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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ABSTRACT
In this report we present the results of refined mapping of the Pierce's disease (PD) resistance locus, *PdR1*, in the 04190 and 9621 population (both with resistance from *V. arizonica* b43-17). The *PdR1* locus is 0.2 and 0.4 cM from flanking markers in 9621 population and 0.9 and 5.0 cM in the 04190 population. BAC library screening is more effective and allows more precise selection of positive BAC clones when flanking distances are within one cM. BAC library development from the b43-17 genotype is complete. We developed two libraries each with one restriction enzyme (*Hind* III and *Mbo* I). The *Hind* III library consists of 34,504 clones with an average insert size of 140 Kb. The *Mbo* I library consists of 23, 000 clones with an average insert size of 130 Kb. We initiated the screening of the *Hind* III BAC library with two markers that flanked *PdR1* and identified 10 positive BAC clones with marker VVCh14-10. A total of 15 positive clones were identified with VVCh14-56. Three of the positive clones were flanked by both markers, implying that they contain the entire region harboring *PdR1*. All positive clones from *Hind* III library were large and range from 120-196 Kb. We are in the process of determining the size of positive clones identified with the VVCh14-56 marker. Ten positive clones are already in the process of BAC end sequencing and results will be presented in 2008. We also developed 48 new markers from the newly released Pinot noir genome sequence and 16 of these markers were polymorphic for both the 9621 and 04190 populations. These markers can be used for MAS screening and as well as mapping. We completed the characterization of the UC Davis Mexican *Vitis* collection, the source of the exceptional resistance to *Xf* that was collected by Dr. Olmo in 1961. We are using these unique selections in our genetic and molecular breeding to produce PD resistant table and wine grape cultivars.

INTRODUCTION
Previous reports described the mapping of resistance to *Xylella fastidiosa* (*Xf*) in four (9621, 0023, 04190, and 04373) populations. The preliminary AFLP-based 9621 genetic map and refined 9621 map with SSR, EST-SSR and ESTP markers have been published (Doucleff et al. 2004; Riaz et al. 2006). A manuscript detailing the genetic mapping of the PD resistance locus, *PdR1*, in three populations (04190, 04373 and 9621 populations all of which derive resistance from the single dominant gene resistance source *V. arizonica* b43-17) and an explanation of segregation distortion mechanisms is in preparation. A manuscript detailing the mapping of QTLs for PD resistance in 0023 population (which derives its multigenic resistance from *V. arizonica* b42-26) is also in preparation. 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 is in press (Riaz et al. 2007). During the last three months, we have refined the position of *PdR1* in the 9621 and 04190 populations by increasing the number of recombinants and adding more markers. We completed mapping of the 04373 and 04191 populations and completed greenhouse screening of 64 genotypes in the 04373 population. We also completed the development of a BAC library from the homozygous resistant b43-17 and started BAC library screening with tightly flanking markers.

This report details the results of three genetic maps (9621, 04190, and 04373), the screening of 48 new markers developed from the newly released Pinot Noir genome sequence, and most importantly the development, characterization and screening of the b43-17 BAC library. A high quality BAC library with good coverage is essential for the isolation of the BAC clones that harbor the *PdR1* resistance gene(s). BAC end sequencing of these clones will allow us to develop a physical map in conjunction with the genetic map, develop more markers around the *PdR1* region, and lead to genetic engineering of susceptible *V. vinifera* grapes with *PdR1* gene candidates to evaluate their function.

OBJECTIVES
1. Develop genetic linkage maps for chromosome 14 around the *Xf* resistance locus, *PdR1*, in three populations 04190 (*V. vinifera* F2-7 x F8909-08), 04191 (*V. vinifera* F2-7 x F8909-17), both segregating for *Xf* resistance, and 04373 (*V. vinifera* F2-35 x *V. arizonica* b43-17).
2. Summarize inheritance of PD resistance from other genetic sources.
3. Develop a BAC library for the homozygous resistant genotype b43-17, parent of F8909-08, F8909-17 and the 04373 population.
4. Utilize map-based positional cloning to identify the resistance gene(s) candidates prior to testing in a transformation system.

