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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Introduction

We hypothesize that novel target MYB transcription factors (VvMYBA6/A7) in grape are effectors of anthocyanin accumulation and potentially glass winged sharpshooter (GWSS) feeding preference determinants important for PD etiology. The model postulates microRNA828 and evolutionarily-related *Trans-Acting Small-interfering locus4* (*TAS4*) activities silence target *VvMYBA6/A7* expression in response to XF infection, mediated through inorganic phosphate (P_i) and plant stress hormone abscisic acid (ABA) signaling crosstalk. Anthocyanin induction in vegetative tissues may serve as antagonists to feeding by GWSS and to colonization by XF. We are currently testing the XF infection/spread hypothesis directly by "knocking out" the key genes using a new genome editing technology- Clustered Regularly Interspaced Short Palindromic Repeats (CRIPSR/Cas9)[1, 2] that the CDFA-PD Board nominated as a feasible, high-priority approach to engineering PD resistance.

We have added a new, independent approach component going forward (pending renewal review by CDFA): testing a facile surrogate tobacco XF infection system developed by the Co-I (De La Fuente)[3] to quickly assess susceptibility to XF infection of a transgenic tobacco line[4] that over-expresses the Arabidopsis homolog of VvMYBA6/A7: *PRODUCTION OF ANTHOCYANIN PIGMENT2/MYB90*. This 'knock-in' experiment can provide information on the validity of the working hypothesis. We will present here our preliminary results of this promising lead that the PI conceived of in Fall 2015, after the initial progress report submission. This over-expression/'knock-in' experiment simulates one facet of the model; namely up-regulation of MYBs (formally by CRISPR knockout of negative regulators MIR828 and TAS4), and impacts both Objectives I and II, as described below.

OBJECTIVES (as funded)

I. Test the miR828, *TAS4*, and target *MYBA6/7* functions in PD etiology and XF infection and spreading by genome editing using CRISPR/Ca9 transgenic technology.
II. Characterize tissue-specific expression patterns of *TAS4* and *MIR828* primary transcripts, sRNAs, and *MYB* targets in response to XF infections in the field.

Description of activities conducted

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

Engineered binary T-DNA *Agrobacterium* vectors to genome edit the grapevine *VvMIR828, VvTAS4ab*, and target *VvMYBA6* /*VvMYBA7* loci were described in the last Progress Report and sent to the Collaborator David Tricoli's lab under APHIS BRS permit # 15-231-102m in Nov., 2015. Additional vectors were constructed to target the *Phytoene Desaturase* (*PDS*) gene of grapevine as a proof-in-principal test of genome editing efficiency by facile visual screening for photobleached sectors during plantlet regeneration. Dr. Tricoli transformed Thompson seedless variety with the *PDS* vectors, because that variety can regenerate more quickly (estimated 6 months; expected plantlet regeneration in May, 2016). Commercially relevant grapevine rootstock 101-14, which produces requisite marker anthocyanins (data not shown), was transformed with *MIR828, TAS4a/b*, and *MYBA6/A7* vectors (**Table I**) in parallel for two separate Agro-induction rounds at the bench (Nov., Dec. 2015). **Figure 1** shows the progress of regeneration from somatic embryos; it is still too early to see with confidence any photobleached sectors for *PDS* constructs, or any purple anthocyanin-pigmented sectors for

Table I. Synthetic guide sequences currently being transformed for CRISPR-Cas9 editing of *VvPDS* (control; GSVIVT01021843001), *VvMYBA6*, *MYBA7*, *MIR828*, and *TAS4a-b* genes. Off targets candidates computed at http://cbi.hzau.edu.cn/cgi-bin/CRISPR

Gene.test	Engineered guide sequence	Relative genome position	Off targets, seed (12)NGG?
VvPDS_1a	TTCACAGTATTCTCAAGTTCTGG	Codon 100, exon 2	**No**
VvPDS_25s	GATGGAGACTGGTATGAGACAGG	Codon 179, exon 4	**No**
VvPDS_41a	TCAAATCGGCTGAATTCCCCTGG	Codon 237, exon 5	**No**
VvMYBA6.1s	GGCCCTTCAGGAGTGCGGAAAGG	Exon1, codon3, sense	**No;** 2 mm in utr VIT_14s0006g01340
VvMYBA7.1s	GGCTCTTTAGGTCTGCGGAAAGG	Exon1, codon3, sense	**No**
VvMYB828.2s	GTTGTTGAGATGCTCATTTGAGG	miRstar, sense	in utr VIT_18s0001g00860
VvTAS4a.6a^*	TTGGTCATGGTGAAGGTCCAAGG	D4 phase, antisense	in utr VIT_08s0040g0280
VvTAS4b.4a^*	GTGGCCATGGTGAAGGTCCGAGG	D4 phase, antisense	**No**

^ also targets other *TAS4ab* locus
* restriction site *Hpy188*I and/or *Ava*II for mutant screening by Cleaved, Amplified Polymorphism (CAP)-PCR.

