

Interim Progress Report for CDFA Agreement Number 18-0296-000-SA: "STRUCTURE-FUNCTION STUDIES ON GRBaV TO ELUCIDATE DISEASE ETIOLOGY"

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INTRODUCTION

Geminiviruses are single-stranded DNA (ssDNA) viruses that cause major losses to many crops throughout the world¹⁻³. *Geminiviridae* constitutes the second largest family of plant viruses. Geminiviruses are characterized by small, circular, ssDNA genomes encapsidated in twinned (hence, the name *Gemini*) icosahedral particles⁴⁻⁶. They are vector-transmissible and infect both monocotyledonous and dicotyledonous plants⁷. The genomes are either monopartite or bipartite with circular DNA molecules of 2.5- 3 kb. Geminiviruses possess a highly conserved common region (CR) of ~200 nucleotides containing an inverted repeat that forms a hairpin loop with an invariant 9-nt sequence 5'-TAATATT¹AC-3'. The viral gene products are required for its replication and transmission. Successful commercialization of engineered viral resistance for certain crops has drawn on invented strategies for blocking virus replication.

Grapevine Red Blotch Virus (GRBV) is a monopartite, grapevine-infecting Grablovirus causing Red Blotch Disease and was first observed in California in 2008⁸. Bahder *et al.*⁹ identified the alfalfa leafhopper *Spissistilus festinus* as the candidate vector that transmits GRBV under laboratory conditions. Disease symptoms manifest as red patches in the middle of the grapevine leaf and in veins and petiole, which coalesce at the end of the growing season¹⁰. GRBV infection results in delayed and uneven berry ripening, higher titratable acids, reduced sugar and reduced anthocyanin content in the berry¹¹, impairing fruit qualities which threaten both table grape and wine industries¹².

Consistent with geminiviruses, GRBV possesses the conserved nonanucleotide sequence and transcription is bidirectional¹⁰. GRBV encodes three ORFs in the virion strand (V1, V2, and V3) and three in the complementary strand (C1, C2, and C3; **Fig. 1**). Similar to mastrevirus (a monopartite geminivirus), GRBV complementary-sense ORF C1 encodes RepA, Replication-associated protein. Another spliced transcript encompassing the C1 and C2 ORFs encodes Rep, the Replication protein^{10, 13-15}. GRBV virion-sense strand ORFs V2 and V3 are predicted to encode movement proteins, whereas V1 ORF encodes coat protein.

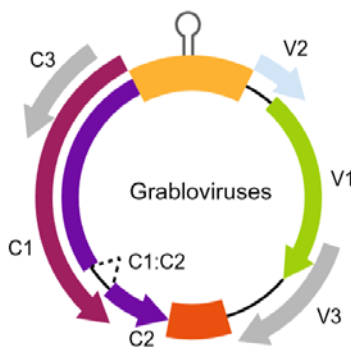


Fig. 1. Genome organization of Grapevine red blotch virus (GRBV), previously called blotch-associated virus (GRBaV).

The functions of the predicted GRBV ORFs are yet to be elucidated experimentally. Understanding the molecular mechanisms by which the virus mounts a successful infection is fundamental and essential to develop cogent engineered resistance strategies. A practical issue is that the few proteins encoded by geminiviruses are multifunctional and likely modulate several host regulatory genes, a mechanism uniquely evolved by the viruses to balance the genome size-constraint emplaced by the capsid. A comprehensive 'omics' profiling experiment on berry development and select metabolite and enzyme quantitations in GRBV-infected grape from two different

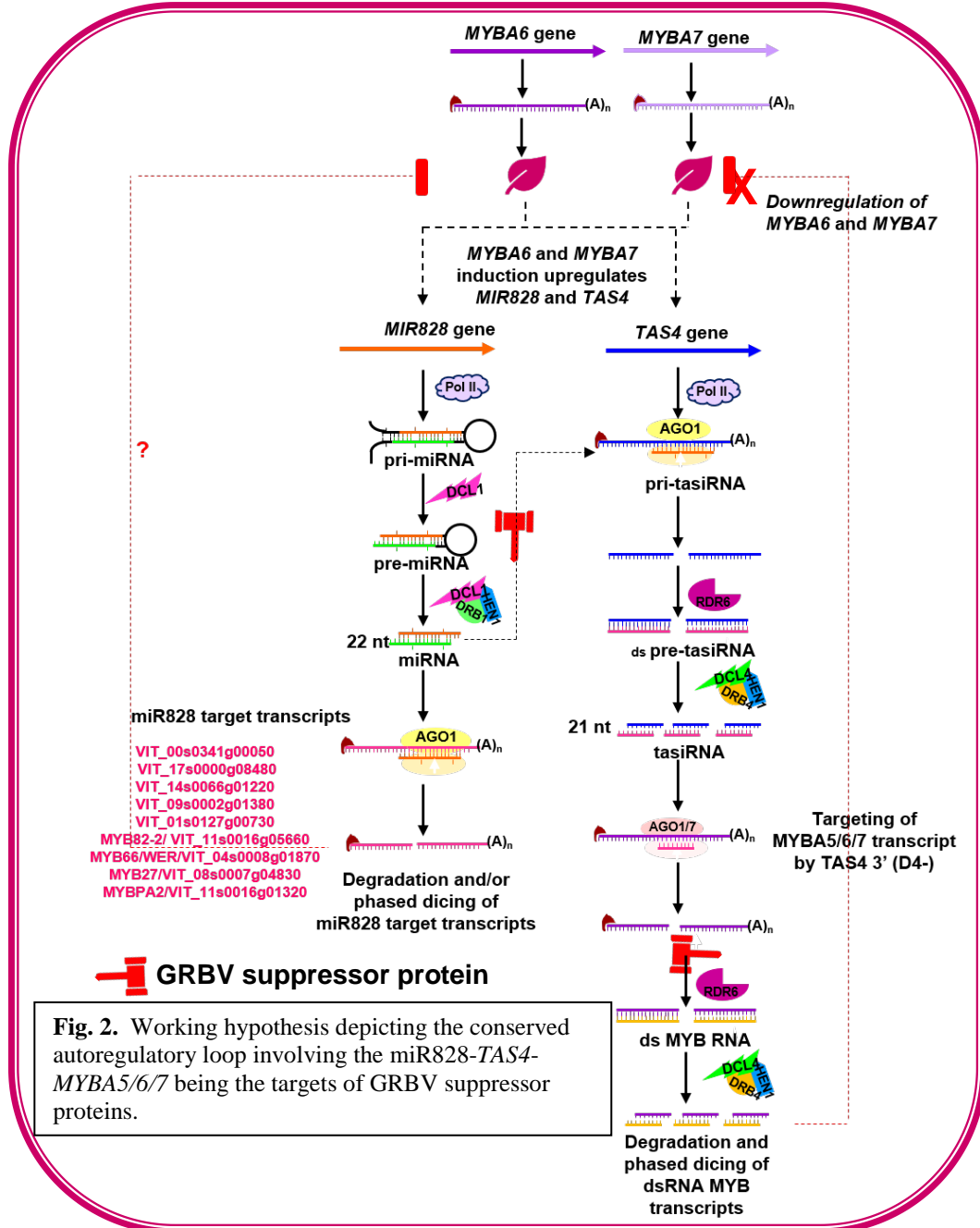
vineyards suggests several host regulatory pathways, in particular phenylpropanoids, are impacted by the virus¹⁶. GRBV infection results in deranged expression of host post-transcriptional machinery, transcription factors, and several hormone biosynthesis and response pathways. Post-transcriptional gene silencing (PTGS) processes involving microRNAs (miRNAs), small-interfering RNAs (siRNAs) and phased siRNAs (phasiRNAs) are known to regulate host immune responses to viruses and microbes, as well as normal plant development and hormonal signaling^{17, 18}. Hence, we postulate GRBV manifests disease by specifically targeting the host PTGS machinery by "suppressor proteins," thereby driving the observed reprogramming of multiple host regulatory and metabolic pathways for its successful replication and transmission.

