SUMMARY FINAL REPORT FOR CDFA AGREEMENT NUMBER 14-0137-SA and 17-0427-000-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". We have surveyed over 250 accessions of *Vitis* species to identify new PD resistant accessions. Small breeding populations were developed with newly identified resistant accessions and a limited mapping strategy utilizing markers from chromosome (Ch) 14 in conjunction with greenhouse PD screening data was used to determine if resistance to PD is different from the previously identified locus *PdR1*. Three new unique resistances sources (T03-16, ANU67 and b41-13) were identified and crosses were made in Spring 2016-2017 to expand populations for map-based identification of the genomic regions that contribute to resistance. We have also identified a new resistance locus, *PdR2*, on Ch8 from b42-26 and markers are being used to combine it with the *PdR1* locus. We have completed the physical map of the *PdR1*a and *PdR1*b locus for b43-17 to clone and characterize resistance genes and the refinement of the *PdR2* locus from b42-26 is underway. Gene sequences of two candidate genes, ORF14 and ORF18, from *PdR1*b were verified and constructs were developed. Transformation experiments with the *PdR1* resistance gene with a native grape promoter were completed with ORF14 and ORF18 and transgenic lines are being tested for 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. These efforts will help us to identify candidate resistance genes by complementation and better understand how they function.

JULY 2017 TO JUNE 2018 ACCOMPLISHMENTS

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.

- We completed genetic mapping and tagging of breeding populations developed from 14 new resistant lines (Riaz et al. 2018).
- The above study identified 3 resistance sources: ANU67, b41-13 and T03-16 that would potentially provide new resistance loci and alternative PD resistance genes.
- Crosses were made to expand population sizes of ANU67, b41-13 and T03-16 resistance sources. A total of 295 seedling plants of the F1 population from b41-13 are established in the field. SSR markers from chromosome 8 and 14 were tested on a small set of parents and progeny, and 35 polymorphic markers were run on the entire population 295 seedlings. Further marker testing is in progress to develop the framework map.
- Greenhouse screening of 122 F1 seedling plants (from the ANU67 and b41-13 resistance sources) is complete and an additional 150 seedlings from the 2016 crosses are in different stages of greenhouse screening with results expected in November 2016.
- Crosses were made with T03-16 and DNA was extracted from 285 seedling plants from its F1 population that are established in the field. A total of 173 seedling plants are in different stages of testing in the greenhouse for which results will be available by the end of 2018.

- In Spring 2018, we carried out DNA extractions and marker tested 3,102 seedling plants from 59 different crosses for *PdR1* (b and c) and *PdR2* loci.

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.
- In order to initiate BAC library screening, we attempted to refine the genetic map and place the locus within a 1 cM genetic window. This is ongoing work and screening of the BAC library will start as soon as the refined mapping is complete for both the Ch8 and Ch10 resistance region.
- In Spring 2017 and 2018, we began using closely linked markers to assist the breeding program in combining *PdR1*b and *PdR2* loci.
- A manuscript detailing genetic mapping in b42-26 and b40-14 is approaching submission.
- We completed the physical map of *PdR1a*, *b* and *c* loci from 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.
- 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 reflect two different haplotypes, therefore cloning and functional characterization of genes from any one haplotype will be sufficient for future work.

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 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 400 samples and the remaining 96 samples are in pipeline.

Objective 4. Cloning *PdR1c* and *PdR2* resistance genes with native promoters.

- The physical map of the *PdR1b* region identified multiple ORFs (open reading frames) 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, 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, 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 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 St George will start in August 2018.ELISA 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 greenhouse screening, marker testing and QTL analysis of breeding populations from new resistance sources including b41-13 and T03-16. Crosses with T03-16 and b41-13 were made to expand the population size for mapping work (initiated for b41-13). We have also 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 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.