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Progress Report — July 2017

REPORT TITLE: Interim Progress Report for CDFA Project 14-0137-SA

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

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INTRODUCTION

A successful resistance-breeding program depends on germplasm that provides strong and different forms of resistance capable of withstanding pests and pathogens overcoming resistance. Identification, understanding and manipulation of novel sources of resistance are prerequisites for successful breeding. This project continues to provide molecular support to the PD resistance grape breeding project – "Breeding Pierce's disease resistant winegrapes" by acquiring and testing a wide range of resistant germplasm, tagging resistance regions with markers by genetic mapping and functionally characterizing the resistance genes from different backgrounds. In earlier versions of this project, genetic markers linked to *Xylella fastidiosa* resistance from *Vitis arizonica* b43-17 and *V. arizonica* b40-14 were used to perform marker-assisted selection (MAS) to accelerate our PD resistant winegrape project and the table and raisin grape breeding of David Ramming. Outcomes from the earlier two projects include genetic maps, and BAC (bacterial artificial chromosome) libraries of the above highly resistant *V. arizonica* accessions. We have also identified a different resistance locus, PdR2, in a *V. arizonica* form from Loreto, Baja California (b42-26) that resides chromosome (Ch) 8. A physical map of the *PdR1* locus was completed for b43-17 and b40-14, and several candidate genes were identified. Two candidate genes were cloned and constructs were developed with native grape promoters for transformation efforts.

The current molecular breeding support project has five key objectives: to identify novel sources of PD resistance for use in broadening the genetic base of PD resistance; to accelerate marker discovery and the identification of new and unique resistance genes; to clone and characterize unique DNA sequences (promoters) that regulate the expression of candidate PD resistant grape genes cloned from the *PdR1b* locus; and to evaluate and compare lines transformed with *PdR1* constructs with native and 35S promoters. 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 allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. PD resistance in the southeastern US *Vitis* species seems to be different than the resistance in *Vitis* from the southwest US and Mexico. We have identified new PD resistant accessions that are genetically and phenotypically different, were collected from different geographic locations, and have different maternal inheritance. Breeding populations from new promising resistant lines have been developed. These populations will be tested to study the inheritance of their resistance. Genetic maps will be developed to identify genomic regions associated with resistance, and genetic markers will be used to enable the combination (stacking) of multiple resistance genes and breeding of winegrapes with durable PD resistance.

Twenty resistant accessions were identified from screening of more than 250 accessions of germplasm collected from the Mexico and southwestern USA. Markers were used to determine their diversity and relationship to each

other. Resistant accessions were used to develop small breeding populations from 2012 to 2015. More than 700 seedlings from breeding populations were marker tested to ensure correct parentage and identity. We used a limited mapping strategy by utilizing markers from Ch14 in conjunction with greenhouse screen data of small breeding populations to determine if resistance to PD is different from the previously identified locus *PdR1*. Three new 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 to expand these breeding populations for map-based identification of genomic regions that contribute to resistance. Physical mapping was completed for b43-17 to clone and characterize resistance genes (*PdR1*a and *PdR1*b see earlier reports). The physical map of the *PdR1*c locus (from b40-14) is also now complete. We are continuously developing and expanding breeding populations from new promising resistant lines.

The identification and characterization of resistance genes and their regulatory sequences will help determine the basis of resistance/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). Alternatively, other well characterized *vinifera*-based promoters; either constitutive (Li et al. 2012) or activated by *X. fastidiosa* (Gilchrist et al. 2007) could be utilized. Development of *V. vinifera* plants transformed with our PD resistance genes and grape promoters might work more effectively and allow us to better understand *PdR1*'s function.

Upstream and downstream sequences as well as 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 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. These efforts will help us to 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, including characterization of novel forms of resistance.
2. Complete a physical map of the *PdR1c* region from the b40-14 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*).
3. Employ whole genome (WGS) sequencing (50X) of recently identified PD resistant accessions and a susceptible reference accession; use bioinformatics tools to identify resistance genes, perform comparative sequence analysis and develop SNP markers to be used for mapping.
4. Clone *PdR1* 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, including characterization of novel forms of resistance.

To make a new variety durably resistant to diseases it is often necessary to combine multiple sources of resistance genes into one background to obtain broad long-lasting resistance. We completed greenhouse testing of over 250 southwestern and northern Mexico *Vitis*, which included accessions collected from multiple collection trips from States bordering Mexico or that were previously collected from Mexico by Olmo. Both SSR (simple sequence repeat) and chloroplast markers were used to establish relationships with known sources of resistance currently being used in the breeding program (Riaz and Walker 2013). We found 20 highly resistant accessions and 14 of these were selected to develop small breeding populations by crossing to highly susceptible *V. vinifera*. In Spring 2016, we extracted DNA from the 704 individuals obtained from these breeding populations that were also greenhouse screened. We carried out a limited mapping strategy by utilizing markers from Ch 14 that are linked to the *PdR1* locus (See previous reports for details of the *PdR1* locus). This strategy allowed us to identify resistance sources whose resistance is similar to *PdR1* and sources that are different among the newly identified

accessions. We selected 12 SSR markers that cover a 3.5 Mb (megabase) region including the *PdR1* locus (Figure 1a), which is located between markers Pd82-1b4 and ORF18-19-03. The genotypic data of all resistant accessions with 22 markers from 19 chromosomes was used to analyze how genetically distinct the resistant accessions were from each other (Figure 1b).

