Report title: Interim Progress Report for CDFA Agreement Number 13-0098-SA
Project Title: Molecular and functional characterization of the putative *Xylella fastidiosa* resistance gene(s) from b43-17 (*V. arizonica*).

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Reporting period: The results reported here are from work conducted from October 2013 to March 2014

INTRODUCTION

New cultivars bred to resist *Xylella fastidiosa* infection and subsequent expression of PD symptoms will provide long-term sustainable control of PD. Disease resistant cultivars can be obtained by conventional breeding through the introgression of resistance from Native American species into elite *vinifera* wine and table grapes. Another approach is "cisgenesis" – the transformation of elite *V. vinifera* varieties with grape resistance genes and their native promoters, cloned from disease resistant American *Vitis* species. The cisgenic approach may have a more limited impact on the genome of the elite *V. vinifera* parent since single genes from the *Vitis* species genome would be added to the elite parent, thus limiting the impact on its fruit and wine quality while making it PD resistant. The cisgene approach in grapes is similar to the natural clonal variation that exists in many wine grape cultivars. This linkage-drag-free approach is attractive, and also allows the opportunity to stack additional resistance genes from other *Vitis* sources, even if these genes originate from the same chromosomal position in different species or accessions (Jacobsen and Hutten 2006). The physical mapping of the resistance region from *V. arizonica/candicans* b43-17, *PdR1*, allowed the identification of potential candidate resistance gene(s). Preliminary comparisons indicated that the *PdR1* region contains multiple tandem repeats of Serine Threonine Protein Kinase with a LRR domain (STPK-LRR) gene family. This category of genes belongs to a group involved in plant resistance. Their defense mechanism is based on compounds involved in the recognition of microbe-associated molecular patterns (MAMP) like compounds, which initiate a defense response (Bent and Mackey 2007). In order to gain insight and to verify the function of resistance gene(s), cloning and functional characterization is required. In this report, we present the progress on the cloning and testing of five candidate resistance genes.

OBJECTIVES

1. Cloning, structural analysis and gene annotation via comparison of the *PdR1b* locus to the susceptible Pinot noir genome sequence using the assembled sequence of the BAC clone H64J14.
2. Expression studies of candidate genes.
3. Complementation tests of candidate gene(s) to test their function using:
   Agrobacterium-mediated transformation of the susceptible *Vitis* cultivars (Chardonnay and Thompson Seedless, and the rootstock St. George); and transformation of tobacco.

RESULTS

Objective 1. Cloning, structural analysis and gene annotation via comparison of the *PdR1b* locus to the susceptible Pinot noir genome sequence using the assembled sequence of the BAC clone H64J14 –
A refined genetic map of chromosome 14, which contains the PD resistance locus, was generated from three grape mapping populations derived from *V. 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, and unassembled sequences, revealed the presence of a high number of transposable elements (TE). Considering the complexity of this region due to the large number of TE tandem repeats, a Fosmid library was generated with an 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 code for receptor-like proteins, a class of resistance proteins. The second assembly led to the identification of two new candidate genes and found that *PdR1b.1* was longer than the sequence previously detected. We have amplified and confirmed the sequences of five candidate genes: *PdR1b.1-2-4-5-6*. *PdR1b.1* is the largest gene (3198 bp), and shares a high degree of homology with *PdR1b.2, 4 and 5*. *PdR1b.6* is significantly different from the other four. It has a kinase domain, which suggests it might be involved in PD resistance in combination with *PdR1b.1* or one of the other candidates. Sequencing of *PdR1b.3* will be continued when more flanking sequence information is available.

Objective 2. Expression studies of candidate genes –
This objective was covered in the last report and no new progress was made.

Objective 3. Complementation tests of candidate gene(s) to test their function using: *Agrobacterium*-mediated transformation of the susceptible *Vitis* cultivars (Chardonnay and Thompson Seedless, and the rootstock St. George); and transformation of tobacco –
Once the gene constructs are completed, they must be tested to see if they contain the resistance genes. This is done by inserting the genes into a susceptible plant and testing to see if the insertion results in resistant plants. 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 *V. rupestris* St. George that have been used for transformation (Agüero et al. 2006).

