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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ABSTRACT
This report presents updated results on the refined mapping of the Pierce's disease (PD) resistance locus, *PdR1*, in the 04190 (397 plants) and 9621 population (900 plants). In both populations, the resistance locus is within a 1cM distance. The flanking markers VVCh14-78 and VVCh14-81 were added to key recombinant plants from both populations and greenhouse screening was repeated to avoid any error. Genetic mapping was initiated in the 07744 (resistance from *V. arizonica* b40-14), 04191 (resistance from F8909-17 *PdR1a*) and 05347 (resistance from *V. arizonica/girdiana* b42-26) populations. A total of 152 markers were completed for 07744 to develop the framework map and greenhouse screening of the 07744 population is complete and underway for the 04191 population. In 07744, preliminary analysis indicated that PD resistance (*PdR1c*) resides on chromosome 14, in the same region where *PdR1a* (resistance from F8909-17) and *PdR1b* (resistance from F8909-08) mapped from the b43-17 background. However, the SSR alleles for resistance are very different between b43-17 and b40-14. Between October 2007 and March 2009 two BAC libraries, each with one restriction enzyme (*Hind* III and *Mbo* I), were completed and the screening of the *Hind* III BAC library with flanking markers was initiated. The Pinot noir genome sequence was used to develop markers to screen the BAC library, and these SSR markers were used to reduce the physical distance to *PdR1*. Two screenings of the libraries identified 24 (with markers VVCh14-56 and VVCh14-10) and 17 positive BAC clones (with marker VVCh14-58). Complete sequencing of two clones (H23P13 and H64M16), representing the two haplotypes of b43-17 was completed. Five clones were positive with VVCh14-56 and VVCh14-58. Clone 'H69J14' (which is bigger than 200Kb) was selected for 454-based sequencing. This clone spans scaffold 21 and nine of the Pinot noir genome sequence. A total of 42,000 sequences were assembled with the help of two different assembly programs. The DNASTAR program was used to obtain assembly at 99% stringency, and yielded more than 79 contigs larger than 5Kb in size. Primers will be designed from the ends to improve the assembly with BAC walking by filling the gaps and verifying the sequences on the ends of contigs. Assembled sequence will be used for the identification of resistance gene(s).

LAYPERSON SUMMARY
Genetic mapping efforts have identified a Pierce's disease (PD) resistance region on chromosome 14 termed *PdR1*, which originated from *Vitis arizonica/candicans* b43-17. This resistance acts as a single dominant gene and we have mapped the two forms from the homozygous parent – *PdR1a* from F8909-17 and *PdR1b* from F8909-08. We have also mapped another form of *PdR1* from *V. arizonica* b40-14, and are examining how the multi-gene PD resistance from *V. arizonica/girdiana* b42-26 maps and relates to *PdR1*. In the future these multiple resistance forms will be combined in our PD breeding program to ensure the strongest resistance possible. The combination of these forms of PD resistance can only be done with the tightly linked genetic markers discovered in these mapping efforts so that the combination of the various forms of resistance can be confirmed in the interbred progeny. These mapping efforts are also essential to physically locating and characterizing PD resistance genes. At present, the chromosome region that *PdR1* exists on has been sequenced and these pieces of sequence are being arranged and compared to the Pinot noir genome sequence and that of other plants to characterize their function and determine which are likely to be involved in PD resistance.

INTRODUCTION
This project continues to genetically map Pierce's disease (PD) resistance in forms of *V. arizonica* (Riaz et al. 2007). These efforts are closely linked to a breeding program focused on developing PD resistant winegrapes (see companion report). The breeding program produces and greenhouse screens the seedling populations upon which this genetic mapping program depends. While the tightly linked genetic markers generated in these mapping efforts are used to optimize and greatly accelerate the PD breeding program (Riaz et al. 2009). These markers are also essential to the successful introgression of resistance from multiple sources, and thus for the production of broader and more durably resistant grapevines (Riaz et al. 2008a). Genetic maps associate DNA markers with phenotypic traits, and allow the linking of these traits with markers positioned relative to each other on linkage groups, which since the sequencing of the Pinot noir genome, are now known to be chromosomes. Fine scale mapping of given regions and careful screening of recombinant progeny (those with a given genetic marker but without resistance, or vice versa, because of a recombination event) is critical to the identification of relatively short genetic regions that can then be sequenced so the genes responsible for PD resistance can be characterized and their function studied (Riaz et al. 2008b).

