FINAL REPORT FOR CDFA AGREEMENT NUMBER 14-0137-SA and 17-0427-000-SA

PROJECT TITLE: Molecular breeding support for the development of Pierce's disease resistant winegrapes.

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REPORTING PERIOD: July 2014 to June 2018

LAYPERSON SUMMARY

Our main focus is to identify and genetically characterize unique PD resistance sources from the southwestern US and Mexican *Vitis* species collections. In order to carry out this task, we create targeted genetic maps that associate regions of chromosomes with PD resistance. These regions (markers) are used to expedite screening for resistance since they can be used to test seedlings for resistance as soon as they sprout. Markers developed from different sources of resistance allow us to combine multiple resistance forms and therefore produce offspring with the likelihood of having more durable resistance. These markers also allow us to identify candidate resistance genes and study how they function by engineering them into susceptible grape varieties to better understand the genes and the resistance.

INTRODUCTION

This project provides molecular support to the PD resistance grape breeding project – "Breeding Pierce's disease resistant winegrapes" by acquiring and testing a wide range of resistant germplasm, tagging resistance regions with markers by genetic mapping and functionally characterizing the resistance genes from different backgrounds. To meet the key objectives of the program, we have surveyed over 250 accessions of *Vitis* species growing in the southern US and Mexico in an effort to identify new PD resistant accessions. Analysis using population genetics tools has allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. Fourteen promising resistant accessions were identified from this germplasm. Markers were used to determine their genetic diversity and relationships to each other. Small breeding populations were developed and more than 700 seedlings were marker tested to ensure that they had the correct parentage and identity. We used a limited mapping strategy by utilizing markers from chromosome (Ch) 14 in conjunction with greenhouse screen data of small breeding populations to determine if the resistance to PD in these 14 accessions is different from the previously identified locus *PdR1* (Riaz et al. 2018**)**. Three unique resistance sources (T03-16, ANU67 and b41-13) were identified as having a different resistance region than Ch14. More crosses were made in Spring 2016-2017 to expand these breeding populations for map-based identification of genomic regions that contribute to resistance.

The identification and characterization of resistance genes and their regulatory sequences will help determine the basis of resistance/susceptibility in grape germplasm. In addition, these genes and their promoters could be employed in production of 'cisgenic' plants. Cisgenesis is the transformation of a host plant with its own genes and promoters (Holmes et al. 2013). We have completed the physical map of *PdR1*a and *PdR1*b locus for b43-17 to clone and characterize resistance genes (see earlier reports). The physical map of the *PdR1*c locus (from b40-14) is also complete. Development of *V. vinifera* plants transformed with our PD resistance genes and grape promoters might work more effectively and allow us to better understand how the *PdR1* resistant gene functions.

Upstream and downstream sequences as well as the gene sequences of two candidate genes, ORF14 and ORF18, from *PdR1*b were verified and constructs were developed to test their function. Transformation experiments with the *PdR1* resistance gene with a native grape promoter were completed with ORF18 and transgenic lines are being developed and maintained for later resistance verification. A large scale multiple time point gene expression project was completed in the greenhouse and RNA extractions were completed for over 400 samples. We used

qPCR to test the expression of candidate genes. Embryogenic callus cultures of *V. vinifera* cvs. Chardonnay and Thompson Seedless and *V. rupestris* St. George are being maintained to test the function of gene sequences. These efforts will help us identify candidate resistance genes by complementation and better understand how they function.

OBJECTIVES

1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the PD resistance breeding program, carry out genetic mapping of two new highly resistant lines b41-13 and T03-16 for use in stacking PD resistance genes.
2. Complete a physical map of the PdR2 region from the b42-26 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*) and b40-14 (*PdR1c*).
3. Employ RNA-seq to understand genome-wide transcriptional changes of the pathways regulated by defense-related genes in b40-14.
4. Clone *PdR1c* and *PdR2* resistance genes with native promoters.
5. Compare the PD resistance of plants transformed with native vs. heterologous promoters

RESULTS AND DISCUSSION

Objective 1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the PD resistance breeding program, carry out genetic mapping of two new highly resistant lines b41-13 and T03-16 for use in stacking PD resistance genes.

We completed screening of over 250 southwestern US and northern Mexico *Vitis*, which included accessions, collected from multiple collection trips from States bordering Mexico or previously collected from Mexico by Olmo. Both SSR (simple sequence repeat) and chloroplast markers were used to establish relationships with known sources of resistance currently being used in the breeding program (Riaz and Walker 2013). Small breeding populations were developed with 14 most promising resistant accessions by crossing to highly susceptible *V. vinifera*. A total of 704 individuals obtained from these breeding populations were greenhouse screened and limited mapping strategy with markers from Ch 14 that are linked to the *PdR1* locus (See previous reports for details of the *PdR1* locus) were used. This strategy allowed us to identify resistance sources whose resistance is similar to *PdR1* and sources that are different. The results from this study identified 9 accessions with a major resistance locus within the genetic window where the *PdR1* locus from accession b43-17 was mapped. Results were not conclusive for 2 accessions A14 and b47-32 due to small population size and/or lack of polymorphic markers. The phenotypic data of three accessions, ANU67, b41-13, and T03-16 did not correlate with the resistance markers from chromosome 14 (Table 1). These three accessions were identified as candidates for further work and the development of framework maps with larger populations to detect new unique loci for PD resistance breeding. The major findings of this work were published (Riaz et al. 2018).

Accession T03-16 from the Big Bend region in Texas and b41-13 from Tamaulipas state in Mexico are strong candidates that do not possess *PdR1*. In order to identify the genomic regions housing PD resistance in these two accessions, crosses were made in Spring 2016 and 2017 to expand population sizes. A total of 295 seedling plants of the F1 population from b41-13 are established in the field. We have completed the greenhouse screening of 122 F1 seedling plants. An additional 150 seedlings from a 2016 cross are in different stages of greenhouse screening and the results will be available by November 2018. SSR markers from chromosome 8 and 14 were tested on a small set of parents and progeny, and 35 polymorphic markers were run on the entire population of 295 seedlings. Further marker testing is in progress to develop a framework map and QTL analysis to identify genomic regions linked to PD resistance. Crosses were also made with T03-16 and 285 seedling plants from the F1 population were established in the field. Multiple replicates of seedling plants were propagated for greenhouse screening – 173 seedling plants are now in different stages of greenhouse testing with results expected by the end of 2018.

