Interim Progress Report for CDFA Agreement Number 17-0514-000-SA

Project Title:"Genome editing of *TAS4, MIR828* and targets *MYBA6/A7*: a critical test of *Xylella fastidiosa* infection and spreading mechanisms in Pierce's disease"

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Time period covered by this report: Feb. 16, 2018- July 31, 2018.

Introduction

The overuse of phosphorous (P) fertilizer results in severe environmental pollution. As natural and anthropogenically-induced climatic changes occur, increased P limitation is expected to hinder biological productivity[1]. The inorganic phosphate (P_i) analogue phosphite (Phi) reduces populations of several insect species in the field[2], making it a potentially good fit for integrated pest management programs although this aspect has not been developed, or tested for PD, since its discovery[3]. There is evidence for host plant stress physiology (e.g. visual and/or olfactory cues related to host metabolites) associated with Glassy Winged Sharpshooter (GWSS) deterrence[4]. A few studies have determined that some anthocyanin and derivative tannic compounds can reduce insect feeding[5] , including sap-sucking insects[6, 7], which provides a plausible basis for observed PD infection susceptibility differences between anthocyanless and red cultivars[8-11]. However, similarity in GWSS PD transmission rates among cultivars harboring different bacterial populations in petioles[12], suggests that within-plant variability in pathogen distribution[13] or phase of the life cycle (biofilm versus motile) may be important for vector transmission and/or disease etiology. Quality improvements depend on applying new genetic insights and new technologies to accelerate breeding through improved genotyping and phenotyping methods, and by increasing the available diversity in germplasm[14-16]. The genetic identity of traditional cultivars used for wine discourages breeding approaches because markets and statutes dictate cultivar choice, thus varieties lack recombination and the resultant opportunity to select/screen for adaptability, e.g. PD resistance and P metabolism.

MicroRNAs (miRNAs) and small interfering RNAs (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[17, 18]. Evidence shows that miRNAs and siRNAs operate systemically by moving through vasculature, raising prospects of genetic engineering of grapevine rootstocks for PD resistance in non-genetically modified organism (GMO) scions[19-21]. Microbes and viruses utilize plant miRNAs to facilitate pathogenesis, and plants have co-opted miRNAs for plant innate immunity[22-27]. Although the molecular mechanisms of RNA interference in plant-microbe interactions are poorly understood, there is mounting evidence that plant immunity to microbial pathogens require post-transcriptional gene silencing (PTGS) pathways[28-35]. This suggests broader roles for plant and pathogen sRNAs in environmental responses and evolutionary adaptations[36, 37], which may include microbe and/or vector feeding processes.

The general research objective of this project is to continue to test a coalescent model that specific siRNAs, namely *Trans-Acting small-interfering locus4 (TAS4*) and miR828 produced by the host are key regulators of PD etiology subject to P modulation[38]. The long-term goal is to establish a new technology in grapes that will allow genetic manipulations that will not carry the negative connotation of "GMO." This is because the transgenes are removed by conventional backcrosses of the transgenics, resulting in only mutated endogenous effector genes, analogous to breeding approaches to introgress dwarfing or pathogen resistance genes. The molecular approaches applied here can have significant impacts on

viticulture by: (i) applying deep knowledge from model plant species to grapes; (ii) facilitating optimal selection of parents for breeding and immediate selection of elite progeny with multiple desirable traits, e.g. *MIR828/TAS4/MYB* haplotypes; (iii) circumventing biological and societal limits to genetic engineering (here, by CRISPR/Cas9 technology applied to create new endogenous effector non-GMO haplotypes); (iv) accessing abundant genetic variation[39] (grape varieties currently face severe pathogen pressures and long-term sustainability of industry relies on exploitation of natural genetic diversity); and (v) understanding PD etiology as it relates to P metabolism by the host and pathogen.

Understanding the molecular mechanisms of miR828/*TAS4* in biotic stress responses will provide cogent (e.g. miRNA-based) strategies for engineering stress-tolerance and productivity by increasing P uptake without increasing fertilizer application. We previously put forward a model and summarized the evidence for a role of deranged P_i, altered source-sink distributions of sucrose, and the stress hormone abscisic acid (ABA)[40] in regulating phytoalexin polyphenolic accumulations via miR828, *TAS4*, and their target MYB transcription factors (*viz*. MYBA6/7 and close homologues) important for PD. As an independent, partial test of the hypothesis, we initiated work on transgenic tobacco that overexpresses the Arabidopsis target of *TAS4* siRNA; *AtMYB90/PRODUCTION OF ANTHOCYANIN PIGMENT2/PAP2*. Transgenic plants have a dominant phenotype of purple leaves[41] and functional endogenous genes for Nta-miR828[42] and *NtTAS4ab*[43] hypothesized to interact with the over-expressed MYB effector[44]. Results reported at the 2016 Pierce's Disease Research Symposium and in the project renewal application provided compelling confirmation, as previously shown in Arabidopsis[38, 40], for functional conservation of an autoregulatory loop where target AtMYB90/PAP2 overexpression induces expression of the endogenous negative siRNA regulator Nt*TAS4*-3'D4(-) and its upstream trigger Nt-miR828. The inverse correlations observed between both *Nt-TAS4*-3'D4(-), Nt-miR828, and XF infection status in PAP2-overexpressing tobacco is strong evidence in support of our model. An unexpected result consistent with the *causative* XF model is that XF-infected transgenic genotypes show Nt*TAS4*-3'D4(-) and Nt-miR828 reductions correlate with disease symptom severity.

In addition to the phased, small interfering RNAs (phasiRNAs) generated from TAS4-3'D4(-) targeting of *VvMYBA6/A7*, we have shown an inverse correlation[45] of abundances of phasiRNAs significantly up-regulated by XF infection and significant down regulation of their cognate mRNA targets, namely disease resistance loci Pentatrico-Peptide Repeat (PPR) and Nucleotide-Binding Sequence/Leucine-rich Repeat Receptors (LRRs). Over 150 LRRs out of the 341 such genes annotated in grapevine[46] were differentially regulated by XF infection in our datasets and produced phasiRNAs in inverse proportion to their target mRNA abundances. Such clustering of gene ontology in our RNA-Seq and sRNA data ***very strongly support the working model*** that XF infection results in amplification of phasiRNAs for loci known to control pathogen resistance by silencing target genes. The diversity and conservation of phasiRNA loci across plant taxa[47-50] revealed by our results encompasses orthologues of *MYB*s triggered by miR828 in many species[51-59], including grape[60]; *TAS* effectors *SUPPRESSOR OF GENE SILENCING3 (SGS3)*, *DCL2*[55, 61] and *AGO2* targeted by miR403[62], and the huge families of *LRR* and *PPR*s targeted by miR482[49, 51, 55] and *TAS1-3*/miR390/3627/4376/7122[59, 63], respectively. The collective loss of miRNAs targeting PTGS effectors, PPRs, and LRRs in virus- and bacteria-infected tissues that results in susceptibility[49, 64] demonstrates their functions as master regulators of defense and targets of pathogen virulence effectors.