RESULTS

Objective 1. As mentioned in the previous report, the resistant genotypes F8909-17 and F8909-08 inherited different sister chromatids 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 that this is a region of segregation distortion. However, these markers on the F8909-17 map were not distorted in this region. Two mapping software programs were used; Join Map and TMAP, and marker order did not change. In the 9621 population, the flanking markers are 0.4 cM (VVCh14-56) and 0.2 cM (UDV095) away from *PdR1*. In the 04190 population, the flanking marker distance is 0.9 (UDV095, VVCh14-10) and 5.0 cM (UDV025). In previous reports the flanking marker distance was 2.0 and 6.0 cM away. Marker VVCh14-56 was not polymorphic for this population. We are in process of screening seven more markers developed from the Pinot Noir genome sequence in an effort to find additional polymorphic markers for the 04190 and 04373 populations. The 9621 map was developed from 425 genotypes (26 genotypes were not included due to a lack of screening data, these genotypes are being re-screened). A total of 361 genotypes were used for 04190 population and screening data was available for all of them. The majority of the markers were homozygous for the parental genotype b43-17. A total of 282 progeny from the 04373 population were used to create the 04373 map. This map of chromosome 14 spans 86 cM with a gap of 44 cM between two groups of markers (Figure 1). Sixty-four plants of 04373 population were selected for greenhouse PD screening. All these genotypes were resistant proving that b43-17 is homozygous resistant.

The 04191 population (*V. vinifera* F2-35 x F8909-17) provides genotypes with 50% *vinifera* background for breeding wine and table grapes as well as more recombinant plants for genetic mapping. It also allows the possible confounding impact of resistance coming from D8909-15 to be examined. Currently, there are 212 genotypes in this population. We are in the process of adding only those markers that are tightly linked to *PdR1*. We categorized resistant, recombinant and susceptible genotypes based on marker information, with recombinant genotypes being selected based on flanking markers. The plants were propagated and inoculated with *Xf*. All marker work is complete and mapping analysis will be carried out as soon as greenhouse screen results are available.

To develop new markers that map only to chromosome 14 and most importantly in the region associated with *PdR1*, we utilized the new released draft of the 11X coverage Pinot noir grape genome sequence, available on NCBI. This sequence information is a great resource and its use is not only confined to determining a DNA sequence. It will also allow predictions of gene positions and comparisons of gene functions. We searched the Pinot noir genome sequence database with the cloned sequences of 16 SSR markers tightly linked to *PdR1* and identified 16 contigs that provide coverage of 55.0 Kb (more detail in report June 2007). We developed 48 new primers and tested them on a small set of parental and progeny DNA from the three populations above. A total of 41 markers amplified cleanly and 16 of them were polymorphic in the 04190 and 9621 populations (data not shown). We added VVCh14-10 to entire set of 361 genotypes from 04190 and it co-segregated with UDV095 (Figure 1). We are in process of adding polymorphic markers to the entire 04373 mapping population, which did not have polymorphic markers in the *PdR1* region. These markers are also candidates for use in MAS screening projects.

Objective 2. So far we have used two resistance sources (b42-26 and b43-17). The populations and genotypes examined are noted in Table 1, and their segregation patterns are reported in previous reports. It is easier to manipulate single locus resistance traits in breeding and when attempting to use map based positional cloning of genes. Resistance from b43-17 is inherited as a single gene while resistance from b42-26 and its offspring D8909-15 is quantitatively inherited and appears to involve multiple genes that might be present on multiple chromosomes. Screening of a wide range of *V. arizonica* genotypes revealed other resistant selections, of which b40-14 is another 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, we made crosses with these resistant F1 genotypes to other susceptible and resistant genotypes to verify the single dominant gene mode of inheritance. Seeds are harvested and progress will be presented in interim report 2008. Our plan is to select 188 genotypes from F2 population to develop a genetic map to position this potentially different PD resistance locus.

Objective 3. Genetic analyses determined that b43-17's *Xf* resistance segregates as a major single locus and that the full sibling progeny, F8909-08 and F8909-17, inherited different sister chromatids for chromosome 14. *PdR1* has been mapped in the F8909-17 genome, and it is possible that the PD resistance gene from F8909-08 is a different allele of the same gene or that it may be a different gene. Based on the genetic map information, the exact number of genes involved could not be determined and a physical map of the *PdR1* region is essential. We developed two BAC libraries (each with different restriction enzymes) from the homozygous resistant b43-17. Young leaves were used to isolate high molecular weight DNA. Two restriction enzymes, *Hind* III and *Mbo* I were used to digest the DNA. The development of two libraries was done to reduce the bias in the distribution of restriction sites in the grapevine genome. The use of two restriction enzymes on one library might generate DNA fragments too large or small to clone, and thus not a complete representation of the genome.