PTGS has evolved as a major host defense mechanism against invasive pathogens including viruses. Asymptomatic leaves with abundant viral siRNAs are natural outcomes of most host-virus interactions associated with reduced but persistent viral titer, induction of PTGS in the host, and symptom recovery¹⁹. miRNAs and siRNAs are the specificity "guide" for nucleases of the ARGONAUTE (AGO) class which cleave or otherwise repress protein-coding transcripts in a nucleotide sequence-specific manner^{20, 21}. The presence of a robust viral counter defense mechanism is underscored by the ubiquitous presence of one or more silencing suppressor proteins in the genomes of many plant viruses. The "arms race" between host silencing of pathogen transcripts and silencing suppression by pathogen gene products results in resistance or susceptibility to the pathogen. Numerous geminiviruses encode silencing suppressor proteins that target PTGS, transcriptional gene silencing (TGS), and cellular regulatory genes (**Table I**).

Table I. Suppressor proteins characterized in Geminivirus and their plant targets.				
Virus*	Suppressor	Suppressing PTGS	Suppressing TGS	Cellular pathways
MYMV	AC2	Upregulate host suppressor protein WEL1 ²²		
TGMV BCTV	AL2 L2	Inactivate Adenosine kinase ^{23, 24}		Inactivate a serine-threonine kinase SnRK1 ²⁵
BSCTV	C2	Stabilize S-adenosyl methionine decarboxylase I (SAMDC1) ²⁶		
TGMV CaLCuV BCTV	AL2 AL2 L2		Inactivate Adenosine kinase and stabilize SAMDC1 ²⁷ Inhibit histone Me-transferase SUVH4/KYP ²⁸	
TGMV SCTV	AL2 C2			Elevation of cellular cytokinin levels ²⁹
TYLCSV	C2			Interact with CSN5 and inhibit jasmonate signaling ³⁰
ACMV	AC4	Binds ss miRNA ³¹		
WDV	Rep	Binds ss-and duplexed 21 and 24 nt siRNAs ³²		
TYLCV	V2		Compete <i>NbMET1</i> for binding to hist deacetylase ³³	
*Acronyms: <i>Mungbean yellow mosaic virus</i> : MYMV; <i>Tomato golden mosaic virus</i> : TGMV; <i>Beet curly top virus</i> : BCTV; <i>Beet severe curly top virus</i> : BSCTV; <i>Cabbage leaf curl virus</i> : CaLCuV; <i>Spinach curly top virus</i> : SCTV; <i>Tomato yellow leaf curl Sardina virus</i> : TYLCSV; <i>Tomato yellow leaf curl virus</i> : TYLCV; <i>African cassava mosaic virus</i> : ACMV; <i>Wheat dwarf virus</i> : WDV				

Previous work on the model plant *Arabidopsis* in the PI's lab showed altered source-sink distributions of sucrose and the stress hormone abscisic acid (ABA)³⁴ interact to regulate anthocyanin accumulation via miR828, *Trans-Acting Small-interfering locus4* (*TAS4*), and their target MYeloBlastosis viral oncogene-like (v-MYB) transcription factors, viz. Vvi-MYBA6/7 and close homologues targeted by miR828 in grapevine^{35, 36}. We recently characterized the conserved autoregulatory loop involving miR828 and *TAS4* down-regulates anthocyanin biosynthesis during berry development by targeting *MYB* transcription factors induced by UV light in grape³⁷. The recently published transcriptome profiling study of GRBV-infected host berries identified significant repression rate-limiting ABA biosynthesis loci *NCED2/3* (first described by the PI³⁸) in infected berries¹⁶.

