California Department of Food and Agriculture PD/GWSS
Interim Progress Report – March 2019

REPORT TITLE: Interim Progress Report for CDFA Agreement Number 18-0400-000-SA

PROJECT TITLE: Molecular breeding support for the development of Pierce's disease resistant winegrapes.

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REPORTING PERIOD: Primarily October 2018 to March 2019

INTRODUCTION

The main objectives of this project are to gain an understanding of the inheritance of Pierce's disease (PD) resistance in a wide range of North American grape species, tag resistance regions with markers by genetic mapping and functionally characterize the resistance genes. Molecular tools developed in this project provide support to the PD resistance grape breeding project ("Breeding Pierce's disease resistant winegrapes") by marker testing thousands of seedlings of advanced crosses each year to expedite the release of new resistant grape varieties. To meet the key objectives of the program, we have surveyed over 250 accessions of *Vitis* species growing in the southern US and Mexico in an effort to identify new PD resistant accessions. Analysis using population genetics tools has allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. Fourteen promising resistant accessions were identified from this germplasm. Markers were used to determine their genetic diversity and relationship to each other. Small breeding populations were developed and more than 700 seedlings were marker tested to ensure that they had the correct parentage and identity. We coined the term 'limited mapping strategy' and effectively used it by utilizing markers from chromosome (ch) 14 in conjunction with greenhouse PD screening data of small breeding populations to determine if the resistance to PD in these 14 accessions is different from the previously identified locus *PdR1* (Riaz et al. 2018**)**. Three unique resistance sources (T03-16, ANU67 and b41-13) were identified as having a different resistance region than ch14. More crosses were made in Spring 2016-2017 to expand these breeding populations for map-based identification of genomic regions that contribute to resistance. We have initiated genetic mapping of breeding population with the b41-13 resistance source to identify the genomic region that contributes to the resistance.

Strongly associated markers are the only means to combine the PD resistance from multiple genetic backgrounds into a single elite line. Currently, our breeding program is employing two loci (*PdR1* and *PdR2*) that were identified from two different resistant accessions b43-17 and b42-26, respectively in this project. Tightly linked markers to both loci are in use in the breeding program. The potential identification of new resistance regions from the genetic mapping of b41-13, T03-16, and ANU67, will add to our repertoire of resistant loci and markers to diversify PD resistance in our new varieties.

The identification and characterization of resistance genes and their regulatory sequences will help determine the basis of resistance and susceptibility in grape germplasm. In addition, these genes and their promoters could be employed in production of 'cisgenic' plants. Cisgenesis is the transformation of a host plant with its own genes and promoters (Holmes et al. 2013). We have completed the physical map of *PdR1*a and *PdR1*b locus for b43-17 to clone and characterize resistance genes (see earlier reports). The physical map of the *PdR1*c locus (from b40-14) is also complete. Development of *V. vinifera* plants transformed with our PD resistance genes and grape promoters might work more effectively and allow us to better understand how the *PdR1* resistant gene functions.

Upstream and downstream sequences as well as the gene sequences of two candidate genes, ORF14 and ORF18 (Open Reading Frame), from *PdR1*b were verified and constructs were developed to test their function. Transformation experiments with the *PdR1* resistance gene with a native grape promoter were completed with ORF18 and transgenic lines are being developed and maintained for later resistance verification. A large scale multiple time point gene expression project was completed in the greenhouse and RNA extractions were

completed for over 400 samples. We used qPCR to test the expression of candidate genes. Embryogenic callus cultures of *V. vinifera* cvs. Chardonnay and Thompson Seedless and *V. rupestris* St. George are being maintained to test the function of gene sequences. These efforts will help us identify candidate resistance genes by complementation and better understand how they function.

