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
Development of a framework simple sequence repeats (SSR) genetic linkage map based on the 181 genotypes of 9621 family, which segregates for Pierce's disease (PD) resistance is complete. The current genetic linkage map consists of 236 non-AFLP markers (SSR, EST-SSR and ESTP-RFLP) in 19 linkage groups. The PD resistance locus, *PdR1*, maps to linkage group 14 (LG - essentially a chromosome) of the male parent (F8909-17), which now consists of 30 markers, nine of which are localized within 10 cM (very closely) of *PdR1*. The 9621 mapping population was expanded from 181 to 457 genotypes. A total of 13 markers polymorphic for F8909-17 mapped to LG 14 and were added to 276 segregants (core population set is 457). We also screened an additional 400 seedlings with two markers (one on either side of *PdR1*) and a total of 50 unique recombinant plants were planted in the field. To avoid confounding affects of resistance inherited from D8909-15 (which is also highly resistant, but with a very different form of resistance) the 04-190 population was selected and a map of LG 14 with 220 genotypes was completed. 04-190 is a cross of *V. vinifera* F2-7 (Cabernet Sauvignon x Carignane) x F8909-08 (sibling of F8909-17). We have used F8909-08 extensively in PD resistant wine and table grapes, therefore it is necessary to validate that *PdR1* gene segregates 1:1 in progeny from its crosses. We completed greenhouse screening of 160 genotypes from the 04-190 population to verify the molecular marker results. The *PdR1* resistance locus segregates 1:1 and mapped to the same position with surrounding markers ctg1025882 and VMCNg2b7.2. We also increased the core population of 04-190 from 220 to 395 seedling plants. Leaf tissue for DNA extraction and green cuttings for greenhouse testing and ELISA screening from the additional 175 plants were collected in late summer, and results are expected in early spring 2007.

Efforts to construct a bacterial artificial chromosome (BAC) library from b43-17 (the basis of the *PdR1*) were initiated. A total of 200 green cuttings were collected that resulted in 160 plants that are being cultivated for young etiolated shoot tips that provide an excellent source of DNA for the BAC library. This BAC library is being developed to provide markers from BAC end sequencing for LG 14, so that we can create a physical map of the *PdR1* gene family, which will lead to genetic engineering efforts. We are also working to add resistance gene analogs (RGA) markers, which are generalized genetic sequences involved in a wide range of pest and disease defense responses in plants, to our genetic maps. The addition of these markers may identify common regions of disease resistance and possible functions of the *PdR1* gene family.

In order to understand the stability and segregation of PD resistance from different sources, work on six different mapping populations was completed. We are also continuing mapping efforts in the 0023 population, a cross of D8909-15 x *V. vinifera* B90-116, to identify quantitative trait loci (QTL) and then saturate linkage groups with these QTLs with more markers. This population is important because we have extensive data for cluster and berry traits, and *Xylella fastidiosa* (*Xf*) resistance data for about 200 plants. We completed the characterization of Mexico collection, the source of the exceptional resistance to *Xf* and collected by Dr. Olmo in 1960. We are using these unique selections in our genetic and molecular breeding to produce PD resistant table and wine grape cultivars.

INTRODUCTION
We have been mapping resistance to *Xylella fastidiosa (Xf)* in three (9621, 0023, and 04-190) populations, and to *Xiphinema index*, the dagger nematode in two (9621 and 0023) populations. The preliminary AFLP-based 9621 genetic map has been published (Doucleff et al. 2004). The 9621 population was then mapped with the more informative microsatellites or SSR markers, which provide a more reliable and repeatable framework for initial mapping of candidate genes and QTLs. In addition, tightly linked SSR markers are ideal for marker-assisted selection (MAS) due to their applicability across different genetic backgrounds and ease of use. This year, mapping efforts within the 9621 and 04-190 populations have concentrated on linkage group 14 that harbor the *PdR1* resistance locus (Krivanek et al. 2006; Riaz et al. 2006). The addition of SSR markers to this linkage group was greatly aided by the existence of other SSR-based genetic maps of grape that have been developed within *V. vinifera* populations and by the availability of expressed sequence tag polymorphism (ESTP) markers developed by other grape researchers and available on various genetic databases. e are now initiating construction of a BAC library. A high quality BAC library with good coverage is essential for the isolation of the BAC clones that harbor *PdR1* resistant genes. BAC end sequencing of these clones will allow us to develop a physical map in conjunction to genetic map, develop more markers around the *PdR1* region, and lead genetic engineering of susceptible *V. vinifera* grapes with the *PdR1* gene.

