CDFA PD/GWSS Progress Report
March 2011

I. Project Title: Molecular characterization of the putative *Xylella fastidiosa* resistance gene(s) from b43-17 (*V. arizonica*).

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Reporting period: March 2010 to March 2011

III. Objectives and Description of Activities

Objective 1. Cloning, structural analysis and gene annotation via comparison of *PdR1b* locus to susceptible Pinot noir using the BAC clone H64J14 assembled sequence

A refined genetic map of chromosome 14, which contains the PD resistance locus, was generated from three grape mapping populations derived from *Vitis arizonica/candicans* b43-17. The resistance locus segregates as a single dominant gene and mapped as *PdR1a* in the F1 selection 8909-17 and as *PdR1b* in its sibling F8909-08. Clone H69J14 from a b43-17 BAC library, containing both markers flanking the PdR1b resistance locus, was sequenced using 454 sequencing. Further detailed analysis of the assembled as well as unassembled sequences revealed the presence of a high number of transposable elements (TE). Chromosome 14 is known to have the second largest number of TEs in the sequenced grape genome (Moisy et al. 2008). Transposable elements play a key role for the diversification of disease resistant genes through a process termed TE-induced gene alteration (Michelmore 1995). Considering the complexity of this region due to the large number of tandem repeats of TEs, a Fosmid library was generated with insert size of 35-40kb from the H69J14 BAC clone. The second round of sequencing was performed using fosmid end-sequencing as well as shotgun reads.

The first assembly generated 10 contigs, with a portion of the sequence remaining unassembled. Analysis of assembled and unassembled sequences revealed the presence of four candidate genes, *PdR1b.1–4*, which appear to be receptor-like proteins, a class of Resistance proteins. PdR1b.1-2 and 4 were cloned into a pGEM-T easy vector (Promega) and subcloned into vector pDE00.0113 containing the 35S promoter and ocs3' terminator (Figure 1). The plasmids generated will be digested with AscI and the expression cassette subcloned into the binary vector pDU99.2215, which contains a neomycin phosphotransferase II selectable marker gene (nptII) (Figure 1). The resulting binary plasmids will be used for transformation via *Agrobacterium tumefaciens*. A similar procedure will be followed with PdR1b-3 and two new candidate genes that were identified from the fosmid assembly: PdR1b.5 and 6. The second assembly also showed that PdR1b.1 was longer than the sequence previously found and will need to be cloned again from H69J14.

Sequence analysis and alignments to identify introns and exons on the *PdR1b-1* gene was performed using the GeneQuest module of Lasergene v 8.1 that facilitates the prediction of coding regions using the Borodovsky's Markov method and predicts intron/exon boundaries using species-specific patterns by aligning to known genes. We also utilized the GeneMark (http://exon.biology.gatech.edu/) program using both *Arabidopsis thaliana* and *Medicago truncatula* settings. By using both systems, we identified two small introns from position 1-168 and position 3128-3191 that are 167bp and 361bp, respectively. With *A. thaliana* as model system, the size of predicted protein was 976 amino acids, and with *M. truncatula* it was 964 amino acids. It is interesting to note that all four genes from the 12X assembly of PN40024

carry large introns. We plan to make sequence comparisons and identify protein domains for other putative candidate genes. Experiments are underway with specific primers to amplify the certain regions of the genes using total RNA extracted, clone and sequence the DNA to make sequence comparisons among Pinot noir and 5 different genotypes used for RNA extraction.

The sequence comparison with the non-redundant Swiss protein database identified a leucine-rich repeats (LRRs) region as well as a substrate biding site region in one of the candidate PD resistance genes.