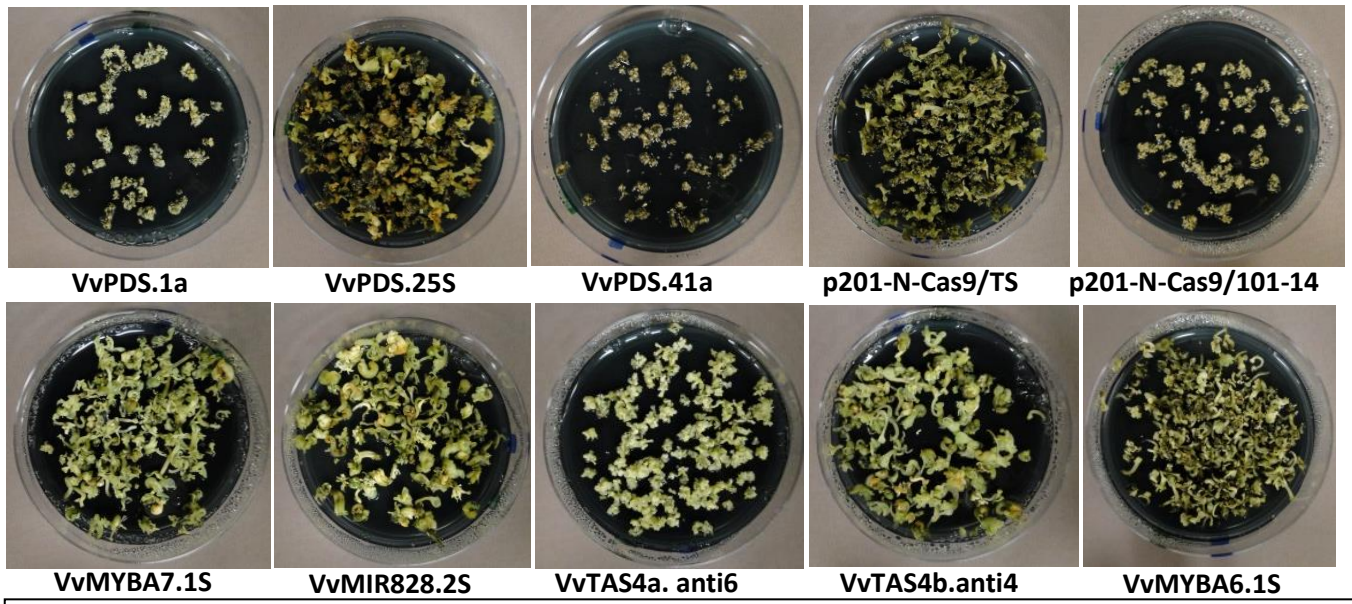

Fig. 1. Regeneration of grapevine transgenics as of 2/26/16.

candidate editing events of *TAS4* and *MIR828*. Validation of editing events going forward will be by PCR cloning and sequencing of target genes, and PAGE-based genotyping[5].

As an independent, partial test of the hypothesis, we have initiated work on transgenic tobacco (gift of N. Layland, USDA) that overexpresses the *Arabidopsis* target of *TAS4* siRNA, *AtMYB90/PRODUCTION OF ANTHOCYANIN PIGMENT2*. Transgenic plants have a dominant phenotype of purple leaves[6] and functional endogenous Nta-miR828[7] and *NtTAS4ab*[8] expression hypothesized to interact with the transgene[4]. **Fig. 2A** shows a preliminary small RNA blot result (Objective II, Method 1: Standard RNA blots and RACE) suggesting, as previously shown in *Arabidopsis*[9, 10], the existence of a conserved autoregulatory loop where target AtMYB90 overexpression induces expression of the endogenous negative siRNA regulator NtTAS4-3'D4(-). The inverse correlations observed between both *Nt*- and *Vv-TAS4*-3'D4(-) and XF infection status in greenhouse and field, respectively (**Fig. 2B**), is evidence consistent with our working model that XF infection deranges miR828/TAS4 expression. Furthermore, the band observed at ~84 nt is predicted to be a post-transcriptional processing intermediate comprised of undiced, double-stranded 3'-D1-D4 *TAS4* species (**Fig. 3**)[11]. Future work will establish this species by RNA-seq (Objective II, Method 2). An unexpected result consistent with the *causative* XF model (i.e. that XF infection triggers accumulation of this negative regulator of anthocyanin production) is that XF-infected homozygous AtMYB90 over-expressor genotype has 2.9-fold elevated NtTAS4-3'D4(-) abundance compared with buffer mock-infected control. Furthermore, this elevated TAS4 siRNA expression correlated with disease symptom severity (Fig. 2A). See Objective II results below for elaboration of this idea.