OBJECTIVES

1. Completely 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).
2. Genetically map PD resistance from other forms of *V. arizonica*: b42-26 (*V. arizonica/girdiana*) and b40-14 (*V. arizonica*).
3. 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.
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. We previously reported that the genetic position of the *PdR1a* resistance locus was between marker VVCh14-56 and VVCh14-70. In the past three months, we have developed three additional SSR markers derived from Pinot noir genome sequence that allowed us to narrow down the physical distance from 300Kb to 200 Kb. These markers (VVCh14-77, VVCh14-78 and VVCh14-81) were added to the composite set of recombinants from the 9621 population as well as to the resistant and susceptible parents used for crosses in 2008 to determine if the resistance allele is unique and not present in susceptible selections. The resistance allele was unique in size, which made these markers very valuable and robust for marker-assisted screening. There are three key recombinants from the tested set of more than 900 plants. For two plants, the recombination event happened between VVCh14-78 and *PdR1a*, and other plant had a recombination event between VVCh14-81 and *PdR1a*. With the addition of new markers, the *PdR1a* locus is within a 1cM window and it completely correlates to the physical distance between the markers that were developed from Pinot noir genome sequence.

F8909-08 possesses the *PdR1b* resistance locus, which is being mapped in the 04190 population. Previously we reported that *PdR1b* maps between VvCh14-02 and VVCh14-70. Additional markers (VvCh14-28/VVCh14-29/VVCh14-30) were added to the entire set of 397 plants in the 04190 population. The greenhouse screen was repeated for key recombinants, which also helped refine the data. In addition, marker analysis identified 14 recombinants from 15 different crosses (1,000 plants) based on resistance from F8909-08. We completed the greenhouse screen on 35 recombinants (including seedlings from *PdR1b* background crosses). The screen identified four key recombinants: in two plants the recombination event occurred between *PdR1b* and VVCh14-02; and in one plant the recombination event occurred between *PdR1b* and VVCh14-70. The greenhouse screen is being repeated for four other recombinants that had inconclusive first test results. In the most updated map, we have placed the *PdR1b* locus between markers VVCh14-81 and VVCh14-78 (**Table 1**). Both of these markers are less than 200Kb apart from each other based on the Pinot noir genome sequence.

The 04191 population (*V. vinifera* F2-35 x F8909-17) has 153 progeny and a population where resistance from F8909-17 (*PdR1a*) can be examined without possible confounding effects from D8909-15 (since D8909-15 has a multigenic resistance from b42-26). The resistance locus *PdR1a* is mapped in the 9621 (D8909-15 x F8909-17) population, and the 04190 population mentioned above, and refined mapping focused only on chromosome 14. The 04191 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, and the 153 plants in 04191 will be greenhouse screened. Greenhouse results will be available in Spring 2010 and the framework map will follow.

Objective 2. Previous mapping and greenhouse screen data from the 0023 population (D8909-15 x *V. vinifera*) with resistance from *V. arizonica/girdiana* b42-26 found that PD resistance is quantitative. The 05347 population (b42-26 x *V. vinifera*) was created to better study this resistance source. A total of 337 markers were tested on a small 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. A total of 70 markers were added on a set of 64 progeny, and many will have marker data soon. The current population size is 165 and crosses were made to increase the population size this Spring. Greenhouse screening results will be available in Summer 2010, and a framework map will be developed on a core set of 165 seedlings; this population is being expanded for the future mapping of this quantitative PD resistance trait.

Vitis arizonica b40-14 is a third promising resistance source with PD resistance that seems to be homozygous and controlled by a single dominant gene. Previously, we reported that all F1 progeny from a cross of *V. rupestris* x b40-14 (the R8918 population) were resistant except three genotypes with intermediate results. Two resistant siblings were used to develop two populations: 07388 (R8918-02 x *V. vinifera*) and 07744 (R8918-05 x *V. vinifera*). We completed DNA extractions from 122 seedlings of the 07744 and 105 seedlings from the 07386. A total of 277 markers were polymorphic for one or the other parent in preliminary marker screening. One hundred fifty two polymorphic markers were completed on the entire set of 122 plants in the 07744 population. Mapping analysis was carried out on each parent separately. The framework map of R8918-05 was produced with 152 markers on 121 genotypes with JOINMAP (3.0). Only three markers were unlinked and the remaining 149 markers were grouped into the expected 19 chromosomes. QTL analysis was performed with MAP QTL (4.0)

and the Kruskal-Wallis approach was used to complete the preliminary analysis. No association with PD resistance was found on any other chromosome except 14 – the same chromosome where *PdR1a* and *PdR1b* map. PD resistance from b40-14 (which we have named *PdR1c*) also maps in the same general region between flanking markers VVCh14-78 and VVIN64 and within 1.5 cM. The LOD threshold for the presence of this QTL was very high (**Table 2**). Next, interval and MQM analysis will be carried out after the selection of markers as cofactors, to determine the level of variance contributed by this b40-14 based resistance locus.

