CDFA PD/GWSS
Progress Report
March 2011

PROJECT TITLE: Map-based identification and positional cloning of *Xylella fastidiosa* resistance genes from known sources of Pierce's disease resistance in grapes.

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REPORTING PERIOD: The results reported here are from work conducted March 2010 to March 2011

LIST OF OBJECTIVES AND DESCRIPTION OF ACTIVITIES:

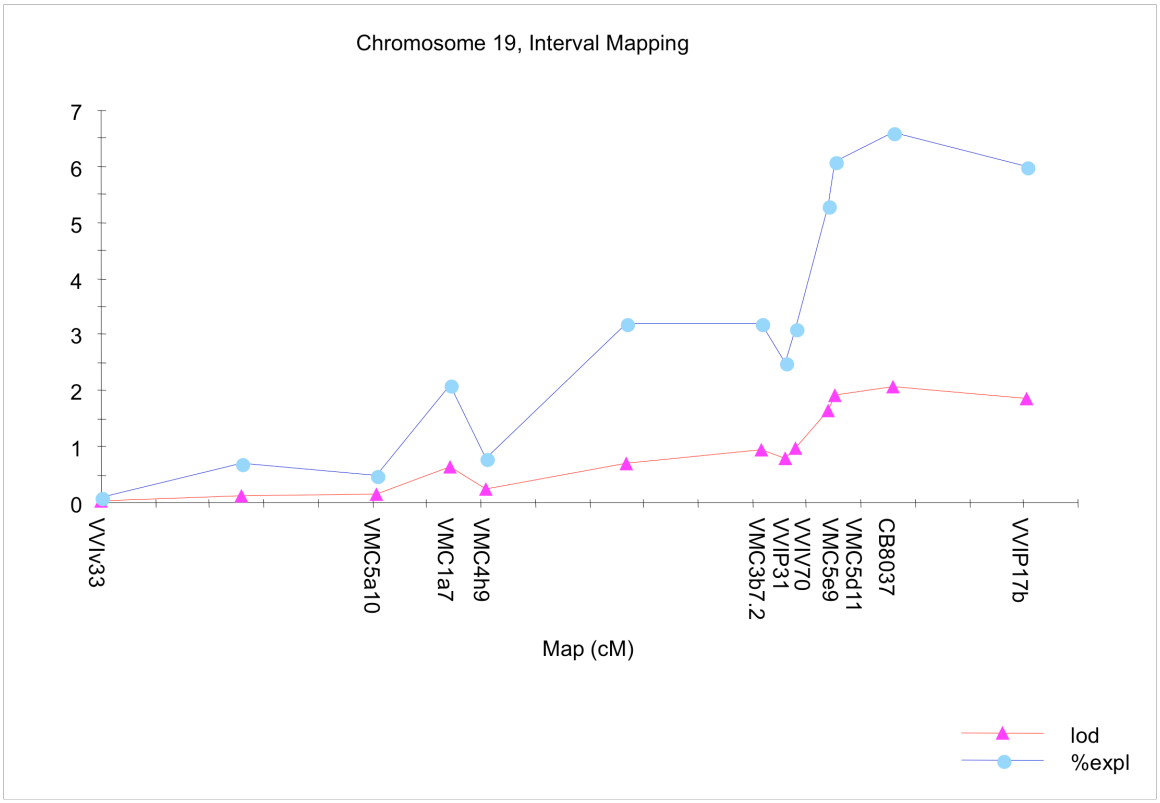
Objective 1. Characterize and refine the PD resistance locus on chromosome 14 by genetically mapping in four populations that derive resistance from *V. arizonica/candicans* b43-17 and its *V. rupestris* x b43-17 progeny F89090-08 (*PdR1b*) and F8909-17 (*PdR1a*): 04190 (*V. vinifera* F2-7 x F8909-08), 9621 (D8909-15 x F8909-17), 04191 (F2-7 x F8909-17), and 04373 (*V. vinifera* F2-35 x *V. arizonica/candicans* b43-17).