In addition to the compelling evidence thus far generated that supports the working model, we generated novel results that Phi impacts XF growth, which underscores the practical value of the project to develop a durable management tool while generating new knowledge about PD etiology and engineered resistance. In the first CDFA award #15-0214-SA (July 2015- Dec. 2017) we initiated production of CRISPR-edited grapevine genotypes targeting *VvMIR828, TAS4a, TAS4b, MYBA6,* and *MYBA7* and described independent evidences[65-72] directly supporting the P stress modulation model (Final Report, https://static.cdfa.ca.gov/PiercesDisease/reports/2018/rock_CDFA_final_report_15-0214SA_submit.pdf). We achieved our initial Objectives within the time frame of two years' funding, and report here our ongoing progress on characterization of the genome-editing effector transgenic grapevine materials for VvMYBA6, MYBA7, and TAS4b.

Objectives of Proposed Research and Path to Application:

I. Test the miR828, *TAS4*, and target *MYBA6/7* functions in PD etiology and XF infection and spreading by genome editing using CRISPR/Cas9 transgenic technology.

II. Characterize tissue-specific expression patterns of *TAS4*, *MIR828* primary transcripts, sRNAs, and *MYB* and other miRNA target genes in response to XF infections in the field and in edited genotypes.

III. Characterize the changes in control versus edited genotypes for (a) xylem sap [P_i], and (b) polyphenolic levels of XF-infected canes and leaves. If results are conclusive based on greenhouse studies, in the future we will conduct field trials and collaborate to carry out insect diet preference/behavioral modification/fitness assays on defended transgenic materials. (c) Test the P_i analogue Phi as a durable, affordable and environmentally sound protectant/safener for PD.

Description of activities conducted to accomplish Objectives

I. Test the miR828, *TAS4*, and target *MYBA6/7* functions in PD etiology and XF infection and spreading by genome editing using CRISPR/Cas9 transgenic technology.

Successful regeneration of plantlets from somatic embryos produced from rootstock 101-14 grape transformations for five CRISPR binary T-DNA vectors (plus empty vector control) in the lab of Cooperator DT was documented in the 15-0214-SA Final Report (https://static.cdfa.ca.gov/PiercesDisease/reports/2018/rock_CDFA_final_report_15-0214SA_submit.pdf). We have received said regenerant transgenic plantlets for six *MYBA6*, six *MYBA7*, two *TAS4b*, and two empty-vector (control) events from the Cooperator under duly issued APHIS-BRS permit # 17-342-101m, and transplanted them in the greenhouse. More regenerants including for the remaining *MIR828* and *TAS4a* effectors are outstanding and forthcoming from a third round of transformations initiated in late 2016.

Fig. 1 shows the results of molecular characterization for those events that have grown to sufficient size to harvest tissue samples. The genomic Southern blots (Fig. 1de), real-time PCR *cas9* expression (Fig. 1f), and RNA blot *cas9* expression (Fig. 1g) evidences support at least three independent events for MYBA6 (lanes d/e1-3), all five tested MYBA7 events, and the TAS4b tested event (panels f,g), with supporting evidence in most cases from PCR amplification of genomic DNAs for the selectable marker *nptII* and effector *cas9* (panels b,c respectively). Further characterization of these and other lines is ongoing including for immunoblot validation of Cas9 protein expression. Characterization of genome editing events of target genes going forward will be by PCR cloning and sequencing and polyacrylamide gel electrophoresis-based genotyping[73].