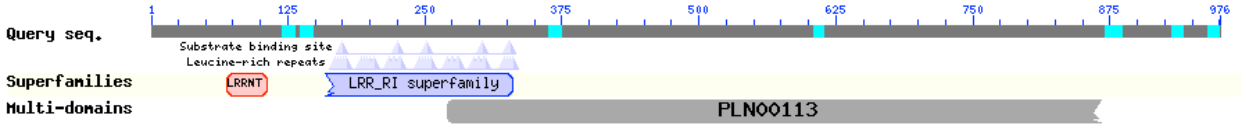
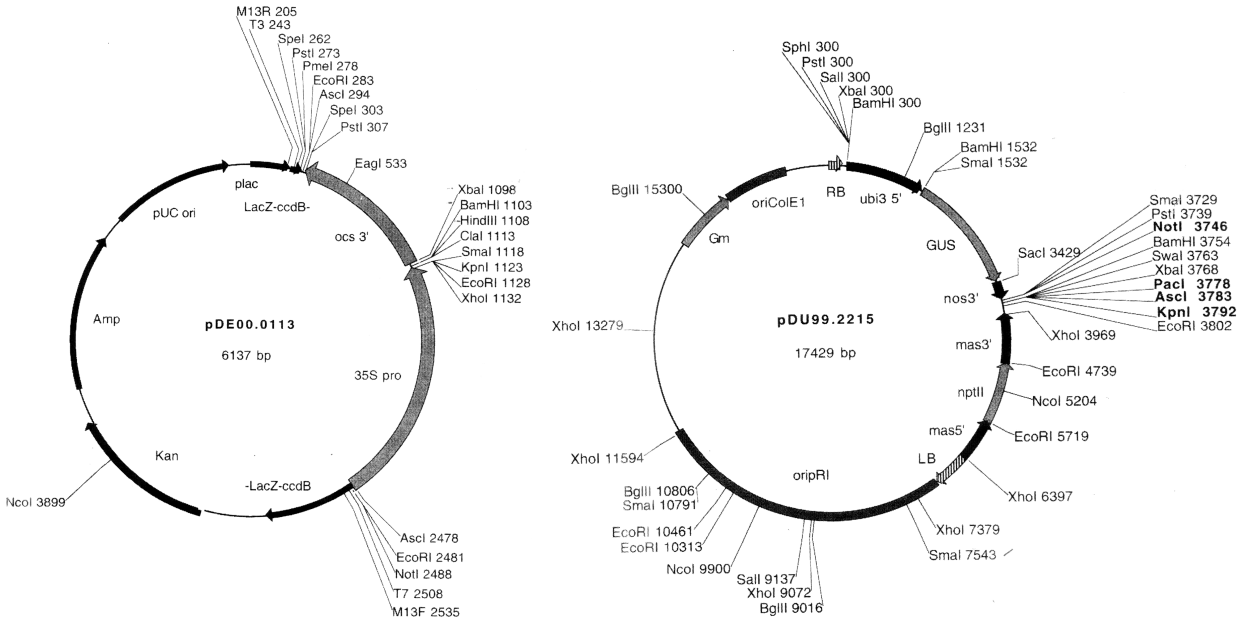


Figure 1. Schematic representation of plasmids used for genetic transformation with *PdR1b* candidates



Objective 2. Expression studies of candidate genes by nested RT-PCR

To conduct the expression studies of the candidate genes, hardwood cuttings were collected in November to generate at least 15 plants of the susceptible *vinifera* control (Chardonnay), resistant and susceptible parents (b43-17 and *V. rupestris* A. de Serres) and two resistant F1 selections (F8909-08 and F8909-17). The stem and leaf tissue of these plants will be used for the total RNA from both resistant and susceptible genotypes using a cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol as described previously (Iandolino et al., 2004).

We used a time course analysis to evaluate expression and to determine when the resistance gene is activated. We isolated the total RNA from leaf and stem tissues from both resistant and susceptible genotypes. Figure 2 presents the results of RNA extracted from the young leaves of the four genotypes before the inoculation of bacteria into the stem. We are currently extracting total RNA from the un-infected stem tissue, which will be used as a control for later experiments of the gene expression. Later this Spring the five genotypes will be inoculated with the *X. fastidiosa* and total RNA will be isolated from leaf and stem tissue every

two-weeks until 14 weeks after inoculation (our standard greenhouse test interval). ELISA will be used to quantify the amount of *X. fastidiosa* in tissues. First-strand cDNA synthesis will be performed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). For nested RT-PCR experiment, the amplified fragments will be designed to include both Kinase and LRR regions. Leaf and stem first-strand cDNAs will be used for the first-round PCR reactions, and first-round PCR products will be used as templates for the nested PCR reactions. The amplification of both PCR reactions as well as visualization of products will be performed as described by Hwang et al. 2010.