Table 1. The 14 resistant accessions used to create 23 breeding populations in an effort to identify PD resistance sources that differ from *PdR1*. Resistant accessions with different sources of resistance are marked as Not 14 in the last column. Accessions marked as LG14 possess the *PdR1* locus. Resistance affinity to Ch14 could not be

determined for the accessions that are marked as Inconclusive due to small population size and less informative markers.

Resistance source	Species description	Populations tested	Number of Screened Genotypes	PD resistance
ANU5	*V. girdiana*	12-314	60	LG14
b40-29	*V. arizonica*, brushy	12-340, 12-341, 14-367, 14-368	29	LG14
b46-43	*V. arizonica*, glabrous with *V. monticola*	12-305, 14-308, 14-321, 14-322, 14-324, 14-336	159	LG14
b41-13	*V. arizonica-mustangensis* and *champinii* hybrid,	13-355	47	Inconclusive
b47-32	*V. arizonica* glabrous with *monticola*,	13-344	13	Inconclusive
SC36	*V. girdiana*	13-348	35	LG14
T03-16	*V. arizonica* glabrous	13-336	62	Inconclusive
A14	*V. arizonica*	14-313	25	Inconclusive
A28	*V. arizonica*	14-347, 14-364	42	LG14
ANU67	*V. arizonica* glabrous	14-362	28	Inconclusive
ANU71	*V. arizonica-riparia* hybrid	14-340	30	LG14
C23-94	*V. arizonica* glabrous and brushy	14-303	44	LG14
DVIT 2236.2	*V. cinerea* like,	14-360	30	LG14
SAZ 7	*V. arizonica*	14-363	52	LG14

We identified a new locus *PdR2* in the *V. arizonica/girdiana* b42-26 background. To create a genetic map of the F1 population 05347 (F2-35 x b42-26), we expanded the population to 352 seedling plants and tested more than 1,000 markers. The level of polymorphism in b42-26 is very low likely because of its geographic isolation and resulting inbred genetic background. The genetic map was developed with 202 markers which grouped to 18 chromosomes. Chromosome 19 was not represented. We tested more than 50 SSR markers that have been mapped on Ch19 in other breeding populations and none of them were polymorphic for b42-26. Table 2 represents the genetic maps of both susceptible *vinifera* F2-35, accession b42-26, and the consensus map. We carried out QTL analysis with this map and identified resistance on Ch8 and Ch10 (Fig. 1). The resistance from Ch8 was also verified on the basis of linked alleles in the pBC1 and pBC2 populations. Using the Cabernet Sauvignon genome sequence, we developed nine new SSR markers for Ch8 and added them to the genetic map. In Spring 2017 and 2018, we began using closely linked markers to assist the breeding program with MAS to stack the *PdR1*b and *PdR2* loci together. A manuscript is detailing genetic mapping in b42-26 and b40-14 is approaching publication.

Table 2. Description of the genetic maps of susceptible *vinifera* parent, resistant accession b42-26 and consensus map.

	F2-35		b42-26		Consensus	
Chromosome	Mapped Markers	Map length (cM)	Mapped Markers	Map length (cM)	Mapped Markers	Map length (cM)
Chr1	11	37.70	16	43.50	19	59.90
Chr2	0	0.00	4	9.50	4	9.50
Chr3	6	28.10	7	43.40	9	44.90
Chr4	12	53.70	12	53.30	14	53.10
Chr5	6	22.20	10	29.20	12	33.10
Chr6	7	45.90	9	54.20	9	45.00
Chr7	3	15.80	9	37.80	9	37.30
Chr8	12	54.90	22	74.10	20	74.40
Chr9	3	21.40	6	27.50	7	28.20
Chr10	9	48.20	10	55.20	10	51.80
Chr11	4	33.90	5	34.80	5	34.40
Chr12	4	5.40	12	40.00	12	39.10
Chr13	8	30.20	13	33.00	15	32.20
Chr14	12	66.20	22	61.50	23	61.80
Chr15	2	31.80	4	48.90	4	51.10
Chr16	8	46.80	9	55.50	10	53.90
Chr17	5	28.90	6	29.70	6	30.10
Chr18	8	61.50	13	94.40	14	95.00
Chr19	0	0.00	0	0.00	0	0.00
Total	120	632.60	189	825.50	202	834.80
Ave marker distance (cM)		2.92		4.37		4.133

