SUMMARY FINAL 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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BRIEF BACKGROUND

This project provides molecular support to the "Breeding Pierce's disease resistant winegrapes" project. In Spring 2019, we extracted DNA and marker tested 2,400 seedling plants from 25 different crosses for the *PdR1* (b and c) and *PdR2* loci that were in all cases combined with powdery mildew resistance. We expanded the population size of T03-16 and b41-13 breeding populations for framework map and completed the genetic mapping and QTL analysis. Results show that their PD resistance resides on chromosome 14 at the same genomic position of *PdR1*. This brings the total to 13 resistant accessions with the *PdR1* locus. We have identified a new resistance locus *PdR2* from the b42-26 (a resistant *V. arizonica/girdiana* plant from Baja California) background and closely linked markers are being used in Marker Assisted Selection (MAS) to combine the resistance loci from these different backgrounds. We have completed the genetic and physical mapping of PD resistance from b40-14 and b43-17. We completed greenhouse screening, of Chardonnay and Thompson Seedless lines transformed with RGA18 and RGA14. Although some transgenic lines responded better than untransformed plants to *Xylella* infection, none reached the level of resistant bio controls. Promising results have been 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.

OBJECTIVES

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.

- In 2018, three resistance sources (ANU67, b41-13 and T03-16) were identified as candidates for alternative PD resistance genes.
- Crosses were made to expand the population sizes of ANU67, b41-13 and T03-16 resistance sources. A total of 295 seedling plants of the F1 population from b41-13 were established in the field.
- 596 markers were tested for b41-13 background and 244 SSR markers were completed to develop the framework map.
- For T03-16, 34 markers were tested and 14 polymorphic markers were completed on larger populations.
- Genetic mapping analysis was completed for both accessions. For b41-13, the map spans all 19 grape chromosomes and for T03-16, it covers chromosome 14 only.
- Greenhouse screening of 250 and 192 seedling plants was completed for F1 seedling plants with the b41-13 and T03-16 background, respectively.
- QTL analysis was completed and for both T03-16 and b41-13 PD resistance was identified on CH 14.
- A manuscript detailing the genetic mapping of PD resistance in b40-14, b41-13 and T03-16 was completed.
- In Spring 2018, we carried out DNA extractions and marker tested 2,400 seedling plants from 25 different crosses for the *PdR1* (b and c) and *PdR2* loci and combining Powdery Mildew resistance.

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 identified a new locus *PdR2* in the *V. arizonica/girdiana* b42-26 background. A genetic map was created with 352 seedling plants and 202 markers that grouped to 18 chromosomes.
- QTL analysis identified resistance on Ch8 and Ch10. The resistance from Ch8 was also verified on the basis of linked alleles in the pBC1 and pBC2 populations.
- Detailed analysis with additional markers will be completed in this year before we initiate the BAC library screening.
- We completed the physical map of *PdR1a*, *b* and *c* loci from the b40-14 and b43-17 backgrounds.
- From the BAC library of b40-14, 30 BAC clones were identified with two probes. Four overlapping BAC clones VA29E9, VA57F4, VA30F14, VA16J22 were sequenced to complete the physical map of the region.
- The physical map of *PdR1b* spans 604 Kb that includes the flanking markers Ch14-77 and Ch14-81, which are used for marker assisted screening.
- From the BAC library of b43-17, clone H43-I23 represents the *PdR1a* haplotype (F8909-17). The length of the assembled sequence was 206Kb. There was complete homology between the over-lapping BAC clone sequences that reflects two different haplotypes, therefore cloning and functional characterization of genes from any one haplotype will be sufficient for future work.
- A total of 21 Open Reading Frames ORFs) were identified in a 604 Kb sequence of the *PdR1b* sequence in comparison to the 18 ORFs in the *PdR1c* sequence.
- The PN40024 sequence was 230 Kb with many gaps; potentially some ORFs are not accounted for. The sequences of Cabernet Sauvignon (CS) within the flanking markers was 527 Kb long.

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

- We completed a time course experiment to monitor the bacterial level in control and inoculated resistant and susceptible plants. The results will help us to plan an experiment capable of answering our biological questions with the maximum statistical power.
- Three resistant and three susceptible plants from the 07744 (with resistance from b40-14) population were selected.
- A time course experiment was carried out in growth chambers with temperature and humidity control to reduce experimental variance. Stem samples were collected for RNA extractions from positions 10cm, 20 cm, 30 cm and 40 cm above the point of inoculation at weekly intervals. Analysis is underway to determine when and where gene expression is optimized. We have completed RNA extractions of 496 samples.

Objective 4. Cloning PD resistance genes with native promoters.