Based on the polymorphic markers for each breeding population, a genetic map was created to determine the relative marker order and then QTL analysis for each population was carried out. The results from this study identified nine accessions with a major resistance locus within the genetic window where the *PdR1* locus from accession b43-17 was mapped. Results were not conclusive for two accessions A14 and b47-32 due to small population size or/and lack of polymorphic markers. The phenotypic data of three accessions, ANU67, b41-13, and T03-16 did not correlate with the resistance markers from chromosome 14. These three accessions were identified as candidates for further work to develop a framework map with larger populations to detect new unique loci for PD resistance breeding. The small breeding populations used in this study effectively identified the presence or absence of a major resistance locus. This approach is being used to enhance the PD resistant winegrape breeding program by rapidly identifying new resistance loci and broadening the genetic base of resistance.

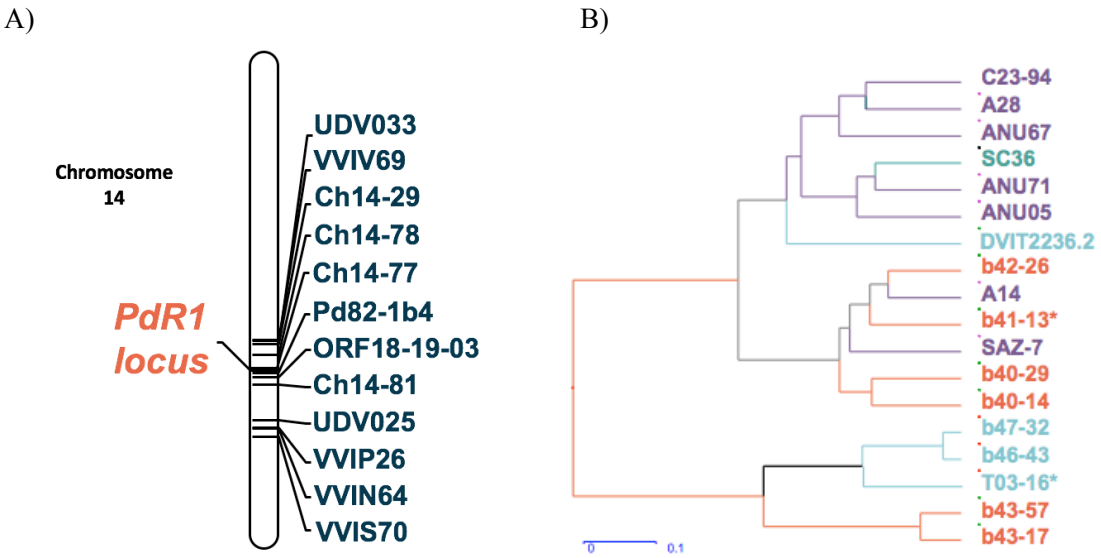


Figure 1. (A) 12 SSR markers that flank 3.5 Mb region on Ch 14. The *PdR1* locus resides between markers Pd82-1b4 and ORF18-19-03. (B) Dendrogram shows genetic relationship between resistant accessions; asterisks show PD resistant accessions with different source of resistance. Purple: accessions from AZ; Green: accessions from CA; Blue: accessions from Mexico; Orange: accessions from TX.

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*. These accessions have great potential for use in the PD grapevine breeding program. In order to proceed and to identify the genomic regions in these two accessions, crosses were made in Spring 2016 to expand population sizes. In three backgrounds, we were not able to determine if resistance is different than *PdR1* due to the small population size (Table 1). We plan to expand the number of individuals in those backgrounds, greenhouse test them for PD resistance and carry out analysis next year to determine whether they possess *PdR1*. These results will get us one step closer to finding a new mechanism of PD resistance that we can use in our breeding program. Table 1 presents the breeding populations that were developed with new resistance sources (For details, see previous reports). We completed propagation of 4-5 replicates for the subset

of crosses mentioned in Table 1. These plants will be inoculated with *X. fastidiosa* in September and the results of the assay will be available in Winter 2018.

Table 1. Resistant accessions used for the 23 breeding populations. Resistant accessions with different sources of resistance are marked as Not 14 in 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**	**Results of Limited mapping strategy**
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 with *V. monticola*	12-305, 14-308, 14-321, 14-322, 14-324, 14-336	159	LG14
b41-13	*V. arizonica-mustangensis* and *champinii* hybrid, red stem with hairy leaves	13-355	47	Inconclusive
b47-32	*V. arizonica* glabrous with *monticola*, small clusters, red stem	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, long cordate leaves, short wide teeth, small flower cluster	14-360	30	LG14
SAZ 7	*V. arizonica*	14-363	52	LG14

We have also identified a new locus *PdR2* in the *V. arizonica/girdiana* b42-26 background. To create a genetic map of the F1 population 05347 (F2-35 x b42-26), we expanded the population to 352 seedling plants and tested more than 1,000 markers. The level of polymorphism in b42-26 is very low likely because of its geographic isolation and resulting inbred genetic background. The genetic map was developed with 163 markers grouped to 17 chromosomes. Chromosome 10 and 19 were not represented. We carried out analysis with this map and identified resistance on Ch8, which was also verified on the basis of linked alleles in the pBC1 and pBC2 populations. The resistance locus is called *PdR2* and it resides between markers FAM82 and VMC 7h2. In Spring 2017, we began using closely linked markers to assist the breeding program with the use of MAS to stack the *PdR1*b and *PdR2* loci together. We are also testing more markers from Ch10 and 19 to get complete representation of the genome for the final genetic map and QTL analysis. A manuscript is approaching publication.