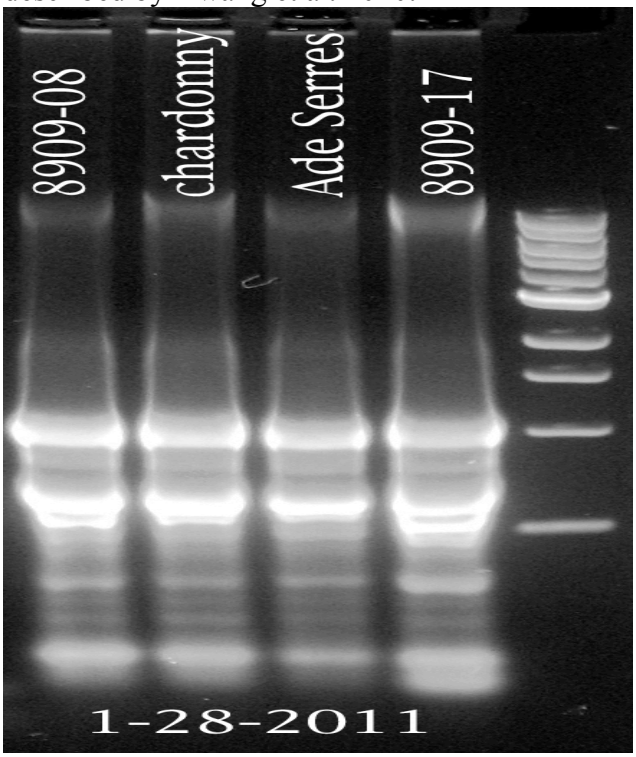


Figure 2. Total RNA extracted from the leaves of susceptible control and resistant plants.

Objective 3. Development of alternative protocols for genetic transformation for the validation of gene constructs.

Once the gene constructs are completed they must be tested to see if they contain the resistance genes. This is done by inserting the gene(s) in to a susceptible plant to test for function. Currently the most widely used method for the production of transgenic/cisgenic grapes is based on *Agrobacterium* transformation followed by regeneration of plants from embryogenic callus. We have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless and Chardonnay and the rootstock Rupestris St. George. These cultures of embryogenic calli can be used readily for transformation (Agüero et al. 2006).

Two alternative transformation techniques via organogenesis have been tested to reduce the time needed to produce transgenic grapes. These methods have been developed in Thompson Seedless and are based on the use of meristematic bulks or etiolated meristems as explants for inoculation with Agrobacterium (Mezzetti et al. 2002, Dutt et al. 2007). The ease of producing and maintaining in vitro micro-propagation cultures from a large number of cultivars makes shoot tip based transformation an effective system. The second method employs genetic transformation of *V. vinifera* via organogenesis (Mezzetti et al 2002). In this method, shoot apical meristem slices are prepared from meristematic bulks for *Agrobacterium*-mediated transformation of grape plants. Using this procedure, transgenic plants can be produced in much

shorter time interval. We have streamlined this procedure and already obtained transgenic shoots using Mezzetti's method in 3 months (Fig. 3). So we expect that the time required for transformation will be shortened to approximately 6 months instead of one year via embryogenic callus. The green fluorescent protein (GFP) was used as a reporter gene for monitoring the occurrence of transformed and chimeric plants. Figure 2 shows both procedures in comparison with transformation via somatic embryogenesis. No plants regenerated from etiolated meristems and the procedures are laborious and time consuming. Transformation via meristematic bulks represents a better method to produce transgenic plants in a shorter period of time, although its efficiency is very low (1 plant in 50 slices). GFP expression in transgenic leaves is uniform; indicating a stable non-chimeric transformation of shoots regenerating in selection medium. Based on these results, transformation of meristematic bulks from Chardonnay and St. George will be tested.

