure 1). This area consists of 10 consecutive rows of 25 vines each (Figure 2). Symptomatic and asymptomatic vines were mapped in this area in 2013 and 2014. In addition, the presence or absence of GRBaV was confirmed in individual vines by PCR in spring and winter 2014 by using leaf and cane material, respectively (Figure 2). Data showed an increase of symptomatic vines from 47% (118 of 250 vines) in 2014 to 67% (168 of 250) in 2015. The presence of GRBaV was confirmed in all symptomatic vines. Similarly, the absence of GRBaV was confirmed in most of the asymptomatic vines with a few exceptions (7 of 250 vines). Based on our monitoring of vines in 2014 and 2015, it is anticipated that the seven asymptomatic vines that tested positive for GRBaV will become symptomatic in 2016. Altogether, these results further support the occurrence of short distance spread of GRBaV in the California vineyard.

A spatio-temporal analysis of a Cabernet franc vineyard in New York in 2013-2015 did not provide any evidence of an increased prevalence of GRBaV over time. These findings suggested that a GRBaV vector does not exist in the New York vineyard ecosystem or it eventually exists at a very low population density or it exists but

does not visit the vineyard. Alternatively, the plant protection program used by the vineyard manager in New York is effective at reducing the vector population.

Close to 100 sentinel vines, i.e. healthy vines for which the mother stocks from which scion budwood and rootstock canes were collected from tested negative for GRBaV, were planted in the Cabernet franc vineyard in California in spring 2015. Some of these sentinel vines are shown at the bottom left of Figure 2. These vines will be used to gain direct evidence of insect-mediated GRBaV spread if they become infected. Sentinel vines replaced existing vines that were weak, regardless of their GRBaV infectious status. The presence of GRBaV will be tested in sentinel vines in fall 2016.

The fact that extensive clustering of diseased vines occurred in one area of the selected vineyard in California (see top middle area of the maps in Figure 1) provided an incentive to investigate the occurrence of alternate hosts in the proximal riparian ecosystem. Samples of oak, willow, walnut, oat, vetch and free-living grapevines were collected and tested for GRBaV in PCR. Among the samples tested, only those from free-living grapevines tested positive for GRBaV. These results suggested that free-living grapevines can potentially serve as an alternate host of GRBaV. They also supported the existence of a hemipteran vector of GRBaV as infected free-living grapevines were at least 150 away from the nearest infected vine in the Cabernet franc vineyard. Fingerprinting of the GRBaV-infected free-living grapevine samples indicated that they corresponded to hybrids of *Vitis californica* x *Vitis vinifera*. Characterizing the GRBaV isolates in free-living grapevines by PCR and sequencing indicated they cluster in phylogenetic clade 2, as did the isolates from the diseased Cabernet franc vineyard, further providing a link between the test vineyard and its proximal riparian area in terms of virus spread.

Insect sticky traps were placed in the area of the selected vineyard in California where clustering of diseased vines is occurring (see top middle area of the maps in Figure 4). Traps were placed on diseased and healthy grapevines from early April to late November in 2014 and 2015 with the goal of catching insects visiting the vineyard. Traps were rotated on a weekly basis. Each trap was analyzed for the presence of insects to establish a census population and identify them at the species level, if possible, by using morphological parameters. Then, a sub-set of each insect family, genus or species that was caught was removed from the traps and tested for the presence of GRBaV by PCR. Results indicated that specimens of four species, among more than 45 species of Dipetra, Apocrita, Coleoptera, Cicadellidae, Thysanoptera, Aphidae, Fulgoroideae, Phylloxera, Aleyrodidae, Membraciade, Blissidae/Lygaeidae, Psyloidea, Psocopetra and Miridae that were caught on sticky traps, consistently carried genetic elements of GRBaV (Table 1).

Table 1. Presence of GRBaV in a subset of insects from a Cabernet franc vineyard in California in which spread of GRBaV is documented.