The genetic position of the *PdR1a* and *PdR1b* resistance loci has been refined between SSR markers VVCh14-56/02 and VVCh14-77 in the maps of two populations 9621 and 04190. These markers have unique allele sizes for the resistant parents that are not found in susceptible *vinifera* parents. This characteristic makes these markers very valuable for marker-assisted selection (MAS) in our grape-breeding program (see companion project report). The physical distance between these markers is 200-230 Kb, and they have been used to test additional plants and identify key recombinants critical to further refinement of the physical map. These rare recombinants document where cross-over events occur and allow more precise positioning of the resistance locus. We have 14 recombinants from more than 1,100 tested plants in the 9621 population and from MAS screening of populations based on resistance from F8909-17. We also have 14 recombinants from the screening of 15 different populations (1,000 plants) based on resistance from F8909-08. We greenhouse screened these recombinants and identified four key individuals. In two plants the recombination event occurred between *PdR1b* and VVCh14-56/02, and in one plant the recombination event occurred between *PdR1b* and VVCh14-77.

The above mapping efforts used the 9621 population (D8909-15 x F8909-17) whose female parent (D8909-15) is also resistant to PD, but its multigenic resistance may have a confounding effect on fine-scale mapping efforts. Thus, the 04191 population (*V. vinifera* F2-35 x F8909-17) is being mapped and is critical for the identification of minor genes that might contribute to PD resistance. We had previously mapped chromosome 14 in this population to verify PdR1 was correctly located, and then expanded the framework genetic map to all 19 chromosomes. A total of 139 SSR markers representing all 19 chromosomes were added to the set of 153 seedlings, of which 141 were greenhouse screened for resistance. Mapping analysis was carried out with 150 genotypes

after eliminating three seedlings that were off-types. Genetic map was constructed with 5.0 LOD and 0.40recombination frequency. 136 markers were grouped on 19 chromosomes (2n=38). QTL analysis was carried out with the natural log of the ELISA values. Two different methods of QTL detection were performed; Kruskal-Wallis and Interval mapping. We reconfirmed a major locus *PdR1a* on chromosome 14 and identified a minor QTL explaining 7% phenotypic variation on chromosome 19 that peaks at marker CB918037 (Fig.1). This is significant finding. Both major and minor QTL explains 82-86% phenotypic variation with 95% confidence interval.

Figure 1. Detail of QTL detected on chromosome 19 via interval mapping in 04191 population.



BLAT comparison of sequences of markers VMC5e9, CB918037, and VVIp17b to the Pinot noir 12X PN40024 genome identified the scaffold 14 region. Currently the distance between the flanking markers is 5.3 and 12.3cM, respectively. This distance corresponds to the 4,000 Kb on the physical sequence based on the alignment of the markers to PN40024. We are now refining this region by utilizing the PN40024 sequence to develop more SSR markers capable of reducing the genetic mapping gap to within 1cM. Seedlings with missing or equivocal phenotypic data and key recombinants will be greenhouse tested a second time. In addition, the 04190 population will also be genotyped with markers from chromosome 19 to determine if the F8909-08 haplotype also carries this minor QTL. We have observed that F8909-17 based genotypes tend to have lower *X. fastidiosa* levels. More work is required to validate and understand the complete genetic basis of PD resistance from the b43-17 background, and how and if minor genes play a role.

Objective 2. Genetically map PD resistance from other forms of *V. arizonica*: b42-26 (*V. arizonica/girdiana*) and b40-14 (*V. arizonica*).
In response to recommendations to broaden resistance, we are characterizing resistance from two additional sources. The goals are to identify additional resistance sources, genetically map them and use tightly linked molecular markers to pyramid resistance from different backgrounds into a single line. We are pursuing two other resistant *V. arizonica* forms: b42-26 *V. arizonica/girdiana* from Loreto, Baja California, and b40-14 *V. arizonica* from Chihuahua, Mexico. Although they are morphologically different than b43-17, they both posses strong PD resistance and greatly suppress *X. fastidiosa* levels in stem tissue after greenhouse screening.

