Renewal Progress Report for CDFA Agreement Number 17-0514-000-SA: "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: Jan. 1, 2018- Feb. 15, 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 Nta-miR828[42] and *NtTAS4ab*[43] expression hypothesized to interact with the transgene[44]. Results reported at the 2016 Pierce's Disease Research Symposium and in the new project 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 Objectives within the time frame of two years' funding (plus six month no cost extension).

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 with 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 filed two weeks ago (https://static.cdfa.ca.gov/PiercesDisease/reports/2018/rock_CDFA_final_report_15-0214SA_submit.pdf). Below is copied correspondence regarding shipment, under duly issued APHIS-BRS permit # 17-342-101m, of the transformed/regenerated plantlets. Shipment has been held up for several days due to need to amend the prior fee-for-service contract. TTU returned to UC Davis the duly signed below-mentioned amendment agreement on Feb. 15, 2018. The cost of plantlet shipping will be borne by the PI from non-CDFA funds, because the timeline is after the end of the prior grant.

From: David M Tricoli [mailto:dmtricoli@ucdavis.edu]
Sent: Monday, February 05, 2018 9:40 AM
To: Rock, Chris
Subject: RE: 22A058 Grant Close Out

Chris,

I went to draft the invoices but discovered that the business agreement has lapsed, so I put in a request to amend the end date. Once that is in place I am hoping I can draft the invoices for the work done in 2015-2017.

David

From: Zahir A Mohammed [mailto:zamohammed@ucdavis.edu]
Sent: Monday, February 12, 2018 2:58 PM
To: Rock, Chris
Cc: Steve Garcia; Vanessa C Rutledge
Subject: Amendment 1_A22548

Good Afternoon,

Attached is amendment #1 for Agreement #A22548 in regards to the Plant Transformation Facility for your review and approval. Please sign and return to the amendment to me at your earliest convenience. If you have any questions please feel free to reach out to me. Thank you.

Very Respectfully,

Zahir Mohammed

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Summary of Objective I accomplishments and results

Successful grapevine transformation and regeneration of CRISPR-Cas9 vector constructs targeting *VvMIR828, TAS4a, TAS4b, MYBA6,* and *MYBA7* and appropriate vector-only control. Validation of editing events going forward will be by PCR cloning and sequencing of target genes, and polyacrylamide gel electrophoresis-based genotyping[73].

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 sent in Dec. 2017 to the Institute of Integrative Genome Biology, UC Riverside a complete set of Illumina libraries with biological replicates for small RNAs, stranded mRNAs, and degradome samples from the 2017 'Calle Contento' Temecula field leaf samples, and the 2016 replicated greenhouse XF tobacco MYB90 overexpression experiment. In addition, we prepared indexed libraries for 'matchstick petiole' samples from the 2017 Temecula field expedition for discovery of differential miRNA expressions associated with diagnostic, yet pleiotropic, PD traits hypothesized to be due to deranged small RNA activities. Two runs of HighSeq500 (400m reads per run) have been completed in Dec. 2017 and the transcriptome libraries dataset obtained. The small RNA libraries dataset awaits demultiplexing and release. This delay was due to the Holiday break when the campus was closed, and in the month of Jan. 2018 due to staff on maternity leave and sick leave. There are eight small RNA libraries pooled and indexed with 18 degradome samples, and 12 stranded mRNA-Seq transcriptome libraries sequenced separately, in hand. This level of complexity will result in ~12 million reads per small RNA library, and ~25 million reads per transcriptome library. All workflow processes and yields were verified as appropriate through the data analysis and genome annotation stages. The statistical power from multiple replicates across years will allow defensible claims at the publication stage, which is in preparation and will be completed when the completed sequencing data is made available in the next few weeks.

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. We also showed 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. These results are further substantiated by prior results for other grape cultivars[74, 75], supporting working model.

We also showed in the Final Report for 15-0214-SA higher anthocyanin concentrations in infected xylem sap. We have generated this month preliminary 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. **Table I** shows the results correlated with digital abundances of XF transcriptome reads from Objective II new results quantified by bowtie[76]. The results directly support the hypothesis that XF infection results in accumulation of anthocyanins in xylem sap and leaves. 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[74, 75]. Phenolic levels in Merlot xylem sap correlate with PD severity compared to other cultivars[77].