PdR1b candidate genes were amplified using Phusion high-fidelity DNA polymerase (Finnzymes), cloned into pGEM-T easy vector (Promega) and sequenced at UC Davis Sequencing Facility. After sequence verification, genes were sub-cloned into binary vector pCambia 1303 (www.cambia.org) containing the 35S cauliflower mosaic virus promoter, the nopaline synthase terminator and an *hptII*-selectable marker gene. *PdR1b.1* was also sub-cloned into binary vector pDU99.2215 containing an *ntpII*-selectable marker gene. The resulting plasmids were transformed into disarmed *A. tumefaciens* EHA105 pCH32 by electroporation and used for transformation of Chardonnay, Thompson Seedless, and St. George.

Pre-embryogenic calli of Thompson Seedless, Chardonnay and St. George transformed with the 5 candidate genes were selected in medium with antibiotics, then sub-cultured to germination medium for plant regeneration. The presence of the genes was checked in callus cultures through PCR and tested again in plants transferred to the greenhouse. For each gene, we attempted to produce, at least 10 independent lines that will be subsequently propagated clonally to 6 plants per line and tested under greenhouse conditions. Table 1 shows the number of independent lines that have been obtained at present. Because the P2 binary plasmid was the last to be constructed, no P2 plants have yet been acclimated to greenhouse conditions yet. Chardonnay has the highest number of transgenic lines and, with the exception of P2, ten lines per gene have been acclimated to greenhouse conditions. Genomic DNA was isolated from these plants with DNeasy Plant Mini Kit (Qiagen). A primer that binds the CaMV 35S promoter and a primer that binds the coding region of each *PdR1b* candidate were used in combination for PCR amplification to verify the presence of the transgene. Candidate genes have amplified successfully in all the plants transferred to the greenhouse (Figure 1).

The first screening was completed in February 2014. It consisted of lines of Chardonnay transformed with *PdR1b.1, 4, 5 and 6*; with 5 independent lines per gene. Each line was multiplied through green cuttings to produce 6 replicates. They were cut back to two buds and re-grown. Inoculations via the pinprick procedure were performed 8 weeks after the grapevines had been cut back, when all plants had reached a height of approx. 1m. Plants were inoculated below and above the node within 5 to 10 cm from the base of the main shoot; using 10 µL of Beringer strain (OD600=0.25) each time. PD resistance was analyzed through phenotype scoring and ELISA. Symptoms of PD were evaluated using a 0-5 score for leaf scorch-leaf loss (LS-LL) and a 0-6 score for cane maturation index (CMI). For ELISA, plants were sampled 12 weeks post-inoculation by taking 0.5 g sections of stem tissue from 30 cm above the point of inoculation (Krivanek and Walker 2005, Krivanek et al. 2005).

All transgenic lines tested in the first screening displayed disease symptoms with different degrees of intensity. Lines P5-7a, P6-14a and P6-35b had low cane maturation index and leaf scorching scores. In addition, line P5-7a

showed the lowest bacteria concentrations among the transgenics, although not as low as the resistant biocontrols (Table 2). qPCR analysis to determine the correlation between the level of transgene expression and symptom/bacteria concentrations is underway.

A second round of screenings was started in March 2014. It includes the remaining five lines of *PdR1b.1, 4, 5* or *6* Chardonnay, plus 5 lines of *PdR1b.6* Thompson Seedless and one line of *PdR1b.6* St. George. A third round of screenings is scheduled to start in July to test of *PdR1b.2* Chardonnay and the rest of the Thompson Seedless and St. George lines. The most promising lines will be re-tested as well.

Several lines of Chardonnay transformed with *PdR1b.6* exhibited an altered phenotype characterized by stunted growth (**Figure 2**).