OBJECTIVES

1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the PD resistance breeding program, carry out genetic mapping of two new highly resistant lines b41-13 and T03-16 for use in stacking PD resistance genes.
2. Complete a physical map of the PdR2 region from the b42-26 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*) and b40-14 (*PdR1c*).
3. Employ RNA-seq to understand genome-wide transcriptional changes of the pathways regulated by defense-related genes in b40-14.
4. Clone PD resistance genes with native promoters.
5. Compare the PD resistance of plants transformed with native vs. heterologous promoters

DESCRIPTION OF ACTIVITIES

Objective 1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the PD resistance breeding program, carry out genetic mapping of two new highly resistant lines b41-13 and T03-16 for use in stacking PD resistance genes.

The screening of additional *Vitis* germplasm collected from southwestern US and northern Mexico provided us 14 new resistant accessions (Riaz and Walker 2013). Small breeding populations and a limited mapping strategy approach allowed us to identify resistance sources whose resistance is similar to *PdR1* and sources that are different. The major findings of this work were recently published (Riaz et al. 2018). We identified 9 accessions with resistance similar to *PdR1* locus on ch14 and three accessions, ANU67, b41-13, and T03-16 where resistance does not reside on ch14 (Table 1). These three accessions were identified as candidates for further work and the development of framework maps with larger populations to detect new unique loci for PD resistance breeding.

Accession T03-16 from the Big Bend region in Texas and b41-13 from Tamaulipas state in Mexico are strong candidates that do not possess *PdR1*. In order to identify the genomic regions housing PD resistance in these two accessions, crosses were made in Spring 2016 and 2017 to expand population sizes. A total of 295 seedling plants of the F1 population from b41-13 are established in the field. We have completed the greenhouse screening of 275 F1 seedling plants. Over 400 SSR markers were tested on a small set of parents and progeny; 53% of the markers were polymorphic. We have completed mapping of 200 of polymorphic markers on a population of 295 seedling plants to develop framework map and carry out QTL analysis to identify genomic regions linked to PD resistance.

Crosses were also made with T03-16 and 285 seedling plants from the F1 population were established in the field. Multiple replicates of seedling plants were propagated for greenhouse screening – 173 seedling plants are now in different stages of greenhouse testing.

Table 1. The 14 resistant accessions used to create 23 breeding populations in an effort to identify PD resistance sources that differ from *PdR1*. Resistant accessions with different sources of resistance are marked as Not 14 in the last column. Accessions marked as LG14 possess the *PdR1* locus. Resistance affinity to Ch14 could not be determined for the accessions that are marked as Inconclusive due to small population size and less informative markers.

Resistance source	Species description	Populations tested	Number of Screened Genotypes	PD resistance
ANU5	*V. girdiana*	12-314	60	LG14
b40-29	*V. arizonica*, brushy	12-340, 12-341, 14-367, 14-368	29	LG14
b46-43	*V. arizonica*, glabrous	12-305, 14-308, 14-321,	159	LG14

	with *V. monticola*	14-322, 14-324, 14-336		
b41-13	*V. arizonica-mustangensis* and *champinii* hybrid,	13-355	47	Inconclusive
b47-32	*V. arizonica* glabrous with *monticola*,	13-344	13	Inconclusive
SC36	*V. girdiana*	13-348	35	LG14
T03-16	*V. arizonica* glabrous	13-336	62	Inconclusive
A14	*V. arizonica*	14-313	25	Inconclusive
A28	*V. arizonica*	14-347, 14-364	42	LG14
ANU67	*V. arizonica* glabrous	14-362	28	Inconclusive
ANU71	*V. arizonica-riparia* hybrid	14-340	30	LG14
C23-94	*V. arizonica* glabrous and brushy	14-303	44	LG14
DVIT 2236.2	*V. cinerea* like,	14-360	30	LG14
SAZ 7	*V. arizonica*	14-363	52	LG14

This project provides molecular support to the companion PD resistance winegrape breeding project by conducting MAS on seedling populations. We are currently in week five of marker testing to support the breeding program. Each week, we receive 400-500 samples for DNA extraction and marker testing. We have established the in-house protocols to complete DNA extractions and marker testing in order to generate and deliver the results in a timely manner to our greenhouse and field team involved in the PD resistance breeding program. In 2019, we marker tested 3,102 seedling plants from 59 different crosses for the *PdR1* (b and c) and *PdR2* loci (Table 2). Marker screening is a time intensive process, but extremely important and makes our breeding program extremely efficient and successful. Each year we spend 12 to 14 weeks in the spring to support the breeding program. Discarding the susceptible plants (those without the marker) saves us huge amounts of time and resources in the field.