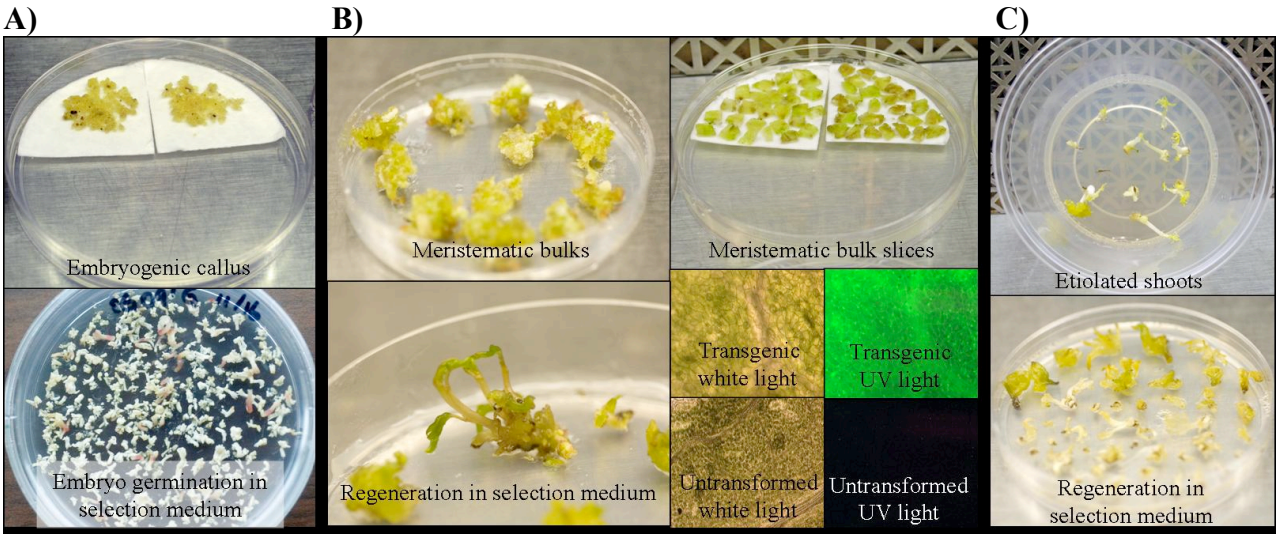


Figure 3. (A) Embryogenic callus developed from anthers are inoculated with *A. tumefaciens*. Transformed embryogenic callus is selected after about 4 months. An additional 4-6 months are needed for embryo germination and plant development. (B) Meristematic bulks, developed from shoot apical meristems, are sliced and inoculated with *A. tumefaciens*. Transformed bulks are selected after about 3 months. An additional 4 months are needed for shoot elongation and rooting. Pictures on the bottom right show green fluorescence protein (GFP) expression in transgenic leaflets. (C) Fragmented shoot apices from etiolated shoots are inoculated with *A. tumefaciens.* Transformed meristems are selected after 2-3 months. An additional 2-3 months are needed for plant regeneration and rooting.

IV. SUMMARY OF MAJOR RESAERCH ACCOMPLISHMENTS:

We maintain and characterize many populations while breeding PD resistant wine grapes, some of which have been used to develop genetic maps. These maps have been used to identify genetic markers that are tightly linked with PD resistance, which have allowed breeding to be greatly expedited through marker-assisted selection. Genetic maps also the construction of physical maps to identify resistance genes (Riaz et al. 2008; Riaz et al. 2009). The physical map of b43-17 resistance region us to identify candidate genes responsible for PD resistance. Comparisons with plant genomes indicated that multiple tandem repeats of the disease resistance gene family Receptor-like proteins with LRRs domains were present in the resistance region. This category of genes is involved in the recognition of microbes and in the initiation of a defense response (Bent and Mackey 2007). We completed the cloning of three of the candidate

genes: PdR1b-1, 2 and 4. We also developed embryogenic callus cultures of the PD susceptible cultivars Chardonnay, Thompson Seedless and Saint George. These embryogenic callus cultures will be used for the transformation/complementation studies to verify candidate PD resistance gene function. To reduce the time span for generating healthy transgenic plants we also tested two different methods that employ organogenesis for *Agrobacterium*-mediated transformation. We were successful in streamlining one method that will allow us reduce the time required to generate transformed plants by 4 months. We also initiated total RNA extraction experiments to allow time course examinations of gene function from leaf and stem tissues. These were successfully completed and we are now ready to evaluate gene function over time in inoculated and un-inoculated plants of the PdR1 containing resistant selections F8909-08 and F8909-17, their resistant parent b43-17, their susceptible parent V. rupestris A. de Serres, and the susceptible control Chardonnay. These plants have been established in the greenhouse and will be inoculated later this Spring.