Greenhouse screening data indicate that resistance from b42-26 is quantitative. A small breeding F1 population 05347 (*V. vinifera* F2-35 x b42-26) was produced in 2005. A subset of 48 genotypes was greenhouse screened – 35 were resistant and 13 were susceptible. A total of 337 SSR markers were tested on the parents and a few progeny. Results found a high level of homozygosity for b42-26 – only 113 markers were polymorphic, 184 markers were homozygous for b42-26, and 40 markers did not amplify. We remade this cross in 2008 and 2009 to increase the population size, and now 239 seedlings in the field. Sixty-four of these have been greenhouse screened, these tests are underway on 100, and the final 75 will be screened in 2011.

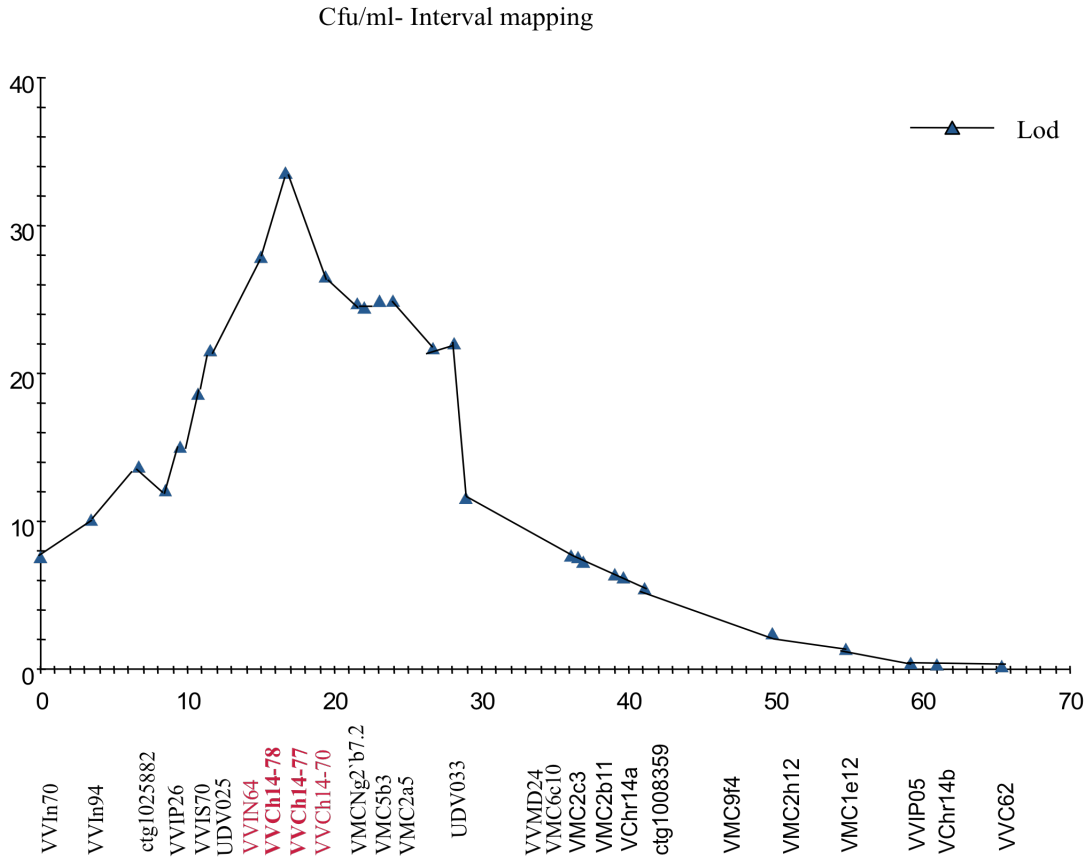
We completed preliminary QTL analysis with the 64 greenhouse screened genotypes with data from 71 SSR markers. A framework genetic map of all chromosomes was not possible with this limited amount of marker data, so we conducted Kruskal-Wallis analysis that allows association of each marker to the phenotypic trait. Because we know the chromosomes the markers reside we can get a rough map from this analysis. The results indicated that markers from chromosome 10 and 14 (and to a lesser extent 2 and 11) are associated with PD resistance. This allows us to focus mapping efforts on markers known to exist on these chromosomes, which will greatly accelerate the identification of genomic regions responsible for b42-26's resistance.