Objective 3 and 4. Two BAC libraries (each from different restriction enzymes) created from the homozygous resistant b43-17 were developed. In the first phase of the project, library screening was carried out with markers (VVCh14-10 and VVCh14-56), both tightly linked to *PdR1*. This process identified 24 positive clones – four of the clones were positive with both markers: H23-P13, H34-B5 and H64-M16 and H45-J22. New markers (both SSR and non-repetitive) were developed from the 695Kb region from the Pinot noir genome sequence covered by markers VVCh14-56 and VVCh14-70/77/78 (see previous reports). This region overlaps two different scaffolds from the Pinot noir genome sequence (9 and 21). Currently, *PdR1* is placed between Ch14-81 and Ch14-78 at a physical distance of ~200Kb. Based on the genetic map from the 9621 population, the physical and genetic distance correlates because 1cM is equivalent to about 216Kb. The second round of BAC library screening was carried out with the Ch14-58 marker. A total of 17 clones were positive, five of them were also positive with the VVCh14-56 marker (see details in previous report). Clone H69J14 was selected for 454 sequencing. A total of 42,000 sequences were generated. Two different programs were used to assemble the sequence. The DNASTAR program seqman allowed assembly at a stringency level of 99%, which generated more than 4,000 contigs representing 38,000 sequences. A total of 79 sequences were bigger than 5 KB. **Table 3** presents the contigs that are bigger than 6 Kb and have sequence similarity to the Pinot noir genome sequence (**Figure 1**). It is important to note that the sequence similarity to scaffold 21 of Pinot noir was almost 98% identical for most of the contigs, however, the b43-17 sequences that overlap with scaffold nine of Pinot noir matched to multiple sites and level of similarity was less. This result suggests that either the b43-17 genomic region with the PD resistance gene(s) is divergent from Pinot noir, or the 8X assembly of Pinot noir's scaffold 9 has lots of gaps and errors. The 12X coverage of Pinot noir genome would be more helpful to conduct meaningful sequence comparison. In the next step, primers will be designed to fill the gaps, improve the coverage and to verify the sequences at the ends of the contigs.

CONCLUSIONS

Genetic mapping efforts have identified valuable genetic markers for marker-assisted selection and enabled rapid progress towards PD resistant winegrapes (see companion report). These mapping efforts have now identified three alleles of *PdR1*: *PdR1a* and *PdR1b* derived from *V. arizonica/candi*cans b43-17; and *PdR1c* derived from *V. arizonica* b40-14. These alleles were found to map within the same general region, but suggest that although PdR1 seems to be a single gene trait, the region may be composed of a number of tightly linked genes. BAC library sequence analysis of b43-7 is resulting in candidate genes suggestions for *PdR1* and these are being compared to the Pinot noir genome sequence and to similar regions in other plants. The genomic characterization of this region will help us determine how this form of PD resistance functions and which genes control it.

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Table 1. Key recombinants from the 9621 (*PdR1a*) and 04190 (*PdR1b*) populations. The genotypes in bold red font are key recombinants with a recombination event between the marker and the resistance locus. "0" indicates a susceptible allele and "1" indicates a resistant allele.

Genotypes with *PdR1a* background	A010	VVCh 14-56	VVCh1 4-81	*PdR1a*	VVCh 14-78	VVCh 14-77	VVCh 14-70	VVCh14 -29	VMCNg2 b7.2
-416	0	0	0	0	0	0	0	1	1
-426	0	0	0	0	0	0	0	1	1
-470	0	0	0	0	0	0	0	1	1
-554	0	0	0	0	0	0	1	1	1
-1064	1	1	1	1	1	1	0	0	0
-8	0	0	0	**0**	**1**	1	1	1	1
-194	0	0	0	**0**	**1**	1	1	1	1
-38	0	0	**0**	**1**	1	1	1	1	1
-15	1	1	1	1	1	1	1	0	0
-23	1	1	1	1	1	1	1	0	0
-901	1	1	1	1	1	1	1	0	0
-915	1	1	1	1	1	1	1	0	0
-919	0	0	0	0	0	0	0	1	1

Table 1. Cont'd.

