FINAL REPORT FOR CDFA AGREEMENT NUMBER 18-0400-000-SA

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

Principal Investigator:	**Collaborator:**	**Cooperating Staff:**
Andrew Walker	Dario Cantu	Summaira Riaz & Cecilia Agüero
Viticulture and Enology	Viticulture and Enology	Viticulture and Enology
University of California, Davis	University of California, Davis	University of California
awalker@ucdavis.edu		

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LAYPERSON SUMMARY

The objectives of this project are to identify and characterize new sources of Pierce's disease (PD) resistance, better understand how resistance functions and how it varies across southwestern US and Mexican grape species, identify and characterize candidate resistance genes, and provide marker support to the breeding program. We develop DNA markers that are tightly linked to PD resistance and use them to select resistant plants at the seedling level. We develop new genetic maps that enable candidate resistance genes to be identified, and have their function verified by engineering them into susceptible grape varieties.

INTRODUCTION

This project provides molecular support to the PD resistant grape breeding program – "Breeding Pierce's disease resistant winegrapes" by acquiring and testing a wide range of potentially resistant germplasm, tagging resistance regions with markers, and genetically 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 highly resistant accessions were identified from this germplasm. Markers were used to determine the genetic diversity of these 14 selections and their 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 Ch 14 locus *PdR1* (Riaz et al. 2018**)**. Three accessions (T03-16, ANU67 and b41-13) were identified to carry out further work with larger populations. More crosses were made in Spring 2016-2017 to expand these breeding populations for map-based identification of genomic regions that contribute to resistance. In this report we present the results of mapping work with the expanded genetic maps of two accessions.

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* resistance 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 PD 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

This project provides molecular support to the companion PD resistance winegrape breeding project by conducting MAS (marker-assisted selection on seedling populations. In Spring 2019, we extracted DNA and marker tested 2,400 seedling plants from 25different crosses for the *PdR1* (b and c) and *PdR2* loci that were in all cases combined with powdery mildew resistance (Table 1). 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 large amounts of time and resources in the field.

Table 1. Marker-assisted testing completed in Spring 2018 to support PD resistance breeding program.

Cross ID	PDR Type	PM Type	Report Group	MAS Test
18-340	PdR1bxb42-26	Rom18U	PD2xPM1	125
18-324	PdR1bxb42-26	Rom18U	PD2xPM1	175
18-320	PdR1b^2xb42-26	Rom18U	PD2xPM1	35
18-314	PdR1bxb42-26	Rom18U	PD2xPM1	70
18-323	PdR1b^2xb42-26	Rom18U	PD2xPM1	140
18-315	PdR1bxb42-26	Rom18U	PD2xPM1	125
18-339	PdR1bxb42-26	Rom18U	PD2xPM1	125
18-316	PdR1bxb42-26	Rom18U	PD2xPM1	25
18-342	PdR1bxb42-26	NC6^2xRom18U	PD2xPM2	75
18-321	PdR1b^2xb42-26	Rom18U	PD2xPM1	100
18-336	PdR1bxb42-26	Rom18UxNC6	PD2xPM2	100
18-338	PdR1bxb42-26	Rom18U	PD2xPM1	100
18-337	PdR1bxb42-26	Rom18U	PD2xPM1	100
18-322	PdR1b^2xb42-26	Rom18U	PD2xPM1	75
18-703	PdR1b	NC6	PD1xPM1	100
18-377	PdR1b	NC6xRen1^2xRom18U	PD1xPM3	100
18-375	PdR1b	Ren1xJB18	PD1xPM2	100
18-325	PdR1bxb42-26	Rom18U	PD2xPM1	75
18-391	PdR1b	NC6	PD1xPM1	150
18-384	PdR1b	Ren1xJB18	PD1xPM2	75
18-376	PdR1b	Ren1xJB18	PD1xPM2	100
18-390	PdR1b	NC6	PD1xPM1	100
18-373	PdR1b	NC6xRen1^2xRom18U	PD1xPM3	70
18-374	PdR1b	NC6xRen1^2xRom18U	PD1xPM3	70
18-371	PdR1b^2xb42-26	NC6xRen1^3xRom18U	PD2xPM3	90
Total				**2400**

In 2018, we reported that limited mapping strategy did not produce the conclusive results for three accessions ANU67, b41-13, and T03-16 with the phenotypic data on small population and markers from chromosome 14 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. Accession T03-16 from the Big Bend region in Texas and b41-13 from Tamaulipas state in Mexico were strong candidates for large populations. Figure 1 presents the collection location information of three accessions.