Table 2. Summary of marker testing completed in Spring 2018 to support our PD resistance breeding program.

PD locus	**Number of crosses**	**Number of seedlings**
PdR1b	24	1350
PdR1b x *PdR2*	28	1450
PdR1c	7	302
Total	59	3102

Objective 2. Complete a physical map of the PdR2 region from the b42-26 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*) and b40-14 (*PdR1c*).

We completed the physical maps of the *PdR1*a, *PdR1*b and *PdR1*c loci from the b40-14 and b43-17 backgrounds. In summary, a BAC (bacterial artificial chromosome) library from b40-14 genomic DNA (see details in previous reports) was screened and 30 BAC clones were identified with two probes, Ch14-56 and Ch14-58. BAC clones that represent *PdR1c* were separated from the other haplotype and four overlapping BAC clones VA29E9, VA57F4, VA30F14, VA16J22 were selected for sequencing. Common probes between the *PdR1c* and *PdR1b* region were used to align the sequences. The assembly of four BAC clones is presented in Figure 1 and 2 and represents the sequence analysis of *PdR1b* and the reference grape genome PN40024 region. A manuscript entitled "The physical map of the PD resistance locus, *PdR1c*" is in preparation.

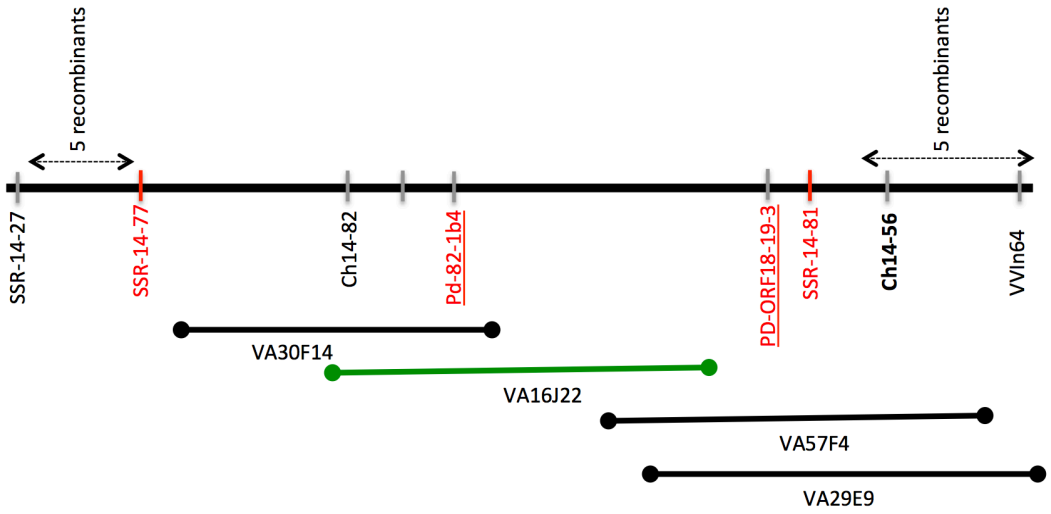

Figure 1. BAC library was developed from genomic DNA of b40-14 and screened with probes. Four overlapping clones were selected for sequencing the complete region. Marker names in red were developed from the sequence of accession b43-17.