Genotypes with *PdR1b* background	VVCh 14-10	VVCh 14-02	VVCh 14-81	PdR1 b	VVCh 14-78	VVCh 14-77	VVCh 14-70	VVCh 14-30	VVCh 14-27
06314-24	0	0	0	0	0	0	0	1	1
06328-05	0	0	0	0	0	0	0	1	1
04190-026	0	0	0	0	0	0	0	1	1
06317-50	1	1	1	1	1	1	1	0	0
04190-383	1	1	1	1	1	1	1	0	0
06317-50	1	1	1	1	1	1	1	0	0
04190-320	1	1	1	?	1	1	1	0	0
04190-065	1	1	1	?	1	1	1	0	0
04190-109	1	1	1	1	1	**1**	**0**	0	0
04190-381	1	1	1	**1**	**0**	0	0	0	0
06711A-60	0	0	0	?	1	1	1	1	1
04190-236	1	1	1	?	0	0	0	0	0
06315-49	1	0	0	0	0	0	0	0	0
06326-23	1	0	0	0	0	0	0	-	-

Table 2. The Kruskal-Wallis analysis LOD values for the PdR1c locus in the 07744 population based on resistance from *V. arizonica* b40-14.

Genetic map	Map locus	K* (df)
0	VVIN70	5.392 (1) **
3.5	VVIn94	9.323 (1) ****
9.5	ctg1025882	16.293 (1) *******
10.4	VVIP26	12.764 (1) ******
10.7	VVIS70	17.315 (1) *******
11.6	UDV025	16.160 (1) *******
15.0	VVIN64	21.081 (1) *******
16.7	VVCh14-78	22.692 (1) *******
16.7	VVCh14-77	22.946 (1) *******
17.7	VVCh14-70	19.350 (1) *******
20.3	VMCNg2b7.2	17.282 (1) *******
21.5	VVMD24	20.496 (1) *******
22.0	VMC5b3	20.631 (1) *******
22.5	VMC2a5	22.915 (1) *******
22.5	VVIV69	21.978 (1) *******
23.2	UDV033	22.857 (1) *******
28.9	VMC6c10	15.577 (1) *******
36.2	VMC2c3	8.872 (1) ****
36.5	VMC2b11	8.057 (1) ****
36.9	VChr14a	7.229 (1) ***
39.0	ctg1008359	8.772 (1) ****
39.8	VMC9f4	9.360 (1) ****
41.1	VMC2h12	8.967 (1) ****
49.8	VMC1e12	3.507 (1) *
59.2	VVIP05	1.714 (1) -
61.1	VChr14b	0.398 (1) -
65.4	VVC62	0.386 (1) -

Table 3. Contig size, level of similarity and match location to Pinot noir contigs 9 and 21. Majority of the contigs that were similar to scaffold 9 matched to different locations. The similarity of the b43-17 sequence is greater when it is closer to scaffold 21.

Contig number	Contig length (Kb)	Number of sequences	Coverage	% Match to Pinot noir	Scaffold	Start position
4154	11,020	158	5.74	98	21	15,201,490
2454	11,011	108	3.55	94	9	15,002,217
2620	10,109	87	3.01	98	21	15,165,403
2801	9,741	82	3.36	97	21	15,183,600
1824	9,375	96	3.55	93	9	15,096,377
1834	9,324	113	4.49	98	21	15,200,996
2554	8,240	78	3.60	92	9	multiple sites,
440	8,177	64	3.14	95	9	15,103,036
673	8,066	73	3.09	98	21	15,175,438
2410	7,779	84	4.04	96	9	15,078,745
3944	7,703	69	3.57	98	21	15,165,453
3654	7,463	61	3.05	98	21	15,180,883
2411	7,288	84	4.36	97	21	15,208,989
3773	7,269	157	8.19	98	21	15,179,580
2341	7,267	102	5.89	-	-	-
1658	7,247	49	2.63	-	-	-
1918	7,230	62	3.14	90	9	multiple sites,
3997	7,226	141	7.2	92	9	multiple sites,
45	7,217	66	3.59	87	9	multiple sites,
1734	6,996	170	9.68	98	21	15,180,952
1885	6,985	87	4.77	-	-	-
496	6,945	90	5.09	99	21	15,181,699
530	6,763	319	19.75	90	9	multiple sites,
3606	6,713	57	3.17	98	21	15,181,366
959	6,647	87	4.8	99	21	15,168,301
420	6,599	99	6.07	92	9	multiple sites,
1259	6,593	155	9.28	-	-	-
2510	6,585	66	3.76	-	-	-
4108	6,545	66	3.91	98	21	15,168,284
1741	6,463	43	2.24	91	9	15,002,306
1216	6,410	184	12.05	94	9	15,070,922
3562	6,354	103	6.42	90	9	15,004,525
2610	6,331	83	4.94	97	21	15,168,523

