MAP-BASED IDENTIFICATION AND POSITIONAL CLONING OF *XYLELLA FASTIDIOSA* RESISTANCE GENES FROM KNOWN SOURCES OF PIERCE'S DISEASE RESISTANCE IN GRAPE

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Reporting Period: The results reported here are from work conducted August 2007 to October 2008.

ABSTRACT
This report presents updated results of refined mapping of the Pierce's disease (PD) resistance locus, *PdR1*, in the 04190 (397 plants) and 9621 population (433 plants) (both with resistance from *V. arizonica* b43-17). The *PdR1* locus is 0.38 and 0.97 cM from flanking markers in 9621 population and 0.39 and 0.23 cM in the 04190 population. Positioning of resistance locus has slightly changed, but marker-assisted screening and physical mapping efforts have not been affected. Between July 2007 and July 2008 two BAC libraries, each with one restriction enzyme (*Hind* III and *Mbo* I), were completed and we initiated the screening of the *Hind* III BAC library with flanking markers and identified 24 positive BAC clones. BAC end sequencing was carried out on 14 of BAC clones and complete sequencing of two clones (H23P13 and H64M16) that represented two haplotypes (either allele) of b43-17 was initiated. With the availability of new greenhouse screen data from both 9621 and 04190 populations and utilization of new SSR markers from chromosome 14 to refine marker data, the placement of *PdR1* locus was slightly shifted. The new flanking markers are VVCh14-56 and VVCh14-70, instead of VVCh14-56 and VVCh14-10. We then began using the Pinot Noir genome sequence to develop homologous markers that could be used for BAC library screening, and developed a set of nine markers (without SSR repeats) for BAC library screening. We are in process of isolating the BAC clones that represent VVCh14-70 end of the genomic sequence.

INTRODUCTION
Previous reports have described the mapping of resistance to *Xylella fastidiosa* (*Xf*) based on b43-17 in four (9621, 04191, 04190, and 04373) populations (Doucleff et al. 2004; Riaz et al. 2006). A complete report on the origin of the "89 group" seedlings, two of which, D8909-15 and F8909-17, are the parents of the 9621 population has been published (Riaz et al. 2007). The genetic mapping of the Pierce's disease resistance locus, *PdR1*, in three populations (04190, 04373 and 9621 populations) with emphasis on chromosome 14 and an explanation of segregation distortion mechanism was also completed (Riaz et al. 2008). A manuscript describing marker-assisted screening for breeding table and wine grape cultivars is in final revision (Riaz et al. 2009) and a report on the multigenic resistance from *V. arizonica* b42-26 is in preparation. We have recently refined the position of *PdR1a* and *PdR1b* in the 9621 and 04190 populations by increasing the number of recombinants that were generated from the ongoing wine and table grape breeding program and the addition of new markers. Greenhouse screening of 64 genotypes in the 04373 population showed that all plants are resistant verifying homozygous resistance in b43-17. We also completed the development of a BAC library from the resistant b43-17 and started BAC library screening with tightly flanking markers. Mapping populations were developed from b40-14 and b42-26 background to allow mapping of PD resistance from these two additional sources. Greenhouse screening of selected F1 genotypes containing b40-14 as a male parent verify b40-14 is homozygous resistant.

This report details three genetic maps (9621, 04190, and 04373), the initial screening of the 07744 and 04191 populations, the development of new markers from the Pinot Noir genome sequence, and most importantly the development, characterization and screening of the b43-17 BAC library. The initial physical mapping in conjunction with the genetic map is also reported and will lead to understanding how these resistance genes function.

OBJECTIVES
1. Completely characterize and refine the *Xf* resistance locus on chromosome 14 by genetic mapping in four populations 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* b43-17).
2. Study the inheritance of PD resistance from other genetic sources (b42-26 and b40-14).
3. Develop a BAC library for the homozygous resistant genotype b43-17 (parent of F8909-08 and F8909-17) and screen the library with closely linked markers.
4. Complete the physical mapping of *PdR1a* and *PdR1b* and initiate the sequencing of BAC clones that carry *PdR1a* gene candidates.