We are now mapping with the complete set of 239 seedlings with 30 to 50 markers on chromosomes 2, 10, 11 and 14, and 4 or 5 on the remaining chromosomes. As stated above, b42-26 is extremely homozygous, which means many of the SSR markers are homozygous and not useful for mapping. Thus, we developed 71 new SSR markers from clone sequences generated from the Vitis Microsatellite Consortium (the original source of SSR markers for grape). These clones had been discarded as not useful for marker development because of the presence of microsatellite repeats at the beginning or end of the sequence, leaving no room for primer design. A total of 238 of these clone sequences were compared to the nearly homozygous 12X Pinot noir genome in order to obtain additional flanking sequences. There were good matches for 71 of the clones and primers were designed for them; 69 of the newly designed primers amplified and 67 of them generated a clean banding pattern with *V. vinifera* DNA samples. We also acquired primer sequences of an additional 200 markers that have not been tested with b42-26.

Marker testing on small set of parents and progeny is underway. We are also adding markers to develop a framework map for the entire population.

Resistance in *V. arizonica* b40-14 seems to be controlled by a single homozygous dominant gene. As mentioned in previous reports 40 F1 progeny from a cross of *V. rupestris* x b40-14 were resistant to PD, while three progeny had intermediate responses. Two resistant siblings were used to develop the 07388 (R8918-02 x *V. vinifera*) and 07744 (R8918-05 x *V. vinifera*) populations. A summary of the genetic mapping and QTL analysis for the 07744 population is presented below: 227 markers were polymorphic for one of the parents; 152 were analyzed on the entire set of 122 plants; a framework map of R8918-05 was produced with MAP QTL (4.0) and the Kruskal-Wallis approach was used to complete the preliminary analysis. PD resistance mapped only on chromosome 14 – the same chromosome where *PdR1a* and *PdR1b* mapped. PD resistance from b40-14 (which we have named *PdR1c*) also maps in the same general region between flanking markers VVCh14-77 and VVIN64 and within 1.5 cM. The LOD threshold for the presence of this QTL was 33 and 82% of the phenotypic variation was explained (Fig. 2). In 2009, crosses were made with resistant F1 selections from 07744 population.

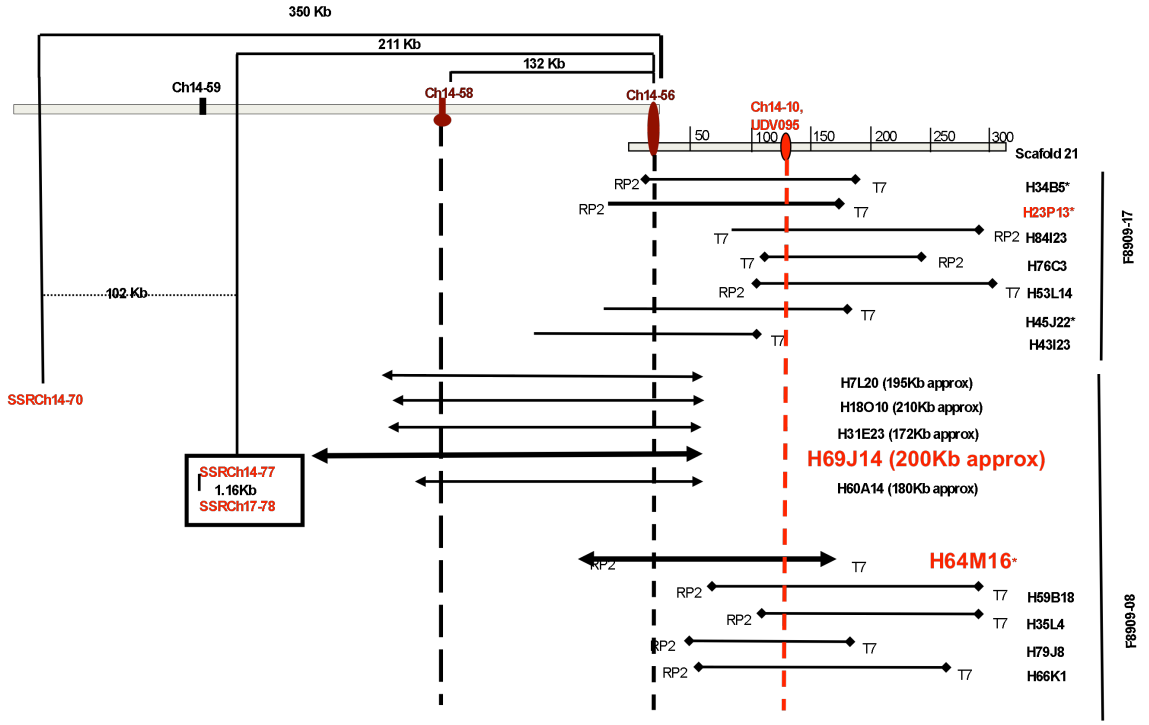
Fig. 2. Interval mapping of *PdR1* indicating a peak at LDD 34.0 with the 95% confidence interval. The X-axis indicates the position of the markers; LOD values are plotted on the Y-axis.