This project also provides molecular support to the companion PD resistance winegrape breeding project by marker testing seedling plants. In Spring 2017, we marker tested 1,895 seedling plants from 23 different crosses for *PdR1* and *PdR2* loci. A total of 1,380 seedlings were tested for both loci and 515 seedlings were tested for

PdR1 locus only. A total of 902 seedling plants from 14 different crosses were tested for veracity. In total, we extracted DNA from 2,797 seedling plants for different PD resistance breeding projects.

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

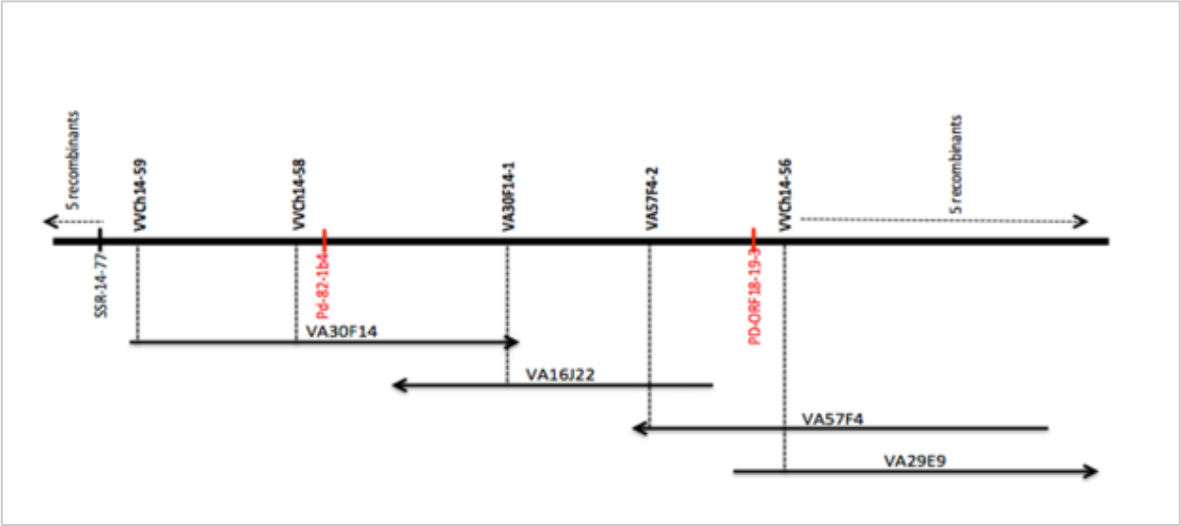
The SSR-based framework genetic map of *V. arizonica* b40-14 was completed. Greenhouse ELISA screen data was used to carry out QTL (quantitative trait locus) analysis and a major PD resistance locus, *PdR1c*, was identified on Ch14 (see previous reports for details). PD resistance from b40-14 maps between flanking markers VVCh14-77 and VVIN64 within a 1.5 cM interval. The genomic location of the *PdR1c* locus is similar to the *PdR1a* and *PdR1b* loci. An additional 305 seedlings were marker tested to identify unique recombinants using new SSR markers developed from the b43-17 sequence to narrow the genetic mapping distance. Four recombinants were identified between Ch14-81 and VVIn64, and one recombinant between the Ch14-77 and Ch14-27 markers. The new markers position the *PdR1c* locus in a 325 Kb (kilobase) region based on the sequence of b43-17.

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 two BAC clones VA29E9 and VA57F4 were selected. The DNA of the selected BAC clones was sequenced using PAC BIO RS II (see previous report).

A third BAC clone was sequenced to expand the region beyond the probe Ch14-58. The previous assembly consisted of two contigs with no overlap. Common probes between the *PdR1c* and *PdR1b* region were used to align the sequences in order to determine length of the gap in the assembly. A fourth BAC clone that overlaps with the VA30F14 and VA57F4/VA29E9 assembly was selected based on use of the new probes. Sequencing of this BAC clone was completed. New probes were designed using the sequence of *PdR1c* region to test for overlapping BACs. The assembly of four BAC clones is presented in Figure 2a and 2b represent the sequence analysis of *PdR1b* and *PdR1c* region. A manuscript entitled "The genetic and physical map of PD resistance locus, *PdR1c*" is in preparation

Figure 2. A) BAC library was developed from genomic DNA of b40-14 and screened with probes. Four overlapping clones were selected for sequencing the complete region. B) 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.

A)