RESULTS AND DISCUSSION
Objective 1. As mentioned in the previous report, the resistant genotypes F8909-17 and F8909-08 inherited different sister chromatids (haplotypes) from the homozygous resistant parent b43-17. It was noted that F8909-08 has a 50 cM region in which marker segregation is distorted and the same markers are distorted in b43-17 indicating a region with suppressed

recombination. However, the same markers on the F8909-17 map were not distorted in this region (Riaz et al. 2008). This report presents an updated map of the 9621 population developed with 433 genotypes and additional markers. The genetic position of the *PdR1a* resistance locus is slightly shifted and it is between marker VVCh14-56 and VVCh14-70 (**Figure 1**). **Table 1** shows the key recombinants from this population. We also marker screened a total of 458 additional plants from the 9621 population with linked markers (VVIP26 and VMC2a5) and a subset of 49 recombinant plants were selected to greenhouse screen, which are key to fine-scale positioning of *PdR1*. Tightly linked markers were added to the set of recombinant plants and three key recombinants were detected. Greenhouse screen results will be available next year. The F8909-17 resistance source was also used in breeding PD resistant grapes and 24 recombinant plants were selected from five different crosses (180 plants) and are being greenhouse screened which result in updates of these genetic maps.

Previously, the 04190 population consisted of 361 progeny and *PdR1b* mapped between markers VvCh14-02 and UDV095/VvCh14-10 within a 0.4 cM distance. We have completed screening and mapping of 36 additional plants from this population. The position of *PdR1* moved from between VvCh14-02 and UDV095/VvCh14-10 to VvCh14-02 and VvCh14-28/VVCh14-29/VVCh14-30. These new markers were developed from the Pinot Noir genomic region that corresponded to the VMCNg3h8 clone sequence (**Table 2**). Using the cloned VMCNg3h8 sequence, we obtained a 99Kb contig and new markers were developed. In the previous published map, VMCNg3h8 was not polymorphic for the 04190 population and that genomic region was not represented. The new markers were added to the base population of 397 plants and map was updated (**Figure 1**). The greenhouse screen was repeated for key recombinants, which also helped refine the data. In addition, marker analysis discovered 23 recombinants from 15 different crosses (1000 plants) that contain F8909-08. These recombinant plants are in the process of being greenhouse screened. Data on these recombinants is critical for fine scale mapping, so greenhouse screens are repeated to rule out all possible mistakes.

A *V. vinifera* F2-35 x F8909-17 cross generated a fourth mapping population, 04191, of 153 progeny. This population provides genotypes with a 50% *vinifera* background for breeding and more recombinant plants for genetic mapping. It also provides a population where resistance from F8909-17 can be examined without possible confounding effects from D8909-15 (since D8909-15 has a multigenic resistance from b42-26). We added markers that are tightly linked to *PdR1* to this set, categorized resistant, recombinant and susceptible genotypes based on marker information, and selected recombinant genotypes based on flanking markers. This population will be critical for the identification of any minor genes that might contribute to resistance. Therefore, we are expanding the framework genetic mapping to all 19 chromosomes. For this purpose, we are initiating greenhouse screen of all 153 plants. The plants were propagated and results will be available by March 2009.

Objective 2. Thus far we have used three resistance sources (b43-17, b40-14 and b42-26 – **Table 3**). It is easier to breed with single locus traits as is the identification of genes using map-based positional cloning. Resistance from b43-17 is inherited as a single gene while resistance from b42-26 and its offspring D8909-15 is quantitatively inherited perhaps by multiple genes on multiple chromosomes. We initiated genetic mapping in the F1 population from the b42-26 background (05347 – **Table 1**). Greenhouse screening of a subset found 48 genotypes were resistant and 13 were susceptible. A total of 337 markers were tested on small a parental data set. Results found a high level of homozygosity for b42-26 (only 113 markers were polymorphic); 184 markers were homozygous for the male parent b42-26, 40 markers did not amplify. We completed 70 markers on a set of 64 genotypes and the remaining polymorphic markers are in process to develop a framework map. This set of 64 genotypes is not an adequate number for this mapping project, and this cross was repeated in Spring 2008 to produce at least 188 plants in the core population.