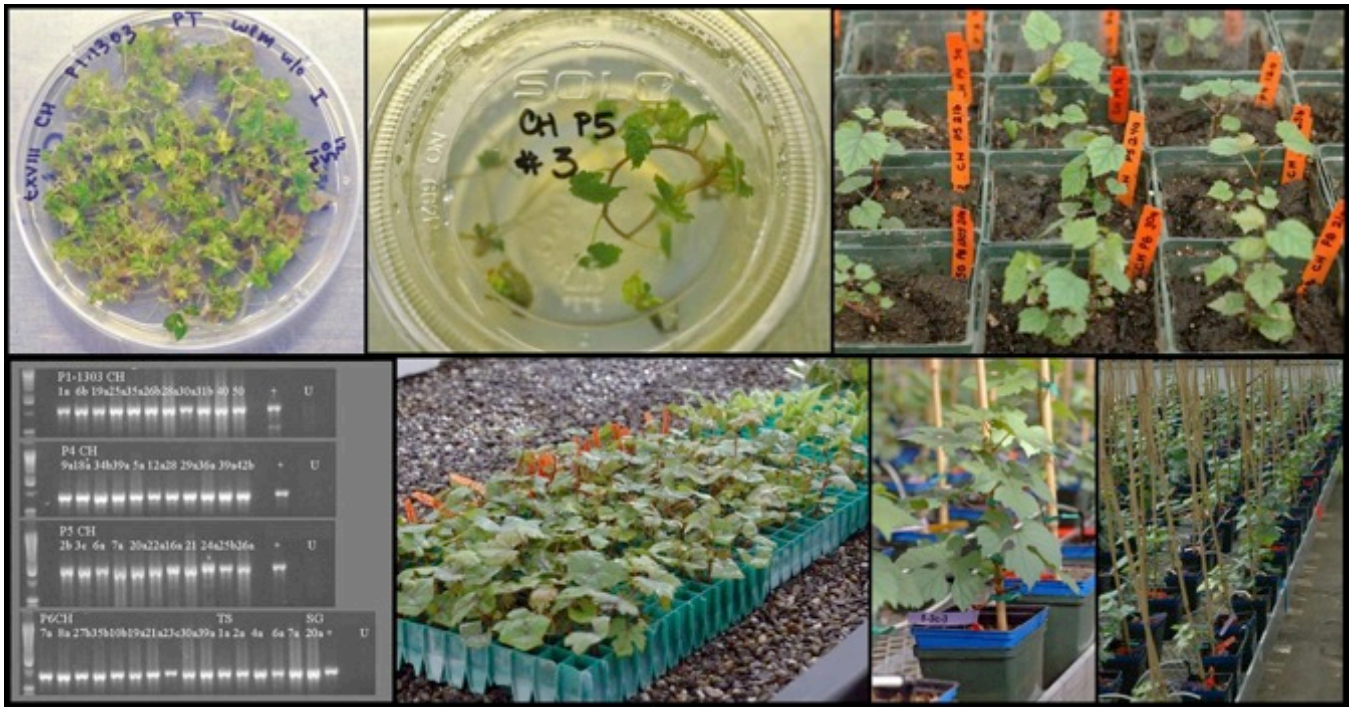

Figure 1. Clockwise from top left: Chardonnay embryos growing in germination medium, regenerated plantlets growing *in vitro*, *in vitro* plants transferred to substrate in greenhouse, transgene detection through PCR, green cuttings in mist bed, plants after cut back.

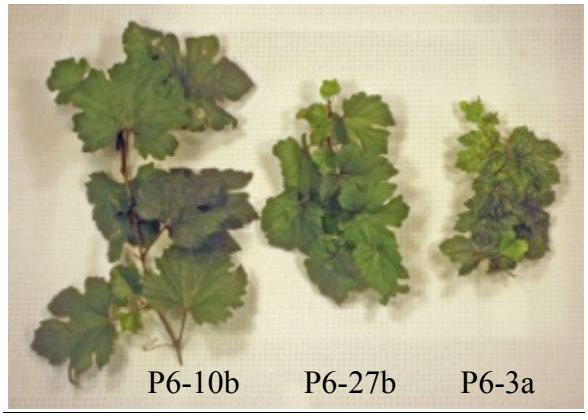

Figure 2. Phenotype of three lines of Chardonnay transformed with *PdR1b.6*, the two lines on the right show a mild and severe dwarf phenotype.