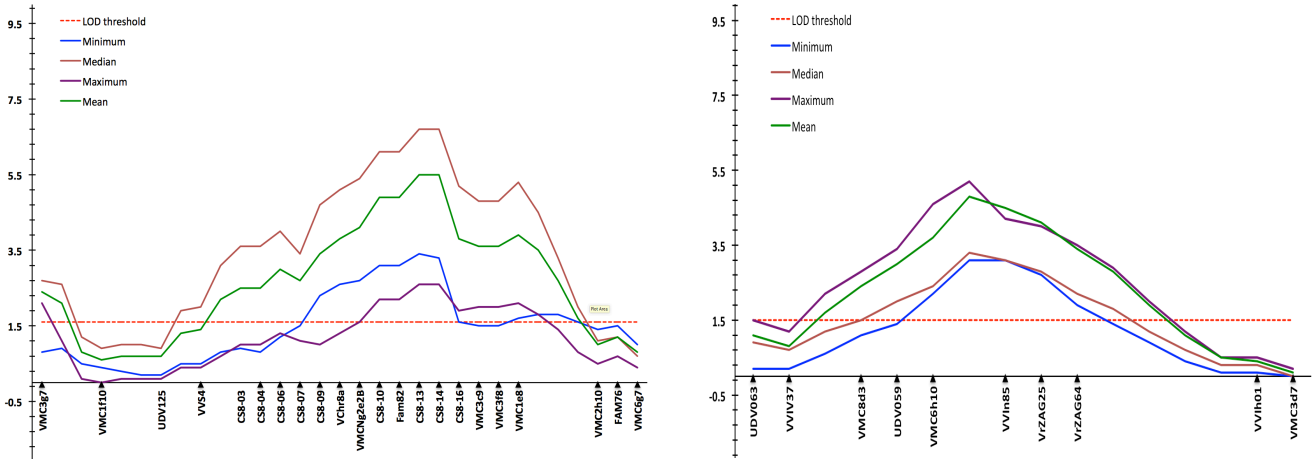

Figure 1. QTL analysis results for Ch8 and Ch10 using the greenhouse screening results for the 05347 population, which segregates for PD resistance from *V. arizonica* b42-26.

This project provides molecular support to the companion PD resistance winegrape breeding project by conducting MAS on seedling populations. In Spring 2018, we extracted DNA and marker tested 3,102 seedling plants from 59 different crosses for the *PdR1* (b and c) and *PdR2* loci (Table 3). Marker screening is a time intensive process, but extremely important and makes our breeding program extremely efficient and successful. Each year we spend 12 to 14 weeks in the Spring to support the breeding program. Planting only resistant plants saves us huge amounts of time and resources in the field.

Table 3. Summary of marker testing completed in Spring 2018 to support our PD resistance breeding program.

PD locus	Number of crosses	Number of seedlings
PdR1b	24	1350
PdR1b x *PdR2*	28	1450
PdR1c	7	302
Total	59	3102

Objective 2. Complete a physical map of the PdR2 region from the b42-26 background and carry out comparative sequence analysis with b43-17 (*PdR1a* and *b*) and b40-14 (*PdR1c*).

We completed the physical maps of the *PdR1*a, *PdR1*b and *PdR1*c loci from the b40-14 and b43-17 backgrounds. In summary, a BAC (bacterial artificial chromosome) library from b40-14 genomic DNA (see details in previous reports) was screened and 30 BAC clones were identified with two probes, Ch14-56 and Ch14-58. BAC clones that represent *PdR1c* were separated from the other haplotype and four overlapping BAC clones VA29E9, VA57F4, VA30F14, VA16J22 were selected for sequencing. Common probes between the *PdR1c* and *PdR1b* region were used to align the sequences. The assembly of four BAC clones is presented in Figure 2 and 3 and represents the sequence analysis of *PdR1b* and the reference grape genome PN40024 region. A manuscript entitled "The physical map of the PD resistance locus, *PdR1c*" is in preparation.

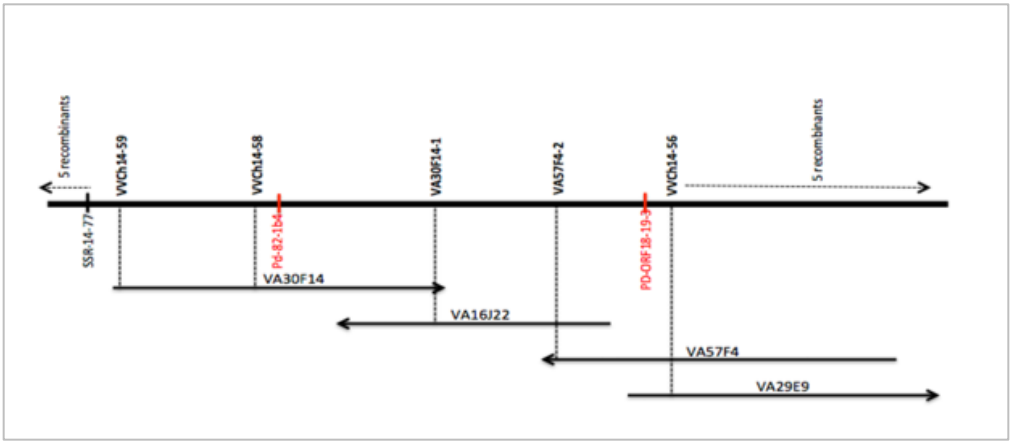

Figure. 2. BAC library was developed from genomic DNA of b40-14 and screened with probes. Four over- lapping clones were selected for sequencing the complete region.

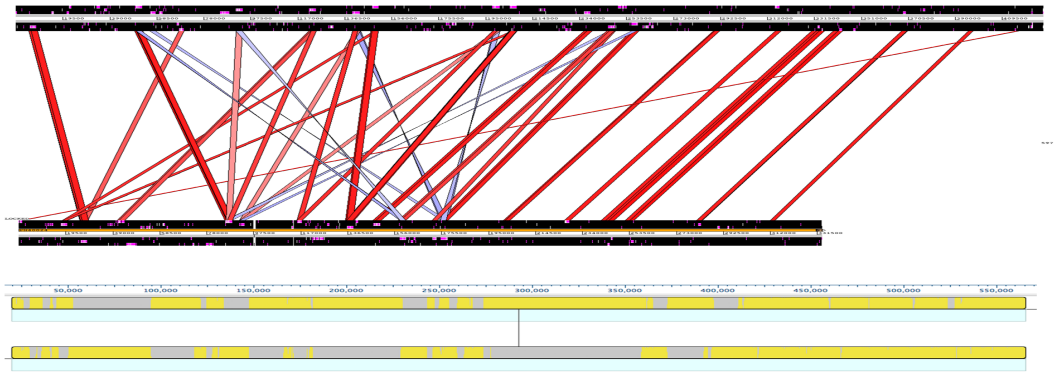

Fig. 3. The sequences of four BAC clones were assembled and full-length open reading frames were identified. Sequences were compared with the reference genome and checked for synteny in that region. Currently analysis is being carried out with the Cabernet Sauvignon genome sequence.