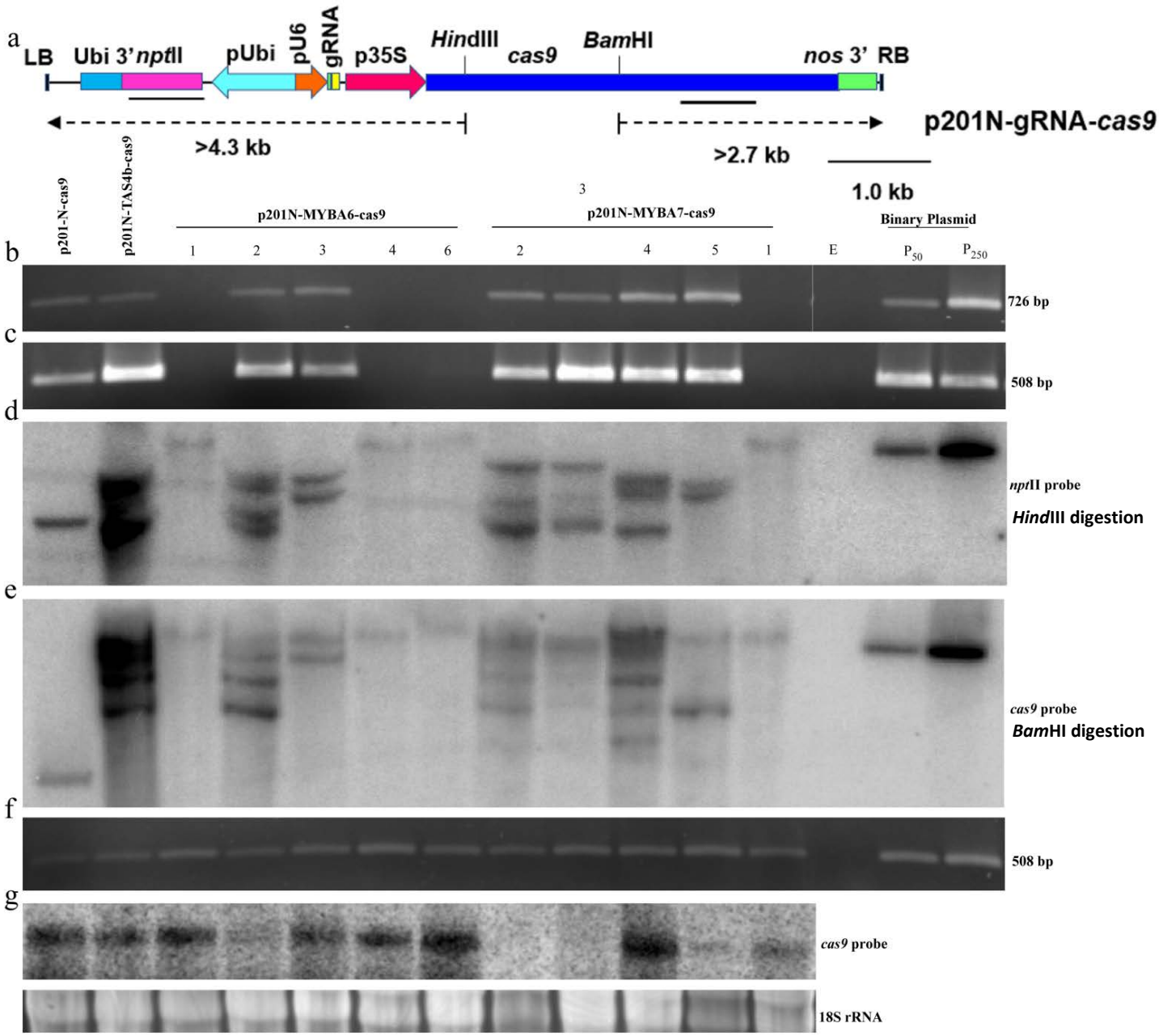

Fig. 1 Analysis of grape plants transformed with p201N-gRNA-cas9. **a)** The T-DNA of the binary vector p201N-gRNA-*cas9*. p201N-gRNA-*cas9* harbors gRNA in p201N-*cas9*. RB: T-DNA border-right, p35S: Cauliflower mosaic virus (CaMV) 35S promoter, *cas9*: CRISPR associated protein 9 human-codon optimized, *nos 3'*: polyadenylation signal of the nopaline synthase gene, pU6: Medicago truncatula U6.6 promoter, gRNA: guide RNA, pUbi: ubiquitin promoter, *npt*II: neomycin phosphotransferase gene, Ubi 3': Ubiquitin polyadenylation signal, LB: T-DNA border-left. Probes used (*npt*II and *cas9*) have been marked in bold lines. The junction fragment sizes >4.3 kb and >2.7 kb has been marked in a dashed arrow. **b)** and **c)** PCR analysis of putative transgenic grape plants **b)** *npt*II primers and **c)** *cas9* primers. DNA (200 ng) from a p201N-*cas9*-transformed, a p201N-TAS4b-*cas9*-transformed, five p201N-MYBA6-*cas9*-transformed and five p201N-MYBA7-*cas9*-transformed putative transgenic plants was used as the PCR template. p201N-cas9 binary plasmid DNA (50 pg and 250 pg) was used as the positive control (P_{50} and P_{250}) E empty lane. **d)** and **e)** Southern blot analysis of grape plants transformed with p201N-gRNA-*cas9* using *npt*II probe and *cas9* probe, respectively. Total DNA from 12 transformed plants (one p201N-*cas9*, one p201N-TAS4b-*cas9*, five p201N-MYBA6-*cas9* and five p201N-MYBA7-*cas9*) that regenerated and rooted under kanamycin selection **d)** DNA (10 µg) was digested with *Hin*dIII and probed with *npt*II coding sequence. Junction fragments of >4.3 kb from transgenic plants are expected to hybridize. The binary plasmid p201N-MYBA6-*cas9* digested with *Hin*dIII (P_{50} and P_{250}) was used as a positive control **e)** DNA (10 µg) was digested with *Bam*HI and probed with *cas9* coding sequence. Junction fragments of >2.7 kb from transgenic plants are expected to hybridize. The binary plasmid p201N-MYBA6-*cas9* digested with *Bam*HI (P_{50} and P_{250}) was used as a positive control. **f)** and **g)** Expression analysis of p201N-gRNA-*cas9* transgenic plants. **f)** RT-PCR analysi of p201N-gRNA-*cas9* transgenic plants with the *cas9*-specific primers. Total RNA extracted from leaves of transgenic plants was used as RT-PCR template. **g)** Northern blot analysis of p201N-gRNA-*cas9* transgenic plants with the *cas9* probe. Total RNA (10 µg) from trasngenic plants were electrophoresed in a 1.2% agarose gel with 1% formaldehyde and probed with *cas9* coding sequence. The 18S rRNA portion of the ethidium-bromide-stained gel is placed at the bottom to show the equal loading of RNA in all the lanes.

Summary of Objective I accomplishments and results

Successful grapevine regeneration of multiple CRISPR-Cas9 vector construct events designed to target *TAS4b, MYBA6,* and *MYBA7,* and appropriate vector-only controls.

II. Characterize tissue-specific expression patterns of *TAS4, MIR828* primary transcripts, sRNAs, and *MYB* and other miRNA target genes in response to XF infections in the field and in edited genotypes.

Summary of Objective II accomplishments

We received three HighSeq500 (~400 million reads per run) datasets in early 2018 for samples submitted in late 2017 to the Institute of Integrative Genome Biology, UC Riverside comprised of Illumina libraries with biological replicates for small RNAs, stranded mRNAs, and degradome samples from the 2017 'Calle Contento' Temecula field leaf samples, the 2016 replicated greenhouse XF tobacco MYB90 overexpression experiment, and for 'matchstick petiole' samples from the 2017 Temecula field expedition. The latter experiment has scope for discovery of differential miRNA expressions associated with a diagnostic, yet pleiotropic and enigmatic PD symptom (abscission of the leaf blade but not at the typical petiole/cane junction) hypothesized to be due to deranged small RNA activities. There are eight small RNA libraries pooled and indexed with 18 degradome samples, and 12 stranded mRNA-Seq transcriptome libraries sequenced separately. **Table I** lists the cumulative grapevine sRNA and degradome library quality control parameters through data pre-processing to remove ribosomal RNAs, t-RNAs, and snoRNAs[74] and genome[75] annotation stages of samples characterized to date. The statistical power from multiple replicates across years will drive defensible claims at the publication stage, which will be completed this year contingent upon sufficient statistical power manifesting from the multiple biological replicates for three years, 2015- 2017.