OBJECTIVES

1. Develop a fine scale genetic linkage map around the *Xf* resistance locus in D8909-15 x F8909-17 (9621) segregating for *Xf* resistance. COMPLETED
2. Add markers associated with *PdR1* from linkage group 14 (9621 map) to 400 additional 9621 individuals (more individuals, more recombinants, more refined genetic map).
3. Screening of additional EST derived SSR markers for which functions are known and shift focus to EST-SSR markers isolated from the resistant genotype D8909-15. COMPLETED
4. Screen resistant gene analogue markers (RGA), if polymorphic add them to the core of 9621 map.
5. Initiate development of a BAC library from the resistant genotype b43-17 (the source of *PdR1*).
6. Study marker segregation linked to *PdR1* in different genetic backgrounds. Initiate genetic mapping of the 04-190 population (*V. vinifera* F2-7 x F8909-08) with markers on linkage group 14. Apply this information to further refine the MAS process and assist the ongoing winegrape breeding efforts. Increase the core population of 04-190 population to 400 plants.

RESULTS

Objective 1

Completed. This project was initiated with an AFLP-based genetic map developed from 116 individuals from the 9621 population (Doucleff et al. 2004). The framework map of the 9621 population is now complete with 236 markers (primarily SSR, 210 mapped and 26 linked). The consensus map spans 1154 cM in 19 linkage groups. LG 14 is largest group with 30 markers. Fifteen markers were closely associated to the PdR1 locus. The average distance between markers is 5.5 cM (Riaz et al. 2006). The framework map contains 60 new functionally associated EST-SSR and EST-RFLP markers that have not been mapped on any other published grape map.

Objective 2

We previously reported on the genetic map based on original core set of 181, which we are expanding to 457 genotypes, the largest population maintained for map-based positional cloning of genes in the grape research community. We used tightly linked markers to screen an additional 276 genotypes and chose a sub set of 60 genotypes (primarily recombinants with a few resistant and susceptible genotypes as controls), currently being screened with results expected fall 2006. This increased number of individuals should help us refine the position of *PdR1* locus. Fine scale placement of markers in relation to a resistance locus is the first step toward the screening of BAC library clones that contain the resistance gene. The "map-based positional cloning of genes" approach relies on solid genetic map and it has been effectively used in many organisms to clone genes of interest. We also screened an additional 400 9621seedlings for two markers flanking *PdR1* to find more recombinants. Fifty recombinants were found, they were planted in the field, and screening is underway to determine the linkage phase of markers to the *PdR1* locus. Markers from LG 14 will be added to these genotypes and they will become part of the core population. A complete map of LG 14 with 450 genotypes will be presented in spring 2007 report.

Objective 3

Completed. The 9621 framework map has more than 40 EST-SSR markers developed by the Genome facility, University of California, Davis; seven out of 30 markers on LG 14 are EST-SSR markers. These markers have been annotated with known functions after being compared to available databases. The nucleotide sequence of these larger fragments of DNA will help with the BAC library screening to isolate clones that harbor the *PdR1* resistance locus. We completed screening of an additional 50 EST-SSR markers with known function and polymorphic markers were added to the 9621 and 04-190 populations.

Objective 4

Resistant gene analogue (RGA) markers have now been reported for many organisms. The theory behind RGAs is that a surveillance system of receptors encoded by R genes reacts in a general way against all pathogens (viruses, bacteria, fungi or nematodes). The R gene products react with the products of *Avr* genes or with general bacterial elicitors. Once binding of a ligand modifies the receiver domain, NBS or STK domains become available for down-stream components of a signal transduction and initiate defense responses. Two reports have been published on grape using degenerate primers of conserved sequences from different classes of R genes to isolate RGA homologs, and then develop these homologs into STS (sequence tagged sites) markers (Di Gaspero and Cipriani 2003, Donald et al. 2002). We chose a subset of 20 RGA-STS primers to screen parental samples for polymorphism. The majority of these RGA markers amplified successfully, however they were not polymorphic. We used a subset of five different restriction enzymes to find restriction site based polymorphism. Three markers were polymorphic with different restriction enzymes. They were added to the core 9621 population, but none resided on LG 14. Additional work on RGA markers is on hold until we find a better system to run the gels, such as a single strand conformational polymorphism gel system.