The assembly of H43-I23 from the b43-17 BAC library that represents the *PdR1a* haplotype (F8909-17) was also completed. The length of assembled sequence was 206Kb. The open reading frames (ORF) of the *PdR1b* region and the BAC clone H69J14 were used to make comparisons. There was complete homology between the over-lapping BAC clone sequences that reflect two different haplotypes. The BAC clone H43I23 has ORF16 to ORF20 and all five ORFs have identical sequences compared to the *PdR1b* haplotype. Based on these results, we concluded that there is complete sequence homology between haplotypes *PdR1a* and *PdR1b* of the *PdR1* locus; therefore cloning and functional characterization of genes from any one haplotype will be sufficient for future work. Complete sequence homology also reflects that the parents of b43-17 must be closely related and may have a first-degree relationship and acquired resistance from shared parents. This also explains why we observed complete homozygosity of SSR markers for the *PdR1* locus in the resistant accession b43-17.

In regard to the physical map of b42-26, first we need to refine the position of the *PdR2* locus and narrow the region to less than 1cM. For this purpose, we developed new markers using the Cabernet Sauvignon genome sequence (described in objective 1) and added them to the genetic map. We are developing more markers to fill gaps around the resistance QTL on chromosome 10 as well, so that library screening can be used to to identify BAC clones that represent both resistance regions.

Objective 3. Employ RNA-seq to understand genome-wide transcriptional changes of the pathways regulated by defense-related genes in b40-14.

RNAseq is a powerful approach to identity transcripts and quantify gene expression while combined using a single high-throughput sequencing assay. A good RNAseq study relies on experimental design (library type, sequencing depth and number of replicates) and a careful execution of the sequencing experiment to ensure that data acquisition is not contaminated with unnecessary biases. We completed a time course experiment to monitor the bacterial level in control and inoculated resistant and susceptible plants to design an experiment capable of answering our biological questions with the maximum statistical power. For this purpose, three resistant and three susceptible plants from the 07744 population with resistance from b40-14 PD (*PdR1c*). Plants were propagated and a time course experiment was carried out in growth chambers with temperature and humidity control to reduce the variance. Stem samples were collected from positions 10cm, 20 cm, 30 cm and 40 cm above the point of inoculation at weekly RNA extractions were performed. Samples were also collected from 30cM above point of inoculation for ELISA screening. We have completed RNA extractions of 400 samples and remaining the 96 samples are in pipeline. Analysis is underway to determine when and where gene expression is optimized. We have completed RNA extractions of 400 samples and the remaining 96 samples are in pipeline.

Objective 4. Cloning *PdR1c* and *PdR2* resistance genes with native promoters.

We employed PAC BIO RSII sequencing approach to sequence H69J14 and three other overlapping BAC clones containing both markers that flank the *PdR1b* resistance locus. The assembled sequence data generated a 604 Kb long fragment without any gaps. Multiple ORFs (open reading frames) of the Leucine-Rich Repeat Receptor Kinase gene family were identified within this region. These genes regulate a wide range of functions in plants including defense and wounding responses for both host and non-host specific defense. With the help of molecular markers, we limited the genetic region to 82 Kb – with 5 ORFs associated with disease resistance and other plant functions described above. ORF sequences found outside the 82 Kb window are also highly similar. Two ORFs V.ari-RGA14 and V.ari-RGA18, within the resistance region boundaries, are the most likely candidates for *PdR1b*. The other 3 sequences, V.ari-RGA15, 16 and 17, are shorter and contain a large number of transposable elements (TE).

Both RGA14 and 18 (resistance gene analogs) have a very similar sequence profile except that RGA18 is 2,946 bp in size and lacks the first 252 bp of sequence that is part of RGA14. Functional analysis of both RGAs revealed that RGA14 lacks a signal peptide in the amino terminal of the protein. This result was verified using 3'RACE (rapid amplification of cDNA ends) to specifically amplify RNA from grapevines transformed with V.ari-RGA14 under the 35S promoter. The results found that mature mRNA does not contain an N-terminal signal peptide necessary for proper membrane localization, thus leaving RGA18 as the strongest candidate. However, this could result from a lack of effect of 35S on splicing. In addition, sequencing of cDNA from b43-17, the original source of resistance, inoculated with *X. fastidiosa*, resulted in the amplification of fragments that comprise sequences identical to RGA14 but different from RGA18. In silico analysis of the upstream regions with PlantCare, a database of plant cis-acting regulatory elements, showed that upstream sequences contain several motifs related to drought and defense responses.

Sequence verification for RGA14 and RGA18 and flanking sequences was completed and fragments that contain the entire coding region plus ~3 Kb upstream and ~1 Kb downstream sequences were synthesized and cloned into pCLB2301NK at Genewiz Inc. pCLB2301NK is an optimized vector (Feechan et al. 2013), capable of carrying large DNA sequences, thus allowing us to insert the candidate genes plus surrounding sequences.

New plasmids, called pCLB2301NK-14 and pCLB2301NK-18, were verified by restriction analysis in our lab (Figure 4). Besides the corresponding 7 Kb fragment, containing RGA14 or RGA18, these plasmids contain a 35S:mGFP5-ER reporter cassette and a kanamycin-selectable marker gene with the NOS promoter.