Previous results determined that *V. arizonica* b40-14 is a promising homozygous resistant genotype. We screened 45 genotypes from an F1 cross of *V. rupestris* x b40-14 and all were resistant except three genotypes with intermediate results. In Spring 2007, these resistant F1 genotypes were crossed to other susceptible and resistant genotypes to verify the single dominant gene mode of inheritance (07744 and 07386 – **Table 1**). We completed DNA extractions from 122 seedlings from 07744 and 105 seedlings for 07386. Marker testing is in the process to create a framework map of the 19 chromosomes, and polymorphic markers will be added to the 07744 population. Greenhouse screening results from these plants will be completed by March 2009. Initially framework genetic maps in F1 and BC1 populations will be developed utilizing 96 to 188 genotypes. Once the resistance locus and QTLs are localized, markers will be added to saturate the linkage groups where the resistance loci reside.

Objective 3 and 4. Two BAC libraries (each with a different restriction enzymes) from the homozygous resistant b43-17 were developed. Library screening was carried out twice with two markers (VVCh14-10 and VVCh14-56), both tightly linked to *PdR1*. This identified 24 positive clones– four of the positive clones were positive with both markers: H23-P13, H34-B5 and H64-M16 and H45-J22. The inclusion of new marker and greenhouse screen information moved the *PdR1* locus between markers VVCh14-56/VVCh14-02 and VVCh14-70 (**Figure 1 and Table 2**), which required the BAC library be screened to find the clones in the genomic region at the end of VVCh14-70 marker. The 14 positive BAC clones that were selected with flanking marker were amplified with marker VVCh14-56, which is polymorphic (with two alleles) for b43-17 and can be used to distinguish and group clones. In an attempt to develop more markers, we utilized the 695Kb region from the Pinot noir genome sequence that covers the marker VVCh14-56 and VVCh14-27/VMCNg2b7.2 (**Figure 1**). It is important to note that this region is from two different scaffolds (9 and 21). A total of 10 primers were developed that spread across 60 to 80Kb of the 695Kb sequence from Pinot noir. Nine of these markers amplified successfully (**Figure 2**). We also developed SSR markers from this region that were placed in between the screening markers (**Figure 2**). Currently the resistance locus resides between Ch14-56 and Ch14-70; a physical distance of 340Kb. Based of the genetic map from 9621 population, the physical and genetic distance correlates as 1cM is equal to about 216Kb. Based on the previous reported position of the *PdR1* locus, we initiated shotgun sequencing of the H23P13 and H64M16 clones. These clones represent two haplotypes of b43-17. A total of 173Kb region of H23P13 clone was assembled after primer walking. Given the new position of *PdR1,* we will be able to utilize the 35Kb region that spans the region from marker VVCh14-56 and beyond, and rescreen the BAC library.

CONCLUSIONS

Results from this project have allowed us to: 1) understand the segregation of PD resistance in two different backgrounds; 2) develop a framework genetic map for *Xf* resistance; 3) select markers for effective marker-assisted selection (MAS) in grape breeding; and 4) begin development of a physical map of genomic fragments that carry the *PdR1* locus, leading to map-based positional cloning of PD resistance genes. MAS has allowed the generation of PD resistant BC3 progeny with 94% of their parentage from elite *V. vinifera* wine grapes in a dramatically shortened time period. We have also constructed a BAC library for b43-17 to isolate the *PdR1a* gene candidates. In order to expand the range of PD resistances by exploiting other resistant accessions, we are studying the inheritance of PD resistance in two other backgrounds. Resistance in *V. arizonica* b40-14 is inherited as a single gene. We are using quantitative trait loci (QTL) analysis in the 0023 and 05347 populations to study PD resistance from *V. arizonica* b42-26 whose resistance is controlled by several genes. The genetic mapping, placement of a variety of resistance genes/traits will allow MAS to broaden resistance and make it more durable. Map-based efforts will also enable us to characterize the variants of PD resistance genes.