Objective 5
Now that we have constructed a strong genetic map for the *PdR1* locus, the next step is to develop a BAC library, which enables the isolation of the *PdR1* resistance gene(s). We choose the resistant genotype b43-17, the *V. arizonica / candicans* source of *PdR1* and *Xf* resistance, to develop this BAC library. The selection of a genotype for development of a BAC library is very critical. From our genetic analyses, we know that *Xf* resistance from b43-17 segregates as a major single locus (*PdR1* segregates in this way in both 9621 and 04-190). However the exact number of genes involved can only be determined from a physical map. Molecular marker studies of b43-17 allele sizes indicate that *PdR1* might be a cluster of very tightly linked genes. Our studies also indicate that b43-17 is heterozygous for three of the markers that are tightly associated to *PdR1*. F8909-08 and F8909-17, which both have strong *Xf* resistance, are progeny of b43-17 and they inherit different resistance alleles with these markers. This information suggests that there might be a cluster of genes associated with resistance and that the F8909-08 and F8909-17 siblings inherited different copies of the resistance genes. This makes b43-17 an even better candidate for BAC library development. We collected 200 cuttings of b43-17 to produce 160 plants. Young leaves, flower clusters and tendrils are ideal for the isolation of high quality DNA, and these tissues from the 160 plants are almost ready for extraction.

Objective 6
Because both parents of the 9621 population are *Xf* resistant, and because the D8909-15 parent contains a different, and as yet unmapped *Xf* resistance loci (derived from *V. arizonica/girdiana* b42-26), more mapping was necessary. This led to the mapping of the 04-190 population, a cross of *V. vinifera* F2-7 (Carignane x Cabernet Sauvignon) x F8909-08. We completed DNA extraction from 220 plants in the 04-190 population and a set of 37 SSR and EST-SSR markers from LG 14 were tested on small subset of eight samples (including both parents) to verify polymorphisms. Markers that were polymorphic for the parents were used on the entire 04-190 set, and the plants were greenhouse screened to verify the molecular marker results. Marker order for LG 14 was consistent between F8909-17 (9621 paternal map) and F8909-08 (04-190 paternal map) except for one marker, VMC6e1 (Figure 1). Both genotypes inherit different resistant alleles of b43-17 that might represent different copies of resistant genes. The F8909-08 LG 14 map spans 92 cM and the closest markers to *PdR1* were six cM on each side. This molecular marker work with two mapping populations developed from full sibling parental genotypes helped us to evaluate the stability, penetration and efficacy of PD resistance. It enabled us to choose easily scored, highly polymorphic markers for use in MAS for breeding PD resistant winegrapes. The results of MAS and genetic map of LG14 for the 04-190 population have been submitted for publication (Riaz et al. 2006, submitted). In addition, we expanded the 04-190 population size from 220 to 400. Leaf tissue for DNA extraction and cuttings for greenhouse testing were recently taken from 175 of these plants. DNA will be extracted from these plants and markers from LG14 will be analyzed, and they will be greenhouse tested. We hope to complete the expanded map of 04-190 population in spring 2007.

We also initiated work to study the expression, penetration; segregation and stability of resistance to PD from different genetic sources to better predict the durability of resistance in crosses. 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 below.

Expected or Known Segregation Patterns:
1. 9621 Population: *PdR1* single locus for F8909-17 and multiple QTLs for D8909-15.
2. 0023 Population: multiple QTLs.
3. 03-300/5 population: *PdR1* resistance segregates 1:1 (single gene model), both marker and greenhouse screen.
4. 04-190 population: *PdR1* segregates 1:1, both marker and greenhouse screen.
5. 04-191 population: *PdR1* resistance segregates 1:1
6. 04-373 population: All plants should be resistant with assumption that b43-17 is homozygous resistant for PD
7. 04-5554 population: progeny are 93.75% *V. vinifera* and an excellent test of *PdR1*expression through four backcross generations to *V. vinifera*.