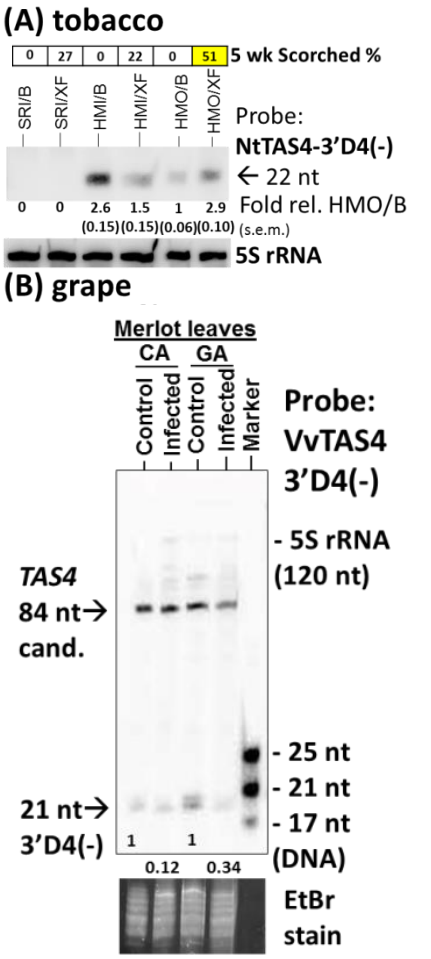

Fig. 2. Effect of XF infections on relative abundances of TAS4-3'D4(-), a negative regulator of *AtMYB90* and *VvMYBA6/A7* from leaves of (**A**) Control ("B"= buffer) non-transgenic SRI and hemizygous (HMI) and homozygous (HMO) AtMYB90-overexpressing tobacco, or (**B**) *V. vinifera* cv 'Merlot' leaves harvested from the field. Numbers below the 21/22 nt band are normalized intensities relative to healthy controls. HMO-infected tobacco plants showed more severe leaf scorching 5 weeks post infection than HMI or SRI (see Fig. 4).

II. Characterize tissue-specific expression patterns of *TAS4* and *MIR828* primary transcripts, sRNAs, and *MYB* targets in response to XF infections in the field.

In a previous progress report we described samples of PD-infected and symptomless Merlot leaves and petioles collected from the Calle Contento vineyard in Temecula CA, and the Black Stock vineyard in Dahlonega, Lumpkin Co., GA. Petiole DNA extracts were assessed by the Co-I's (De La Fuente) lab for XF titres, and correlated leaf materials have been extracted by the PI and used for construction of a first round of Illumina small RNA libraries.

We have adopted a spectrophotometric assay[12] for quantitation of anthocyanins and polyphenolics from PD-infected leaf samples to correlate with molecular phenotypes. **Table II** shows significant differences in accumulation of anthocyanins in XF-infected vs. control leaves from the field samples previously collected and currently used for small RNA library preparation for deep sequencing and statistical analyses of qualitative and quantitative miR828/TAS4 changes in response to XF infection (Objective II, Method 2: siRNA-seq). Presence of XF was confirmed by qPCR and calibrated to CFU/gfw. A trace amount of XF was detected in healthy control samples; this potentially confounding result must be validated by actual plating of petiole extracts to prove XF

colonies can be found in these samples producing qPCR signals at the limit of detection. This work is ongoing in the Co-I lab.