Table I. Quality control parameters* of sequenced sRNA libraries from 2015-2017 Temecula PD-infected samples.

sRNA libraries. Sample/Year	**raw reads (million)**	**%rRNA, tRNA**	**%snoRNA**	**trimmed, clean reads (million)**	**%MIRNAs$**
Leaf, PD2015	7.83	66.29	5.76	3.22	13.30
Leaf, Con2015	2.91	65.27	6.18	1.22	34.49
Leaf, PD2016.1	16.13	81.76	4.41	4.67	29.62
Leaf, PD2016.2	54.08	82.46	4.21	15.56	39.44
Leaf, Con2016.1	5.16	48.08	6.05	2.39	47.89
Leaf, Con2016.2	8.70	46.20	5.64	4.39	35.11
Leaf,PD2017.1	4.54	85.29	2.59	0.53	18.49
Leaf,PD2017.2	10.97	69.26	3.63	3.05	22.18
Leaf, Con2017.1	37.55	65.59	5.64	15.45	14.80
Leaf, Con2017.2	16.38	56.41	3.65	7.83	21.03
Petiole,PD2017	15.51	45.52	4.60	8.34	17.25
Petiole,Con2017	10.31	81.72	5.81	3.45	6.15
Degradome libraries. Sample/Year					
Leaf, PD2016	23.69	72.70	0.08	9.33	trace
Leaf, Con2016	27.49	46.85	0.03	16.04	trace
Leaf, PD2017.1	19.82	0.75	0.05	19.49	trace
Leaf, PD2017.2	21.82	54.09	1.12	11.27	trace

Leaf, Con2017.1	36.40	0.13	0.05	36.00	trace
Leaf, Con2017.2	24.16	5.80	0.38	22.48	trace
Petiole, PD2017	23.11	1.12	0.08	22.63	trace
Petiole, Con2017	25.58	1.21	0.04	25.03	trace
RNA-seq transcriptome libraries. Sample/Year†					
Leaf, PD2016.1	38.67	1.10	0.33	38.24	trace
Leaf, PD2016.2	33.96	0.69	0.15	33.73	trace
Leaf, Con2016.1	39.52	0.79	0.06	39.21	trace
Leaf, Con2016.2	26.07	11.28	0.08	23.13	trace
Leaf, PD2017.1	17.68	3.14	0.01	17.46	trace
Leaf, PD2017.2	45.12	7.39	0.04	37.63	trace
Leaf, Con2017.1	29.42	1.23	0.01	28.49	trace
Leaf, Con2017.2	24.91	16.56	0.07	23.06	trace
Petiole, PD2017	36.30	0.07	0.03	36.26	trace
Petiole, Con2017	37.30	0.58	0.02	37.07	0.01

*datasets mapped to *Vitis vinifera* 12X genome sequence, version NCBI RefSeq GCF_000003745.3 [75] with bowtie [85] after trimming adapter with fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).
$ mapped to miRBase22 plant MIRNA hairpins (http://www.mirbase.org/)
† mapped to ref transcriptome with kallisto-sleuth [82].

A fourth set of PD-infected and candidate control samples (> four biological replicates) was collected from the 'Calle Contento' vineyard in Temecula CA on July 23-25, 2018. These samples can be characterized by Illumina sequencing and included at the publication stage, if warranted based on results in process for three consecutive sample years.

In the prior 2017 July Progress Report we documented the down-regulation of miR398, miR399, miR828, and *TAS4ab* expressions from two independent XF challenge experiments with transgenic tobacco over-expressing AtPAP2/MYB90 (target of TAS4 siRNA) and evidence for the importance of the miR828/TAS4/MYB autoregulatory module[40] in response to XF. We also reported preliminary evidence from grapevine PD 2015 libraries for concordant down regulation of miR156 *SBP* targets, miR162 target *DICER, SUPPRESSOR OF GENE SILENCING3*, miR168 target *ARGONAUTE*, miR399 target *VvPHTs*, and *PdR1* candidate Leucine-Rich Repeat receptor (2017 March Progress report) in strong support of the model. The tobacco-as-surrogate model system component of the study is mostly completed except degradome validation of miRNA activities on target mRNAs, which will be completed in parallel with ongoing grapevine miRNA/mRNA/degradome library analysis on multiple years of field samples. Here we report current analysis of differentially expressed *MIRNA*s and phasiRNA-producing loci from three years of PD and control sample Merlot field materials to date. **Figure 2** shows principal component analysis (PCA) of the differences in PD field samples versus controls across three years for 200,000 phasiRNA loci and 219 *MIRNA* loci called de novo by ShortStack and validated for all annotations in miRBase22[76]. The good clustering of PD versus controls for dimensions of treatment and replicates across years that encompass >60% of all variation demonstrates a robust experimental design for statistical inference.

Previous studies in soybean, tobacco, and Arabidopsis documented an association with Leucine-Repeat Receptor phasiRNA production by miR482/2118/*TAS5* and miR6019/6020 modules that correlate with virus susceptibility[49, 55, 64, 77, 78], but the broader functional significance of phasiRNA production in general, and in biotic stress and PD in particular, is unknown. We have obtained evidence that phasiRNA production from novel PHAS protein-coding genes and ncRNA loci is strongly correlated with PD infections: the percentage of PHAS loci (ShortStack Dicer phase scores > 30) for well-expressed grape mRNAs and ncRNAs is ~6.3% (n=1,200 out of 99k moderately expressed clusters), but for multiple-test

corrected differentially expressed loci in PD symptomatic leaves the percentage is ~10% ($p < 0.0008$; data not shown). The significance of this observation will become clearer as we mine those PHAS loci for their biology and by discovering their miRNA effectors by running PhaseTank[79] on the degradome libraries. This observation establishes a key role of phasiRNAs in PD host response and supports our working model of PD etiology mediated by sRNAs.