Our working model (**Fig. 2**) is that GRBV infection interferes with the normal PTGS pathways of the host by the activity of viral-encoded suppressor proteins. miRNAs/tasiRNAs/phasiRNAs regulate a large array of host gene expression at the post-transcriptional level and transcriptional levels. Viruses utilize plant miRNAs to facilitate pathogenesis, and plants have co-opted miRNAs for plant innate immunity^{21, 39-41}. Their collective loss in virus-infected tissues that results in susceptibility^{42, 43} demonstrates their functions as master regulators targeted by pathogens. Broader roles for plant sRNAs in evolutionary adaptations^{44, 45} may include virus vector feeding processes and preferences. Under P_i starvation, reduced ABA and sugar regulate the expression of miRNAs that facilitate anthocyanin biosynthesis by MYB-bHLH-WD40 ternary transcriptional complexes. Increases in MYB-



bHLH-WD40 results in up-regulation of miR828 via the conserved auto-regulatory loop^{34, 37} involving miR828/TAS4 to regulate MYBA5/6/7 levels and thereby anthocyanin levels (**Fig. 2**). We hypothesize the red blotch phenomena observed in GRBV-infected grape leaves is a consequence of viral suppressor proteins targeting the miR828/TAS4/MYBA5/6/7 autoregulatory loop which fine tune anthocyanin levels by a "rheostat" feedback³⁷.

A recent paper reported GRBV effects on berry development¹⁶. **Table II** provides preliminary evidence drawn from this publicly available berry transcriptome data which supports our model. As per our hypothesis we observe a near-statistically significant downregulation of *Vvi-TAS4c* at veraison and post-veraison in GRBV-infected berries, indicating the *miR828-TAS4-MYB* pathway is a specific target of GRBaV. This is supported by the strong up-regulation of *MYBA6* at harvest, the target of a deeply conserved TAS4c tasi-RNA 3'D4(-) along with several other MYBs^{36, 37} shown to function in the phenylpropanoid/flavonol pathway and targeted by miR828. Interestingly, we observe up-regulation of *AGO*, *DICER2* and *SUPPRESSOR OF GENE SILENCING3* (*SGS3*) transcripts, all major effectors of the PTGS machinery and themselves subject to PTGS and spawning of amplified phasiRNAs^{46, 47}. It will be very interesting to determine if transitivity of these loci is deranged by GRBV; we hypothesize a repression of host sRNA effector machinery upon virus infection, but the evidence is that the host is compensating by overexpressing PTGS effector pathways. This could be because the data we analyzed is from different developmental stages of berry ripening, which is also under post-transcriptional regulation. These preliminary results underscore the need to perform transcriptome and sRNA analysis from different tissues of field-infected grapevines including girdled petioles at the sites of vector feeding to decipher the targets of GRBV (<http://ucanr.edu/blogs/blogcore/postdetail.cfm?postnum=20473>).

Table II. Analysis of publicly available transcriptome data [^] for GRBV-infected berries across development											
developmental stage:			pre-veraison		veraison		post-veraison		harvest		
target; sRNA effector	gene ID	Phase Score	beta ~LFC	pval	beta ~LFC	pval	beta ~LFC	pval	beta ~LFC	pval	
GRBaV genome	JQ901105.2	n.d.	6.26	1.91E-15	NA	NA	NA	NA	6.76	3.47E-32	
Vvi-TAS4c; miR828	chr1:2961251:2961747	3375	NA	NA	-1.01	0.13	-1.01	0.13	0.38	0.53	
AGO1a; miR168/530	VIT_17s0053g00680	n.d.	0.06	0.55	0.17	0.04	0.17	0.04	0.16	0.05	
AGO1b; miR168/530	VIT_19s0014g01840	n.d.	0.26	0.47	0.43	0.04	0.43	0.04	0.08	0.75	
MYBA6, TAS4	VIT_14s0006g01290	22.2	NA	NA	NA	NA	NA	NA	1.25	0.09	
MYB ^{PAL1} ; miR828	VIT_00s0341g00050	476	0.52	0.01	0.12	0.39	0.12	0.39	0.13	0.31	
MYB; miR828	VIT_17s0000g08480	1330	0.62	0.09	0.33	0.35	0.33	0.35	NA	NA	
MYB; miR828	VIT_04s0079g00410	24.6	0.39	0.01	0.17	0.04	0.17	0.04	-0.06	0.46	
AGO2a; miR403	VIT_10s0042g01180	50	0.61	0.02	0.36	0.07	0.36	0.07	0.82	0.02	
AGO2b; miR403	VIT_10s0042g01200	n.d.	0.04	0.81	0.03	0.81	0.03	0.81	-0.16	0.29	
DCL2; unknown	VIT_04s0023g00920	33.8	0.39	0.25	0.47	0.03	0.47	0.03	0.11	0.57	
SGS3; unknown	VIT_07s0130g00190	177.4	0.04	0.69	0.23	0.01	0.23	0.01	0.16	0.06	
DCL1; miR162	VIT_15s0048g02380	n.d.	-0.05	0.62	0.05	0.54	0.05	0.54	-0.21	0.15	

[^] Oakville vineyard dataset (ref. 16) analysed by kallisto/sleuth.

OBJECTIVES OF PROPOSED RESEARCH AND PATH TO APPLICATION:

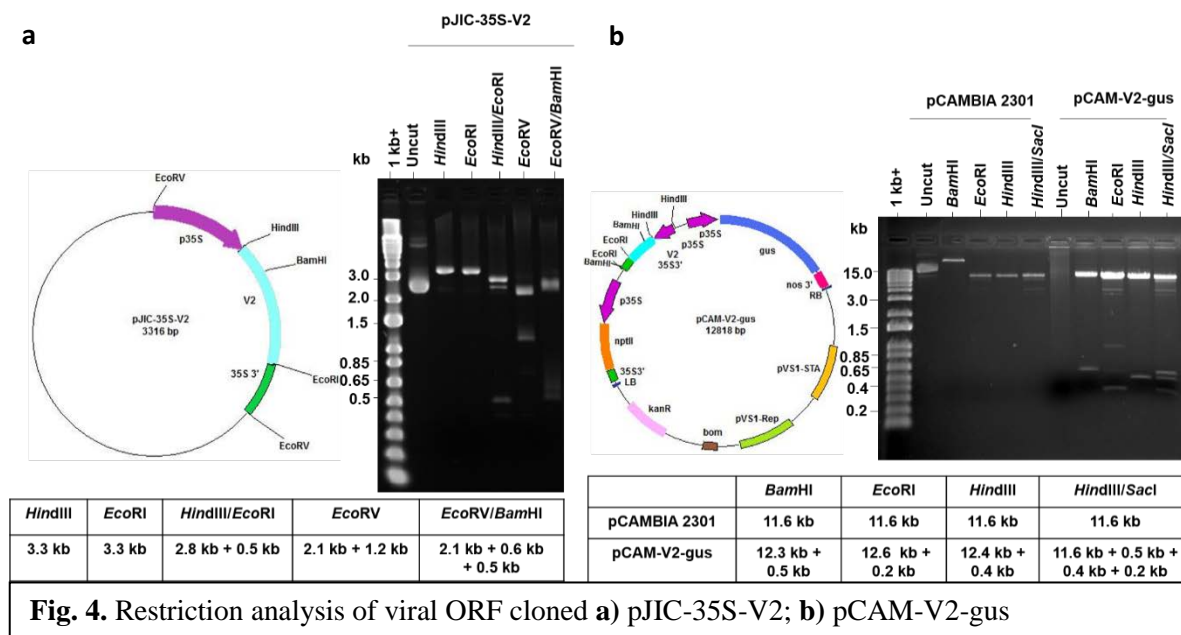
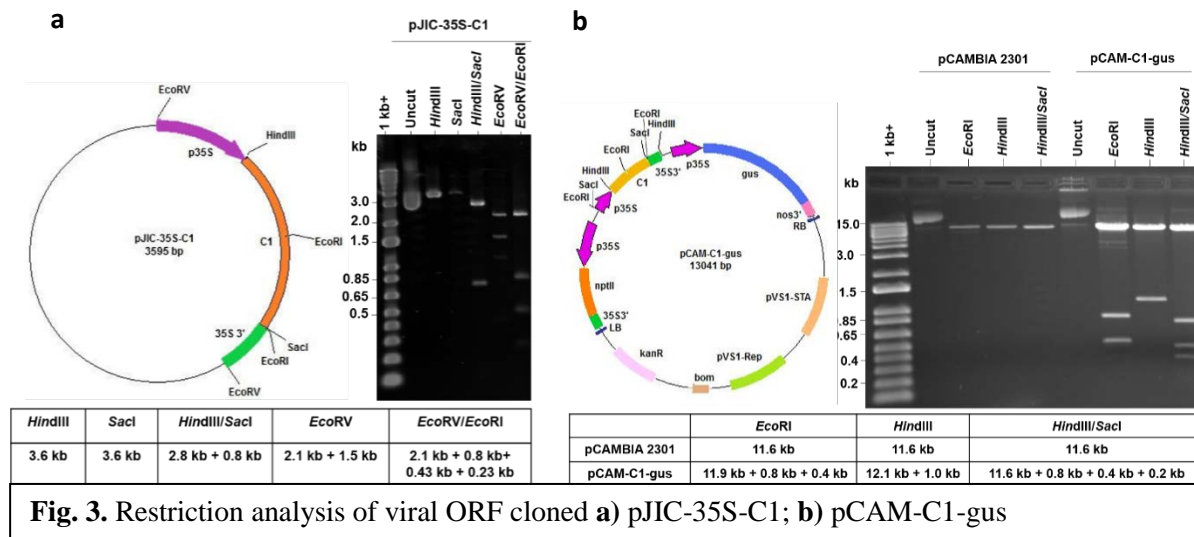
- I. Characterize hypothesized silencing suppressor protein(s) encoded by GRBaV to establish the molecular mechanism by which GRBaV (and GLRaV by inference) cause disease by derangement of host microRNAs (miRNAs), *trans*-acting small interfering (tasi-) RNAs, and phased-tasi-RNAs (phasi-RNAs).
- II. Identify the host grapevine targets of GRBaV suppressor proteins
- III. Creation of model system transgenics for future characterization of the host targets of GRBaV suppressor proteins

DESCRIPTION OF ACTIVITIES CONDUCTED TO ACCOMPLISH OBJECTIVES

Objective I.

We have focused most of our efforts to date to characterize GRBV suppressor proteins because Objectives II and III are predicated on Objective I success. We PCR-amplified GRBV genes *V1*, *V3*, *C1*, and *C3* with engineered *HindIII*/*SacI* flanking sites, and *V2/C2* with *HindIII*/*EcoRI* sites templated from genomic DNA extracted from GRBV-infected grape leaf tissue collected in 2016 from 'Calle Contento' vineyard (cv. Merlot) in Temecula CA, and from cv. Pinot Noir vines from Jacksonville, OR in July 2018 with the assistance of Cooperator K.C. Achala. The PCR fragments were successfully cloned into the corresponding sites of pJIC-35S vector⁴⁸. We confirmed the clones by Sanger sequencing and by restriction enzyme site mapping (**Fig. 3a and 4a**; pJIC-35S-C1 and pJIC-35S-V2 shown as representative). The pJIC-35S-ORF cassettes (except pJIC-35S-V3, which

has an internal *EcoRV* site) were excised as *EcoRV* fragments and cloned into the *Sma*I site of binary transformation vector pCambia2301. The V3 cassette was separately cloned as blunt-ended PCR fragment engineered with alternative linkers. The clones and their orientations were confirmed by multiple restriction site mapping (Figs. 3b and 4b; e.g. pCAM-C1-*gus* and pCAM-V2-*gus* shown as representative).



