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Progress Report
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Renewal Progress Report for CDFA Agreement Number 10-0277 Amend 01

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

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LAYPERSON SUMMARY

By-products of our PD resistance breeding program are populations from which we can construct genetic maps, identify tightly linked DNA markers, and use these markers to expedite selection of resistant individuals. Genetic maps also allow the construction of physical maps capable of identifying resistance genes (Riaz et al. 2008; Riaz et al. 2009). The physical mapping of the PD resistance gene from *Vitis arizonica/candicans* b43-17 resistance region allowed us to identify six candidate genes that may be responsible for PD resistance. Comparisons with other 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 the six candidate genes: *PdR1b.1 – 6* and confirmed their sequences. We also developed embryogenic callus cultures of PD susceptible *V. vinifera* Chardonnay and Thompson Seedless and the susceptible rootstock *V. rupestris* St. George so that we could engineer the gene candidates into these susceptible backgrounds to verify candidate PD resistance gene function. *PdR1b.1, 4, 5* and *6* have been used in transformation of tobacco and grape. The remaining candidates, *PdR1b.2* and *3* will be next. Tobacco plants transformed with *PdR1b.1* and *6* are ready to be tested against *Xylella fastidiosa* in the greenhouse. 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.

INTRODUNCTION

New cultivars bred to resist *X. 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 with 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 gene from *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. This approach in grapes is similar to natural clonal variation represented by different known winegrape cultivars. This approach is also linkage-drag-free is attractive since it allows movement of the resistance gene without associated genetic regions which may

be related to lower quality. It 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 map of the resistance region from *V. arizonica/candicans* b43-17, *PdR1b*, allowed the identification of 6 potential candidate resistance genes. Preliminary comparison indicated that the *PdR1*b 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 lead to the initiation of 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 progress on the cloning and functional characterization of these 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. Genetic transformation for the validation of gene constructs / Development of alternative protocols
    a) *Agrobacterium*-meditation transformation of the susceptible *Vitis* cultivars (Chardonnay and Thompson Seedless, and the rootstock St. George).
    b) Transformation of tobacco.

RESULTS AND DISCUSSION

Objective 1. 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). 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 in the diversification of disease resistance genes through a process termed TE-induced gene alteration (Michelmore 1995). 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 be receptor-like proteins, a class of resistance proteins. The second assembly allowed the identification of two new candidate genes and showed that *PdR1b.1* was longer than the sequence previously found. As a consequence, we repeated the amplifications from BAC H69J14, using primers that hybridize to regions flanking the ORFs. We have re-amplified and confirmed the sequences of all six candidate genes *PdR1b.1 - 6*. *PdR1b.1* is the largest gene, sharing a high degree of homology with *PdR1b.2, 3, 4*, and *5*. *PdR1b.6* is significantly different from the rest (Table 1). It has a kinase domain that suggests it might be involved in PD resistance in combination with *PdR1b.1* or one of the other candidates.

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, which 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 the 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 these regions

of the genes using total RNA extracted, then clone and sequence the DNA to make sequence comparisons among Pinot noir and 5 different genotypes used for RNA extraction.

Table 1. Size and degree of homology between *PdR1b* candidate genes

	Size (bp)	% Homology with *PdR1b.1*	% Homology with *PdR1b.1 over-lapping region*
PdR1b.1	3198	-	-
PdR1b.2	2946	87%	95%
PdR1b.3	2787	83%	95%
PdR1b.4	2580	80%	98%
PdR1b.5	2235	53%	77%
PdR1b.6	2052	21%	-

Objective 2. 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). Stem and leaf tissues were 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). Two other methods were tested to get better yield and quality of total RNA with less genomic DNA. The best yield of total RNA was obtained by the procedure described by Reid et al. 2006.

We used a time course analysis to evaluate expression and to determine when the resistance gene(s) is activated. Total RNA was extracted from the leaves and stem tissue of un-inoculated plants, and from plants 1, 3 and 5 weeks after inoculations with the *X. fastidiosa*. ELISA screening was carried out after 12 weeks to quantify the amount of *X. fastidiosa* in tissues. Figure 1 presents the results of RNA extracted from the young leaves of the four genotypes before the bacterial inoculations and stem tissue two weeks after inoculations. First-strand cDNA synthesis will be performed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). We tested three different genes to act as housekeeping gene controls (actin, chitinase and B-actin). The actin gene (F-actgctgaactggaaattgt; R- acggaatctctcagctccaa) as described Vasanthaiah et al. 2008 worked very well in our system. We designed specific primers for quantitative PCR from two of our candidate genes (PdR1.1 and PdR1.6) and carried out test comparative RT-PCR to check the effectiveness of the primers and the whole setup. We observed differences in the level of expression of the genes indicating that our RT-PCR system is working. Experiments that are more detailed are underway to monitor the gene expression of all six candidate PD resistant genes.