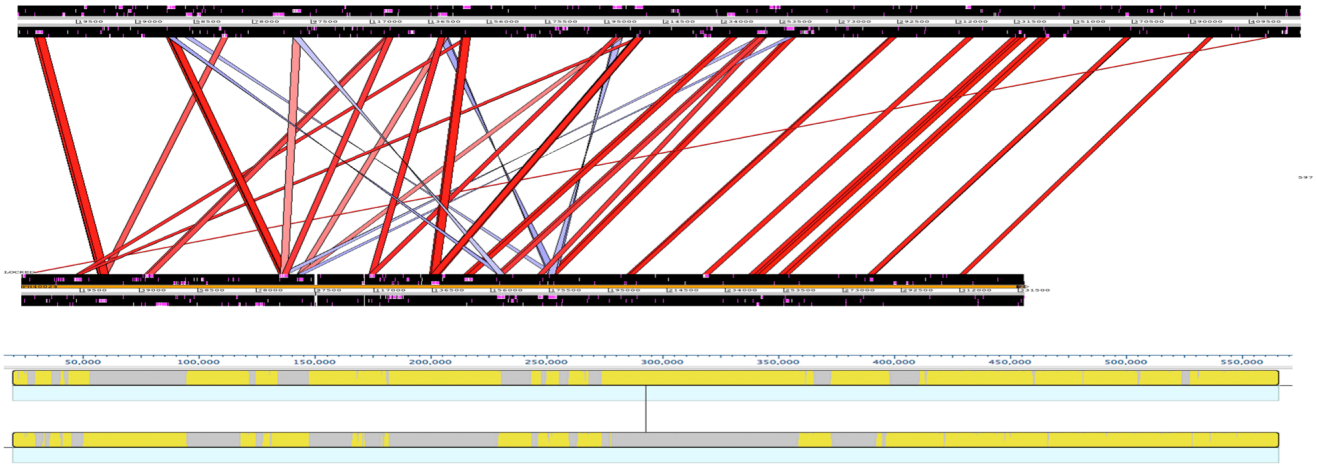

Figure 2. The sequences of four BAC clones were assembled and full-length open reading frames were identified. Sequences were compared with the reference genome and checked for synteny in that region. Analysis is being carried out with the Cabernet Sauvignon genome sequence.

The assembly of H43-I23 from the b43-17 BAC library that represents the *PdR1a* haplotype (F8909-17) was also completed. The length of assembled sequence was 206Kb. The open reading frames (ORF) of the *PdR1b* region and the BAC clone H69J14 were used to make comparisons. There was complete homology between the overlapping BAC clone sequences that reflect two different haplotypes. The BAC clone H43I23 has ORF16 to ORF20 and all five ORFs have identical sequences compared to the *PdR1b* haplotype. Based on these results, we concluded that there is complete sequence homology between haplotypes *PdR1a* and *PdR1b* of the *PdR1* locus; therefore cloning and functional characterization of genes from any one haplotype will be sufficient for future work. Complete sequence homology also suggests that the parents of b43-17 must be closely related and may have a first-degree relationship and acquired resistance from shared parents. This also explains why we observed complete homozygosity of SSR markers for the *PdR1* locus in the resistant accession b43-17.

In regard to the physical map of b42-26, we need to refine the position of the *PdR2* locus and narrow the region to less than 1cM. For this purpose, we developed new markers using the Cabernet Sauvignon genome sequence (described in objective 1) and added them to the genetic map. We are developing more markers to fill gaps around the resistance QTL on chromosome 10 as well, so that library screening can be used to identify BAC clones that represent both resistance regions.

Objective 3. Employ RNA-seq to understand genome-wide transcriptional changes of the pathways regulated by defense-related genes in b40-14.

RNAseq is a powerful approach to identity transcripts and quantify gene expression while combined with a single high-throughput sequencing assay. A good RNAseq study relies on experimental design (library type, sequencing depth and number of replicates) and a careful execution of the sequencing experiment to ensure that data acquisition is not contaminated with unnecessary biases. We completed a time course experiment to monitor the bacterial level in control and inoculated resistant and susceptible plants to design an experiment capable of answering our biological questions with the maximum statistical power. To accomplish this, three resistant and three susceptible plants from the 07744 population with resistance from b40-14 PD (*PdR1c*) were used. Plants were propagated and a time course experiment was carried out in growth chambers with temperature and humidity control to reduce the variance. Stem samples were collected from positions 10cm, 20 cm, 30 cm and 40 cm above the point of inoculation and weekly RNA extractions were performed. Samples were also collected from 30cM above point of inoculation for ELISA screening. We have completed RNA extractions of 496 samples.