Species/Family	Common Name	Number Tested	GRBaV detected	Percent Positive
Spissistilus festinus	Three cornered alfalfa treehopper	25	12	**48%**
Cixiidae	Cixiid planthoppers	8	4	**50%**
Colladonus reductus	*Colladonus reductus*	23	14	**61%**
Osbornellus sp.	*Osbornellus* sp.	31	13	**42%**
Thysanoptera	Thrips	12	0	0%
Aleyrodidae	Whiteflies	52	0	0%
Psylloidea	Psyllids	25	0	0%
Deltocephalus sp.	*Deltocephalus sp.*	15	0	0%
Erythroneura elegantula	Western grape leafhopper	41	0	0%
Erythroneura variabilis	Variegated leafhopper	22	0	0%
Euscelis sp.	Brown leafhopper	33	0	0%
Daktulosphaira vitifoliae	Grape phylloxera (winged adults)	22	0	0%
Sophonia orientalis	Two-spotted leafhopper	5	0	0%
Aphididae	Aphids	46	1	2%
Scaphytopius magdalensis	Sharp-nosed leafhopper	45	3	7%
Empoasca sp.	Potato leafhopper	28	1	4%
Graphocephala atropunctat	Blue-green sharpshooter	23	1	4%

These four species are members of the Membracidae (*Spissistilus festinus*), Cicadellidae (*Colladonus reductus* and *Osbornellus* sp.) and Cixiidae (unidentified species). These findings suggest that these four species can

acquire GRBaV in the vineyard. Testing the capacity of these three hemipteran insects at transmitting the virus to healthy grapevines in the greenhouse is critical to ascertain their role as vector.

Testing the capacity of the four vector candidates at transmitting GRBaV to healthy grapevines was initiated in the greenhouse using *Spissistilus festinus*. First, specimens of *Spissistilus festinus* from alfalfa fields in Yolo County and Fresco County in California were collected and established on alfalfa seedlings at Cornell. Then, groups of 10 individuals were deposited on GRBaV-infected potted vines that were obtained by agroinoculation. After 3-5 days of acquisition, groups of 2-4 individuals were transferred to healthy potted vines and allowed to feed for 5-6 days. Transmission assays were replicated three times. Subsets of *Spissistilus festinus* were tested for the presence of GRBaV after the acquisition and transmission steps. Data showed that most of the specimens tested positive for GRBaV in multiplex PCR after the 1-5 days acquisition step. These results were consistent across several transmission experiments. Also, some specimens tested positive for GRBaV 2-3 weeks after the 1-5 days transmission step, indicating that *Spissistilus festinus* can acquire the virus from infected vines in the greenhouse and keep it for extended time after acquiring it. This is consistent with a persistent transmission of GRBaV. Three to five months post-transmission, recipient vines (6 of 22) became infected with GRBaV in replicated experiments, supporting the capacity of *Spissistilus festinus* at acquiring and transmitting GRBaV. These results confirm those recently reported by Bahder et al. (2016b) using a colony of *Spissistilus festinus* established in the laboratory. Together with our insect trap studies, the control transmission experiments revealed three cornered alfalfa treehopper as a vector of epidemiological important.

To address objective #2 and improve diagnostics for GRBaV, a robust real time PCR methodology was developed using infected and healthy vines grown in the greenhouse and vineyards. This assay is being used to characterize the titer of the virus in infected plants and to determine the optimal plant tissue and time of the year to collect samples for a reliable diagnosis. In parallel, strategies to produce an antiserum are refined through RNAseq approaches. This work will provide insights into the expression strategies of the GRBaV genome during the infection process. This knowledge is critical to understand how viral genes are expressed in infected plants because efforts to develop an antiserum against the structural coat protein have failed so far (Perry and Fuchs, unpublished).