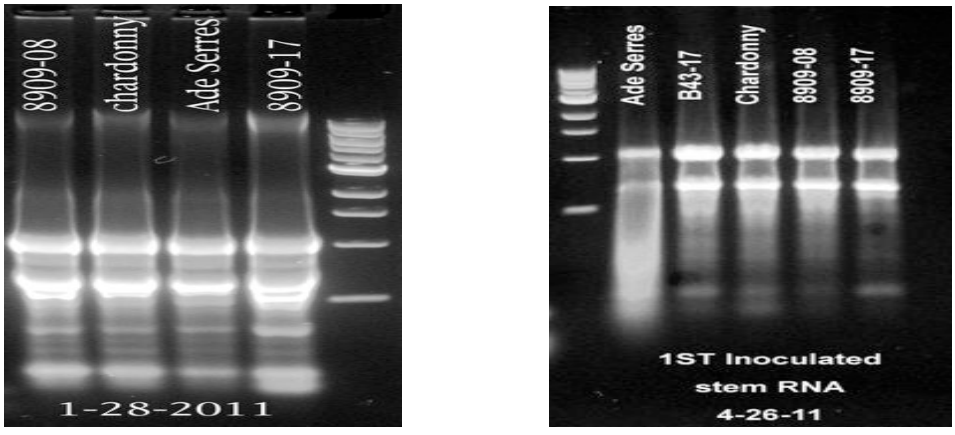


Figure 1. Total RNA extracted from the leaves and stem tissue of susceptible control and resistant plants

Objective 3. Genetic transformation to validate the function of gene constructs / Development of alternative protocols. Now that most of the gene constructs have been completed, they must be tested to see which contain the resistance genes. This is done by inserting the genes into a susceptible plant and testing to see if the insertion makes it resistant. 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. These cultures of embryogenic calli can be used readily for transformation (Agüero et al. 2006).

We have sublconed *PdR1b.1* into the binary vectors pCAMBIA-1303 (www.cambia. org) and pDU99.2215 (Figure 2) and *PdR1b.4, 5* and *6* into pCAMBIA-1303. pCAMBIA-1303 was included in the experiments because it carries a hygromicin resistance gene that improves the selection of transformants (D. Tricoli, pers. comm.). An additional advantage is that it allows subcloning of the gene in one step, by replacing the *gus* gene with the gene of interest. The resulting plasmids were used for transformation via *Agrobacterium tumefaciens* of Chardonnay, Thompson Seedless, St. George and tobacco SR1. A similar procedure will be followed with the remaining two genes. The transformation experiments are at different stages of development: 1) the most advanced are tobacco plants transformed with *PdR1b.1* and *6,* which have been multiplied *in vitro* and acclimated to greenhouse conditions for testing against *Xylella fastidiosa* as described by Francis et al., 2008 (Figure 3a); 2) tobacco plants transformed with *PdR1b.4* and *5* are being multiplied in vitro (Figure 3b); 3) pre-embryogenic calli of Thompson Seedless, Chardonnay and St George transformed with *PdR1b.1* and *6* that developed in selection medium with antibiotics have been subcultured to germination medium; we expect to obtain plants in about 4 months (Figure 3c); 4) pre-embryogenic calli of grapes transformed with *PdR1b.4* and *5* are being cultured in selection medium (Figure 3d); and finally 5) we working to subclone of *PdR1b.2* and *3* into the binary plasmid pCAMBIA-1303. A summary of progress for each candidate gene is shown in Table 2.

Two alternative transformation techniques via organogenesis have been tested with the goal of reducing the time needed to produce transgenic grapes. These methods have been developed in Thompson Seedless and are based on the use of meristematic bulks (MB) or etiolated meristems (EM) as explants for inoculation with *Agrobacterium* (Mezzetti et al. 2002, Dutt et al. 2007). In this latter method, *Agrobacterium* is inoculated on slices of the MBs. Using this procedure, transgenic plants of Thompson Seedless expressing GFP were produced in 3 months. No plants were regenerated from etiolated meristems and the procedure was laborious and time consuming. We have produced meristematic bulks of Chardonnay and St. George (Figure 4). They were inoculated with *Agrobacterium* carrying *PdR1b.4* in pCAMBIA-1303. We are testing 3 initial levels of hygromicin, 5, 10 and 15 ug/ml. Hygromicin concentration will be increased gradually up to 25 ug/ml with each subculture (Figure 5).