Hind III *and Mbo* I *libraries:* The *Hind* III BAC library consisted of a total of 34,560 clones that are stored in ninety 384 wells plates. The average insert size is about 140 Kb. The BAC cloning vector is pCC1 (Epicenter) and Invitrogen DH10B T-R competent cells were used. This library provides almost 12X genome coverage of grape genome (W=NI/G, where W is the

genome coverage, N is the total number of BAC clones, I is the mean length of DNA insertion and G is the genome size of grape). With only 3X coverage, the chance of finding a particular genomic sequence in a library is approximately 95%. The *Mbo* I library consisted of 23,040 clones with average insert size of 130 Kb. The same cloning vector and competent cells were used. This library provides about an 8X coverage of grape genome. With both libraries we have average of 10X genome coverage. Both libraries were also gridded onto 22.5 × 22.5 cm2 Hybond N+ filters. Clones were double-spotted using a 4x4 array. The grid pattern allowed all clones to fit on two filters for each library.

Library screening: The *Hind* III BAC library was screened with hybridization using ECL Direct Nucleic Acid Labelling and Detection System (Amersham, GE Healthcare, Buckinghamshire, UK). Standard protocol for screening was used as provided with the kit. Screening was carried out twice with two markers (VVCh14-10 and VVCh14-56), which are tightly linked to *PdR1* (Figure 1). VVCh14-10 was developed from Pinot noir genome sequence. We blasted the UDV095 clone sequence and obtained a 50,000bp contig that was used to develop more markers. VVCh14-10 segregated and amplified cleanly in the 04373 and 04190 populations. There were a total of 10 positive BAC clones. BAC DNA was isolated and verified by PCR-screening (Figure 2). To determine the size of the positive BAC clones, bacteria containing the positive BAC clones were cultured overnight and clones were isolated. BAC DNA was digested with restriction enzyme *Not* I (New England Biolabs) and analyzed with Chef gel DRIII system (BioRad, Calif.) run for 15.5 hours at 14C with 6 v/cm and pulse 5-35 s (Figure 3). Eight of the positive clones have more than 140Kb inserts. Two clones H23-P13 and H64-M16 had two additional bands. *Not* I is a GC rich eight-base cutter and there are two *Not* I sites on the vector, and releases about 8.1 Kb fragments with additional cuts at other *Not* I sites in the insert. Other bands represent additional *Not* I sites that are inside the grape genomic DNA insert. H23-P13 has an insert that is 145 Kb + 20 Kb + 25 Kb = 190 Kb. Similarly, H64M16 is probably ~155 Kb (100 + 25 +30).

The *Hind* III library was also screened with the other flanking marker VVCh14-56 using the same procedures. A total of 15 positive clones were selected based on an X-ray image. We are in process of PCR screening to verify and size the positive clones. Interestingly, three of the positive clones that were selected based on the VVCh14-10 screening were also positive for the VVCh14-56 marker. These three clones are H23-P13, H34-B5 and H64-M16 and they are 190Kb, 160Kb and 155Kb in size, respectively. VVCh14-10 and VVCh14-56 flank the PdR1 locus and the identified clones should contain the complete *PdR1* region. BAC end sequencing of these clones is in process and results will be presented in 2008.

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 MAS for grape breeding; 4) begin development of a physical map of genomic fragments that carry *PdR1*; and finally 5) work towards map-based positional cloning of genes. We focused on chromosome 14 in a variety of genetic backgrounds to verify the single gene nature of *PdR1* expression, and are using quantitative trait loci (QTL) analysis in the 0023 population to study PD resistance from b42-26. These genetic linkage maps will enable us to characterize and clone different variants of PD resistance genes, and ultimately lead to the genetic transformation of susceptible grape varieties with grape resistance genes. PD resistance makers generated in this study are also used in our breeding program to optimize and expedite selection, allowing us to screen larger populations and make more rapid progress in the production of resistant winegrapes.