Furthermore, in transgenic tobacco lines a correlation was observed for anthocyanin accumulations for both hemizygous state (HMI) of the AtMYB90 transgene and elevated anthocyanins in response to XF infection, compared to homozygous state (HMO) and non-transgenic control (SRI)(Table II). Remarkably and unexpectedly, the homozygous AtMYB90 overexpressing lines showed lower anthocyanin accumulations compared to hemizygous lines (Table II), which correlated with disease severity of leaf scorch observed over time (**Fig. 4**) and where all genotypes had similarly high XF titres (**Fig. 5**). This compelling experiment is currently being repeated by the Co-I's lab and will be replicated in the PI's lab, as soon as his lab is certified for Biosafety Level II work with XF. An application for the same is under review by USDA APHIS (permit #P526-160120-034). Our initial smRNA libraries from select grapevine samples (Objective II, Method 2: small RNA-seq) are currently being analyzed by the Institute for Integrative Genome Biology at UC Riverside. We have purchased an Illumina trueseq stranded mRNASeq kit for mRNA-seq (Obj. II, Method 2) and library construction will begin this month.

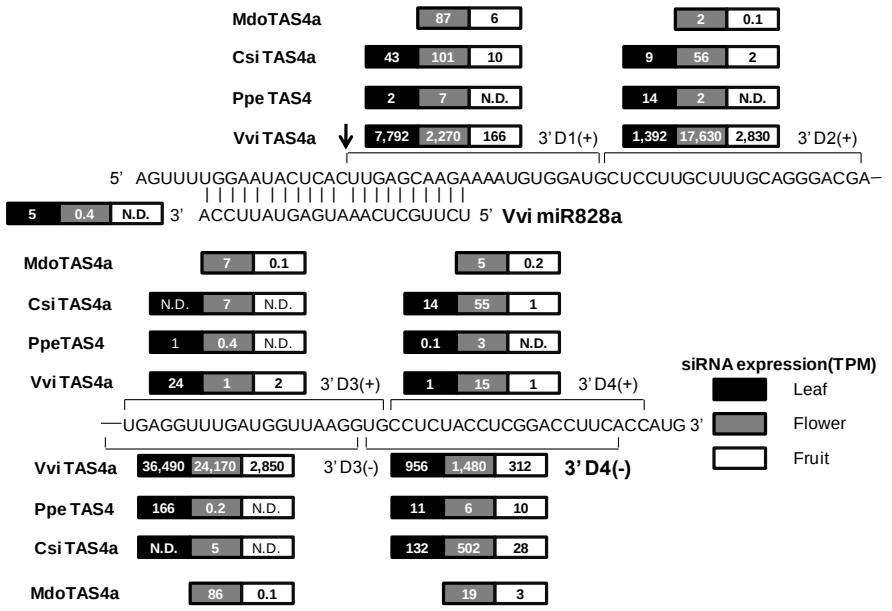

Fig. 3. *TAS4* siRNAs and miR828 are expressed strongly (transcripts per million [TPM]) in leaves and flowers but not fruits of apple, citrus, peach, and grape, providing evidence that *TAS4*-3'-D4(-) and miR828 targeting *MYB* mRNAs function as negative effectors of polyphenolic biosynthesis. Data from NCBI GSE28755 (citrus, grape), GSE36065, and GSE38535. Vv-miR828a expression confirms observations by Pantaleo et al. [11].

Table II. Correlation of anthocyanins with XF titres in grapevine Merlot leaves and petioles, respectively, from Temecula CA & Black-stock GA., and from transgenic tobacco genotypes challenged in the greenhouse with or without XF. **n.d.:** not detected by qPCR. n.a.: not analyzed. * n = 3.

Sample	XF titre (CFU/gfw)	µmoles cyanidin-O-Glu/mgfw equivalents (± s.e.m.)	P value
XF-infected vine, CA	1.4 x 10^8	**74.4** (±12.6; n = 7)	<0.02
healthy vines, CA	1.4 x 10^3	**13.7** (± 4.6; n = 3)	
XF-infected vine, GA	n.a.	**27.2** (±1.6; n = 5)	$<10^{-5}$
healthy vines, GA	n.a.	**7.4** (± 1.2; n = 5)	
tobacco SR1, XF-infect	See Fig.5	**0.7** (± 0.2)*	
tobacco SR1, buffer	**n.d.**	**0.7** (± 0.4)*	
MYB90-OX HMI, XF-inf	See Fig.5	**7.8** (± 0.4)*	0.004
MYB90-OX HMI, buffer	**n.d.**	**2.0** (± 0.9)*	
MYB90-OX HMO, XF-inf	See Fig.5	**3.4** (± 0.5)*	
MYB90-OX HMO, buffer	**n.d.**	**2.7** (± 0.1)*	