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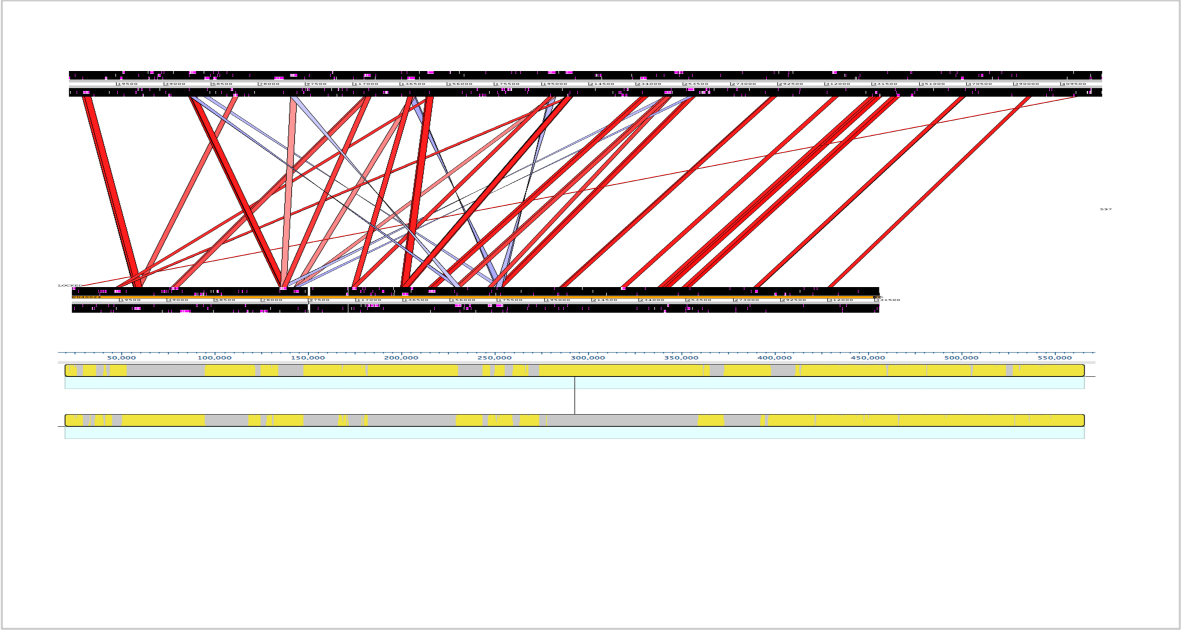
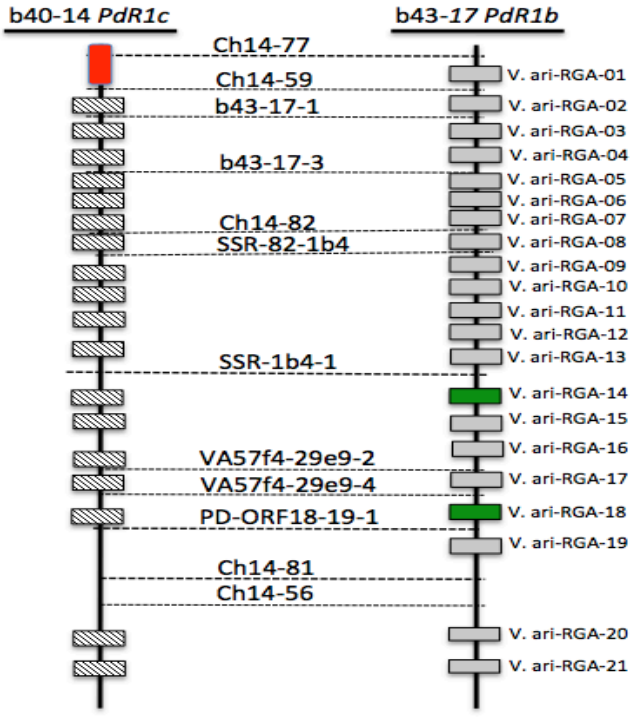


Figure 3. Sequence analysis of the *PdR1b* and *PdR1c* regions. In *PdR1c*, the assembled sequence is 426Kb. Two of the resistance genes are outside the genetic window with marker Ch14-81. The red regions represent the gap between the Ch1459 and Ch14-77 markers in the assembly.



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 over-

lapping BAC clone sequences that reflect two different haplotypes. The BAC clone H43I23 has ORF16 to ORF20 and all five ORFs have identical sequences to the *PdR1b* haplotype. Based on these results, we concluded that there is complete sequence homology between haplotype a, and b 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 reflects 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.

Objective 3. Employ whole genome sequencing (WGS) (50X) of recently identified PD resistant accessions and a susceptible reference accession; use bioinformatics tools to identify resistance genes, perform comparative sequence analysis and develop SNP markers to be used for mapping.

In this proposal and as detailed in previous reports, we proposed to use WGS to genetically map two new resistant accessions, b46-43 and T03-16, which have very strong *X. fastidiosa* resistance in repeated greenhouse screens. Next generation sequencing using Illumina HiSeq and MiSeq platforms to carry out SNP discovery and identification of SNP markers linked to resistance would only be used with those resistant lines for which we have strong greenhouse screen information, information on the heritability of their PD resistance, and the potential to screen the population using a limited mapping strategy.

The *V. arizonica* accession b46-43 is homozygous resistant to PD. Multiple crosses to *V. vinifera* were made to develop BC1 populations in 2014 and 2015. Breeding populations were tested with markers to verify the integrity of the crosses. Greenhouse screening of the BC1 populations with b46-43 and other resistant sources was completed (see companion project report) and results were used in conjunction with markers from Ch14 to evaluate the correlations between markers and resistance. Preliminary results indicate that there is a major PD resistance locus on Ch14. However, our breeding program has already identified two other accessions that have a major PD resistance locus on this chromosome. In order to optimize the development of broadly resistant PD winegrapes, we need to use PD resistance sources that map to different regions so that we have the greatest chance of stacking resistance genes from multiple and diverse sources. Test results suggest that b46-43 is not a unique source of PD resistance since it maps to the same location as *PdR1*, although it does have very strong resistance to *X. fastidiosa*. In the light of these results, we will not pursue WGS to map in the b46-43 background.