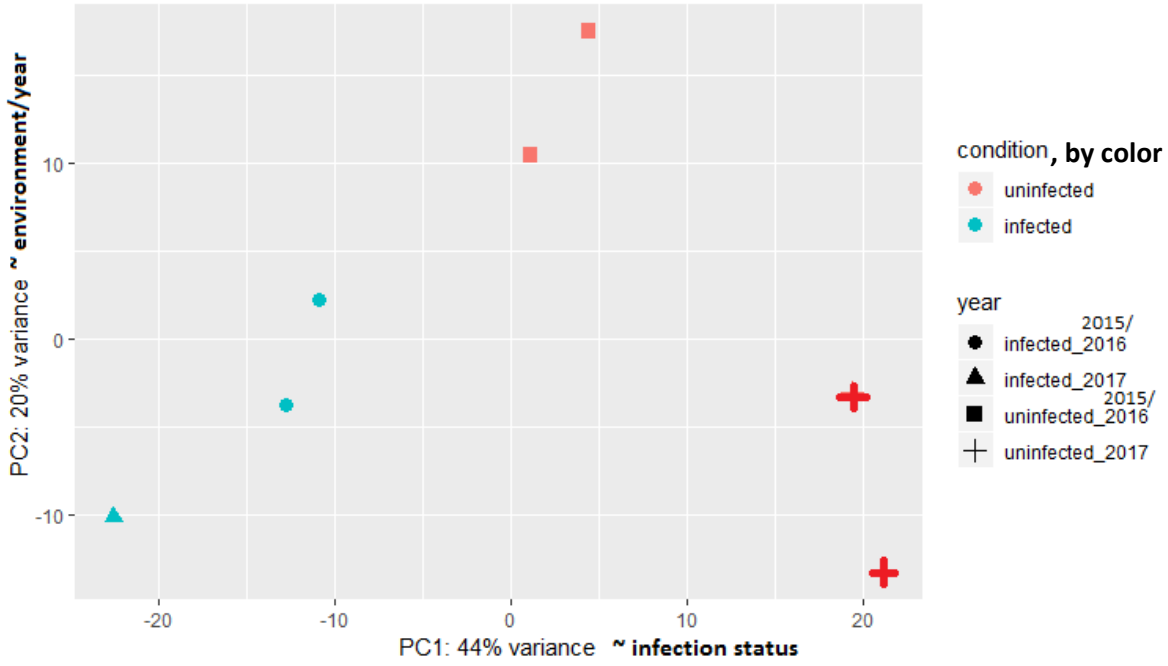

Fig. 2. Principal component (PC) analysis of seven grape leaf libraries from Temecula CA field samples representing sRNA-generating loci subjected to differential expression analysis. The percentage of variation is depicted in the PC1 and PC2 axes. Based on clustering of samples, PC1 represents the major dimension of Pierce's disease symptoms that was the basis for sample collection and PC2 is inferred to capture the environmental variation across years.

Table II lists in descending order of statistical significance (false-discovery rate < 0.05) the top *MIRNA*s and select phasiRNA-producing loci differentially expressed in field samples manifesting PD symptoms. Their known biological functions and previous reports[65, 80, 81] of up regulation in XF-infected grapevine corroborates our preliminary results presented in previous progress reports and strongly establish the validity of our working model. The top DE miRNAs in our analysis are miR397 and miR408, which independently target laccases important for lignin biosynthesis, a novel finding that provides insight into the molecular mechanism underlying the enigmatic textbook symptom of 'green-island bark' on XF-infected canes. Also relevant is the finding that miR858, which has been shown in Rosids and cotton to target other homologous MYBs than those MYBs targeted by miR828 involved in lignin and secondary metabolite biosynthesis[53, 54, 57], is also differentially expressed in response to XF. This compelling result is consistent with observed down regulation of miR408 with concordant increase in target *PLANTACYANIN*, and deranged expression of miR399 and miR827 that independently target phosphate transporters and phosphate homeostasis F-box effectors in Arabidopsis[67] and citrus infected with bacterial pathogens[25, 71, 72] including XF[66]. An interesting observation that warrants further study is that *TAS4c* is up-regulated by XF infection. We have observed in degradome analyses of ultraviolet light-mediated induction of miR828 and *TAS4* activities that TAS4c 3'-D4(-), which has a divergent nucleotide sequence from TAS4ab D4(-) species, shows slicing activity against *TAS4ab* primary transcript (data not shown). Thus the up-regulation of *TAS4c* in response to XF may be evidence of a homeostatic feedback

loop by *TAS4c* to negatively control *TAS4ab* activities that antagonize anthocyanin effectors *MYBA5/6/7* (Sunitha *et al*., submitted). This would fit with the model that XF pathogenicity towards its host is via sRNAs targeting anthocyanin and lignin metabolism.

Table II. Differential expression of phasi sRNAs from select protein-coding genes and *MIRNA*s clusters in PD symptom field leaf samples, Temecula CA 2015- 2017. Up regulated loci in **bold**.

Locus Annotation	**baseMean expression**	**log_2Fold Change**	**P value**	**PhaseScore 21nt register**
Phe-Ammonia Lyase VIT_06s0004g02620	323	**6.11**	0§	NA†
Raffinose synthase VIT_11s0016g05770 [see 80]	861	**6.10**	0§	NA
Anthocyanidin synthase VIT_02s0025g04720	287	**6.89**	4.4E-11	NA
Chalcone synthase3 VIT_05s0136g00260	230	**5.43**	3.5E-08	NA
Xylella fastidiosa genome sRNAs	2731	**3.70**	1.7E-04	NA
TAS4b, targets MYBA5/6/7 → triggers phasiRNAs	5815	-2.96	6.7E-03	16018.6
vvi-miR397a-3p, targets laccases	29	-3.31	7.1E-03	828.7
vvi-miR408-3p, targets laccases	117	-2.32	7.2E-03	817.9
vvi-miR391-5p, targets *TAS3*, pentatricopeptide rpt	86	-2.65	8.1E-03	599.7
vvi-miR858, targets MYBs associated with lignin	9	-3.45	1.0E-02	14.6
MYB VIT_14s0066g01220, target of miR828 → triggers phasiRNAs	39	-3.08	1.1E-02	4416.8
vvi-miR399i, targets phosphate transporters	104	-3.13	1.3E-02	307.3
vvi-miR394c-5p, targets F-box	19	-3.36	1.4E-02	189.6
MYBA6 VIT_14s0006g01290, target of *TAS4* 3'-D4(-) tasiRNA → triggers phasiRNAs	6	-3.49	1.9E-02	69.0
vvi-miR827-3p, targets phosphate signaling F-box	807	-1.68	6.3E-02	547.6
TAS4c, targets MYBA5/6/7 → triggers phasiRNAs	230	**0.19**	n.d.§	4060.5
vvi-miR828-star (mature below detection limit)	1	-1.44	0.38	16.6
TAS4a, targets MYBA5/6/7 → triggers phasiRNAs	23428	-0.51	0.59	29108.5