Objective 4. Cloning PD resistance genes with native promoters.

With the help of molecular markers, we limited the genetic region that contains the *PdR1b* resistance locus to 82 Kb. Five ORFs of the Leucine-Rich Repeat Receptor Kinase gene family, associated with disease resistance, were identified within the resistance region boundaries. Two ORFs V.ari-RGA14 and V.ari-RGA18 are the most likely candidates for *PdR1b*. The other 3 sequences, V.ari-RGA15, 16 and 17, are shorter and contain a large number of transposable elements (TE). Fragments that contain the entire coding region of V.ari-RGA14 and V.ari-RGA18 plus ~3 Kb upstream and ~1 Kb downstream sequences were synthesized and cloned into pCLB2301NK (Feechan et al. 2013) at Genewiz Inc to produce plasmids pCLB2301NK-14 and pCLB2301NK-18. (See Final Report for CDFA Agreement Number 14-0137-SA and 17-0427-000-SA for details).

Objective 5. Comparing the PD resistance of plants transformed with native vs. heterologous promoters.

We have established *Agrobacterium* mediated transformation systems followed by regeneration of plants from embryogenic callus and meristematic bulks (Agüero et al. 2006, Xie et al. 2016). *Agrobacterium tumefaciens* strain EHA 105 pC32 was chemically transformed with pCLB2301NK-14 or pCLB2301NK-18 and subsequently used to transform embryogenic calli of *V. vinifera* cvs. Chardonnay (CH), Thompson Seedless (TS) and the rootstock *V. rupestris* St. George (SG). The evaluation of 44 transgenic plants of CH and TS (10-11 lines per genotype x 2 constructs) showed that all transgenic lines displayed disease symptoms with different degrees of intensity; TS being considerably more susceptible than CH. Although some lines exhibited reduced symptoms or lower bacteria concentrations, none reached the levels of the resistant biocontrols (See Final Report for CDFA Agreement Number 14-0137-SA and 17-0427-000-SA for details).

Plant regeneration of transgenic SG has been more challenging (Table 3), however promising results were obtained with one RGA14 line that was inoculated in August 2018. Cane Maturation Index Means of untransformed and transgenic were 4.9 and 1.7 respectively, while Leaf Scorching Index Means were 4.5 and 3. Figure 3 shows re-growth in transgenics after cutting back of plants for sampling 12 wks after inoculation (right). None of the untransformed resumed their growth (left). Shoot lignification 30 cm above POI is shown at the bottom of Figure 3. ELISA tests also produced significant differences between untransformed (410,000 cfu/ml) vs transgenic (120,000 cfu/ml). This findings agree with results from sequencing of cDNA from b43-17, the original source of resistance, inoculated with *X. fastidiosa*, showing the amplification of fragments that comprise sequences identical to RGA14 but different from RGA18. Currently, we are testing one SG RGA18 line, which was inoculated on January 18, 2019. The rest of the SG lines are dwarf and grow very slowly, consequently more

in vitro transformations have been initiated. Co-transformations with both pCLB2301NK-14 and pCLB2301NK-18 are also underway.

Table 3

Genotype	No. lines	No lines PCR positive
pCLB2301NK-18		
St. George	4	4 (1 inoculated Jan 2019)
29-42	3	not tested
pCLB2301NK-14		
St. George	1	1 (1 inoculated Aug 2018)
29-07	6	not tested