Transformation of pre-embryogenic cultures of MBs have been performed with *A. tumefaciens* EHA105 pCH32, carrying binary plasmids with *PdR1b* coding sequences. Overnight cultures of the bacteria in LB medium + antibiotics are diluted to 10^8 cells·ml^{-1} using liquid co-cultivation medium. Pre-embryogenic calli are placed on a sterile glassfiber filter (GFF) overlaid on co-cultivation medium. The *Agrobacterium* culture is poured over the callus and excess is blotted with sterile filter paper after 5 min. MB slices are dipped in bacteria suspension for 10 minutes. Pre-embryogenic callus or MB are then transferred onto fresh co- cultivation medium. After 48 h in the dark, MB or callus pieces, sub-divided into clumps of about 2 mm in diameter, are cultured on selection medium containing 100 ug/ml kanamycin or 15-25 ug/ml hygromicin.

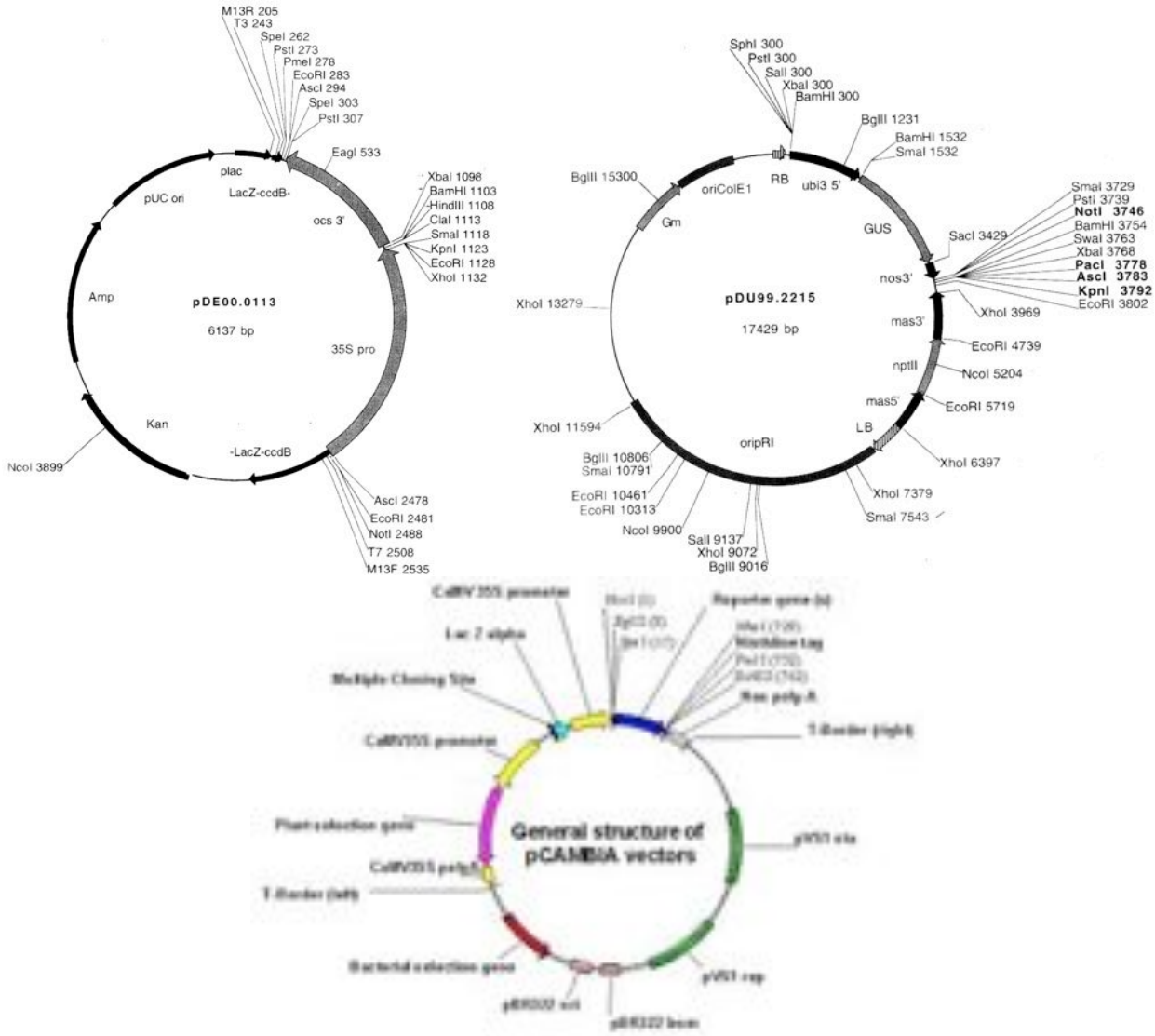
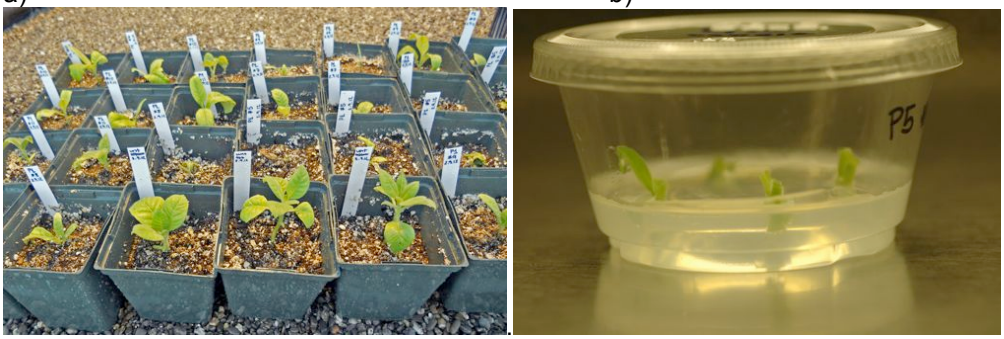