(a) (b) (c)

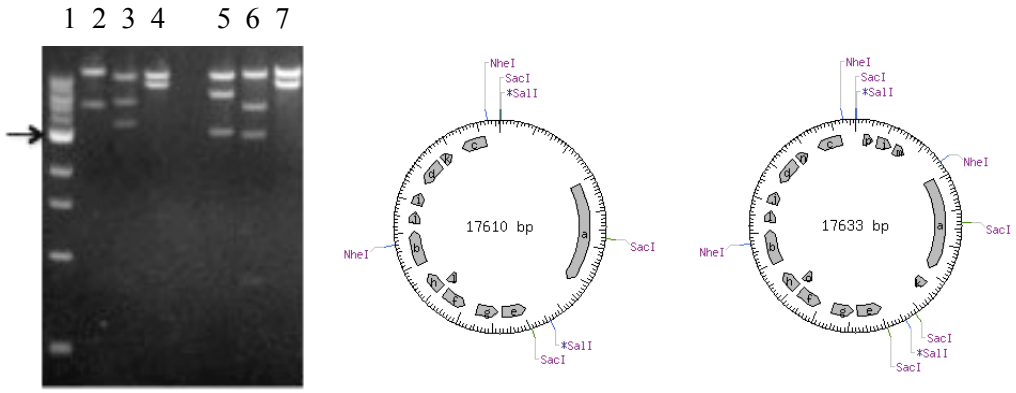

Figure 4. (a) Restriction analysis of plasmids pCLB2301NK-14 (lanes 2, 3, 4) and pCLB2301NK-18 (lanes 5, 6, 7) after digestion with Nhe1 (lanes 2, 5), Sac1 (lanes 3, 6) and Sal1 (lanes 4, 7). Gel image includes a 1Kb ladder (lane 1) with the 3 Kb fragment having increased intensity to serve as a reference band. The results on the gel match the predicted sizes inferred from the plasmid information; (b) pCLB2301NK-14 restriction map; (c) pCLB2301NK-18 restriction map.

Objective 5. Comparing the PD resistance of plants transformed with native vs. heterologous promoters.

We have established an *Agrobacterium* mediated transformation system followed by regeneration of plants from embryogenic callus. We have streamlined the protocol and have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* Thompson Seedless (TS), Cabernet Sauvignon, Chardonnay (CH) and the rootstock *V. rupestris* St. George (SG) (Agüero et al. 2006). In an earlier phase of this project, we transformed these varieties with five candidate genes containing the 35S cauliflower mosaic virus promoter, the nopaline synthase terminator and an *hptII*-selectable marker gene (see previous reports for details). We completed testing and found that the transgenic plants did not confer PD resistance or tolerance. These results are in accordance with the latest assembly obtained using PAC BIO SRII system. They show that only one of the sequences tested, V.ari-RGA14, lays within the more refined resistance region of 82 Kb.

We have also developed meristematic bulks (Xie et al. 2016), which we are using as an alternative explant for genetic transformation, of PD susceptible genotypes selected from the 04-191 population, which are 50% *vinifera*, 25% b43-17 and 25% *V. rupestris* A. de Serres (as in the original population used for *PdR1b* mapping). These genotypes can provide an additional genetic background for analysis of expression of *PdR1* candidate genes. Two of these genotypes, designated 29-42 and 47-50 exhibited great potential for the development of MB and transformation experiments with *Agrobacterium* have been initiated.

Transformations with *Agrobacterium tumefaciens* carrying binary plasmids that contain hygromicin (pCLB1301NH) or kanamycin (pCLB2301NK) selectable marker genes showed that both antibiotics are effective selection agents for embryogenic calli. However. MB regeneration has mainly occurred when selecting with kanamycin, confirming our previous observation that MB are highly sensitive to hygromicin. Thus, pCLB2301NK was chosen to carry RGA14 and RGA18 expanded sequences and named pCLB2301NK-14 and pCLB2301NK-18 thereafter.

Agrobacterium tumefaciens strain EHA 105 pC32 was chemically transformed with pCLB2301NK-14 or pCLB2301NK-18 and subsequently used to transform embryogenic calli of *V. vinifera* cvs. Chardonnay, Thompson Seedless and the rootstock *V. rupestris* St. George. Transformation experiments with pCLB2301NK-18 and pCLB2301NK-14 were initiated in March and July 2016 respectively, after synthesis and cloning was completed. In addition, *Agrobacterium* is being used to transform meristematic bulks (MB) of PD susceptible genotypes selected from the 04-191 population. Table 4 shows the number of independent lines regenerated so far. Transformation was checked through PCR, and transformed plants were transferred to the greenhouse. Primers that bind the promoter and coding regions of RGA14 or RGA18 were used for amplification. DNA fragments amplified successfully in all the lines tested (Figure **5)**. Transformation was also verified by

fluorescence microscopy to visualize GFP, since pCLB2301NK-18 and pCLB2301NK-14 also contain a 35S:GFP5-ER cassette (Figure 6).

Table 4. Number of independent lines regenerated after transformation with *Agrobacterium* carrying pCLB2301NK-18 or pCLB2301NK-14.

ype	**No. lines *in vitro***	**No lines in greenhouse**
pCLB2301NK-18		
Chardonnay	13	11
Thompson Seedless	30	11
St. George	4	4
29-42	1	-
pCLB2301NK-14		
Chardonnay	20	11
Thompson Seedless	18	10
St. George	4	4

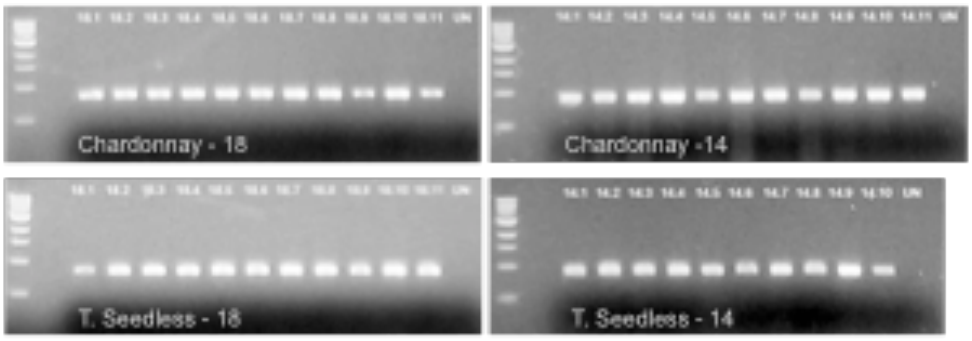

Figure 5. Transgene detection of RGA18 and 14 through PCR (UN: untransformed Chardonnay or Thompson Seedless, 1-10 or 11: transgenic lines). Gel image includes a 1Kb ladder (left lane) with the 3 Kb fragment having increased intensity to serve as a reference band. The results on the gel match the predicted sizes inferred from the sequence information.