Table 1. Number of independent lines produced until March 2014; lines in the greenhouse are shown in parentheses.

	Chardonnay	Thompson Seedless	St George
P1 pDU 99.2215	4 (3)	0	0
P1 pCambia1303	15 (10)	5 (2)	13 (10)
P2 pCambia1303	4 (0)	0	0
P4 pCambia1303	20 (10)	7 (2)	4 (4)
P5 pCambia1303	13 (10)	5 (2)	2 (2)
P6 pCambia1303	18 (10)	8 (7)	1 (1)

Table 2. Greenhouse screen results for Chardonnay transformed with *PdR1b.1, 4, 5 and 6.* First six genotypes correspond to negative control and resistant biocontrols.

Genotype	Reps	Geometric mean (cfu/ml)	Mean (ln cfu/ml)	Std Error (ln cfu/ml)	CMI Mean	CMI Std Err	LS-LL Mean	LS-LL Std Err
CH uninoc	6	9.897	9.2	0.0	0.2	0.2	0.9	0.2
b43-17	6	10.232	9.2	0.0	1.8	0.2	2.7	0.4
Roucaneuf	6	16.592	9.7	0.5	0.0	0.0	0.7	0.2
U0505-01	6	27.356	10.2	0.5	0.8	0.7	1.7	0.3
Blanc du Bois	5	43.478	10.7	0.8	2.2	0.4	2.4	0.2
U0505-35	6	107.291	11.6	0.8	0.2	0.2	2.2	0.3
5-7a	6	705.527	13.5	1.0	0.3	0.2	2.2	0.5
1-19a	6	1,518.601	14.2	0.5	1.3	0.6	2.7	0.3
4-9a	5	1,559.694	14.3	0.4	1.0	0.3	2.4	0.2
6-14a	6	1,764.363	14.4	0.4	1.5	0.5	2.2	0.3
1U-20a	6	1,794.075	14.4	0.4	1.1	0.5	2.7	0.3
4-39a	4	2,032.953	14.5	0.4	1.6	0.9	2.8	0.3
5-6a	6	2,303.638	14.7	0.4	2.0	0.4	2.7	0.2
1U-10c	6	2,421.748	14.7	0.5	2.0	0.7	2.7	0.3
5-2b	6	2,421.748	14.7	0.5	1.8	0.6	2.2	0.3
4-34b	5	2,520.581	14.7	0.3	1.5	0.6	2.8	0.4
6-8a	5	2,520.581	14.7	0.4	3.6	0.4	2.8	0.4
5-20a	6	3,007.741	14.9	0.4	2.0	0.7	2.5	0.3
1-6b	5	3,078.645	14.9	0.3	3.4	0.2	2.4	0.2
Chardonnay	6	3,324.068	15.0	0.4	2.0	0.5	2.5	0.3
1-35a	6	3,379.708	15.0	0.3	2.5	0.6	2.7	0.3
Untransf-2	6	3,436.623	15.1	0.4	3.8	0.2	2.5	0.2
U0505-22	6	3,494.497	15.1	0.5	2.0	0.7	1.6	0.3
5-22a	6	3,673.664	15.1	0.4	1.1	0.5	2.3	0.4
Untransf-1	6	3,926.661	15.2	0.3	2.0	0.6	2.5	0.2
6-7a	3	4,127.985	15.2	0.5	2.0	0.6	3.0	0.6
6-35b	3	4,268.188	15.3	0.4	1.5	0.5	1.7	0.3
1U-18b	5	4,325.334	15.3	0.3	2.4	0.5	2.2	0.4
1-1a	5	4,685.579	15.4	0.3	1.7	0.4	2.4	0.2
1-25a	6	5,213.177	15.5	0.1	2.3	0.8	2.7	0.2
4-18a	5	5,722.979	15.6	0.1	1.1	0.5	3.0	0.3
5-3c	4	6,261.936	15.7	0.1	1.5	1.0	3.0	0.4
6-27b	5	6.582.993	15.7	0.0	3.8	0.2	3.0	0.3

