BIOLOGY AND SPREAD OF GRAPEVINE RED BLOTCH-ASSOCIATED VIRUS

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ABSTRACT
In the search of alternative hosts of Grapevine read blotch-associated virus (GRBaV), free-living grapevines in riparian areas proximal to diseased vineyards were found infected with GRBaV in Napa Valley in California (Perry et al., 2016). The GRBaV isolates from free-living grapevines, some of which were fingerprinted as hybrids of *Vitis californica* x *Vitis vinifera* cv. Sauvignon blanc, belong to phylogenetic clade II, as did most of the GRBaV-infected vines in proximal Cabernet franc and Merlot vineyards. The presence of GRBaV in free-living grapevines suggests the existence of a vector. Among the four hemipteran species identified as potential candidate vectors in the Cabernet franc vineyard where spread of GRBaV is documented, *Cerasus 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 causal agent called Grapevine red blotch-associated virus (GRBaV). 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 are infected with GRBaV, suggesting their potential role as alternative host. Among insects visiting the California vineyard, four species were found to carry the virus, suggesting a role as potential vector. Subsequent work in the greenhouse showed that the three cornered alfalfa treehopper, *Cerasus festinus*, transmits GRBaV from infected to healthy vines. This result suggests that 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). GRBaV is a putative member of a new genus in the family *Geminiviridae* (Varsani et al., 2014; Sudarshana et al., 2015). It has a single-stranded DNA genome that codes for six open reading frames (Al Rwahnih et al., 2013; Krenz et al., 2012; 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).

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. The two groups of isolates are involved in the etiology of the disease but it is not known if mixed infections by representative isolates of each group can have a synergistic effect and exacerbate the negative impact on production.

GRBaV was documented in major grape-growing US States (Krenz et al., 2014). The virus was also reported in British Columbia (GenBank JX559642.1) and Ontario (Fuchs, unpublished observations) in Canada, 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., 2015; 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.

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. 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 GRBa) 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. A comparative analysis of the infection rate of GRBaV in the selected vineyard in California between 2014 and 2015 indicated a 1.5% increase, suggesting the possibility of virus spread. 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 as a result of either vine-to-vine transmission within the selected vineyard or of an influx from adjacent vineyards. Similar work in a New York vineyard did not provide evidence of GRBaV spread.

Close to 100 sentinel vines, i.e. healthy vines for which the mother stocks tested negative for GRBaV, were planted in the Cabernet franc vineyard in California in spring 2015. These vines will be tested for the presence of GRBaV in 2016 and used to gain direct evidence of insect-mediated GRBaV spread if they become infected.

Insect sticky traps were placed in the northwest area of the selected vineyard in California. Traps were placed on vines 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 insects 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 the majority of 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). These four species are members of the Membracidae (*Ceresa festina*), Cicadellidae (*Colladonus reductus* and *Osbornellus* sp.) and Cixiidae (unidentified species). These findings suggest that these four species can acquire GRBaV in the vineyard.

Table 1. Presence of GRBaV in a subset of insects caught on sticky traps in a Cabernet franc vineyard in California in which spread of GRBaV is documented.

Order	Species/Family	Common Name	Number Tested	GRBaV detected	Percent Positive
Hemiptera	*Ceresa festina*	Three cornered alfalfa hopper	25	12	48%
Hemiptera	Cixiidae	Cixiid planthoppers	8	4	50%
Hemiptera	*Colladonus reductus*	*Colladonus reductus*	23	14	61%
Hemiptera	*Osbornellus* sp.	*Osbornellus* sp.	31	13	42%
Thysanoptera	Thysanoptera	Thrips	12	0	0%
Hemiptera	Aleyrodidae	Whiteflies	52	0	0%
Hemiptera	Psylloidea	Psyllids	25	0	0%
Hemiptera	*Deltocephalus sp.*	*Deltocephalus sp.*	15	0	0%
Hemiptera	*Erythroneura elegantula*	Western grape leafhopper	41	0	0%
Hemiptera	*Erythroneura variabilis*	Variegated leafhopper	22	0	0%
Hemiptera	*Euscelis* sp.	Brown leafhopper	33	0	0%
Hemiptera	*Daktulosphaira vitifoliae*	Grape phylloxera (winged adults)	22	0	0%
Hemiptera	*Sophonia orientalis*	Two-spotted leafhopper	5	0	0%
Hemiptera	Aphididae	Aphids	46	1	2%
Hemiptera	*Scaphytopius magdalensis*	Sharp-nosed leafhopper	29	1	3%
Hemiptera	*Empoasca sp.*	Potato leafhopper	28	1	4%
Hemiptera	*Graphocephala atropuncta*	Blue-green sharpshooter	23	1	4%

Testing the capacity of the four hemipteran insects that are identified as candidate vectors at transmitting the virus to healthy grapevines was initiated in the greenhouse using *Ceresa festina* specimens. First, *Ceresa festina* 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 *Ceresa festina* were tested for the presence of GRBaV after the acquisition and transmission steps. Data showed that 8 out of 10, and 6 out of 12 individuals tested positive for GRBaV in multiplex PCR after the acquisition and transmission steps, respectively. These results were consistent across several transmission experiments. Also, some individuals tested positive for GRBaV two (3 of 5) or even three weeks (2 of 3) following the transmission step, indicating that *Ceresa festina* 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 months post-transmission, 2 of 4 recipient vines from the first transmission experiment became infected with GRBaV, as shown by PCR, supporting the capacity of the three cornered alfalfa treehopper to acquire and transmit GRBaV, and suggesting a role as a vector.

To address objective #2 and improve diagnostics for GRBaV, efforts are on going with regard to the development of a robust real time PCR methodology and the refinement of the strategy needed to produce an antiserum. This work is critical because preliminary efforts to develop an antiserum against the structural coat protein have failed (Perry and Fuchs, unpublished). A real time PCR was developed and optimized using infected and healthy vines grown in the greenhouse and vineayrds.

Objective #3 on the pathogenicity of the two groups of GRBaV was addressed earlier.

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 Cornell Recent Advances in Viticulture and Enology conference on November 4, 2015 at the IRL Conference Center in

Ithaca (60 participants), NY, (ii) the Napa Continuing Education Class Series 3 on November 10, 2015 in Yountville, CA (250 participants), and (iii) a webinar on Grapevine red blotch disease: What you need to know' organized by Regional IPM Centers, February 26, 2016 (participants = 310).

CONCLUSIONS

Analysis of the spatio-temporal distribution of symptomatic, infected vines suggested spread of GRBaV in a vineyard in California but not in New York. Free-living grapevines proximal to the diseased vineyard in California can be infected with GRaBV, providing additional evidence of spread. The use of insect sticky traps followed by the analysis of a sub-set of insect species for the presence of GRBaV enabled us to identify four candidate vectors, among which, *Ceresa festina*, the three cornered alfalfa treehopper was shown to acquire the virus fro infected vines and transmit it to healthy vines. This finding suggests a role *Ceresa festina* as a vector of GRBaV.

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