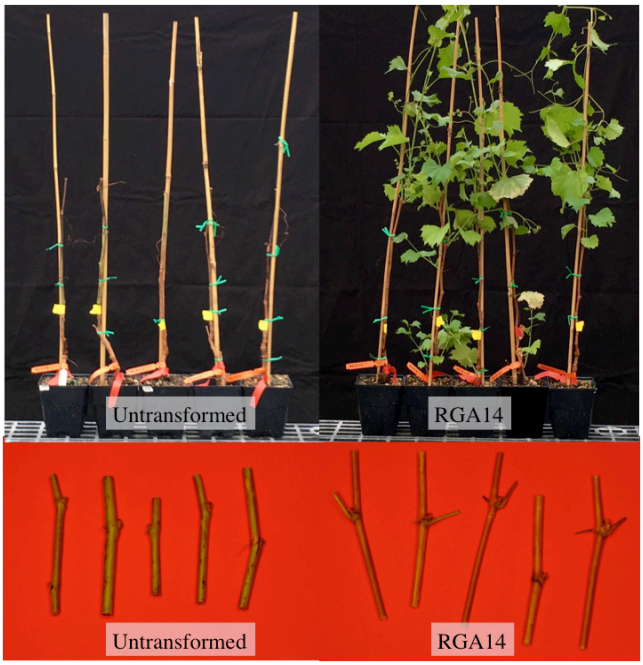

Figure 3

Transformation of meristematic bulk of genotypes selected from the 04-191 population, which are 50% *vinifera*, 25% b43-17 and 25% *V. rupestris* A. de Serres are also being evaluated. Two of these genotypes, designated 29-07 and 29-42 have produced plants that show GFP fluorescence (Table 3) and will be acclimated to greenhouse conditions for further testing. Evaluating these lines plus additional transgenic SG could help to clarify the role of genetic background on *PdR1b* resistance.

In addition, we are generating meristematic bulks of b43-17, and U0505-01, which is 87.5% *vinifera*, positive for *PdR1b* molecular markers and highly resistant to PD, for reverse genetic analysis using CRISPR-Cas systems.

CONCLUSIONS

We completed greenhouse screening, marker testing and QTL analysis of breeding populations from 15 new resistance sources including b46-43 and T03-16. We identified T03-16 and b41-13 as possessing resistance on a different region than chromosome (Ch) 14. Crosses were made to expand these breeding populations for framework map development in order to identify other genomic regions of resistance. Given that the

incorporation of multiple resistances should make resistance more durable, our goal is to identify new sources of resistance that do not reside on Ch14 and facilitate stacking (combining) of these resistance sources with *PdR1* from b43-17 using genetic markers. We have identified a new resistance locus *PdR2* from the b42-26 background and closely linked markers are being used in MAS to stack resistance loci from these different backgrounds. We have completed the genetic and physical mapping of PD resistance from b40-14. This resistance source maps within the *PdR1b* locus, but it may be an alternative gene within this complex replicated locus. We completed greenhouse screening, of CH and TS lines transformed with RGA18 and RGA14. Although some transgenic lines responded better than untransformed plants to *Xylella fastidiosa* infection, none reached the level of resistant bio-controls. Promising results were obtained with one line of SG RGA14. Testing of RGA14 and 18 in SG and other genetic backgrounds, as well as more information about RGA15, 16 and 17 will help to clarify the meaning and importance of these results.

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Walker, A., A. Tenscher and S. Riaz. 2018. Breeding Pierce's disease resistant winegrapes. CDFA PD/GWSS Board Symposium Poster, San Diego, CA Dec. 12

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Agüero, C.B., S. Riaz, A. Tenscher and M.A. Walker. 2018. Molecular breeding support for the development of Pierce's disease resistant winegrapes – genetic transformation with *PdR1b* candidates. CDFA PD/GWSS Board Symposium Poster, San Diego, CA Dec. 12

Presentations that included PD breeding and genetics of resistance

Walker, M.A. 2018. PD causes and cures. Lecture and tasting. D. Roberts Grower Meeting, Santa Rosa, Jan 12.

Walker, M.A. 2018. Developing PD resistant wine grapes. Lecture and Tasting. Chateau Elan, Braselton, GA. Georgia Wine Producers Meeting, Jan 23

Walker, M.A. 2018. Understanding plant material selection for vineyard redevelopment: Including rootstock and plant material selection and soil pest and virus considerations, South State Gallo Growers Meeting, Fresno, CA Feb 15.

Walker, M.A. 2018. Understanding plant material selection for vineyard redevelopment: Including rootstock and plant material selection and soil pest and virus considerations, North State Gallo Growers Meeting, Lodi, CA Feb 16.