Objective 3 and 4. Develop a BAC (bacterial artificial chromosome) library for the homozygous resistant genotype b43-17 (parent of F8909-08, and F8909-17) and screen the library with closely linked markers. Complete the physical mapping of *PdR1a* and *PdR1b* and initiate the sequencing of BAC clones that carry *PdR1a* gene candidates.

Two BAC libraries were created from the homozygous resistant b43-17. Screening of the library with markers VVCh14-10, VVCh14-56 and VVCh-58, which are closely linked to *PdR1*, identified 41 positive clones – 4 of the clones were positive with VVCh14-10, VVCh14-56 markers (H23-P13, H34-B5 and H64-M16 and H45-J22) and 5 of them were positive with the VVCh14-56 and VVCh14-58 marker (Figure 3).

Figure 3. The arrangement of BAC clones and relative positions in comparison to the Pinot noir (PN40024) sequence. Three markers in red were used to screen the BAC library. The H69J14 clone has been sequenced via 454 and Sanger shotgun method. H64M16 clone has been sequenced by the Sanger method only. Both clones overlap for about 60Kb. These two clones represent the haplotype *PdR1b* region of b43-17.



Two clones were selected for sequencing with an overlap of 60Kb and a spanned region of 340Kb. A shotgun library of BAC clone H64M16 was Sanger sequenced. Clone H69J14 was selected for 454 sequencing. A total of 42,000 sequences were generated and two different programs were used to assemble the sequence. However, the sequenced region was highly enriched with repetitive elements, which complicated the assembly. Newbler software as well as the Lasergene program SEQMAN do not work well with sequences that contain many repeated regions. In order to generate longer sequence fragments, a shot gun library was constructed for clone H69J14; 384 sequences were generated in both directions to develop paired ends in order to fill the gaps between

the contigs from the 454 sequence data. We then masked the repetitive region from all the sequences (both H69J14 and H64M16 clones) to allow assembly with the MIRA assembler program. These steps improved the assembly, but the contig number was still too high for primer walking. Moreover, all the major contigs had masked repetitive regions on both ends indicating that the primer design effort would not generate sequence specific results capable of bridging the gaps. To overcome the repetitive elements and produce longer sequences, a Fosmid library was generated with an insert size of 35-40Kb. We are in the process of assembling the contigs using both fosmid and shotgun reads. Because the fosmid clones are 35-40Kb inserts, the paired ends will help us to determine the orientation and distance between different small size contigs.

The improved 12X assembly of the Pinot noir (PN400204) sequence recently became available, and greatly improves upon the 8X assembly. Detailed analyses based on the NCBI protein search identified four tandem repeats of serine threonine protein kinase with a leucine-rich repeat domain gene family in the resistance region (Table 1). The four genes in this region have large introns indicating that they may carry transposable element-like sequences. We utilized CENSOR software to screen query sequences against a reference collection of repeats to generate a report classifying all detected repeats (Table 1). All four genes carry DNA transposons as well as LTR retrotransposons documenting the complexity of the region.

Table 1. Details for the transposable elements, sizes of exons and introns in the four genes present in the Pinot noir (PN40024) 12X genome sequence correlated to the *PdR1* region of b43-17. The analysis of the sequence was carried out with CENSOR program, which compares the sequence to known repeat regions from *Vitis*, Maize, Wheat and Pine.