- The physical map of the *PdR1b* region identified multiple ORFs of the Leucine-Rich Repeat Receptor Kinase gene family. With the help of molecular markers, we limited the genetic region to 82 Kb – with 5 ORFs associated with disease resistance and other plant functions described above.
- Two ORFs V.ari-RGA14 and V.ari-RGA18, within the resistance region, are the most likely candidates for *PdR1b*. Both RGA14 and 18 (resistance gene analogs) have a very similar sequence profile except that RGA18 is 2,946 bp in size and lacks the first 252 bp of sequence that is part of RGA14.
- Functional analysis of both RGAs revealed that RGA14 lacks a signal peptide in the amino terminal of the protein. This result was verified using 3'RACE (rapid amplification of cDNA ends) to specifically amplify RNA from grapevines transformed with V.ari-RGA14 under the 35S promoter.
- We completed the sequence verification for RGA14 and RGA18 and flanking sequences and cloned into pCLB2301NK at Genewiz Inc.

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

- We have established and streamlined an *Agrobacterium* mediated transformation system followed by regeneration of plants from embryogenic callus.
- We have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless, Cabernet Sauvignon, Chardonnay and the rootstock *V. rupestris* St. George.

- We have also developed meristematic bulks (MB), an alternative explant for genetic transformation, of PD susceptible genotypes selected from the 04-191 population (50% *vinifera*, 25% b43-17 and 25% *V. rupestris* A. de Serres). These genotypes can provide an additional genetic background for analysis of expression of *PdR1* candidate genes. Two of these genotypes, designated 29-42 and 47-50 exhibited great potential for the development of MB and transformation experiments with *Agrobacterium* have been initiated.
- Transformations with *Agrobacterium tumefaciens* carrying binary plasmids that contain hygromicin (pCLB1301NH) or kanamycin (pCLB2301NK) selectable marker genes showed that both antibiotics are effective selection agents for embryogenic calli. However, MB regeneration has most often occurred when selecting with kanamycin, confirming our previous observation that MB are highly sensitive to hygromicin.
- Transformation experiments with A*grobacterium 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, Thompson Seedless and the rootstock *V. rupestris* St. George.
- In addition, *Agrobacterium* is being used to transform meristematic bulks (MB) of PD susceptible genotypes selected from the 04-191 population. Transformation was checked through PCR, and transformed plants were transferred to the greenhouse.
- Transformation was also verified by fluorescence microscopy to visualize GFP (Green Fluorescent Protein), since pCLB2301NK-18 and pCLB2301NK-14 also contain a 35S:GFP5-ER cassette.
- Chardonnay and Thompson Seedless transgenic lines were multiplied from green cuttings and inoculated with the Beringer strain of *X. fastidiosa* in August 2017 (RGA18 lines) and March 2018 (RGA14 lines).
- Twelve weeks after inoculation, PD symptoms were evaluated using a 0-5 score for leaf scorch-leaf loss (LS-LL) and a 0-6 score for the cane maturation index (CMI).
- For ELISA, plants were sampled by taking 0.5 g sections of stem tissue from 30 cm above the point of inoculation. For gene expression analysis, plants were sampled by taking 0.5 g sections of stem tissue from 50 cm above the point of inoculation.
- Testing of the RGAl4 lines is underway. Thompson Seedless was considerably more susceptible than Chardonnay.
- qPCR analysis to determine the correlation between the level of transgene expression and GFP fluorescence/PD symptoms/bacteria concentrations has been inconclusive. Untransformed Chardonnay infected with *X. fastidiosa* also exhibits low Ct numbers and cDNA sequencing has revealed that genes with high homology with RGA14 and RGA18 are being expressed.

In conclusion, we completed the greenhouse screening, marker testing and QTL analysis of breeding populations from new resistance sources including b41-13 and T03-16 and determined that PD resistance resides on chromosome 14. We have also identified a new resistance locus, *PdR2*, from the b42-26 background and closely linked markers are being used in MAS to combine resistance loci from these different backgrounds. We have completed the genetic and physical mapping of PD resistance from b40-14 and b43-17 and initiated the refined mapping of the *PdR2* locus that will be physically mapped. Finally, we verified the sequence of two candidate genes from the *PdR1b* locus, completed transformations with RGA18 and RGA14 and obtained transgenic lines for complementation tests in the greenhouse. Although some transgenic lines responded better than untransformed plants to *Xylella* infection, none reached the level of resistant biocontrols. Testing of RGA14 and 18 in a genetic background other than *vinifera*, as well as more information about RGA15, 16 and 17 will help to clarify the meaning and importance of these results.