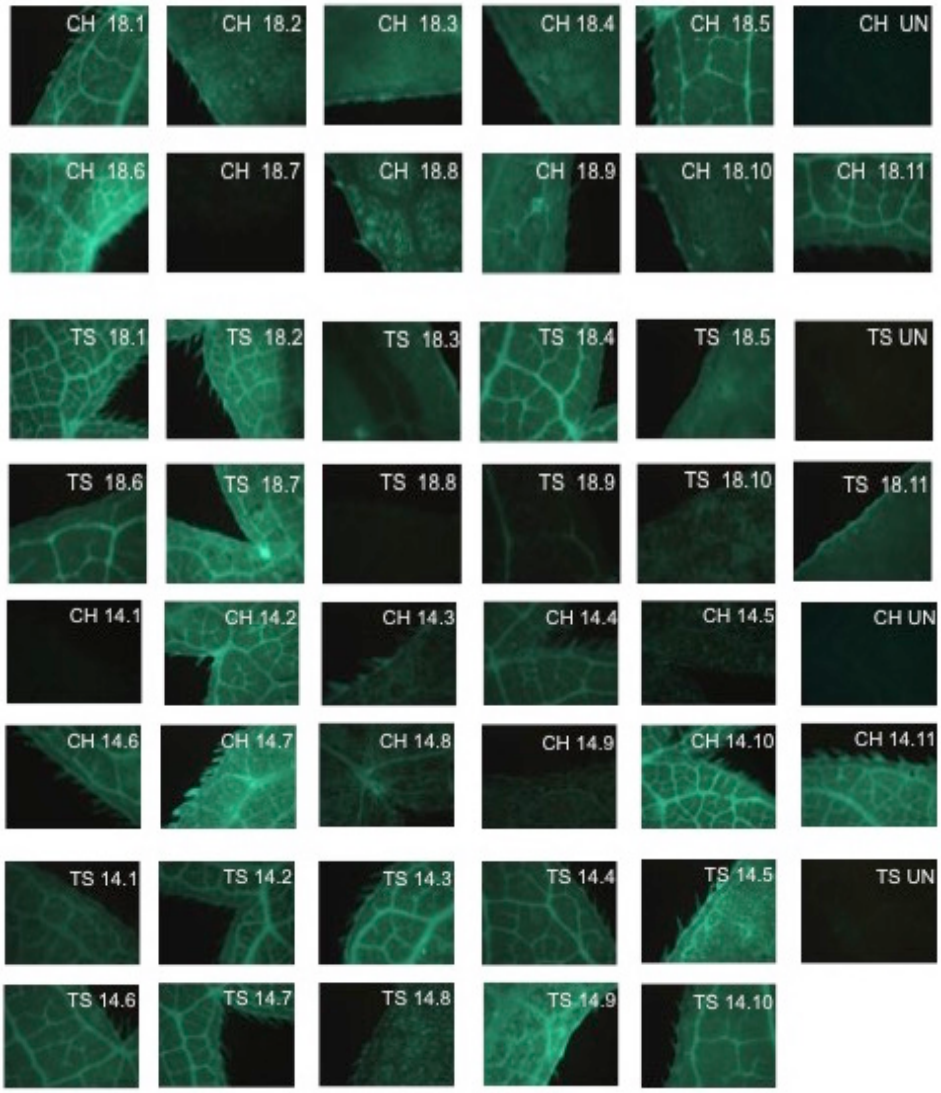

Figure 6. GFP fluorescence in untransformed Chardonnay (CH UN), Thompson Seedless (TS UN), and RGA18 and RGA14 transgenic lines (1-10 or 11).

V.ari-RGA18 lines: Chardonnay and Thompson Seedless transgenic lines in the greenhouse were multiplied from green cuttings and were inoculated with the Beringer strain of *X. fastidiosa* in August 2017 (RGA18 lines) and March 2018 (RGA14 lines). Twelve weeks after inoculation, PD symptoms 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 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). For gene expression analysis, plants were sampled by taking 0.5 g sections of stem tissue from 50 cm above the point of inoculation. Testing of St George will start in August 2018.

Tables 5 and 6 show that transgenic lines displayed disease symptoms with different degrees of intensity. On one hand, lines CH 18-2 and CH 18-7 showed low cane maturation index. On the other, most CH 14 lines, especially CH 14-1 and CH 14-2 exhibited low leaf scorching (Figure 7). Bacteria concentration in CH 18-2 and CH 18-7 was lower than in the untransformed control but not as low as the resistant biocontrols (Table 3). ELISA testing of the RGAl4 lines is underway. Thompson Seedless was considerably more susceptible than Chardonnay.

Table 5. Greenhouse screen results for Chardonnay and Thompson Seedless transformed with V.ari-RGA18. Top six genotypes correspond to negative control and resistant biocontrols. CH 0 and TS 0 are untransformed Chardonnay and Thompson Seedless, respectively.