We completed the map of only Ch14 for the BC1 mapping population, completed screening in the greenhouse for 121 seedling plants. QTL analysis results indicated that locus explains only ~42% phenotypic variation indicating that there might be another locus on a different chromosome (Fig. 4)

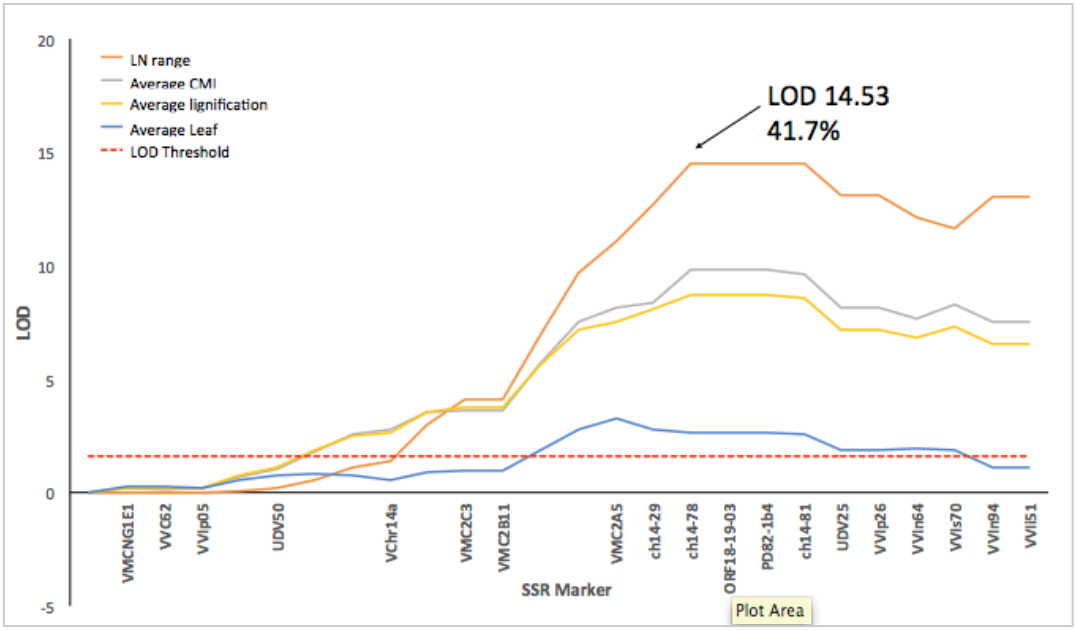


Figure 4. QTL analysis results of interval mapping of the pBC1 14399 population for Ch14. The arrow represents the maximum LOD for marker ch14-78 and the percent-explained variation for PD resistance. Red dotted line is LOD threshold for a significant QTL call. All mapped markers are on the x-axis

Figure 5 presents the correlation of different phenotypic parameters we have used to screen the pBC1 population. We are currently repeating the greenhouse screen and expanding the mapping effort to develop a framework map of all chromosomes to identify any other genomic region that contribute to the resistance.

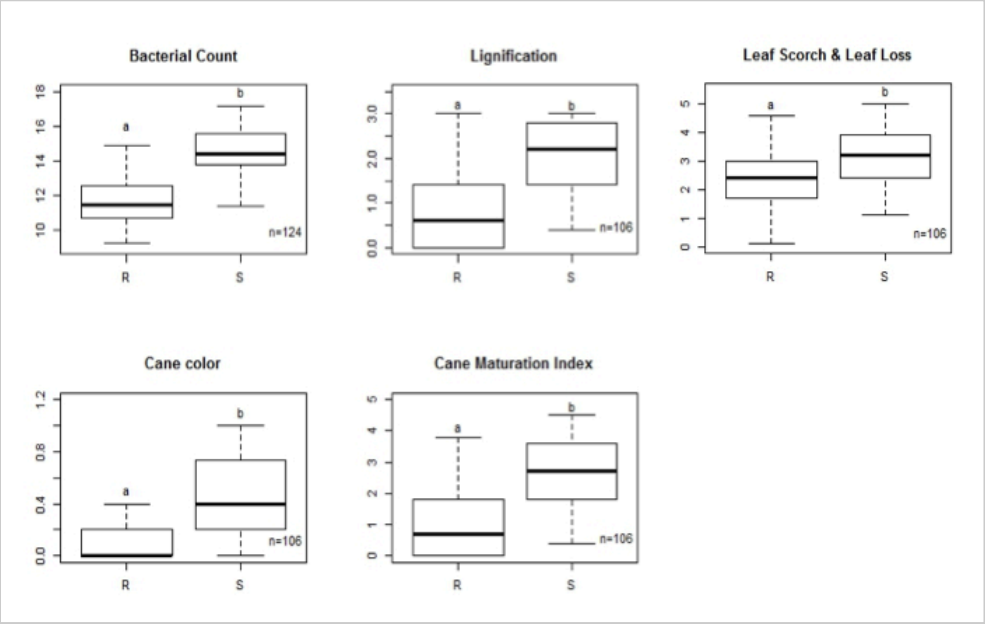


Figure 5. Comparison between resistant (R) and susceptible (S) genotypes in each measured phenotypic parameter. Significant differences with Tukey's test are indicated with letters a and b. The letter 'n' denotes the number of genotypes screened.

Objective 4. Cloning of *PdR1* genes with native promoters.

We employed PAC BIO RSII sequencing approach to sequence H69J14 and three other overlapping BAC clones containing both markers flanking the *PdR1b* resistance locus. The assembled sequence data generated a 604 Kb long fragment without any gaps. Multiple ORFs (open reading frames) of the Leucine-Rich Repeat Receptor Kinase gene family were identified. These genes regulate a wide range of functions in plants including defense and wounding responses for both host and non-host specific defense. 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. ORF sequences found outside the 82 Kb window are also highly similar. Two ORFs V.ari-RGA14 and V.ari-RGA18, within the resistance region boundaries, 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).