Objective IIIc. We showed results in the Final Report validating the LD_{50} < 3 mM [Phi] for inhibition of plate growth of XF. Ongoing work is testing similar concentrations of foliar applications of Phi as safener for tobacco plants challenged with XF in the greenhouse.

Table I. Quantification of XF titres by quantitative real-time PCR and RNA-seq in 2016 greenhouse replicated XF challenge experiment with AtMYB90-overexpressing transgenic tobacco, and Temecula CA 2017 field samples.

Sample	**Log_{10}, cfu/gfw qRT-PCR (± s.e.m.)**	**Leaf XF RNA-seq Reads/10^6 host reads**	***P*- value**
Control leaf petioles, 2017 Temecula	5.21 (0.17)	1.8 (n=2)	---
PD symptom leaf petioles, 2017 Temecula	6.82 (0.40)	10.2 (n=1)	0.006*****
SRI non transgenic-Buffer control	7.30	3.0	
Hemizygous transgenic-Buffer	7.30	0	
Homozygous transgenic-Buffer	7.32	0.8	
SRI non transgenic-XF infected	12.4	88	
Hemizygous transgenic-XF infected	12.0	129	
Homozygous transgenic-XF infected	12.1	299	0.03†

* significantly different from qRT-PCR control, Student's two-sided t-test, n=5, equal variance assumed.
† significantly different from RNA-Seq buffer controls, Student's one-sided t-test.

Publications produced and presentations made that relate to the funded project

Rock CD, Azad F, Sunitha S, Maia I, Nunes-Laitz AVN, Domingues D. "The auto-regulatory loop involving miR828, *TAS4*, and target MYB transcription factors: basic and applied studies." Sao Paulo Research Foundation (FAPESP)-TTU Conference: "SPRINT STEM Across Continents." TTU International Cultural Center, Lubbock TX. Sept. 21, 2017. Oral presentation.

Rock CD. (2017) Phenylpropanoid metabolism. In: eLS (Encyclopedia of Life Science). John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0001912.pub2

Rock CD, Azad F, Sunitha S, Traore SM, De La Fuente L, Tricoli D. "Genome editing of *TAS4*, *MIR828*, and targets *MYBA6/A7*: A critical test of *Xylella fastidiosa* infection and spreading mechanisms in Pierce's disease." 2017 CDFA Pierce's Disease Control Program Symposium Proceedings. Pp. 70-78. https://www.cdfa.ca.gov/pdcp/Documents/Proceedings/2017CDFAWinegrapePestResearchRpts.pdf

Rock CD, Azad F, Sunitha S, Traore SM, De La Fuente L, Tricoli D. "Genome editing of *TAS4*, *MIR828*, and targets *MYBA6/A7*: A critical test of *Xylella fastidiosa* infection and spreading mechanisms in Pierce's

disease." 2016 CDFA Pierce's Disease Control Program Symposium Proceedings. Dec. 12-14, 2016. Pp. 121-129. Courtyard San Diego Liberty Station Hotel, San Diego CA. Oral presentation. https://www.cdfa.ca.gov/pdcp/Documents/Proceedings/2016ResearchProgressRpts.pdf

Rock CD, De La Fuente L, Sunitha S, Traore SM. "Genome editing of *TAS4*, *MIR828*, and targets *MYBA6/A7*: A critical test of *Xylella fastidiosa* infection and spreading mechanisms in Pierce's disease." 2015 CDFA Pierce's Disease Control Program Symposium Proceedings. Pp. 164-170. https://www.cdfa.ca.gov/pdcp/Documents/Proceedings/2015ResearchProgressRpts.pdf

U.S. Patent application #61,641,045 (filed 05/1/12) converted to regular application #13874962 (filed 05/01/13) "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" by C.D. Rock and Q.-J. Luo. USPTO Application published 3/13/14 Publication No. US-2014-0075596-A1. Texas Tech University Office of Technology Commercialization Docket # TTU D-0862, D-0876, D-1327.

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 the PI 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 is compelling and supports 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, petioles, and cane bark has been completed. Further analysis of new sequence data forthcoming in the next weeks 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 by disrupting host *MIR828* and four downstream effector genes for anthocyanin regulation to determine their 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 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 are set to take place in early 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 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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