Publications pending/in preparation

Sukumaran S, Tricoli D, Rock CD. Efficacy of CRISPR/Cas9 in grapevine based on select guide sequences targeting *Phytoene Desaturase*. *Computational and Structural Biotechnology Journal*, in prep. Solicited research article by Editor Gianni Panagiotou. Deadline for manuscript submission: July, 2016

Sukumaran S, Traore SM, Azad F, De La Fuente L, Rock CD. small RNA profiles in grapevine variety Merlot infected with *Xylella fastidiosa*. In prep. Solicited research article for special edition of *Frontiers in Plant Science*: 'Omics and systems approaches in grapevine fruit composition to understand responses to environmental

factors and agronomical practices.` Eds: José Tomás Matus, Simone Diego Castellarin, and Giovanni Battista Tornielli. Deadline for manuscript submission: August, 2016.

Invited seminar by C Rock: Dept of Genetics, Botucatu Institute of Biosciences, Sao Paulo State University-Botucatu, Brazil. Nov. 4, 2015. "Plant Polyphenolics, small RNAs, and Darwin's 'Abominable Mystery.'"

Rock CD, Luo Q-j. U.S. Patent application #61,641,045 (filed 05/1/12) converted to regular application #13874962 (filed 05/01/13) "siRNAs Compositions and Method for Manipulating Berry Ripening." USPTO Application published 3/13/14 Publication No. US-2014-0075596-A1.

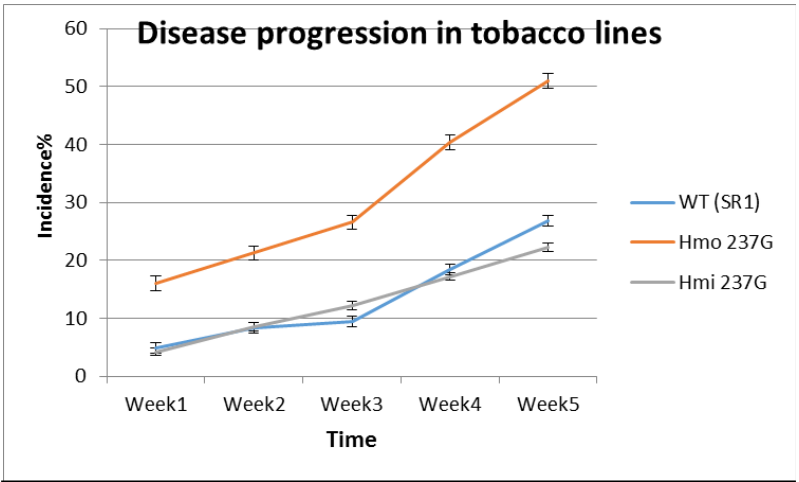

Fig 4. Comparison of the disease progression in transgenic tobacco lines challenged with XF in the greenhouse suggests homozygous AtMYB90-overexpressing plants (Hmo) are more susceptible than hemizygous (Hmi) and the wild type SR1 lines, consistent with a causative model for XF infection triggering silencing of MYB expression and decreased anthocyanin accumulation (see Table II).

Research relevance statement

The general research objective (within the scope of Year1 seed funding) is to test the hypothesis that specific *trans*-acting small interfering RNAs (*ta*-siRNAs) produced by grape are regulators of the Pierce's Disease process. 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 vector transgenes can be removed by conventional backcrosses to the transgenic lines, resulting in only mutated endogenous effector genes in progeny. These proof-in-principle experiments could result in a new paradigm for host-vector-pathogen interactions in PD for the advancement of the grapevine biotechnology and breeding sectors.