† These clusters of sRNAs were not called by ShortStack as having a dominant DICER activity size class
§ not determined by DESeq2 due to automatic independent filtering of assumed outliers defined by Cook's distance [82]

RNAseq data was mapped to the reference transcriptome with kallisto-sleuth[82]. We obtained 1,329 differentially expressed genes (793 up, 536 down; data not shown) with expression above a threshold (>30 reads mapped to a transcript per library on average), a log2-fold-change (LFC) of > |2|, and multiple-testing Bonferroni-adjusted $p < 0.05$ for statistical significance. This is comparable to the 1,240 (977 up, 263 down) DE genes reported for a similar greenhouse XF challenge experiment by the Dandekar group[80]. For the 13 genes claimed differentially regulated by XF infection and quantification validated by RT-PCR (in Figs. 3 and 6B of Dandekar's *FPS* paper)[80], we observe a good concordance with our results (correlation coefficient=0.75, $p < 0.05$), however only eight of our results are statistically significant, suggesting differences exist between the published greenhouse results and our field and/or leaf samples, read depths of libraries (see Table I), and/or methods (**Table III**). When the Dandekar group Supplemental Datasets are made available by the publisher in due course (the paper is only just published while the supplemental materials are not yet available), we will be able to conduct a genome-wide correlation of our results with the published claims using our independent methods[82] to ascertain whether those methods and results are comparable to ours or otherwise, which will shed light on questions about sample/experimental variability. **Table IV** compares our MapMan[45] RNAseq Gene Ontology

classification results of 1,240 top XF DE genes with the top 1,240 DE genes reported by the Dandekar group for greenhouse XF challenge RNAseq[80].

Table III. Comparison between recently reported DE of 14 genes in XF-challenged greenhouse leaves versus our RNAseq DE calculations on four Temecula CA field sample biological replicates harvested in 2016/17. Significant LFC values in **bold.** Note the sole discordant result for Fold Change sign (line in *italics*) is for a very low-expressed gene and is therefore discounted.

Annotation	**GeneID**	**Dandekar LFC**	**our LFC***	**Mean expressed**	**our padj**
PR-2/beta1,3-glucanase	VIT_06s0061g00100	6.64	6.43	4659	**2.75E-21**
PR-1	VIT_03s0088g00810	5.32	2.03	635	1.27E-01
PR-8; chitinase	VIT_05s0094g00200	1.58	2.30	21	**4.68E-02**
HSP18	VIT_08s0058g00210	4.32	4.21	6	**2.60E-04**
HSP17	VIT_04s0008g01520	3.58	4.97	50	**5.51E-14**
HSP4	VIT_07s0031g00670	2.32	1.90	303	**9.23E-04**
Nucleoredoxin-1	VIT_01s0127g00520	2.81	1.58	91	**3.23E-02**
Peroxidase	*VIT_00s1677g00010*	*1.14*	*-0.10*	*4*	*9.58E-01*
ferritin5	VIT_13s0067g01840	-1.00	-0.58	1066	4.17E-01
Sucrose synthase	VIT_07s0005g00750	3.46	1.62	7345	**4.40E-02**
Pectin lyase	VIT_14s0066g01060	1.72	0.23	24	8.25E-01
UDP-glycosyltransferase	VIT_17s0000g04750	1.07	0.37	857	6.13E-01
Xyloglucan-endotransglucosylase	VIT_06s0061g00550	2.32	4.71	174	**4.31E-04**
thaumatin-like protein	VIT_18s0001g14480	2.00	0.76	589	6.58E-01
	Pearson of LFCs, R=	0.75		**Binomial p-val†**	**0.05**

* kallisto-sleuth method [82].
† bionomial distribution probability of 13 successful LFC values being the right sign in 13 tests when DE up regulated =79% probability (true for 12 of 13 genes; a conservative estimate) based on results reported in [80].

Table IV. Comparison of Gene Ontology over-represented terms metrics of project field sample PD RNAseq versus published greenhouse XF challenge RNAseq results [80]

MapMan Gene Ontology term	**MapMan fold over-represented field RNAseq**	**Field expt MapMan pval**	**Greenhouse RNAseq PANTHER Gene Ontology term**	**Greenhouse PANTHER fold enrichment**	**Greenhouse expt PATHER pval**
phenylpropanoid metabolism	576	0.00001	phenylpropanoid metabolic process	5.85	0.0012
flavonoid metabolism	2610	0.049	flavonoid biosynthetic process	5.23	0.0016
TCA/organic acid transformation	6.3	0.002	carboxylic acid transport	4.95	0.016
cell wall	124	0.15	cell wall organization or biogenesis	2.68	0.0035
glycolysis	5.4	0.002	carbohydrate catabolic process	3.56	0.034

UDP glucosyl and glucoronyl transferase	28	0.014	UDP-glucosyltransferase activity	4.73	0.037
transport -p and v-ATPase H+ exporting ATPase	20	0.009	transmembrane transporter activity	2.16	0.008

III. Characterize the changes in control versus edited genotypes for (a) xylem sap [Pi], and (b) polyphenolic levels of XF-infected canes and leaves. If results are conclusive based on greenhouse studies, in the future we will conduct field trials and collaborate to carry out insect diet preference/behavioral modification/fitness assays on defended transgenic materials. (c) Test the Pi analogue Phi as a durable, affordable and environmentally sound protectant/safener for PD.

Summary of Objective III accomplishments and results

Objectives IIIa,b. We previously reported in the July 2017 Interim Progress Report results for mass spectrometric quantification of cyanin and malvin in xylem sap from the Temecula June 2017 field samples, and anthocyanins in leaves, showing significant differences between infected and control samples for the latter. These results are further substantiated by prior results for other grape cultivars[83, 84], supporting working model. Spectrophotometric quantification of the 2018 field samples from Temecula are in process.

We also showed conclusively P_i quantifications by two methods of fully expanded leaves and canes in 2016 and 2017 Temecula PD samples that support the hypothesis that XF infection results in significantly lower [P_i] (about 60% decrease) in host leaves and xylem sap that correlate with elevated anthocyanins quantified in PD xylem sap by mass spectrometry and leaves by spectrophotometry. Thus we have accomplished Obj. IIIa and will publish the results in due course.