All six C1-3/V1-3 binary transformation vectors were electroporated into *A. tumefaciens* strain EHA105. The electroporation was confirmed by PCR with respective ORF primers (Fig. 5). To evaluate if GRBV

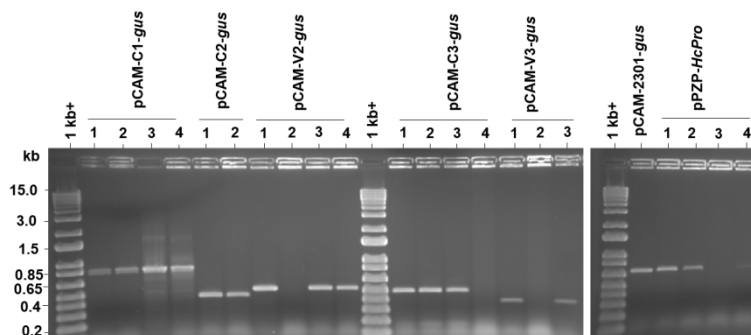


Fig. 5. PCR confirmation of binary vectors electroporated into *A. tumefaciens* with respective ORF primers.

possesses viral silencing suppressor proteins, *N. benthamiana* line 16c, developed in the laboratory of Sir David Baulcombe⁴⁹ expressing *A. victoria* jellyfish Green Fluorescence Protein (GFP) was used as the test system. In this system, RNA silencing of the *gfp* transgene can be triggered by transient expression of a *gfp* (trigger)-expressing vector. The over-expression of transiently transformed *gfp* (trigger) construct normally results in RNAi-mediated co-suppression of the stably integrated *gfp* transgene. Consequently, the agroinfiltrated leaf will exhibit loss of GFP protein and UV-excited fluorescence, and instead red auto-fluorescence from chlorophyll (chl) is observed as a positive control result. When a silencing suppressor protein gene construct is co-infiltrated along with *gfp* (trigger), the test infiltrated zone will exhibit rescue of green fluorescence as marker of suppression of silencing.

Six-week-old *N. benthamiana* 16c plants were agroinfiltrated with the *A. tumefaciens* strain harboring the p35S-*gfp* (pBI-mgfp5-ER; the 'trigger') either alone (*mgfp*; a positive control for silencing), with pCAMBIA-2301 empty vector (another positive control for silencing to compare two constructs delivered versus one), or with co-infiltration of one of the respectively six test GRBV constructs (substituted for pCAMBIA-2301 empty vector). Potyvirus HcPro⁵⁰ construct co-infiltration served as positive control for silencing suppression. Five days post infiltration, local GFP silencing of infiltrated leaves was observed under long wave UV light as red chl fluorescence (**Fig. 6**; *mgfp*; *mgfp*+pCAM-2301). To evaluate the silencing suppression effect of co-expressed GRBV genes, the 16c plants were agroinfiltrated with 1:1 test mixture of the *A. tumefaciens* strains harboring p35S-*gfp* (trigger) plus one p35S-V1/p35S-V2/p35S-V3/p35S-C1/p35S-C2 or p35S-C3 construct, respectively. As expected, bright green fluorescence was observed in the infiltrated zones (on either side of the central midvein) with *mgfp* plus *HcPro* co-inoculation (**Fig. 6**; *mgfp*+*HcPro*). GRBV C1, C3, V1 and V3 construct co-infiltrations did not result in green fluorescence, demonstrating their expression did not suppress the silencing triggered by *mgfp* (**Fig. 6**; *mgfp*+C1; *mgfp*+C3; *mgfp*+V1; *mgfp*+V3). In the presence of GRBV C2 and V2 expression from co-infiltrated constructs, the infiltrated area was **not silenced** by the *gfp* trigger (infiltration zones display green