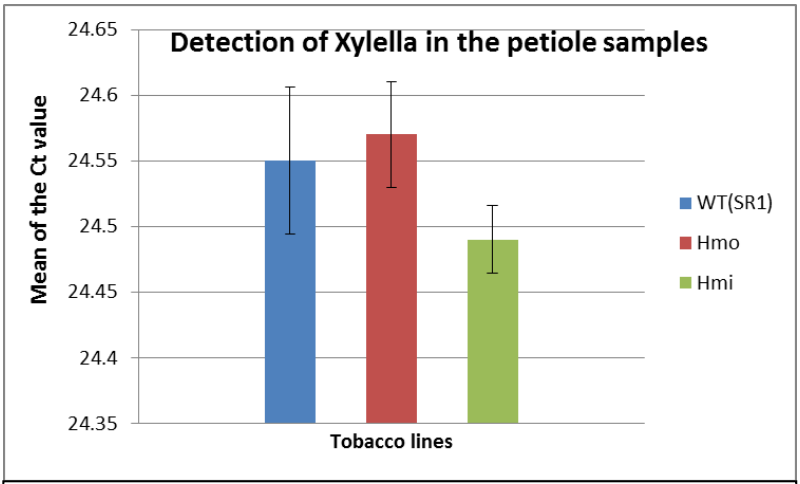

Fig 5. Detection of *Xylella fastidiosa* in petiole samples from the tobacco lines challenged with XF in the greenhouse (SR1/wild type, Hmo and Hmi). All the tobacco lines supported about the same populations of bacteria (~10^8 CFU/gfw [data not shown]) based on qPCR. No XF was detected in buffer-innoculated plants (not shown).

Layperson summary of project accomplishments

We are on track to achieve our Objectives. In future applications to PD/GWSS, contingent upon satisfactory progress towards Objectives 1 and 2, we will characterize the changes in control versus edited genotypes for xylem inorganic phosphate (P_i) and polyphenolic levels of XF-infected and Pi-treated stems, leaves, and berries. We will conduct XF challenge experiments with genome-edited transgenic plants. We will also endeavor to conduct insect diet preference and XF growth assays with candidate polyphenolics that arise from our results. It is noted that no host genes are yet known that normally function to *enhance* host susceptibility; altering host gene (e.g. PD resistance) activities may result in increased susceptibility to infections. Thus engineering PD resistance is likely to be by incremental advances from characterizing molecular mechanisms.

Status of funds

Salaries funds have all been committed- a new Ph.D. student (Congyu LU) has been offered an RAship funded by the project. The deadline for acceptance of the offer on the table to begin studies in Fall, 2016 is April 15th, 2016. If the candidate grad student accepts the offer, a no-cost extension will be made to the sponsor to spend the remaining salary funds by Dec 31, 2016. As of Feb. 2016, there remains $1,457 in travel (to be used in July 2016 under a no-cost extension request, in the event, to collect more PD-infected samples in Temecula CA and Sonoma Co.), $2,200 in publication charges (already budgeted for two manuscripts in prep), $8,567 in sequencing fee-for-service (to be paid to UC Riverside Institute for Integrative Genome Biology facility upon invoicing for sequencing currently in the pipeline), and ~$1,500 in Maintenance and Operations. The budget allocated for the Co-I L. De La Fuente ($20,851) is being used to pay partially the salary of the postdoc S. Traore and supplies for greenhouse experiments with transgenic tobacco plants (including qPCR kits). We are on track to spend all funds by the project end date, with the proviso that a no-cost extension is requested 60 days before the end date (April 30th) and subsequently granted by the sponsors.

Summary and status of intellectual property associated with the project

The PI has disclosed a "Subject Invention" and reported it to NIH (see publications list above). The pending patent application awaits First Office Action by the USPTO. 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 two erstwhile collaborators: Jennifer Doudna/Emmanuelle Charpentier et al. at UC Berkeley/University of Vienna (PCT/US2013/032589; priority date May 25, 2012) and Feng Zhang at MIT (USPTO# 8697359, issued April 15, 2014)[13]. The IP landscape surrounding CRISPR/Cas9 is wider than that part of it controlled by these inventors (with respective start-up companies Intellia, CRISPR Therapeutics, and Editas, respectively). The Doudna/Charpentier et al. inventors have filed an interference petition against Zhang. It is unclear whether non-exclusive licenses will be negotiated between these parties to facilitate freedom to operate by their respective companies and others. Research by other groups and biotech firms are the basis for numerous filed patent applications on genome editing tools that might be considered prior art, or that haven't yet entered the public domain. There appear to be upwards of ~1,000 published PCT applications that are related to CRISPR/Cas9. CRISPR/Cas9's easy reproducibility in different organisms 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. Companies wishing to practice the CRISPR/Cas9 technology now can seek a non-exclusive license from The Broad Institute for the issued Zhang patent, and non-exclusive licenses are available for some other important CRISPR-based patents for the purpose of portfolio building to cover specific applications of the CRISPR/cas system (potentially like being proposed here) and for negotiating possible cross-licensing arrangements.

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