We previously showed in the Final Report for 15-0214-SA higher anthocyanin concentrations in infected xylem sap, and in the 2018 Renewal Progress results for XF titers in concordant petioles samples from those same prior leaf samples by real time-PCR, as well as for the 2016 replicated greenhouse XF tobacco MYB90 overexpression experiment correlated with digital abundances of XF transcriptome reads quantified by bowtie[85]. These results together directly support the hypothesis that XF infection results in accumulation of anthocyanins in xylem sap and leaves. Thus we have accomplished Obj. IIIb and will publish the results in due course. Similar results have been reported for procyanidins and other polyphenolics in xylem sap two months post-XF infection in Thompson seedless and several winegrape cultivars[83, 84]. Phenolic levels in Merlot xylem sap correlate with PD severity compared to other cultivars[86].

Objective IIIc. Supporting our previous results (shown in 2017 March Progress Report) that XF infection induces miR828 and *TAS4* expression in tobacco, **Fig. 3** is a blot probed with the transgene of RNA extracted from the 2106 repeat greenhouse XF challenge experiment that further establishes the importance of the autoregulatory feedback loop in XF host response based on PAP2/AtMYB90 induction upon XF infection, because even in the absence of the transgene (the SR1 non-transgenic control line) the endogenous PAP2/MYB90 orthologue *ANTHOCYANIN2* is inferred to hybridize with the probe due to high homology with *PAP2/MYB90*. The result clearly shows *AN2* to be up-regulated several fold in the SR1-treated sample in response to XF infection.

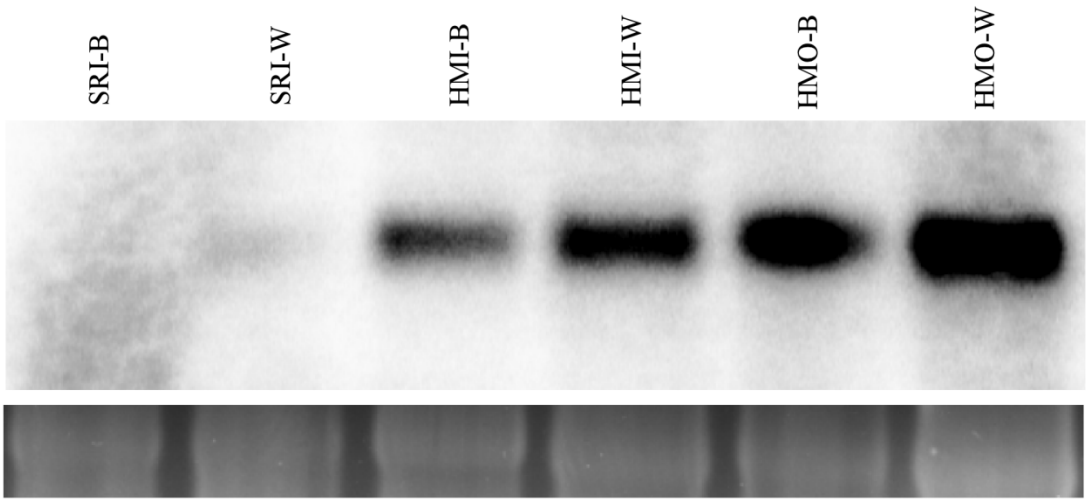

Fig. 3 Northern blot analysis of *Arabidopsis thaliana AtMYB90* tobacco transgenic plants. Total RNA (10 µg) from trasngenic plants were electrophoresed in a 1.2% agarose gel with 1% formaldehyde and probed with *Myb90* 3' fragment coding sequence. SR1: Untransfomed control plant; HMI: Hemizygous; HMO: Homozygous; B: Buffer; W: *Xylella* infected. The 18S rRNA portion of the ethidium-bromide-stained gel is placed at the bottom to show the equal loading of RNA in all the lanes.

Results presented in the 2017 Final Report provided additional validation of preliminary results showing that the LD_{50} < 3 mM [Phi] for inhibition of plate growth of XF. Based on these pilot experiments we conducted a greenhouse XF challenge experiment from April until July 2018 with phosphite treatments as test. We encountered technical problems with 1) plant growth in the absence of fertigation (we did not want phosphite effects to be confounded by excess nutrient conditions and thus withheld application of NPK fertigation), and 2) with the third time point (experiment endpoint) RT-PCR XF titre assay that requires us to repeat the experiment. Below is described the results for five tobacco plants of each genotype (SR1 non-transgenic, HMI heterozygous transgenic, and HMO homozygous transgenic overexpressing AtPAP2/MYB90[41, 44]) challenged with XF in the greenhouse. The results in **Table V** demonstrate the technical methods and experimental procedures give reproducible results in our hands, because we validate and extend the prior results documented in the Feb. 2016 Progress Report that the transgenic lines have lower XF titres that correlate with transgene copy number, yet higher leaf scorch symptom severity in the homozygous transgenic line (data not shown). We will conduct a larger phosphite test for XF antagonism going forward by bracketing the parameters of phosphite concentrations and interaction with amounts of fertigation supplement during post-inoculation growth and development.

Table V. Results of XF challenge of greenhouse-grown transgenic tobacco plants (n=5) overexpressing AtPAP2/MYB90 assayed at two and seven weeks post infection (WPI) for bacterial titre by RT-PCR.

Genotype	**2 WPI**	**7 WPI**	**p value† vs control, 2 WPI**
	cfu/gfw		
SR1 non transgenic	2.3E+07	3.0E+09	--
HMI heterozygous transgenic	6.4E+06	2.1E+07	0.07
HMO homozygous transgenic	5.6E+06	9.4E+06	0.05
† two sided Student's t-test, unequal variance assumed			

Publications produced and presentations made during the interim time period that relate to the funded project

Rock, C.D. "Career opportunities in agricultural genomics and genetics." Keynote address to Agronomy Society Annual Field Day, Brigham Young University-Idaho, Rexberg ID. July 6, 2018. Presented project progress in context of potential impacts on Idaho winegrape and potato production. (Potato is infected by both XF and a different bacterium [Candidatus Liberibacter solanacearum]; both bacteria are associated with "Zebra Chip" and "purple top" maladies).

Relevant to CDFA project, but funded by alternative source: Sunitha S, Loyola R, Alcalde JA, Arce-Johnson P, Matus JT, Rock CD. 2018. "The role of UV-B light on small RNA activity during grapevine berry development." Under review, *G3: Genes, Genomes, and Genetics*. Available at BioRiv https://www.biorxiv.org/content/early/2018/07/24/375998. Funding: FAPESP-SPRINT Brazilian-TTU Joint Program and TTU-VPR Open Access Publication Initiative.