REFERENCES

Doucleff, M., Y. Jin, F. Gao, S. Riaz, A.F. Krivanek and M.A. Walker. 2004. A genetic linkage map of grape utilizing *Vitis rupestris* x *Vitis arizonica*. Theor. Appl. Genet. 109:1178-1187

Krivanek, A.F.,S. Riaz and M.A. Walker. 2006. Identification and molecular mapping of *PdR1*, a primary resistance gene to Pierce's disease in *Vitis*. Theor. Appl. Genet. 112:1125-1131

Riaz, S., A.F. Krivanek, K. Xu and M.A.Walker. 2006. Refined mapping of the Pierce's disease resistance locus, *PdR1*, and *Sex* on an extended genetic map of *Vitis rupestris* x *V. arizonica*. Theor. Appl. Genet. 113:1317-1329.

Riaz, S., S. Vezzulli, E.S. Harbertson, and M.A. Walker. 2007. Using molecular markers to correct grape breeding errors and determine the true identity of novel sources of resistance to *Xiphinema index* and Pierce's disease. Am. J. Enol. Vitic. (*In Press*).

Riaz, S., A.C. Tenscher, R. Graziani, R. Zhou, and M.A. Walker. 2007. Molecular breeding: Marker-assisted selection for Pierce's disease and powdery mildew resistance n grapevine. Abstract. National Viticulture Research conference, University of California, Davis

Riaz, S., A.C. Tenscher, A.F. Krivanek and M.A. Walker. 2007. Optimized grape breeding with marker assisted selection for the Pierce's disease resistance gene, *PdR1. In preparation*.

Riaz, S., A.C. Tenscher, Ruben J, Pao S, Graziani R, and M.A. Walker. 2007. Segregation distortion factors in genus Vitis. *In preparation.*

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Table 1. 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*
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 (both 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