It is much easier to manipulate resistance when it is inherited as a single major locus, both in terms of traditional breeding and for map based positional cloning of genes. Therefore, it is essential to understand how resistance from different sources segregates in populations. The greenhouse and marker testing of the six populations in Table 1 (9621, 0023, 03300, 04-190, 04191, and 04373), which derive *Xf* resistance from our two highly resistant backgrounds (b42-26 and b43-17), indicates that resistance coming from b43-17 segregates as single major locus and it is very unique to this genotype. Resistance from b42-26, in the 0023 population, is quantitatively inherited and appears involve multiple genes that might be present on multiple chromosomes. We used six SSR markers, tightly associated to *PdR1* in F1 populations of b42-26 and b40-14 (another resistant *V. arizonica* genotype). Both genotypes were heterozygous for all six markers and both alleles of six markers were associated to resistance in the F1 progeny. This is a very important finding, indicating that *Xf* resistance involves different mechanisms of resistance, different genes, and that resistance genes are specific to certain genotypes. Understanding of the single locus resistance mechanism in b43-17, will help us to elucidate the complex mechanisms of resistance in b42-26 and b40-14. The addition of multiple markers to the 0023 population is nearly complete. The map will contain about 230 markers, and should provide enough coverage for QTL analysis. Once linkage groups with QTLs for PD resistance are identified, we can focus on those linkage groups and saturate them with more markers.

We have completed analysis of the Olmo Mexican *Vitis* Collection, verifying the identity of these complex species and the extent to which *Xf* resistance and the *PdR1* locus exist in the population; a manuscript is in preparation. This work resolved confusion between the original campus collection and the USDA National Clonal Repository collection. Fifty-one genotypes were with the six SSR markers linked to *PdR1*, and they were greenhouse screened for *Xf* resistance. We are correlating this data to identify new resistance alleles for breeding purposes, and determine the distribution of known resistance alleles in the entire set. A set of 24 SSR markers was added to the 51 genotypes to study correlations among taxonomic descriptions, geographic location and *Xf* resistance. This manuscript is also in preparation.

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
04-190	F2-7 x F8909-08
04191	F2-7 x F8909-17
04373	F2-35 x b43-17

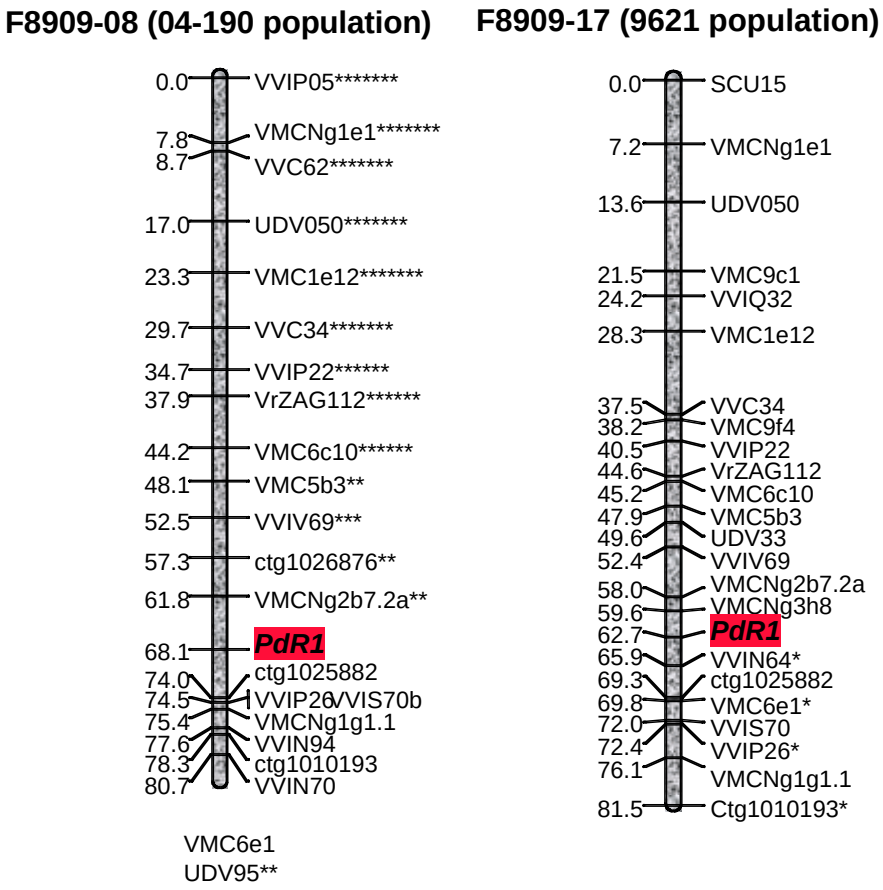

Figure 1. Genetic map of LG14 of two populations (9621 and 04-190).

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 resistance genes; and finally 5) work towards map-based positional cloning of genes. We are focusing on LG 14 in a variety of genetic backgrounds to verify the single gene nature of *PdR1* expression, and are using QTL analysis in the 0023 population to study resistance from b42-26. These genetic linkage maps will enable us to characterize and clone different variants of genes conferring resistance to PD, 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.