Walker, M.A. 2018. Grape breeding update. Current Issues in Viticulture, UC Davis, Feb 21.

Walker, M.A. 2018. Rootstock breeding update. CDFA IAB Nursery Board meeting, UC Davis, Apr 11.

Walker, M.A. 2018. Grape breeding update and PD wine tasting. UC Davis for the PD/GWSS Grower Advisory Board, April 23.

Walker, M.A. 2018. UCD PD breeding program update and tasting. Temecula Winemakers Meeting, Wilson Creek winery, Temecula, June 8.

Walker, M.A. 2018. Grape breeding at UC Davis. Lebanon Table Grape Growers Group, July 17.

Walker, M.A. 2018. Grape breeding update. CGRIC Nursery Meeting, July 24.

Walker, M.A. 2018. Fanleaf Field Day, discuss plot and breeding – Healdsburg, CA, Aug. 16 .

Walker, M.A. 2018. Rootstock breeding program update. CDFA IAB meeting, UC Davis, Nov 14.
Walker, M.A. 2018. New/replanted vineyard establishment concerns. UCD/On the Road Presentations, Escondido, CA, Nov 29.
Walker, M.A. 2018. Current and future objectives of the grape breeding program at UCD. Recent Advances in Viticulture and Enology, UC Davis, Nov 30
Walker, M.A. 2018. Current and future objectives of the UCD grape breeding program. Foundation Plant Services Annual Meeting, UC Davis, Dec. 4
Walker, M.A. 2018. PD resistant winegrape breeding program update. CDFA PD/GWSS Board Symposium, San Diego, CA Dec. 12
Walker, M.A. 2019. An update on the performance of the GRN rootstocks. Daniel Roberts Client Meeting, Jan 18
Walker, M.A. 2019. How to select rootstocks. Viticulture Short Course, Napa, Feb 13.
Walker, M.A. 2019. Grape vine pruning demo and instruction, UC Davis for Folsom Lake College, Feb 23.
Walker, M.A. 2019. Stacking PD resistance genes for durable resistance. Current Advances in Wine and Grape Research, UC Davis, Feb. 27
Walker, M.A. 2019. Current and future objectives of the grape breeding program at UC Davis, Salinas Farm Advisor Office, On the Road Presentation, March 8
Walker, M.A. 2019. Grape rootstock breeding update. California Grape Rootstock Improvement Commission, Coalinga, CA March 11.
Walker, M.A. 2019. The grape breeding program at UC Davis: where it's been and where it's going. CSU Fresno, March 20.
Walker, M.A. 2019. An update on the performance of the GRN rootstocks, Lakeport, On the Road Presentation, March 28.

RESEARCH RELEVANCE
The goal of this research is to identify new PD resistance sources so that resistance can be broadened and made more durable, to identify genomic regions and develop markers to facilitate marker-assisted breeding, to support the companion breeding project by marker testing progeny populations, to combine multiple resistance sources into single elite selections, and to functionally characterize genes to understand how they confer resistance.

LAYPERSON SUMMARY
Our main focus is to identify and genetically characterize unique PD resistance sources from the southwestern US and Mexican *Vitis* species collections. In order to carry out this task, we create genetic maps that associate regions of chromosomes with PD resistance. These regions (markers) are used to expedite screening for resistance since they can be used to test seedlings as soon as they germinate. Markers developed for different sources of resistance allow us to combine multiple forms of resistance and therefore produce offspring with the likelihood of having more broad and durable resistance. These markers also allow us to identify candidate resistance genes and study how they function by engineering them into susceptible grape varieties to better understand the genes and the resistance.

STATUS OF FUNDS: These funds are scheduled to be spent by the end of the grant.

INTELLECTUAL PROPERTY: PD resistant varieties will be released through the Office of Technology Transfer (Patent Office) of the University of California, Davis.

FUNDING AGENCY
Funding for this project was provided by the CDFA PD/GWSS Board. Additional support from the Louise Rossi Endowed Chair in Viticulture is also gratefully acknowledged.