Figure 2. Vectors used in genetic transformation with *PdR1b* candidate genes. *PdR1b.1* was subcloned into pDE00.0113 and then the expression cassette was moved into binary plasmid pDU99.2215. *PdR1b.1, 4, 5,* and *6* were subcloned directly into binary plasmid pCAMBIA 1303 in the place of the reporter gene. Because the two binary plasmids have different plant selection genes, it is possible to co-transform tobacco with *PdR1b.1* and *6*

a) b)



c) d)

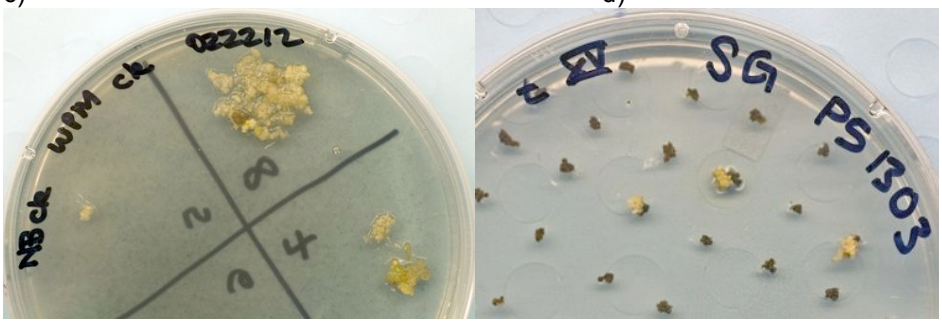


Figure 3. a) Transgenic tobacco plants in the greenhouse and b) multiplied in vitro using uninodal cuttings; c) calli in germination medium, d) pre-embryogenic calli in selection medium

Table 2. Progress status of transformation experiments

	Cloned into pGEM/sequence confirmation	Cloned into binary plasmid	Grape transformed	Tobacco transformed/plants
PdR1b.1	x	x	x	x / plants
PdR1b.2	x			
PdR1b.3	x			
PdR1b.4	x	x	x	x
PdR1b.5	x	x	x	x / plants
PdR1b.6	x	x	x	x / plants

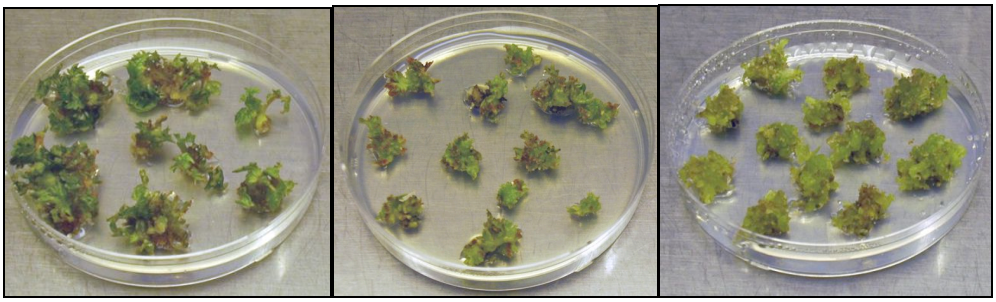


Figure 4. Meristematic bulks of Chardonnay (left), St. George (middle) and Thompson Seedless (right).