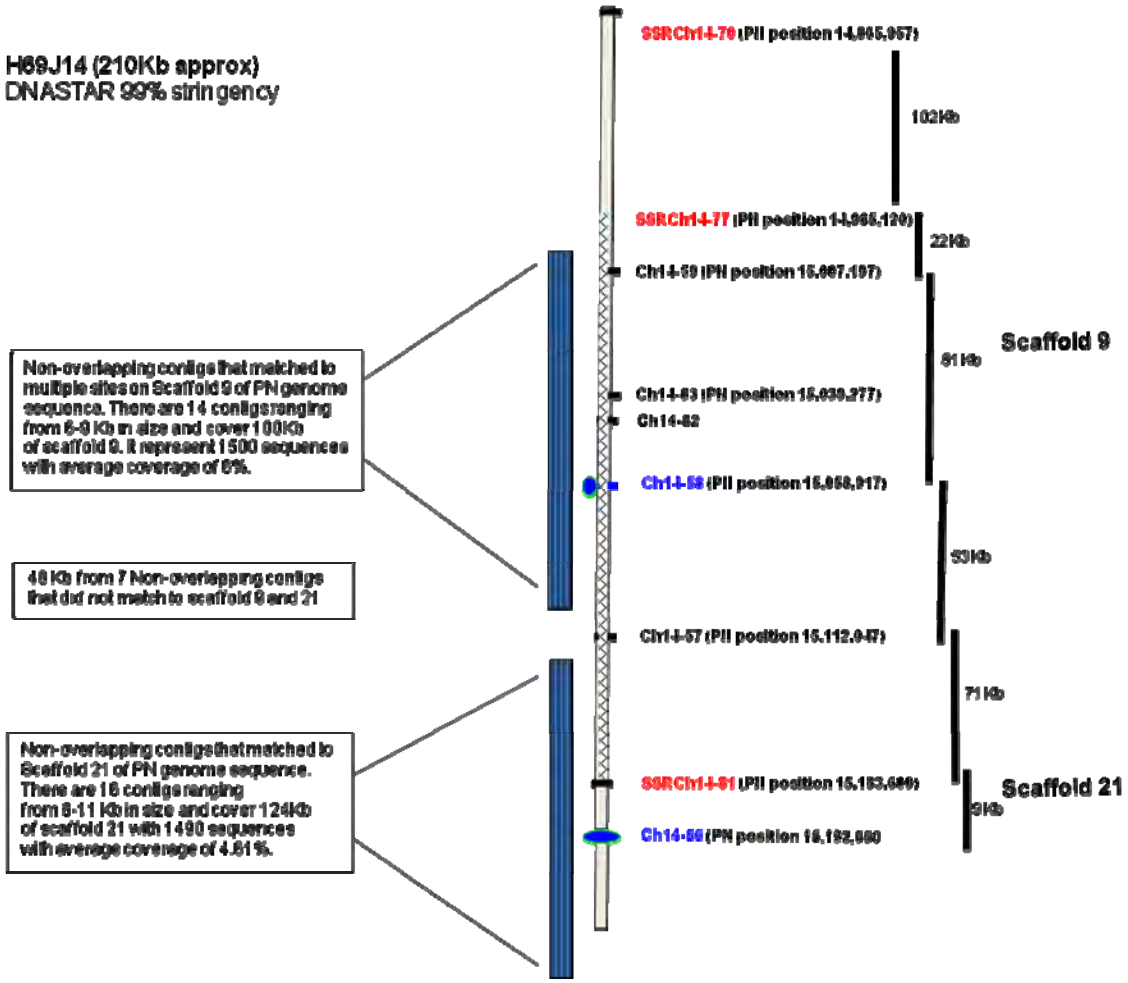

Figure 1. Assembly detail of the H69J14 BAC clone that spans two scaffolds from the Pinot noir genome (scaffold 21 and 9). Currently the PD resistance locus resides between SSR markers Ch14-81 (on scaffold 21) and Ch14-77 (on scaffold 9). The relative position and distance of all the markers that have been used in mapping and library screening are on the right. Non-overlapping contigs were grouped based on their position on the Pinot noir. Primers will be developed for the contig ends to enable BAC walking in order to fill the gaps and verify the sequences.

Section 6: Economics