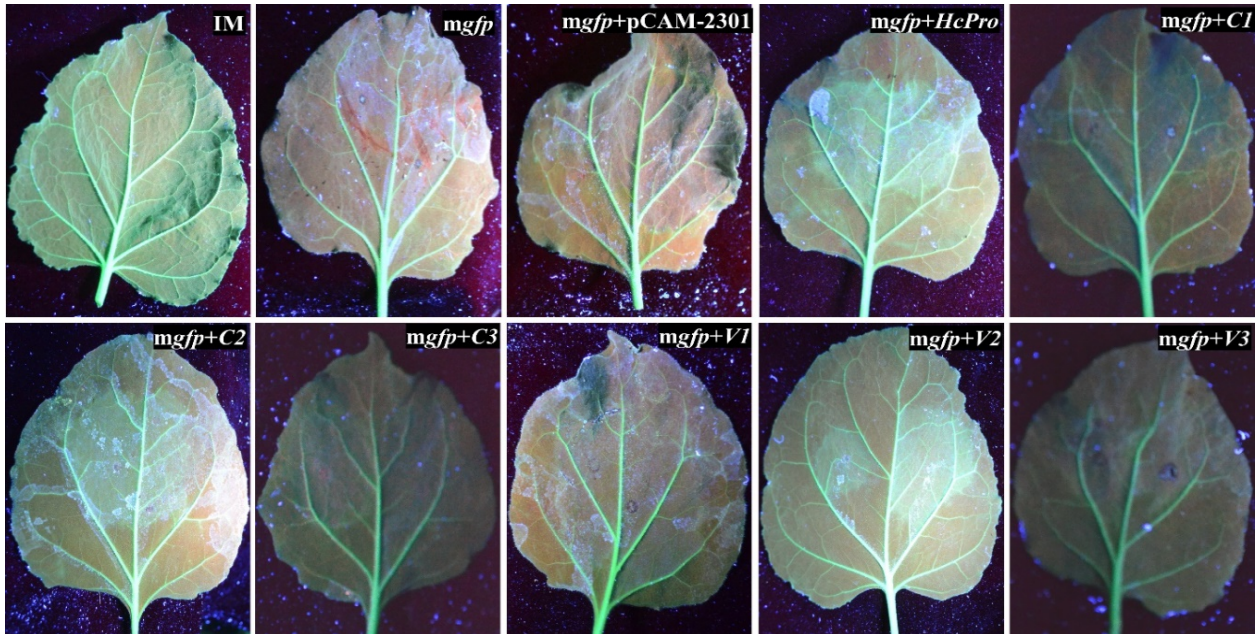


Fig. 6. Agroinfiltration of the *Nicotiana benthamiana* 16c-GFP to demonstrate silencing suppression by HcPro and test GRBV genes. *Agrobacterium*-mediated transient expression in *N. benthamiana* leaves. **IM**: Mock-agroinfiltrated with infiltration medium. ***mgfp***: Agroinfiltrated with the *A. tumefaciens* strain harboring p35S-*gfp* as control for *GFP* transgene silencing. ***mgfp*+pCAM-2301**: Co-agroinfiltrated with *A. tumefaciens* strains harboring p35S-*gfp*+pCAMBIA 230 (negative control). ***mgfp*+*HcPro***: Co-agroinfiltrated with *A. tumefaciens* strains harboring P35S-*gfp* + Potyvirus *HcPro* (Positive control for PTGS). ***mgfp*+C1**: Co-agroinfiltrated with *A. tumefaciens* strains harboring P35S-*gfp* + GRBV C1. ***mgfp*+C2**: Co-agroinfiltrated with *A. tumefaciens* strains harboring P35S-*gfp* + GRBV C2; Note PTGS suppression evidence, like HcPro. ***mgfp*+C3**: Co-agroinfiltrated with *A. tumefaciens* strains harboring P35S-*gfp* + GRBV C3. ***mgfp*+V1**: Co-agroinfiltrated with *A. tumefaciens* strains harboring P35S-*gfp* + GRBV V1. ***mgfp*+V2**: Co-agroinfiltrated with *A. tumefaciens* strains harboring P35S-*gfp* + GRBV V2. Note PTGS suppression evidence, like HcPro. ***mgfp*+V3**: Co-agroinfiltrated with *A. tumefaciens* strains harboring P35S-*gfp* + GRBV V3.

fluorescence, like observed for HcPro silencing suppression positive control (**Fig.6**; *mgfp*+C2; *mgfp*+V2). Thus, we have established that GRBV C2 and V2 proteins are candidate suppressor proteins. This result has been repeated, providing compelling evidence for C2 and V2 functioning as GRBV silencing suppressor proteins.

Objective I completion requires an independent proof of silencing by assaying the molecular markers for the process. For the first round of agroinfiltration, RNA blot analysis of the RNAs extracted from agroinfiltrated leaf tissue was performed using *gfp* gene as probe. The agroinfiltrated area was harvested by visualization and dissection under UV light and total RNA was extracted and leaf samples were pooled from 5-8 technical replicates. Leaves from mock-infiltrated sections accumulated *gfp* transcript (**Fig. 7**), proving there was no silencing. Agroinfiltration of P35S-*gfp* triggers silencing of GFP protein expression as consequence of complete absence of *gfp* transcript due to sRNA-induced slicing of the 2 kb mRNA by host silencing machinery (**Fig. 7**). Co-infiltration with *mgfp*+HcPro resulted in green fluorescence and return of steady state abundance of *gfp* transcript, as expected. Co-infiltration with *mgfp*+GRBV V2 resulted in green fluorescence and comparable accumulation of *gfp* transcript to that of control HcPro, providing additional independent evidence that V2 is a silencing suppressor gene.

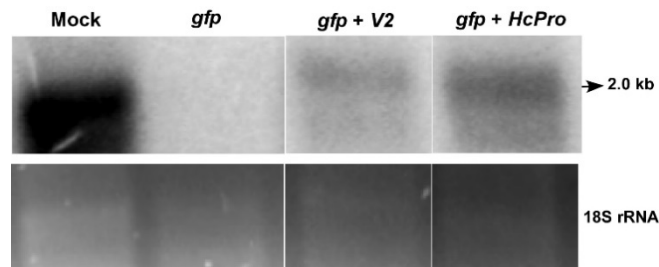


Fig.7 Northern blot analysis with *gfp* probe. Total RNA from the infiltrated areas of mock infiltrated leaves, P35S-*gfp* infiltrated leaves (*gfp*), P35S*gfp*+V2 (*gfp*+V2) and P35S*gfp*+HcPro (*gfp*+HcPro) infiltrated leaves was probed with the *gfp* coding sequence. The 18S rRNA portion of the ethidium bromide-stained gel is placed at the bottom.

We repeated the agroinfiltration assay and the phenotypic results were the same as presented in **Fig. 6**. We extracted total RNA and performed quantitative Real-Time PCR (qPCR) with *gfp* primers. We used actin primers as internal control to normalize for amplification efficiency. The efficiency of three sets of *gfp* primers and a pair of actin primers was tested by plotting a standard curve of 2⁻-fold dilutions of template cDNAs from V2- and C2-agroinnoculated excised leaves. The *gfp* primers with 110% (**Fig. 8a**) and actin primers with 104% (**Fig. 8b**) were used for subsequent relative expression comparisons using V2- and C2-specific primer pairs.

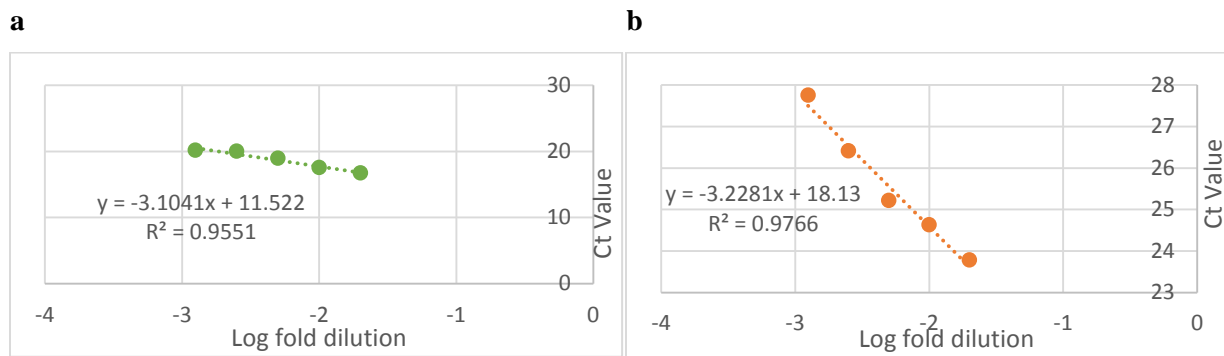


Fig. 8. Primer efficiencies with **a)** *gfp* primer and **b)** actin primer pairs.