Both RGA14 and 18 (resistance gene analogs) have a very similar sequence profile except that RGA-18 is 2,946bp in size and lacks the first 252 bp of sequence that is part of RGA14. Functional analysis of the protein sequence of both RGAs revealed that RGA-14 lacks a signal peptide in the initial part of the sequence. 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. The results found that mature mRNA does not contain a signal peptide, necessary for proper membrane localization, at the beginning of the sequence thus leaving RGA-18 as the strongest candidate. Sequence verification for RGA14 and RGA18 and flanking sequences was completed and fragments that contain the entire coding region plus ~3 Kb upstream and ~1 Kb downstream sequences were synthesized and cloned into pCLB2301NK at Genewiz, Inc. pCLB2301NK is an optimized vector (Feechan et al. 2013), capable of carrying large DNA sequences, thus allowing us to insert the candidate genes plus surrounding sequences.

New plasmids, called pCLB2301NK-14 and pCLB2301NK-18, were verified by restriction analysis in our lab (Figure 6). Besides the corresponding 7 Kb fragment, containing RGA14 or RGA18, these plasmids contain a 35S:mGFP5-ER reporter cassette and a kanamycin-selectable marker gene with the NOS promoter.

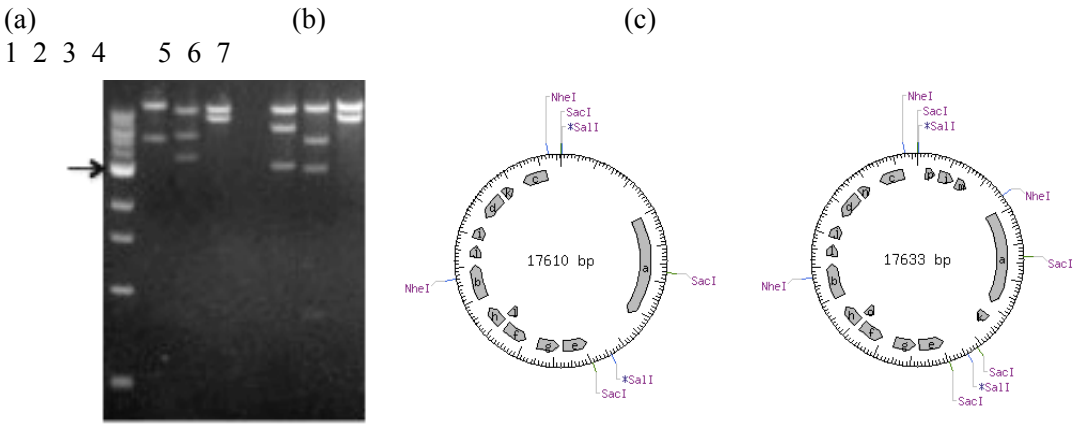


Figure 6. (a) Restriction analysis of plasmids pCLB2301NK-14 (lanes 2, 3, 4) and pCLB2301NK-18 (lanes 5, 6, 7) after digestion with Nhe1 (lanes 2, 5), Sac1 (lanes 3, 6) and Sal1 (lanes 4, 7). Gel image includes a 1Kb ladder (lane 1) with the 3 Kb fragment having increased intensity to serve as a reference band. The results on the gel match the predicted sizes inferred from the plasmid information; (b) pCLB2301NK-14 restriction map; (c) pCLB2301NK-18 restriction map.

We sequenced genotype U0505-22, which is used as a biocontrol in our greenhouse screenings. This genotype was originally selected for the presence of *PdR1b* markers in our breeding program. However, U0505-22 is susceptible to PD despite being positive for the markers, which then offers the opportunity to explore the changes that could explain this behavior at the DNA level. Primers were designed to produce 3 Kb fragments that include sequences upstream and downstream of RGA-14 or RGA-18 in order to increase the specificity of the amplification and facilitate cloning. Results obtained with U0505-22 showed the amplification of fragments of the predicted size, but with sequences that differ from RGA14 and RGA18 in several bases. On the other hand, sequencing of cDNA from b43-17, the original source of resistance, 16 days after inoculation resulted in the amplification of fragments with sequences identical to RGA-14 without the first the bp and to RGA-18 with a 500 bp deletion close to the 5' end.

A large experiment with resistant and susceptible plants using multiple replicates, and time points for control (un-inoculated) and inoculated plants (see details in previous report) was completed. To date, we have completed RNA extractions from 450 samples in the above-mentioned experiment. We have also designed primers and determined primer efficiency for gene expression studies with both RGA14 and RGA18. Two different primer pairs with efficiency of greater than 90% were selected to carry out preliminary analysis with un-inoculated and inoculated samples of Chardonnay and F8909-17 (source of *PdR1*). Preliminary results with samples from six time points indicates that the expression level of both RGA14 and RGA18 in F8909-17 increases after day 8 in comparison to un-inoculated, which peaks at day 23 and then decreases. Un-inoculated and inoculated susceptible Chardonnay did not show any expression. Gene expression and cDNA sequence analysis is underway.

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

We have established an *Agrobacterium* mediated transformation system followed by regeneration of plants from embryogenic callus. We have streamlined the protocol and have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless (TS), Cabernet Sauvignon, Chardonnay (CH) and the rootstock *V. rupestris* St. George (SG) (Agüero et al. 2006). In an earlier phase of this project, we transformed these varieties with five candidate genes containing the 35S cauliflower mosaic virus promoter, the nopaline synthase terminator and an *hptII*-selectable marker gene (see previous reports for details). We completed testing and found that the transgenic plants did not confer PD resistance or tolerance. These results are in accordance with the latest assembly obtained using PAC BIO SRII system. They show that only one of the sequences tested, V.ari-RGA-14, lays within the more refined resistance region of 82 Kb. The 3'RACE technique was used to amplify RNA from V.ari-RGA14 transformed grapevines and results showed that mature mRNA does not contain

the signal peptide, necessary for proper membrane localization, at the beginning of the sequence. However, this could result from a lack of effect of 35S on splicing.