Gentoype	cfu/ml	ln cfu/ml	Std Error (cfu/ml)	CMI Mean	CMI Std Err	LS-LL Mean	LS-LL Std Err
CH uninoc	10,034	9.2	0.00	0.0	0.00	0.0	0.00
b43-17	23,416	10.1	0.32	2.1	0.48	2.9	0.48
U0505-01	37,499	10.5	0.62	0.3	0.15	1.8	0.41
U0505-35	100,798	11.5	0.83	0.3	0.33	1.5	0.43
Blanc du Bois	194,385	12.2	0.46	2.1	0.49	1.9	0.49
Roucaneuf	245,426	12.4	0.78	0.3	0.33	0.8	0.40
U0505-22	760,190	13.5	0.27	2.3	0.71	2.6	0.43
CH 0	6,119,118	15.6	0.42	3.6	0.60	1.6	0.53
CH 18-1	4,636,369	15.3	0.18	1.0	0.63	1.8	0.20
CH 18-2	2,078,921	14.5	0.42	0.8	0.58	2.4	0.51
CH 18-3	6,152,629	15.6	0.04	2.6	0.81	2.4	0.40
CH 18-4	4,686,410	15.4	0.06	3.4	0.82	0.9	0.11
CH 18-5	5,562,260	15.5	0.10	1.6	0.24	2.8	0.58
CH 18-6	4,888,719	15.4	0.18	1.0	0.55	3.0	0.32
CH 18-7	1,786,455	14.4	0.60	0.0	0.00	1.8	0.58
CH 18-8	6,500,000	15.7	0.00	2.2	0.58	3.2	0.37
TS 0	6,500,000	15.7	0.00	4.2	0.20	5.0	0.00
TS 18-1	6,500,000	15.7	0.00	4.6	0.40	4.6	0.40
TS 18-2	6,500,000	15.7	0.00	4.0	0.32	5.0	0.00
TS 18-3	6,500,000	15.7	0.00	4.0	0.32	4.4	0.24
TS 18-4	6,500,000	15.7	0.00	5.0	0.45	4.8	0.20
TS 18-5	6,500,000	15.7	0.00	4.0	0.00	4.6	0.24
TS 18-6	6,500,000	15.7	0.00	4.2	0.66	4.2	0.20
TS 18-7	6,500,000	15.7	0.00	3.6	0.75	4.6	0.24
TS 18-8	6,500,000	15.7	0.00	5.0	0.45	5.0	0.00
TS 18-9	6,500,000	15.7	0.00	4.2	0.37	4.8	0.20
TS 18-10	6,500,000	15.7	0.00	4.6	0.40	5.0	0.00
TS 18-11	6,500,000	15.7	0.00	4.2	0.73	4.8	0.20

Table 6. Greenhouse screen results for Chardonnay and Thompson Seedless transformed with V.ari-RGA14. CH-0 and TS-0 are untransformed Chardonnay and Thompson Seedless, respectively.

Genotype	CMI Mean	CMI Std Err	LS-LL Mean	LS-LL Std Err
CH 0	1.9	0.46	3.1	0.29
CH 18-9	2.4	0.51	3.6	0.37
CH 18-10	0.7	0.70	2.7	0.41
CH 18-11	1.7	0.62	2.1	0.37
CH 14-1	1.7	0.37	1.3	0.25
CH 14-2	1.6	0.64	2.0	0.16
CH 14-3	1.2	0.72	2.5	0.59
CH 14-4	3.2	0.58	2.3	0.34
CH 14-5	3.7	0.60	3.1	0.29

CH 14-6	2.5	0.94	2.2	0.25
CH 14-7	2.7	0.80	1.4	0.46
CH 14-8	1.4	0.48	1.8	0.30
CH 14-9	2.5	1.04	2.0	0.16
CH 14-10	3.3	0.46	1.7	0.60
CH 14-11	1.8	0.54	1.8	0.41
TS 0	4.8	0.20	4.7	0.20
TS 14-1	4.7	0.30	4.8	0.20
TS 14-2	4.4	0.10	4.5	0.27
TS 14-3	4.3	0.20	5.0	0.00
TS 14-4	4.7	0.20	4.9	0.10
TS 14-5	3.8	0.40	4.8	0.20
TS 14-6	4.1	0.33	4.9	0.10
TS 14-7	4.2	0.37	4.1	0.37
TS 14-8	5.1	0.24	4.8	0.20
TS 14-9	4.7	0.20	4.7	0.20
TS 14-10	4.4	0.24	5.0	0.00

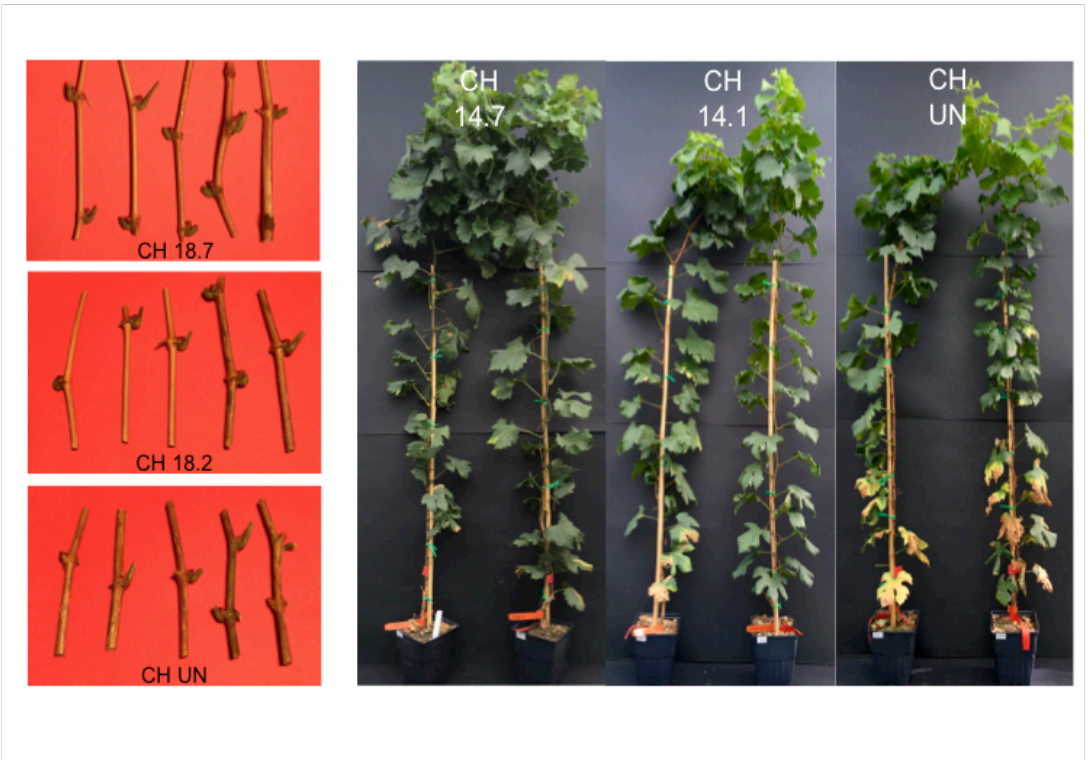

Figure 7. Left: lignification observed in nodes collected 40 cm above POI, 3 months after inoculations in transgenic lines CH 18.2, CH 18.7 and untransformed Chardonnay (CH UN). Right: scorching observed in basal leaves, 3 months after inoculations in transgenic lines CH 14.1, CH 14.7 and untransformed Chardonnay (CH UN).