Agroinfiltration of P35S-*gfp* is expected to degrade the *gfp* transcript, and although red fluorescence and loss of detectable *gfp* mRNA was observed (**Figs. 6, 7**) the high sensitivity of qPCR showed some amount of *gfp* transcript was still present (**Fig. 9**) in positive control *mgfp* agroinnoculated leaf samples when compared to the Mock-infiltrated plant *GFP* mRNA level (set to unity). This unexpected observation suggests that the silencing trigger did not completely degrade the *gfp* transcript made from the agroinfiltrated P35S-*gfp*. Similar results were observed⁵¹ by the Co-I in *gfp*-triggered silencing experiments to test Mung bean Yellow Mosaic Virus suppressor

proteins, where the silencing by controls was not complete. Although, C2 and HcPro showed relative increases in *gfp* transcript above trigger alone control as expected for silencing suppressors (**Fig. 7**), V2 did not show a relative increase in *gfp* transcript. We attribute the variations we observed from the expected results to errors in the sample collection technique. Dissecting out the infiltration zone under UV light is laborious and quite tricky; it is likely that we had accidentally collected certain regions of non-infiltrated leaf samples in our sample pool. We are repeating the agroinfiltration experiment and we will be collecting leaf samples very precisely (0.5 cm inside from the apparent edges of the infiltration zones) to address this technical issue with sample collection.

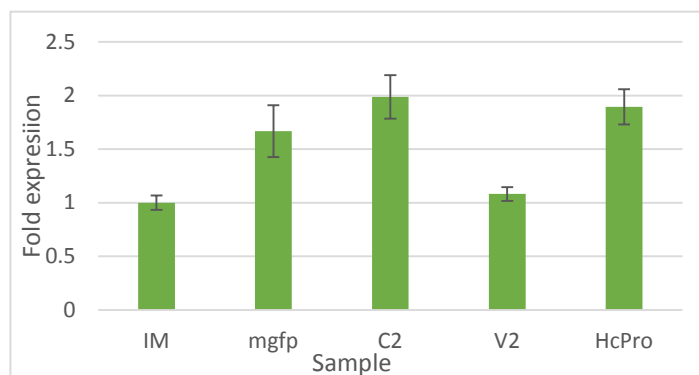


Fig.9. quantitative Real-time PCR with *gfp* primers as high sensitivity assay for silencing and function of viral candidate silencing suppressor genes C2 and V2. *mgfp* mRNA levels were quantified from cDNAs made from total RNA extracts of leaf infiltration zones dissected 5 days post-infiltration. Relative expression was determined against the mean of mock-inoculated samples. Values are means \pm SE of three technical repeats.

Summary of Objective I accomplishments:

Taken together our protein, mRNA blot and qPCR results have provided conclusive evidence that GRBV genes C2 and V2 are suppressor proteins. We are repeating the agroinfiltration assay to verify the claim and will publish the results thereafter.

Objective II: Identify the host grapevine targets of GRBaV suppressor proteins

We are taking a systems approach to address the molecular mechanisms by which GRBV causes symptoms by quantitative genome-wide analyses of host messenger RNAs, miRNAs, sRNAs, and phasiRNAs deranged in field samples of vineyard leaves manifesting GRBV infection symptoms versus healthy plants. We are in the process of deep sequencing at the Institute of Integrative and Genome Biology at UC Riverside matched libraries made from control healthy and GRBV-infected leaf total RNA and sRNA extracts from samples collected in Temecula CA in 2016. Genome-wide systems analysis of such datasets can reveal the specific host genes in vegetative tissues deranged by the pathogen and provide leads for understanding the underlying mechanisms, e.g. specific miRNA effectors of host gene regulatory networks controlling plant immunity. We have in hand PCR-verified GRBV-infected and control samples collected in July 2018 from the Calle Contento vineyard (cv. Merlot) in Temecula CA and from Pinot Noir cultivars collected in Jacksonville, OR. We are in the process of generating a second year set of sRNA-seq and RNA-seq libraries for deep sequencing. We will collect new materials in late summer 2019. The 2018 Oregon mid-season samples in hand did not manifest symptoms and prior PCR results from tagged vines were the basis for collecting infected versus healthy controls. Several years of biological replicate libraries across environmental variables will add statistical power to our analyses, contingent upon funding.

Summary of Objective II accomplishments:

We have established baseline datasets for interpretation of test sequencing experiments, in progress. The results from multiple years of field sampling will afford powerful statistical inference of the targets of GRBV suppressor proteins.

Objective III: Creation of model system transgenics for future characterization of the host targets of GRBaV suppressor proteins

We have extensive experience in *Arabidopsis*^{52, 53} and tobacco transformation, and have initiated axenic leaf disc tissue culture of the transgenic tobacco line 237⁵⁴ that overexpresses AtMYB90 anthocyanin biosynthesis effector. We will then 'supertransform' the homozygous and heterozygous transgene genotypes in tissue culture with suppressor protein-expression constructs (**Figs. 3,4**) to critically and easily test in regenerated plants the functional efficacy of overexpressed GRBV V2 and C2 proteins to silence the readout anthocyanin accumulation and molecular signatures of the underlying endogenous autoregulatory miR828/TAS4/ANTHOCYANIN2 MYB loop pathway³⁵ activated by AtMYB90 overexpression. We are in the process of obtaining a USDA-BRS permit to ship binary constructs in hand (**Figs. 3,4**) for V2 and C2 suppressor protein expression to Dr. David Tricoli at UC Davis Ralph Parsons Plant Transformation facility, who will generate transgenic tobacco expressing the V2 and C2 candidate suppressor genes as fee-for-service. Based on results to date for structure-function of GRBV V2 and C2 genes (Objective I), and host sRNA and mRNA sequencing (Objective II), we are confident for rapid progress and ultimate success of Objective III, the overexpression of the C2 and V2 suppressor proteins in *Arabidopsis* and tobacco in order to empirically and directly characterize by biochemical approaches those sRNAs specifically bound by V2 and C2 with high affinity. This latter objective is beyond the scope of one year of support but on track for completion of transgenics starting materials at the end of the current award.