Gene ID (12X genome of PN40024)	**Size (bp)**	**# Exons and introns**	**Size w/o introns (bp)**	**Repeat class categories**	**Fragments**	**Length**
GSVIVT01033116001	7,729	6, 5	2,496	DNA transposon	1	294
GSVIVT01001802001	33,894	10, 9	3,360	Transposable Element	31	13,969
				DNA transposon	18	2,747
				EnSpm	3	485
				Harbinger	3	305
				Helitron	3	474
				MuDR	5	640
				hAT	1	408
				LTR retrotransposon	12	11,164
				Copia	3	406
				Gypsy	8	10,613
				Non-LTR Retrotransposon		
				L1	1	58

GSVIVT01001803001	11,310	10, 9	3,309	Transposable Element	8	2,453
				DNA transposon	7	2,182
				Harbinger	1	377
				MuDR	4	1,121
				hAT	2	684
				LTR Retrotransposon	1	271
				Gypsy	1	271
GSVIVT01001804001	12,165	11, 10	2,691	Transposable Element	9	2,778
				DNA transposon	8	2,520
				Harbinger	1	383
				MuDR	5	1,450
				hAT	2	687
				LTR Retrotransposon	1	258
				Gypsy	1	258

A direct comparison of the H69J14 clone sequence to the PN40024 sequence is not advisable because it is possible that the arrangement of repetitive elements is significantly different between the two genomes. When the b43-17 resistance region sequences were matched to scaffold 68 of the Pinot noir sequence, they matched to multiple sites and the level of similarity was reduced. These results suggest that the *PdR1* region of the b43-17 genome is divergent from PN40024 and that transposable elements may play a major role in these sequence differences. Our main emphasis is assembling the complete region in order to make comparisons to the susceptible sequence. This will help us understand the causes of sequence divergence and the evolution of the PD resistance gene family. Previous studies have indicated that the sequence of chromosome 14 is known to carry members of 13 different families of DNA transposons and retrotransposon (Moisy et al. 2008).

After we added the fosmid sequences and reassembled the region, we identified six candidate genes from the partial assembly of the H69J14 clone sequence. In a previous report, we described four candidate genes. Figure 4a presents the percent identity of the six candidate genes to each other. PdR1b-3 and 5 are 99.3% similar in sequence and PdR1b-1 and 4 are 99.6% identical; PdR1b-5 is 78% similar to the others. On the other hand PdR1b-6 is very unique. A large similarity of the gene sequence will be challenging for the gene expressions studies, as it will be difficult to design primers that amplify a specific gene and not the other family member. We are planning to employ nested PCR with the help of divergent restriction sites to overcome this issue. Figure 4b provides a comparison to the four genes identified from the Pinot noir sequence. The closest match with PN-2001 with the PdR1b-5 (83.4%)

4a.

Percent Identity

divergence

	1	2	3	4	5	6		
1		99.6	94.8	78.2	94.9	38.3	1	PdR1b-4.seq
2	0.4		94.9	78.2	95.0	38.2	2	PdR1b-1.seq
3	4.9	4.7		78.8	99.3	38.0	3	PdR1b-3.seq
4	25.5	25.4	24.0		78.5	38.2	4	PdR1b-5.seq
5	5.2	4.9	0.4	24.9		38.1	5	PdR1b-2.seq
6	138.5	138.8	138.5	137.1	138.6		6	PdR1b-6.seq
	1	2	3	4	5	6		