qPCR analysis to determine the correlation between the level of transgene expression and GFP fluorescence/PD symptoms/bacteria concentrations has been inconclusive. Untransformed Chardonnay infected with *X. fastidiosa* also exhibits low Ct numbers (Figure 8, primers 14.3 and 18.5) and cDNA sequencing has revealed that genes with high homology with RGA14 and RGA18 are being expressed.

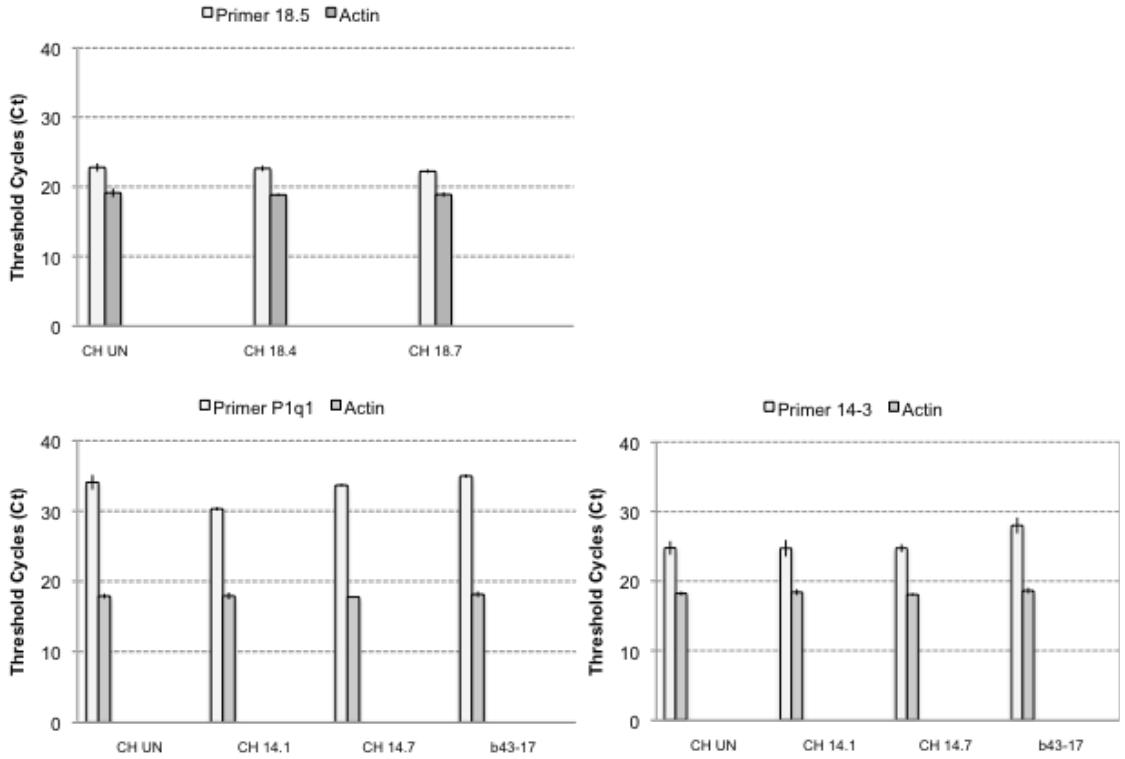

Figure 8. RGA18 and RGA14 expression in transgenic lines CH 18.4, CH 18.7, CH 14.1, CH 14.7, untransformed CH and b43.17. Error bars represent standard deviation of the mean (n=3). UN: Untransformed. Primers 18.5 and 14.3 amplify a fragment in the 3' region of the RGA18 and RGA14 respectively, while P1q1 amplifies a fragment in the first 252 bp of RGA14. Higher Ct values correspond to lower expression levels. Actin is used as reference gene.

CONCLUSIONS

We completed greenhouse screening, marker testing and QTL analysis of breeding populations from 15 new resistance sources including b46-43 and T03-16. We identified T03-16 and b41-13 as possessing resistance on a different region than chromosome (Ch) 14. Crosses were made to expand these breeding populations for framework map development in order to identify other genomic regions of resistance. Our primary goal is to identify new sources of resistance that do not reside on Ch14 so we can facilitate stacking of these resistance sources with *PdR1* from b43-17, since the incorporation of multiple resistances should make resistance more durable. We have also identified a new resistance locus *PdR2* from the b42-26 background and closely linked markers are being used in MAS to stack resistance loci from these different backgrounds. We have completed the genetic and physical mapping of PD resistance from b40-14. This resistance source maps within the *PdR1b* locus, but it may be an alternative gene within this complex replicated locus. Finally, we verified the sequence of two candidate genes from the *PdR1b* locus, completed transformations with RGA18 and RGA14 and obtained transgenic lines for complementation tests in the greenhouse. Although some transgenic lines responded better than untransformed plants to *Xylella* infection, none reached the level of resistant biocontrols. Testing of RGA14 and 18 in a genetic background other than *vinifera*, as well as more information about RGA15, 16 and 17 will help to clarify the meaning and importance of these results.

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FUNDING AGENCY

Funding for this project was provided by the CDFA PD/GWSS Board. Additional support from the Louise Rossi Endowed Chair in Viticulture supporting work with grape species is also gratefully acknowledged.