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FUNDING AGENCIES

Funding for this project was provided by the CDFA PD/GWSS Board. Supplemental funding from the Louis P. Martini Endowed Chair in Viticulture is also gratefully acknowledged.

Table 1. The key recombinants from the 9621 population. The genotypes in bold red font are key recombinants with a recombination event between the marker and the *PdR1a* resistance locus. "0" indicates a susceptible allele and "1" indicates a resistant allele.

9621 Genotype	UDV095	A0101	VVCh14-56	*PdR1a*	VVCh14-70	VVCh14-29	VMCNg 3h8	VMCNg 2b7.2
-36	0	0	0	0	0	1	1	1
-45	0	0	0	0	0	1	1	1
-259	0	0	0	0	0	1	1	1
-320	0	0	0	0	0	1	1	1
-363	0	0	0	0	0	1	1	1
-376	0	0	0	0	0	1	1	1
-400	0	0	0	0	0	1	1	1
-416	0	0	0	0	0	1	1	1
-426	0	0	0	0	0	1	1	1
-470	0	0	0	0	0	1	1	1
-08	0	0	0	0	1	1	1	1
-194	0	0	0	0	1	1	1	1
-554	0	0	0	0	1	1	1	1
-629	0	0	0	0	1	1	1	1
-28	0	0	0	1	1	1	1	1
-38	0	0	0	1	1	1	1	1
-15	-	1	1	1	1	0	0	0
-23	1	1	1	1	1	0	0	0
-31	1	1	1	1	1	0	0	0
-337	1	1	1	1	1	0	0	0
-345	1	1	1	1	1	0	0	0
-360	1	1	1	1	1	0	0	0
-337	-	1	1	1	1	0	0	0
-397	1	1	1	1	1	0	0	0
-409	1	1	1	1	1	0	0	0
-428	1	1	1	1	1	0	0	0
-505	-	1	1	1	1	0	0	0
-595	1	1	1	1	1	0	0	0
-697	1	1	1	1	1	0	0	0

Table 2. List of new markers that were developed from Pinot noir genome sequence and were utilized on 4 different populations.

Name	PN contig id	New marker	Amp size	04190	9621	04373	04191
A010	VV78X214158.8	VVCh14-02	170	Y	Y	N	Y
		VVCh14-56			Y		Y
UDV095	VV78X004565.11	VVCh14-09	170	Y		Y	
		VVCh14-10	210	Y	N	Y	N
VMCNg2b7.2	VV78X072246.8	VVCh14-27	193	Y	Y	Y	Y
VMCNg3h8	VV78X190796.4	VVCh14-28	167	Y	Y	Y	Y
		VVCh14-29	200	Y	Y	N	Y
		VVCh14-30	206	Y	Y	N	Y
		VVCh14-70	193	Y	Y	N	Y

Table 3. Parentage and species information for populations and genotypes being used to map PD resistance.

Population / Genotype	Species / Parentage
b42-26	*V. arizonica/girdiana*
b43-17	*V. arizonica/candicans*
b40-14	*V. arizonica*
D8909-15	*V. rupestris* A. de Serres x b42-26
F8909-08 and F8909-17	*V. rupestris* A. de Serres x b43-17
F2-7 and F2-35 (females)	*V. vinifera* (Carignane x Cabernet Sauvignon)
9621	D8909-15 x F8909-17
0023	F8909-15 x *V. vinifera* B90-116
03300/5	101-14Mgt (*V. riparia* x *V. rupestris*) x F8909-08
04190	F2-7 x F8909-08
04191	F2-7 x F8909-17
04373	F2-35 x b43-17
05347	F2-35 x b42-26
07744	R8918-05 x *V. vinifera* Airen
07386	R8917-02 x *V. vinifera* Airen