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BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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ABSTRACT
We continue to make strong progress breeding Pierce's disease (PD) resistant winegrapes. We have incorporated marker-assisted selection (MAS) for the PD resistance gene, *PdR1* (see companion report), into our breeding and reduced the seed to seed breeding cycle to three years, allowing very rapid progress towards PD resistant winegrapes. This year's crosses were focused on broadening the *V. vinifera* winegrape base in our breeding lines. We produced thousands of seed with 87.5% and 75% *vinifera* progeny. Many of our current populations have the *PdR1* allele from F8909-08; we made many crosses this year to include the alternate *PdR1* allele from F8909-17. Crosses were also made to produce a new mapping population for a collaborative project with the USDA-Parlier to allow mapping of PD resistance from the resistant Florida selection BD5-117, which will help with comparative evaluations of PD resistance genes. The best sources of PD resistance allow very low levels of *Xylella fastidiosa* (*Xf*) to develop in xylem vessels. If these were grafted onto phylloxera resistant rootstocks, the *Xf* in them might kill the rootstocks. Thus, we have made crosses to produce PD and nematode resistant rootstock, and can use MAS for both *PdR1* and the *Xiphinema index* resistance gene, *XiR1*. We made crosses to develop a number of additional mapping populations for fine-scale mapping efforts with *PdR1*. We also replicated advanced 87.5% Syrah and Chardonnay selections with *PdR1* to produce enough fruit for wine evaluation studies next fall. Finally, we are studying wine making and quality parameters at the 1L, 20L and 2,000L levels to determine which quality parameters are predictive at all scales and which will be best suited to large scale winemaking tests on single vine selections.

INTRODUCTION
This project is directed at breeding Pierce's disease (PD) resistant winegrapes with *Vitis vinifera* fruit quality and the ability to greatly suppress *Xylella fasidiosa (Xf)* populations and movement within the vine while preventing PD. California's *V. vinifera*-based vineyards are susceptible to PD and resistant varieties provide the best long-term solution to this disease. PD resistance exists in a number of *Vitis* species and in the related genus, *Muscadinia*. In addition, many resistant cultivars exist, which derive their resistance from these sources, but they lack *V. vinifera* fruit quality and the genetics of their resistance is complex, and considered to be controlled by at least three independently inherited genes (Mortensen 1968). This complex genetics greatly limits the number of resistant progeny they produce when crossed to *V. vinifera* cultivars, which dramatically slows breeding progress. However, we have discovered a unique form of resistance to *Xf* that is controlled by a single dominant locus (*PdR1*) derived from forms of *V. arizonica* (Riaz et al. 2006 and see companion report in this Proceedings by Walker and Riaz), and are using this resistance source and *PdR1* markers to rapidly backcross PD resistance into high quality *V. vinifera* winegrapes via marker assisted selection. At the same time we continue to incorporate other resistance sources to broaden the base of PD resistance.

We are uniquely poised to undertake this important breeding effort. We have developed rapid screening techniques for *Xf* resistance and have optimized ELISA and PCR detection of *Xf* (Buzkan et al. 2003, Buzkan et al. 2005, Krivanek et al. 2005a 2005b, Krivanek and Walker 2005). We have unique and highly resistant *V. rupestris* x *V. arizonica* selections, as well as an extensive collection of southeastern grape hybrids, that allow the introduction of extremely high levels of *Xf* resistance into commercial grapes. We have seed that is 87.5% *V. vinifera*, from winegrape cultivars, with resistance from our b43-17 *V. arizonica/candicans* resistance source. There are two sources of *PdR1*, siblings from b43-17. These selections – F8909-08 and F8909-17 have been introgressed into a wide range of winegrape backgrounds over multiple generations. We are also maintaining a number of lines with resistance from southeastern United States (SEUS) species. Although these lines have complex genetics and we have not been able to develop markers associated with their resistance, we maintain these lines for later crosses to broaden PD resistance.

OBJECTIVES
1. Breed PD resistant winegrapes through backcross techniques using high quality *V. vinifera* winegrape cultivars and *Xf* resistant selections and sources characterized from our previous efforts.
2. Continue the characterization of *Xf* resistance and winegrape quality traits (color, tannin, ripening dates, flavor, productivity, etc.) in novel germplasm sources, in our breeding populations, and in our genetic mapping populations