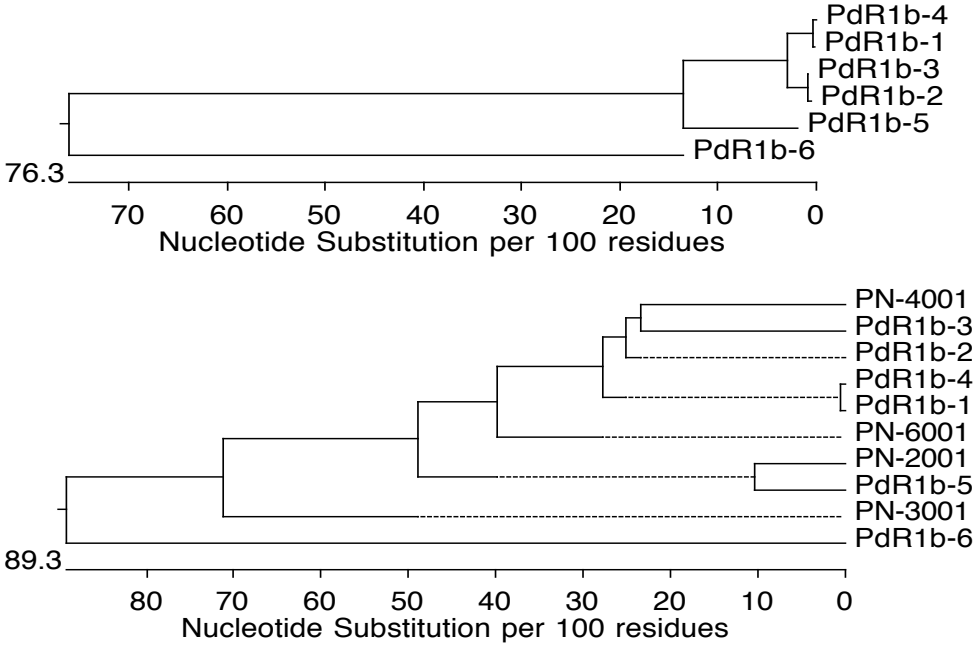
4b.

Percent Identity

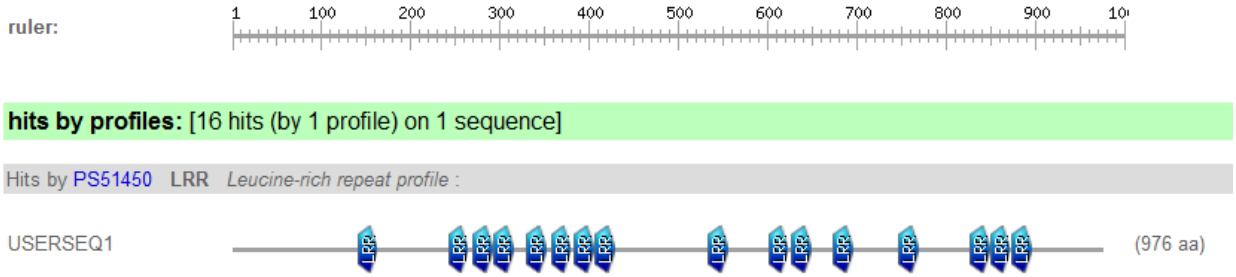
divergence

	1	2	3	4	5	6	7	8	9	10		
1		57.8	43.9	65.3	68.4	60.2	62.5	83.4	63.3	38.3	1	PN-2001.seq
2	62.7		39.1	56.4	59.3	53.7	55.9	62.4	56.1	36.5	2	PN-3001.seq
3	105.3	128.4		50.7	61.8	64.6	67.7	52.1	66.9	33.7	3	PN-4001.seq
4	47.1	66.3	81.5		78.7	78.7	77.8	69.3	78.9	37.2	4	PN-6001.seq
5	41.5	59.1	53.7	25.1		99.6	94.8	78.0	94.9	38.2	5	PdR1b-4.seq
6	57.2	72.5	48.1	25.2	0.4		94.9	78.1	95.0	38.3	6	PdR1b-1.seq
7	51.8	66.2	41.7	25.9	4.9	4.7		78.6	99.3	37.8	7	PdR1b-3.seq
8	18.5	52.1	76.3	39.3	25.8	25.7	24.3		78.3	38.3	8	PdR1b-5.seq
9	50.5	66.3	43.5	24.7	5.2	4.9	0.4	25.1		38.0	9	PdR1b-2.seq
10	136.4	144.2	166.7	145.3	138.9	138.2	139.6	134.7	138.5		10	PdR1b-6.seq
	1	2	3	4	5	6	7	8	9	10		

Fig 5. Alignment tree of the six candidate PD genes separately and with four genes of Pinot noir.



We utilized different tools from www.expasy.org/tool/ to conduct pattern and profile searches of the PD resistance genes. There is very strong evidence of a LRR region in five of the candidate genes. Following figure displays 16 LLR regions in PdR1b-1 gene.