Summary of Objective III accomplishments:

Initiation of tissue culture for stable transformation of a facile system (AtMYB90 overexpressing tobacco line 237) to assess V2 and C2 functions in planta. The significance is synergy with other Objectives: sRNA and total RNA blots probed for the putative host target miRNAs in C2 and V2- over-expressing lines will provide evidence of sufficiency for hypothesized V2 and C2 functions to silence anthocyanin synthesis mediated by host phasiRNAs. We predict concordance in expression profiles of putative targets in over-expression lines with that of sequencing data (Objective II) which could independently validate the sRNA targets of GRBaV suppressor proteins.

PUBLICATIONS AND PRESENTATIONS MADE THAT RELATE TO THE FUNDED PROJECT

The email acknowledgement below is for a manuscript under review not directly related to this project, but results have established a baseline dataset for leaf tissues to be leveraged for Objective II. The work was funded by CDFA Agreement Number 17-0514-000-SA to the PI.

From: tpc@msubmit.net [mailto:tpc@msubmit.net]

Sent: Tuesday, March 19, 2019 11:45 PM

To: Rock, Chris

Subject: TPC2019-RA-00188 Manuscript Received

Dear Dr. Rock:

On 20-Mar-2019, the manuscript entitled "microRNAs associated with phosphate starvation and secondary metabolism are down-regulated in *Xylella fastidiosa*-infected grapevine" by Christopher Rock, Sukumaran Sunitha, Leonardo De La Fuente, Md. Fakhru Azad, Sy Traore, and David Tricoli was submitted to *The Plant Cell* by the corresponding author.

The manuscript has been assigned the Paper #: TPC2019-RA-00188.

The manuscript will undergo a quality control check by journal staff and the corresponding author will be contacted within a few days if anything else is needed at this stage.

Sincerely,

Annette Kessler

Peer Review Manager, *The Plant Cell*

Sunitha S, Rock CD, Azad MF, Tricoli D, De La Fuente L, Traore S. "A conserved autoregulatory feedback loop is targeted by grape pathogens." Plant-2019: International Conference on Plant Science Research. March 4-5, 2019. DoubleTree by Hilton Baltimore BWI Airport. Oral presentation.
<https://unitedscientificgroup.com/conferences/plant/whova.html>

Sunitha S, Loyola R, Alcalde JA, Arce-Johnson P, Matus JT, Rock CD (2019) The role of UV-B light on small RNA activity during grapevine berry development. *G3: Genes/Genomes/Genetics* 9: 769-787. This paper is not directly related to the project, but lays the groundwork for Objective II with baseline characterization of host

miRNAs regulated during berry development and in response to abiotic light and oxidative stresses. The work was funded by FAPESP-SPRINT Brazilian-TTU Joint Program and TTU-VPR Open Access Publication Initiative awards to the PI.

Sunitha S, Weligodage H, Jin G, Rock CD. "Structure-function studies on Grapevine Red Blotch Virus to elucidate disease etiology." 2018 CDFA Pierce's Disease Research Symposium Proceedings. Pp. 229-240. Dec. 17-19, 2018. Kona Kai Resort, San Diego, CA. Oral presentation.
<https://www.cdfa.ca.gov/pdcp/Documents/Proceedings/2018ResearchProgressRpts.pdf>

RESEARCH RELEVANCE STATEMENT, INDICATING HOW THIS RESEARCH WILL CONTRIBUTE TOWARDS FINDING SOLUTIONS TO RED BLOTCH VIRUS IN CALIFORNIA

The Board has suggested genetic modification of genes involved in diffusible signals (here, applies to viral suppressor proteins) and host chemical specificity for disease etiology (here applies to host target small RNAs). The Objectives are fundamental to proofs-in-principle that the hypothesis is correct; future research can address the Board's directive to develop chemical inducers of host resistance. It is likely that miRNAs and tasi-RNAs operate systemically by moving through vasculature, raising prospects of genetic engineering of grapevine rootstocks for GRBaV resistance in non-genetically modified organism (GMO) scions⁵⁵⁻⁵⁸.

LAYPERSON SUMMARY OF PROJECT ACCOMPLISHMENTS

We have accomplished all of Objective I and half of Objective II, and have initiated Objective III. We are close to understanding how GRBV causes disease: through its dual silencing suppressor proteins C2 and V2. The knowledge gained should inform and present cogent strategies (e.g. transgenic and/or genome-edited grapevine resistant to viruses) for combatting the emerging virus threats to a multibillion-dollar industry. Results are also informative as to suggest possible conserved mechanisms underlying Leaf Roll-associated Virus (GLRaV) and other viral disease states.

STATUS OF FUNDS

Funds are being expended on track as budgeted.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY ASSOCIATED WITH THE PROJECT

The PI has disclosed a "Subject Invention" (USPTO patent application #13/874,962; May 1, 2013 "Regulating plant development and secondary metabolite biosynthesis useful for e.g. treating Pierce's disease due to *Xylella fastidiosa* infection by providing plant cells with anthocyanin effector, and regulating expression of genes") of potential relevance to this project in the event enablement of the invention can be applied towards other pathogens of grapevine like GRBV, GFLV, and/or GLRaV. The PI and Co-I have filed an invention disclosure to our institution on enablement of the patent application that claims *MIR828*, *TAS4*, and *MYB* targets. If title is elected TTU will share reagents via a Materials Transfer Agreement. Interested parties are referred to David Snow, Director and IP Manager, www.texastech.edu/otc, ph. 806-834-4989.

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