Research relevance statement, indicating how this research will contribute towards finding solutions to Pierce's disease in California.

Our prior novel results demonstrating that Phi impacts XF growth underscores the practical value of the project to develop a durable management tool while generating new knowledge about PD etiology and engineered resistance. The Board has suggested knocking out genes involved in diffusible signals and host chemical specificity for PD etiology by CRISPR (pp. A1-3); this is precisely what this project is pursuing. Knocking out any host gene (e.g. PD resistance or P stress effector) may result in increased susceptibility to infections. Thus engineering PD resistance is likely to be by incremental advances from characterizing hypothesized and modeled molecular mechanisms.

Layperson summary of project accomplishments

The bacterium XF is the cause of PD in grapes and is a major threat to fruit, nut, olive, and coffee groves. The most damaging effect of PD other than death of the vine is the reduction of production, and shriveling of fruits. Obvious symptoms in grapevine are characteristic bands/rings of anthocyanin (red pigment) accumulation in distal zones adjacent to necrotic leaf blades. Anthocyanins can reduce insect feeding, and induction in vegetative tissues may serve as antagonists to feeding by GWSS and to colonization by XF. The etiology of pleiotropic PD symptoms such as 'matchstick petioles' and 'green cane islands' is not understood. Prior work showed that XF infection causes a significant decrease in leaf elemental phosphorus (P) content, but the bioavailable form of P (e.g. phosphoproteins, lipids, nucleic acids, subcellular compartmentation, etc.) underlying this phenomenon is unknown. The myriad host responses to XF are hypothesized to be due to deranged host inorganic phosphate (P_i) -regulated miRNA activities (both P_i and miRNAs are diffusible signals in plants). The data generated in two initial years of PD/GWSS support was compelling and a renewal application was funded for 2018. Result continue to strengthen support of our testable model of phosphate-regulated miRNAs synergizing with *MIR828*/*TAS4* to regulate anthocyanin levels. Deep sequencing of miRNAs and their targets in XF-infected leaves and petioles has been completed from three years of field collection and the datasets quality-assured. Further analysis of the sequence data and new samples collected in 2018 will allow a systematic and comprehensive view of gene activities and their roles in etiology of PD. A CRISPR/Cas9 genome-editing approach has generated transgenic plants to directly test the model of anthocyanin regulation to determine the effector genes' roles in susceptibility to XF, and whether they function to impact GWSS feeding preferences. We are also testing a corollary of the working hypothesis: whether a durable, affordable, and environmentally sound 'safener/protectant' analogue of P_i (phosphite; reduced P_i), which alters host and microbe phosphate homeostasis, can impact XF growth and host PD etiology. This aspect could result in development of a novel management tool for PD complementary to the primary high-priority genome editing approach to engineer PD resistance. Genome editing is akin to breeding in that it can produce non-"genetically modified organism" (GMO) grapevines and rootstocks after outcrossing the transgene locus.

These proof-in-principle experimental results offer a new paradigm for PD management with potential translational benefits for other crops.

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) and reported it to the National Institutes of Health. As described in the July 2016 CDFA Progress Report for 15-0214-SA, the PI disclosed to his institution (Docket D-1327, Sept. 18, 2016) the enabling sequences that form the basis of a proposed Continuation in Part for original claims 12-21, subject to rejoinder as indicated in Manual of Patent Examining Procedure § 821.01 through § 821.04. Original broad claims 1-11 were elected by TTU and subsequently rejected by USPTO Examiner under 35 USC section 112, citing SCOTUS *Myriad* case, to wit: "claimed invention does not rise to a level that is markedly different in structure from what exists in nature." A Response to Office Action was filed by the PI's Office of Technology Commercialization on Oct. 18, 2016 but was not sustained. A future Continuation-in-Part patent application for original claims 12-21 can be prosecuted drawing on Docket D-1327 full description of the methods and compositions of NEW structural variants that do NOT occur in nature. 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. For commercialization of transgenic dicot plants, including grapevine made by the Agrobacterium co-cultivation method, the patent (USPTO# 8273954) will need to be licensed from Monsanto/Bayer for industrial partners to have freedom to operate.

CRISPR/Cas9 foundational technology is being prosecuted for patent protection by inventors at UC Berkeley/University of Vienna (PCT/US2013/032589; priority date May 25, 2012) and MIT/Broad Institute (USPTO# 8697359, issued April 15, 2014). In May 2017, the patent office issued another key CRISPR patent to Vilnius University in Lithuania which was filed earlier than the UC-B application, so patent law could dictate that the Vilnius patent takes precedence. The U.S. Patent Trial and Appeal Board ruled in Feb. 2017 that patents granted to MIT were significantly different from patents UC-B/Vienna had applied for, and hence would stand. UC-B has challenged the Patent Board's ruling in the U.S. Court of Appeals for the Federal Circuit. In Oct. 2017 MIT/Broad filed a fresh set of counter arguments to the appeal, claiming UC-B lacks legal standing to appeal. Oral arguments took place on April 30, 2018. A ruling is expected later in 2018. UC-B researchers have been granted a patent for CRISPR applications to eukaryotic cells by the European Patent Office and the United Kingdom, and other USPTO applications for broad patent protection of UC-B IP will move ahead with the resolution to the infringement case. It is unclear whether non-exclusive "bundling" or shared entitlements licenses will be negotiated between these parties to facilitate freedom to operate. The ease of reproducibility in different organisms for CRISPR/Cas9 is the technology's most exciting hallmark and could suggest that, in patent terms, it is "obvious" that CRISPR would work in non-prokaryotic organisms including plants. The patent situation for CRISPR/Cas9 is uncertain and will likely remain so for several years. There are more than 1,880 families of CRISPR patent according to IPStudies, a consulting firm near Lausanne, Switzerland. More than 100 new CRISPR families- each a group of related intellectual-property claims- are published each month. Companies wishing to practice the CRISPR/Cas9 technology now can seek a non-exclusive license from The Broad Institute for the issued MIT patent, and/or from Caribou Biosciences (www.cariboubio.com) for the UC-Berkeley/University of Vienna IP. Non-exclusive licenses are available for other CRISPR-based patents.

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