Tobacco transformation

To speed the functional analysis, MSc student Carolina Bistue also transformed the tobacco variety SR1, which was recently demonstrated to be a susceptible host for *X. fastidiosa* and is much easier and quicker to transform and test (Francis et al. 2008). Transgenic tobacco plants carrying each candidate gene (9-10 independent lines per gene) were produced at the UC Davis Transformation Facility and multiplied *in vitro* in our lab. No significant differences were observed in stem Xf counts between untransformed controls and transformed plants 12 weeks post inoculation. However, candidate genes *PdR1b.1* and *PdR1b.6* displayed significantly lower symptoms compared to the untransformed controls. Expression analysis by real-time PCR confirmed expression of both genes. These results have been presented in previous reports.

Genetic transformation via organogenesis

Inoculation with *A. tumefaciens* of meristematic bulks (MB) is being tested as an alternative transformation technique via organogenesis to reduce the time needed to produce transgenic grapes (Mezzetti et al. 2002). In our lab, transgenic plants of Thompson Seedless expressing GFP were produced in 3 months using MB and kanamycin as the selective agent. Based on these results, Thompson Seedless MB slices were inoculated with *A. tumefaciens* carrying *PdR1b.5* in pCAMBIA 1303 using 3 initial levels of hygromicin: 5, 10 and 15 ug/ml. Since no regeneration was produced at any of the concentrations tested, experiments assaying 0 ug / ml in the first subculture after inoculation, followed by 2.5 ug /ml hygromicin were initiated. Two, out of 50 initial MB, regenerated at this lower concentration, efforts to establish regenerated plants are underway.

The partial success obtained with the use of hygromicin and the production of MB of Chardonnay and St. George, have led PhD student Xiaoqing Xie to test different hormone ratios to adapt the process to these cultivars and study the use of different antibiotics. She has developed protocols to produce MB of Chardonnay, St. George and 101-14 (Figure 3) and has transformed them with *A. tumefaciens* carrying plasmids pCambia 1303 and pCambia 2303 to compare the use of hygromicin and kanamycin as selectable markers.

Transformation of pre-embryogenic cultures or MB via Agrobacterium has been described in previous reports

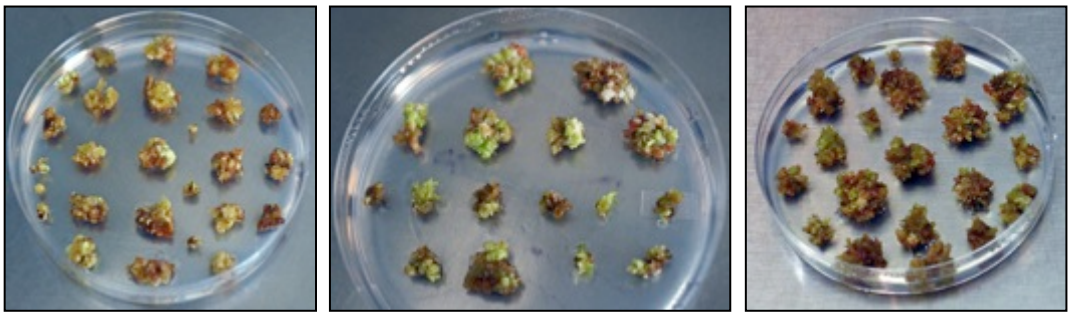

Figure 3. Meristematic bulks of St. George (left), 101-14 (center) and Chardonnay (right)