In addition to the embryogenic calli of Thompson Seedless (TS), Chardonnay (CH), Cabernet Sauvignon (CS) and *Vitis rupestris* St. George (SG) we have available for transformation, we developed meristematic bulks (MB) of these genotypes plus 101-14 Mgt for transformation via organogenesis (Figure 7). Slices of MB can regenerate transformed shoots in a much shorter period of time than somatic embryos. We have tested different media and selective agents and established protocols for the initiation, maintenance and genetic transformation of MB from these 5 genotypes (Xie et al. 2016). Meristematic bulk induction in non-*vinifera* genotypes is less efficient but still high, with about 80% of the explants producing MB after 3 subcultures in medium containing increasing concentrations of cytokinins.

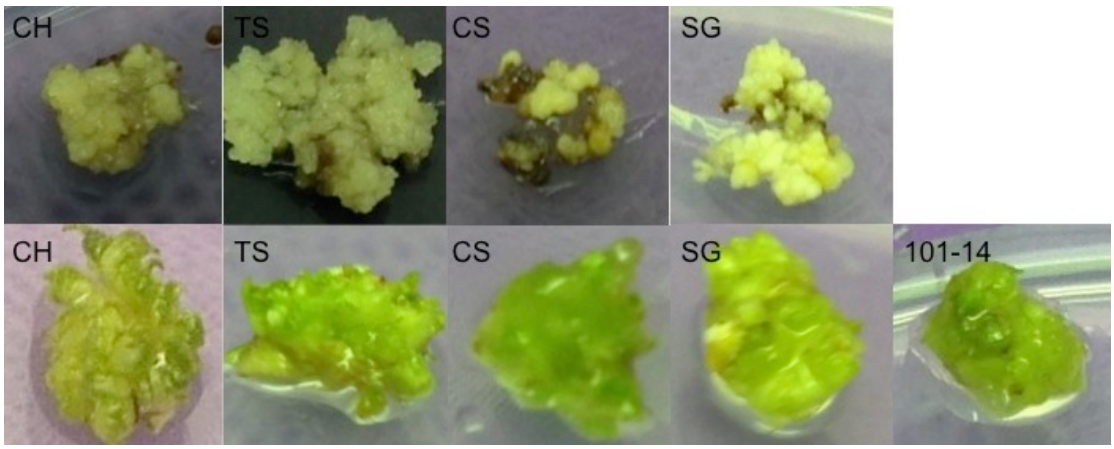


Figure 7. Embryogenic cultures (top) and meristematic bulks (bottom) of CH, TS, CS, SG and 101-14

In order to include native promoters and terminators in constructs for future genetic transformations, we verified sequences upstream and downstream of V.ari-RGA14 and 18, the two most likely *PdR1b* candidates. Sequence verification was completed up to 4-6 Kb in the upstream region and 1 Kb in the downstream region. In silico analysis of the upstream regions with PlantCare, a database of plant cis-acting regulatory elements showed that upstream sequences contain several motifs related to drought and defense responses.

Previous 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 mainly occurred in selection with kanamycin, confirming our previous observation that MB are highly sensitive to hygromicin. Thus, pCLB2301NK was chosen to carry RGA14 and RGA18 expanded sequences and named pCLB2301NK-14 and pCLB2301NK-18 thereafter.

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, Thompson Seedless and the rootstock *V. rupestris* St. George. Transformation experiments with pCLB2301NK-18 and pCLB2301NK-14 were initiated in March and July 2016 respectively, after synthesis and cloning was completed. In addition, *Agrobacterium* was used to transform meristematic bulks (MB) of PD susceptible genotypes selected from the 04-191 population, which are 50% *vinifera*, 25% b43-17 and 25% *V. rupestris* A. de Serres (as in the original population used for *PdR1b* mapping). 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 (Figure 8) and transformation experiments with *Agrobacterium* have been initiated.

Table 2 shows the number of independent lines regenerated up to date, while Figure 8-e shows the most advanced cultures growing in greenhouse. V.ari-RGA18 lines in the greenhouse were multiplied from green cuttings and

will be inoculated with *X. fastidiosa* in August 2017. It is expected that V.ari-RGA14 lines will be tested in November 2017. Lines in the greenhouse have tested positive for the presence of transgene by PCR. Transgene expression will be analyzed in petioles of infected plants 2 weeks after inoculation.

Table 2. Number of independent lines regenerated after transformation with Agrobacterium carrying pCLB2301NK-18 or pCLB2301NK-14.