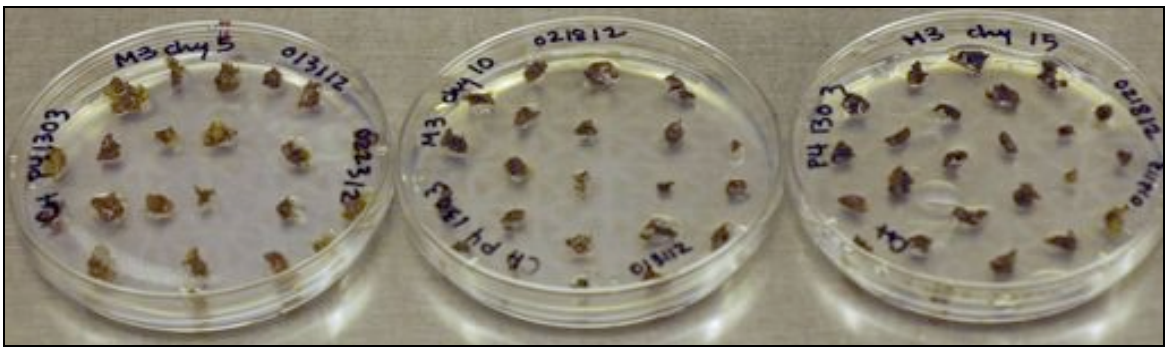


Figure 5. Meristematic bulks of Chardonnay cultured in selection medium containing 5 (left), 10 (middle) and 15 (right) ug/ml hygromicin

CONCLUSIONS and RELEVANCE

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. *Agrobacterium*-based transformation can be used with grape, but initiating transformable and regenerating tissue is often a problem with grape. We have cloned the six *PdR1b* candidate genes and four of them have been used in genetic transformation of tobacco and pre-embryogenic callus of Chardonnay, Thompson Seedless and St. George to produce transgenic plants for use in testing the *PdR1b* candidates. Work is underway to complete the transformation with the last two genes. Plants of transformed tobacco with *PdR1b.1* and *6* have been obtained and are ready to be tested against *Xylella* in the greenhouse. 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 in a much broader range of genotypes. If *PdR1* gene candidates function they could be transformed into a wider range of winegrapes with this technique.

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

Walker M.A., Riaz S., Agüero C., Bistue C. 2011. Molecular characterization of the putative *Xylella fastidiosa* resistance gene(s) from b43-17 (*V. arizonica/candicans*). Poster presentation Pierce's Disease Research Symposium, Dec. 13-15, Sacramento.

Presentations

UC Davis grape breeding program, Croatian Grape Growers and Wine Makers Group, UC Davis, July 13

UC Davis grape breeding program, University of Florida Horticulture Graduate Students Association visit, UC Davis, August 10

Using marker-assisted selection to optimize breeding for resistance to powdery mildew, American Vineyard Foundation Research Forum, UC Davis, February 24

Grape growing and breeding at UCD, Culinary Institute of America Foodies Tour, UC Davis, March 11

Grape research and careers, Early Academic Outreach Program, UC Davis, March 15

UCD grape breeding program, Lodi/Woodbridge Grape Growers Meeting, Lodi, CA, March 18

Sustainable winegrape growing, UC Berkeley Haas Business School Top Tech Program, Mondavi Winery, Oakville, CA, April 9

Grape breeding at UC Davis BOKU : University of Natural Resources and Life Sciences, Vienna, Austria, June 13

PD resistant winegrapes are approaching wine quality and field testing, 62nd Annual Meeting of the American Society of Enology and Viticulture, Monterey, CA, June 22

Grape breeding at UCD. Hopland Growers Meeting, Hopland, CA November 21

Grape breeding progress. Daniel Roberts Growers Meeting, Santa Rosa, CA December 5

Resistance Round Table: Breeding for resistance to grape pests and diseases. Annual Meeting of the Association of Applied IPM Ecologists, Napa, CA, December 16

Grape breeding progress update. Wilbur Ellis Viticulture Team, Santa Rosa, CA, February 9

Breeding for resistance to grape diseases. Ag Unlimited Annual Meeting, Napa, CA, March 1

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

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

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