To address objective #3 and determine if either of the two groups of GRBaV isolates show greater virulence and pose an increased threat to vineyard production, we engineered infectious clones of a representative GRBaV isolate of each of the two phylogenetic clades. Partial dimer constructs of the genome of GRBaV isolates NY358 and NY175 were engineered and cloned into a binary plasmid for mobilization into *Agrobacterium tumefaciens* strains LBA4404 or C58. Isolates NY175 from *V. vinifera cv.* Merlot and NY358 from *V. vinifera cv.* Cabernet franc belong to GRBaV phylogenetic clades I and II, respectively (Krenz et al., 2014). These clones were used in agroinoculation experiments using healthy, tissue culture-grown vines of *Vitis vinifera* cvs. Cabernet Sauvignon, Cabernet franc, Syrah, Chardonnay, Pinot noir and Pinot gris, and rootstock genotypes SO4 and 3309C that tested negative for GRBaV by PCR. Tissue culture-micropropagated grapevines (30-40 per genotype) showing 4-6 leaves (Alzubi et al., 2012) were selected for agroinoculation experiments using vacuum-assisted infiltration. Alternatively, grapevine tissue was gently pricked with needles dipped in a solid agrobacterium culture grown on a Petri plate. A β−glucuronidase gene construct containing an intron was used as control to optimize conditions for agroinfiltration-mediated delivery of DNA. Constructs of both genomic RNAs of Grapevine fanleaf virus (GFLV) were used as negative control in agroinfiltration experiments. Following agroinfiltration and/or pricking, plants were maintained at 25±2°C and 33-45 $mEm^{-2}s^{-1}$ (16-h photoperiod) in a tissue culture growth room for 2-3 months prior to establishment in a greenhouse for symptom observations and testing. The presence of GRBaV was tested by PCR in newly developed leaves of agroinoculated grapevines by using specific primers designed in the putative coat protein and replicase-associated genes, and the 16S ribosomal RNA used as a housekeeping gene (Krenz et al., 2014). Plants were tested 3-10 months post-agroinfiltration and some of them were also tested after one or two dormancy periods. The full-length genomic sequence of some of the GRBaV progeny was determined in a few selected agroinfected plants by rolling circle amplification, cloning and sequencing.

A number of treated vines of Cabernet Sauvignon, Cabernet franc, Syrah, Pinot noir, Pinot gris and Chardonnay showed red blotch-like symptoms at 1-3 months post-treatment. Foliar symptoms consisted of interveinal reddening in red-berried cultivars and chlorotic spots in the white-berried cultivar Chardonnay. Unlike for wine grape cultivars, agroinoculated SO4 became symptomatic (chlorosis and cupping) only after one dormancy

period, whereas agroinoculated 3309C remained asymptomatic. Some of the grapevines agroinfiltrated with the NY358 construct (28-76%) tested positive for GRBaV by PCR. All the PCR-positive plants were symptomatic, while the negative plants were asymptomatic. None of the plants treated with GFP (0 of 237), GFLV-derived constructs (0 of 476) or untreated plants (0 of 56) exhibited red blotch-like symptoms, nor those that were assayed tested positive for GRBaV in PCR. The virus detected in symptomatic, agroinoculated vines was further characterized by sequencing. Sequence analysis indicated a 99.6-99.9% identify with the partial dimer construct used as inoculum in agroinfection assays, indicating that the recovered GRBaV variant is nearly-identical to the engineered inoculum. Similar results were obtained from agroinfiltration experiments with the NY175 construct. These findings were consistent with our hypotheses that GRBaV is the causal agent of red blotch disease and that GRBaV isolates from the two phylogenetic clades are equally infectious. In agroinfiltrated plants, the detection of GRBaV correlated with symptoms and virus progeny nearly identical in sequence to the inoculated partial dimer genomic construct was obtained from agroinfiltrated plants.

To address objective # 4 and disseminate information to farm advisors and the industry, research results were be communicated to farm advisors, extension educators, crop consultants, researchers, vineyard managers and regulators at winter school meetings in California and New York. The targeted venues were (i) the Eastern Winey Exposition on March 19, 2015 (120 participants), (ii) the Cornell Recent Advances in Viticulture and Enology conference on November 4, 2015 at the IRL Conference Center in Ithaca (60 participants), NY, (iii) the Napa Continuing Education Class Series 3 on November 10, 2015 in Yountville, CA (250 participants), and (iv) a webinar on Grapevine red blotch disease: What you need to know' organized by Regional IPM Centers, February 26, 2016 (participants = 310).

CONCLUSIONS

Isolates of each of the two phylogenetic clades of GRBaV cause red blotch disease symptoms in *Vitis vinifera* following agroinoculation, confirming their etiological role, while infection is latent is rootstocks with the exception of SO4. Analysis of the spatiotemporal distribution of symptomatic, infected vines documents spread of GRBaV in a vineyard of Cabernet franc in California but not in New York. Some free-living grapevines proximal to the diseased vineyard in California are infected with GRaBV. The analysis of a sub-set of insect species caught on sticky traps for the presence of GRBaV enabled us to identify four vector candidates, among which, *Spissistilus festinus*, the three cornered alfalfa treehopper was shown to acquire the virus from infected vines and transmit it to healthy vines. This finding suggests *Spissistilus festinus* as a GRBaV vector of epidemiological importance.

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