Genotype	No. lines *in vitro*	No lines in greenhouse
pCLB2301NK-18		
Chardonnay	12	8
T. Seedless	30	11
St. George	3	-
pCLB2301NK-14		
Chardonnay	19	-
T. Seedless	18	-
St. George	3	-

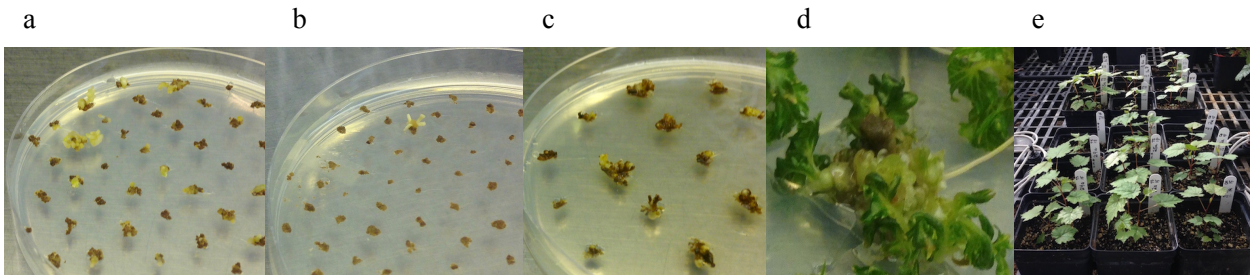


Figure 8. Embryo regeneration from embryogenic callus in (a) Thompson Seedless and (b) St. George. Shoot regeneration from meristematic bulks in (c) St. George. Meristematic bulk development in (d) genotype 47-50 from the 04-191 population. First independent lines transplanted into the greenhouse (e).

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. Our primary goal is to identify new sources of resistance that do not reside on Ch14 so we can facilitate stacking of these resistance sources with *PdR1* from b43-17, since the incorporation of multiple resistances should make resistance more durable. 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. This resistance source maps within the *PdR1b* locus, but it may be an alternative gene within this complex replicated locus. Finally, we verified the sequence of two candidate genes from the *PdR1b* locus, completed transformations with ORF18 and ORF14 and obtained transgenic lines for complementation tests in the greenhouse scheduled to start in June. This effort is also

identifying the promoters of these genes so that we can avoid the use of constitutive non-grape promoters like CaMV 35S.

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PRESENTATIONS

Talks at Grower Meetings (Extension/Outreach) August 2016 to July 2017

Grape breeding at UCD. Chilean table grape growers association, UCD Oct 3

Grape breeding above and below ground. Cal Poly San Luis Obispo, CA Oct 6

Grape breeding update. CDFA Industry Advisory Board, UC Davis, Nov 1.

PD resistant wines – lecture and tasting. Sacramento Private School Auction Prize, with Darrel Corti. UCD, Nov. 13

Breeding PD Resistant Winegrapes. Texas A&M, Driftwood, TX, Nov, 18

What are the next steps for the PD resistant wine grape breeding program? Vineyard Health Seminar, UCD, Nov. 29

PD Breeding program update. FPS Annual Meeting, UCD, Dec. 1, 2016

Progress in the Grape Breeding Program, Recent Advances in Viticulture and Enology, UCD, Dec. 9

Classical and molecular breeding to combat PD. CDFA PD / GWSS Board Annual Meeting, San Diego, CA, Dec. 13

The origin of grapes and grape breeding. 3rd Intl Symposium on Viticulture, Hermosillo, Mexico Jan 27

Origin of grapes and grape breeding. Daniel Roberts Grower Group Meeting, Santa Rosa, CA Jan 30

Breeding PD resistant winegrapes. Talk and tasting. DEVO Dept. Enology and Viticulture Student Organization, UC Davis, Feb. 12

Vineyard sustainability – rootstocks, irrigation, disease. Santa Cruz, Chile March 22

Breeding PD resistant winegrapes. Talk and tasting. Dept Vit & Enol. Executive Leadership Board, Santa Rosa, CA, May 5

The GRN rootstocks and breeding progress. San Pedro Winery, UC Davis, June 5

Breeding PD resistant winegrapes. Viticulture 101D.Adaptive Winemaking Extension Class, UC Davis, July 28

Presentations at Scientific Meeting

Fayyaz, L., S. Riaz and M.A. Walker. 2017. Map-based positional cloning of genes for powdery mildew resistance from the Chinese species *Vitis piasezkii*. ASEV National Meeting, Bellevue, WA, June 28

Huerta, K., S. Riaz and M.A. Walker. 2017. Evaluation of genetic diversity in wild *Vitis* material from northern and central Mexico. ASEV National Meeting, Bellevue, WA, June 28

Uretsky and Walker. 2017. A preliminary examination of taxonomic and geographic relationships among accessions of *Vitis berlandieri* and associated taxa. ASEV National Meeting, Bellevue, WA, June 28

Walker, M.A. 2017. The southwestern *Vitis*: a grape breeding motherlode. Merit Award lecture. ASEV National Meeting, Bellevue, WA, June 29

Walker, MA. 2017. Vinifera hybrids and resistance to Pierce's disease. Lecture and tasting. ASEV-East Annual Meeting Charlottesville, VA, July 12

RESEARCH RELEVANCE

The goal of this research is to understand the genetics of PD resistance and provide genetic support to our PD resistance breeding of wine grapes. We successfully mapped the resistance genes from a form of *V. arizonica* and used the linked markers to greatly expedite our breeding program. We are now searching for additional forms of PD resistance in other species from a variety of geographic locations across the southern US and Mexico, with the goal of combining resistance from several species together to ensure durable resistance.

LAYPERSON SUMMARY

We continue to identify and genetically characterize unique PD resistance sources from our southwestern US and Mexican *Vitis* species collections. 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 for resistance as soon as they sprout. We are developing these markers for different sources of resistance so that we can combine multiple resistance forms and therefore produce offspring with more durable resistance. These markers also allow us to identify resistance genes and engineer them into susceptible grapes, which we are doing to better understand the genes and the resistance.

STATUS OF FUNDS: These funds are schedule 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
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