CONCLUSIONS

The last step in the characterization of a resistance gene is to verify that the isolated gene functions in a host plant. This process requires that the gene is transformed into a susceptible host and challenged by the disease agent. We have transformed grape and tobacco with five *PdR1b* candidate genes. Results obtained with *Xylella* inoculations of tobacco point to 2 potential sequences that might be involved in the resistance. We are currently testing the transgenic grapes. The classical methods of gene introgression have the disadvantage of potential linkage drag (inclusion of unselected genes associated with a trait) and the time required for time-consuming backcrosses and simultaneous selection steps. Cisgene micro-translocation is a single-step gene transfer without linkage drag; as well as a possible means of stacking resistance genes in existing winegrape varieties

PUBLICATIONS AND PRESENTATIONS

Agüero C.B., Bistué C., Riaz S., Xie, X., and Walker M.A. 2013. Molecular characterization of the putative *Xylella fastidiosa* resistance gene(s) from b43-17 (*Vitis arizonica/candicans*). Proceedings 2013 Pierce's Disease Symposium. December 16-18, 2013, Sacramento.

Riaz S, Walker MA 2013. Phylogeographic analysis of resistance to Pierce's Disease in North American and Mexican species. 64th Annual Meeting of the American Society for Enology and Viticulture, Monterey, CA

Bistue C, Aguero CB, Riaz S, Walker MA. 2013. Testing *Vitis arizonica* candidate genes for Pierce's disease resistance in *Nicotiana tabacum* SR-1. 64th Annual Meeting of the American Society of Enology and Viticulture and Enology. Monterey, CA

Breeding PD resistant winegrapes. Talk and Tasting (2 Sessions), Napa Grape and Wine Expo, Napa, CA, November 14, 2013.

Breeding PD resistant winegrapes. Talk and Tasting. Martini/Gallo North Coast Winemaking Group, UC Davis, CA, December 9, 2013.

PD resistant winegrapes nearing release. CDFA PD Research Symposium. Sacramento, CA, December 16-18, 2013.

UCD Grape Breeding. Chilean Growers and CORFO, UC Davis, February 4, 2014

Grape Breeding at UC Davis. ISHS Workgroup tour and presentation, UC Davis, February 7, 2014

PD resistant winegrapes coming soon. Unified Grant Management Seminar, UC Davis, February 12, 2014

RESEARCH RELEVANCE

This research is focused on demonstrating whether PD resistance genes developed from genetic and physical mapping efforts function when transformed into susceptible host plants. These transformations are underway in tobacco (an easily used model system) and susceptible grape (Chardonnay, Thompson Seedless and St. George). These studies will lay the foundation to understanding how these resistance genes work, and may provide a tool to genetically engineer grape resistance genes into susceptible grapevines.

LAYPERSON SUMMARY

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 classical breeding to be greatly expedited through marker-assisted selection. Genetic maps allow the construction of physical maps to identify resistance genes (Riaz et al. 2008; Riaz et al. 2009). The physical map of the b43-17 resistance region allowed 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 LRR domains were present in the resistance region. This category of genes is involved in the recognition of microbes and in the initiation of defense responses (Bent and Mackey 2007). We completed the cloning of five candidate genes: *PdR1b.1, 2, 4, 5* and *6* and confirmed their sequence. We also developed embryogenic callus cultures of PD susceptible Chardonnay and Thompson Seedless and rootstock St. George for genetic transformation to verify candidate PD resistance gene function. *PdR1b.1, 2, 4, 5* and *6* have been used in transformation of tobacco and grape. Transgenic tobacco plants have been tested against Xf in the greenhouse and promising results have been obtained with 2 candidate genes. Transgenic grape plants have been acclimated to greenhouse conditions and are being tested against Xf; screening of the first set of plants of Chardonnay was completed in February 2014. Two other sets of transformed Chardonnay are scheduled for testing over the next 12 months. Although the current transgenic grape plants were produced using the traditional procedure, we are also testing another technique to speed the development of transgenic tissue from meristematic bulks that will allow *PdR1* gene candidates to be tested faster.

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

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

LITERATURE

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