V. PUBLICATIONS:

Cheng, D.W., H. Lin, Y. Takahachi, M.A. Walker, E.L. Civerolo and D.C. Stenger. 2010. Transcriptional regulation of the grape cytochrome P450 monooxygenase gene CYP736B expression in response to *Xylella fastidiosa* infection. BMC Plant Biology 10:135 doi:10.1186/1471-2229-10-135

Yang, L., H. Lin, Takahashi, Y., Chen, F., Walker, M.A. and Civerolo, E. 2011. Proteomic analysis of grapevine stem in response to *Xylella fastidiosa* inoculation. Physiological and Molecular Plant Pathology (doi:10.1016/j.pmpp.2010.11.002)

Riaz, S., A.C. Tenscher, D.W. Ramming and M.A. Walker. 2011. Using a limited mapping strategy to identify major QTLs for resistance to grapevine powdery mildew (*Erysiphe necator*) and their use in marker-assisted breeding. Theoretical and Applied Genetics 122:1059-1073

VI. 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.

Grape growing in California. South African Grape Growers and Winemakers, UC Davis, CA Aug 23, 2010.

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.

VII. RESEARCH RELEVANCE STATEMENT:

The goal of this project is to prove the function of candidate resistance genes identified in our PD/GWSS Board project entitled "Map-based positional cloning of Xf resistance genes". We

have optimized a grape regeneration system and are prepared to examine gene expression via RNA analysis from leaf and stem tissue. We are most interested in determining which of these gene candidates are responsible for resistance and by comparative studies learn more about where other such genes exist in the grape genome, how they are controlled, if other genes are needed for their effective expression, and whether their function will be stable over time.

VIII. LAY SUMMARY OF CURRENT YEAR'S RESULTS:
This project's goal is to test the function of the genes identified through our genetic and physical mapping efforts. These efforts have located the region on a grape chromosome where the PD resistance gene from *V. arizonica/candicans* b43-17 exists. This region has genetic sequences that are known to be involved in disease resistance in other plants. We have 5 candidates and to test them we need to transform (genetically engineer) them into susceptible grape cultivars. To do this we need to have grape cells that can be transformed and then re-grown into new plants. We optimized several systems to do this and have reduced the time to obtain transformed plants by 4 months. We have prepared several of these candidate genes will soon to engineer them into embryogenic callus of Chardonnay and St. George.

IX. STATUS OF FUNDS: These funds are scheduled to be spent by the end of the grant.

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

LITERATURE CITED

Agüero CB, Meredith CP, Dandekar AM (2006) Genetic transformation of *Vitis vinifera* L. cvs Thompson Seedless and Chardonnay with the pear PGIP and GFP encoding genes. Vitis 45:1-8

Bent AF and Mackey D (2007) Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. Ann. Rev.Phytopath. 45, 399–436

Dutt, M., Li, Z.T., Dhekney, S.A., and Gray, D.J. 2007 Transgenic plants from shoot apical meristems of *Vitis vinifera* L. 'Thompson seedless' via Agrobacterium-mediated transformation. Plant Cell Rep. 26: 2101-2110

Iandolino, A.B.; Goes Da Silva, F.; Lim, H.; Choi, H.; Williams, L.E. And Cook, D.R. (2004) High-quality RNA, cDNA, and derived EST libraries from grapevine (*Vitis vinifera* L.). Plant Molec. Biol. Rep. 22: 269-278.

Jacobsen, J and Hutten, R (2006) Stacking resistance genes in potato by cisgenesis instead of introgression breeding. In: N.U. Haase and A.J. Haverkort, Editors, *Potato Developments in a Changing Europe*, Wageningen Academic Publishers (2006), pp. 46–57

Mezzetti, B., Pandolfini T., Navacchi, O., Landi, L. 2002 Genetic transformation of *Vitis vinifera* via organogenesis, BMC Biotechnol. 2:18

The French-Italian Public Consortium for grapevine Genome Characterization (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449: 463-467

Riaz S, Tenscher AC, Rubin J, Graziani R, Pao SS and Walker MA (2008) Fine-scale genetic mapping of Pierce's Disease resistance loci (*PdR1a* and *PdR1b*) and identification of major segregation distortion region along Chromosome 14 in grape. Theor. Appl. Genet. 117:671-681.

Riaz S, Tenscher AC, Graziani R, Krivanek AF, Walker MA (2009) Using marker assisted selection to breed for Pierce's disease resistant grapes. Am. J. Enol. Viticult. 60:199-206.