There is no signal sequence in the protein sequence, which indicates that the resistance gene product is not secreted. There is also no indication of a coiled-coil, which suggests that the PD resistance gene is not a member of the CC-NB-LRR class of resistance proteins. The protein sequences do carry transmembrane domains, however they lack the kinase domain. Interestingly, the PdR1b-6 gene candidate, which was very different from the other candidates and from the Pinot noir genes, seems to have a protein Kinase domain. These are very interesting preliminary examinations of these gene candidates, but further work is needed.

Currently we are in the process of cloning 3 of the candidate genes and developing constructs for transformation experiments to determine which of these gene candidates confers resistance to PD. (See companion report "Molecular characterization of the putative *Xylella fastidiosa* resistance gene(s) from b43-17 (*V. arizonica*)).

INTELLECTUAL PROPERTY: The resistance genes identified in this research will be handled by PIPRA, UC Davis.

LAYPERSON SUMMARY

Genetic mapping from two different forms of *V. arizonica* has identified a region on chromosome 14 that is responsible for PD resistance, which we termed *PdR1*. We have mapped two forms of *PdR1* from *Vitis arizonica/candicans* b43-17, and have mapped a third form, *PdR1c*, that originated from *V. arizonica* b40-14. These forms are single dominant genes for PD resistance. Recently, we discovered a minor gene on chromosome 19 that is associated with *PdR1* and may be important in its regulation. We are also examining another resistance *V. arizonica/girdiana* b42-26, which is controlled by multiple genes. Fine-scale mapping is underway to determine if markers are tightly enough linked to these multiple resistance genes to be used for marker-assisted selection. At this point we have identified quantitative trait loci (QTLs) on chromosomes 10 and 14 and to a lesser extent on 2 and 11. We plan to combine these multiple resistance sources

in our breeding program to ensure broad and durable resistance to PD. Genetic markers to these forms of resistance will make this possible and allow the confirmation and tracking of interbred progeny. These mapping efforts are also essential to physically locating and characterizing PD resistance genes. At present, the chromosome region where *PdR1* exists has been sequenced and the pieces of sequence were arranged and compared to the Pinot noir genome sequence. This comparative analysis indicates that the susceptible Pinot region carries 4 genes. The Pinot noir region was compared to the sequences we have from the resistant b43-17 and we identified 6 candidate resistant genes. These candidates appear to be members of typical resistance gene families and we preparing them for transformation experiments to test whether they induce PD resistance in susceptible Chardonnay.

This project provides the genetic markers critical to the successful classical breeding of PD resistant wine, table and raisin grapes. Identification of markers for PdR1 has allowed us to reduce the seed-to-seed cycle to 2 years and attain the 97% vinifera PD resistant level. These markers have also led to the identification of 6 genetic sequences that house the PD resistance gene, which we will soon be testing to verify their function. These efforts will help us better understand how these genes function and could also lead to PD resistance genes from grape that would be available to genetically engineer PD resistance in *V. vinifera* cultivars.

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PRESENTATIONS ON RESEARCH:

California viticulture and problems. John Deere Corp. UC Davis visit, April 1, 2010.

Beneficial outcomes of the UCD grape breeding program. Monterey County Grape Day, Salinas, CA, Apr. 13, 2010.

Sustainable agriculture in the vineyard. UC Berkeley Haas Business School Course, Calistoga, CA Apr. 17, 2010.

UCD grape breeding program (rootstocks, PD and powdery mildew). Fosters/Beringer Grower Representatives and Technical Staff, Santa Rosa, CA Apr. 23, 2010.

UC Davis grape breeding program. Grape Growers and Wine Makers from Croatia, UC Davis, July 13, 2010.

UC Davis grape breeding program. University of Florida Graduate Students / ASHS visit, UC Davis, CA Aug. 10, 2010.

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Breeding PD resistant grapevines. Annual Pierce's Disease Research Symposium, San Diego, CA, Dec. 16, 2010.

Optimizing the breeding of wine grapes for resistance to powdery mildew. Current Wine and Wine Grape Research 2011, UC Davis, Feb. 24, 2011.