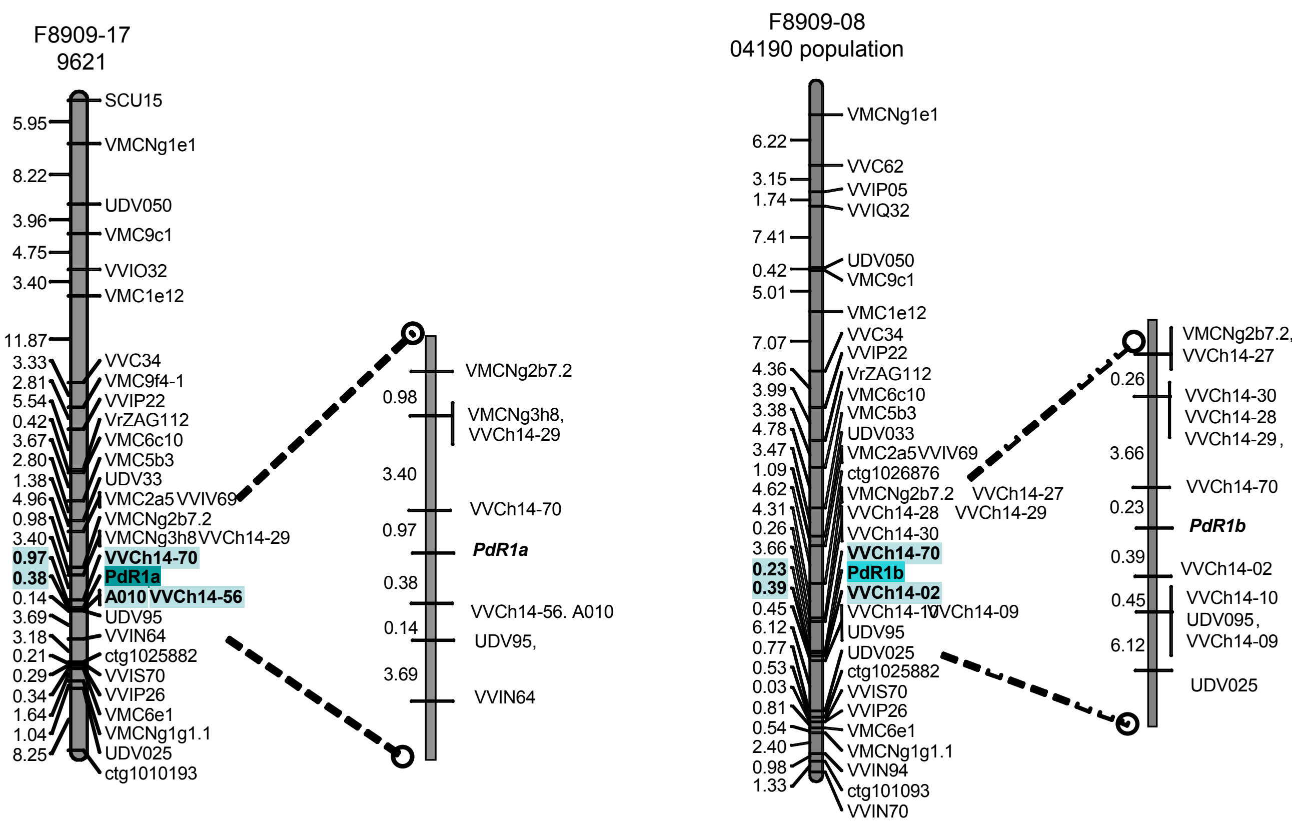

Figure 1. SSR-based genetic map of chromosome 14 from the 9621 (left) and 04190 (right) populations.

Fig. 2. Detail of physical map of chromosome 14 for the region that harbor resistance to PD. Preliminary screening was carriedout with Ch14-10 Ch14-56. Currently the PdR1 locus reside between Ch14-56 and Ch14-70. Five clones are also positive with screening marker Ch14-58 (in green). Only Ch14-70 marker was polymorphic and could be mapped in three populations.