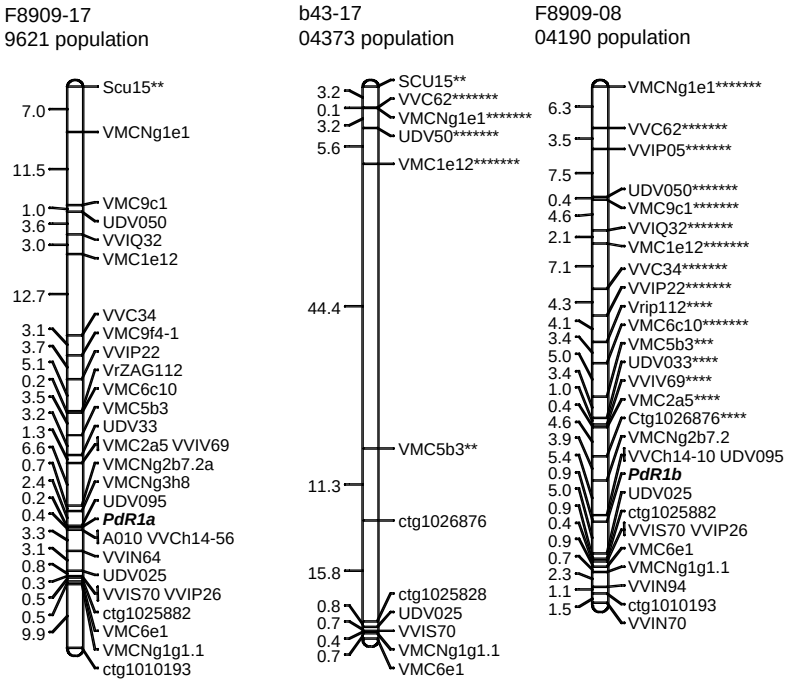

Figure 1. Genetic maps of F8909-17, b43-17 and F8909-08 genotypes

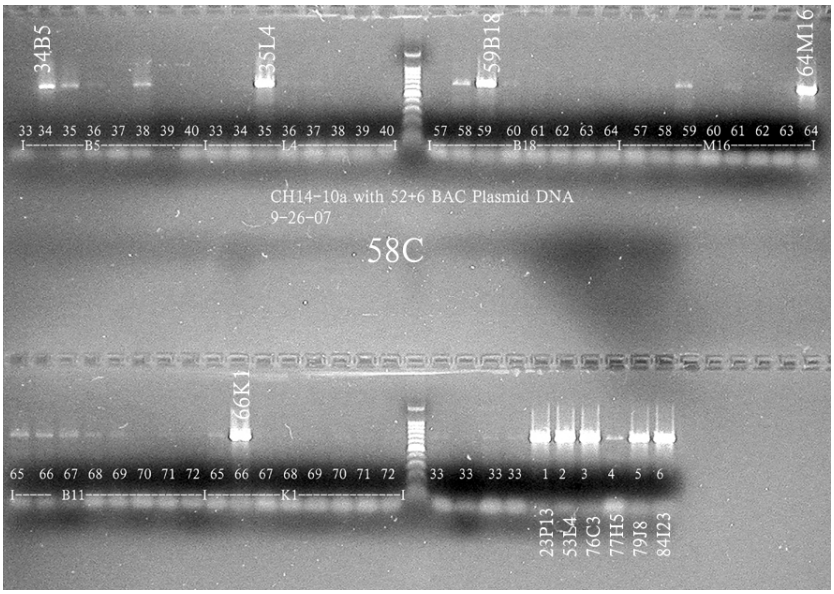

Figure 2. PCR screening of positive BAC clones with VVCh14-10 marker.

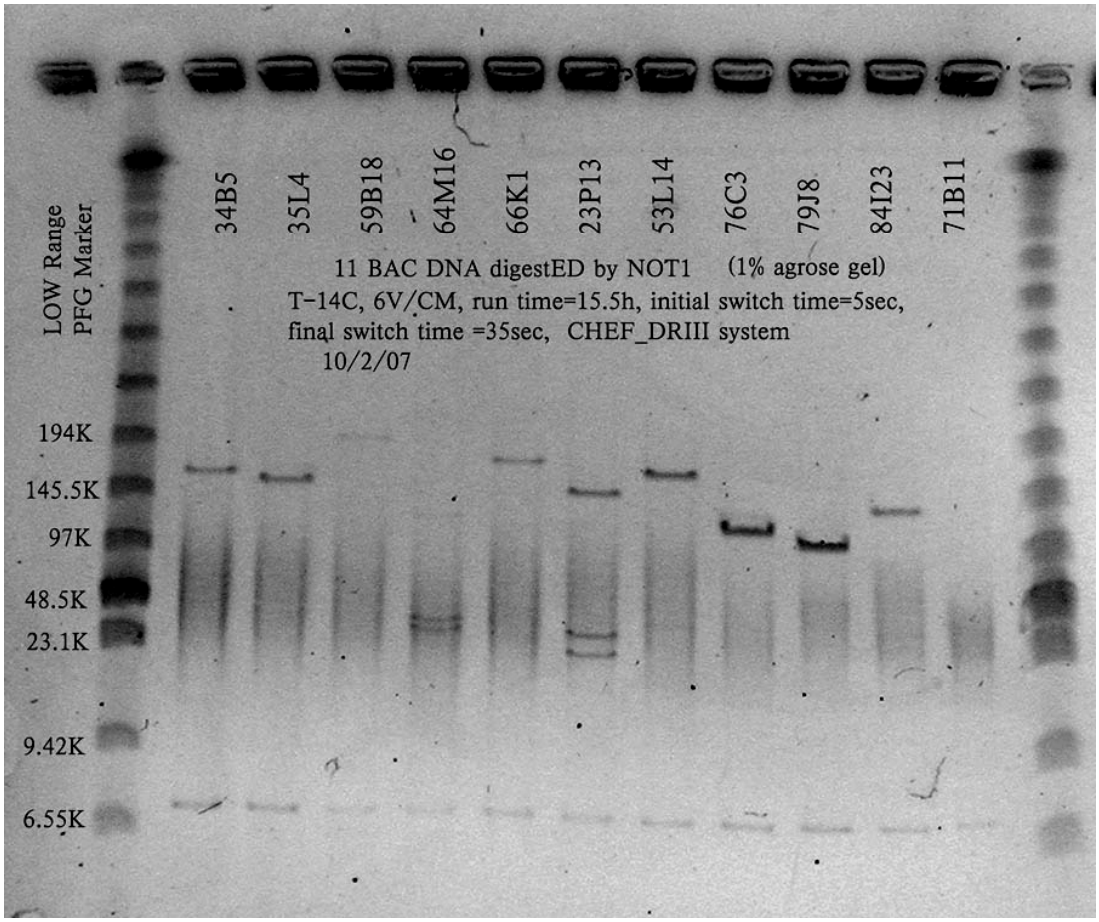

Figure 3. Size determination of Positive BAC clones.