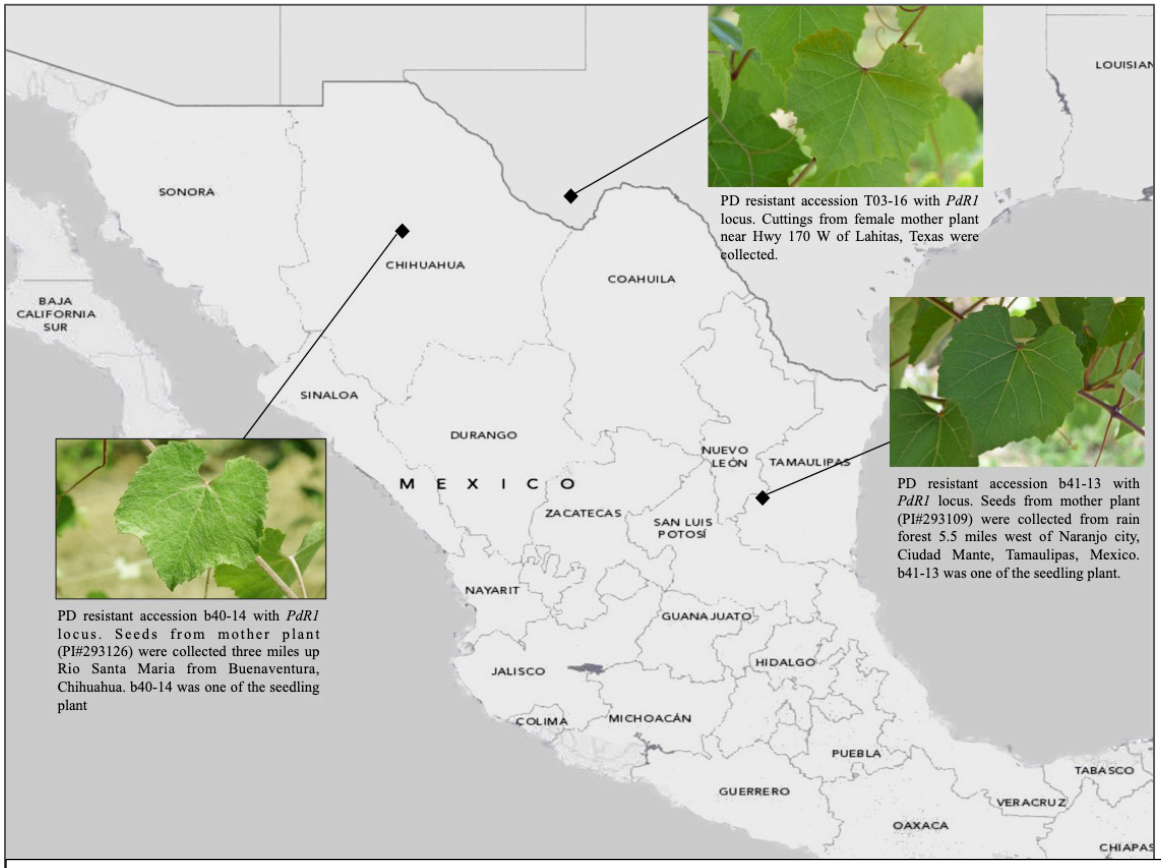


Figure 1. Collection location information of three accessions evaluated in this study. Two accessions were collected from Mexico and one was collected from Texas.

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. Crosses were also made with T03-16 and 285 seedling plants from the F1 population were established in the field. Table 2 provide summary of population size, number of markers tested for polymorphism for b41-13 and T03-16, and markers that were completed on large population to develop the genetic map. We also completed the manuscript "**Genetic mapping of Pierce's disease resistance in Germplasm collected from the Southwestern US and Mexico"** that is ready for submission. Simple sequence repeat (SSR) marker-based framework maps that cover all 19 grape chromosomes were developed for b40-14 and b41-14 while the genetic map for T03-16 was developed only for chromosome 14. The QTL analyses identified that these three additional accessions also have PD resistance on the chromosome 14 within the genetic window of the *PdR1* locus, bringing the total to 13 accessions that have identified with PD resistance on chromosome 14. The apparent lack of additional genomic regions conferring resistance to PD and the widespread distribution of the *PdR1* locus in wild germplasm collected from the Southwestern US and Northern Mexico indicates that wild grapes developed resistance in response to the disease at or very near its center of origin and that gene flow occurring over millennia has spread it to wider regions of southwestern US and Mexico. Table 3 provides the summary statistics of the genetic maps of b41-13, T03-16 and b40-14.

Table 2. List of accessions used in the study with collection information, population identification (Id) and number of seedlings tested in the study. Markers were tested on a set of eight samples including parents and progeny. Only polymorphic markers were added to the entire population for each genetic background

Accession	Collection Location	Population Id and size for genetic mapping	No. of tested markers	No. of amplified markers	No. of polymorphic markers	No. of completed markers
b40-14	80km n Chihuahua, Mexico	07744, 120	607	449	323	225
b41-13	Near Ciudad Mante, Mexico	16337, 250	596	543	295	244
T03-16	Hwy 170 W of Lahitas, Texas	(13302, 13336, 16304, 17344), 192	34	34	14	14

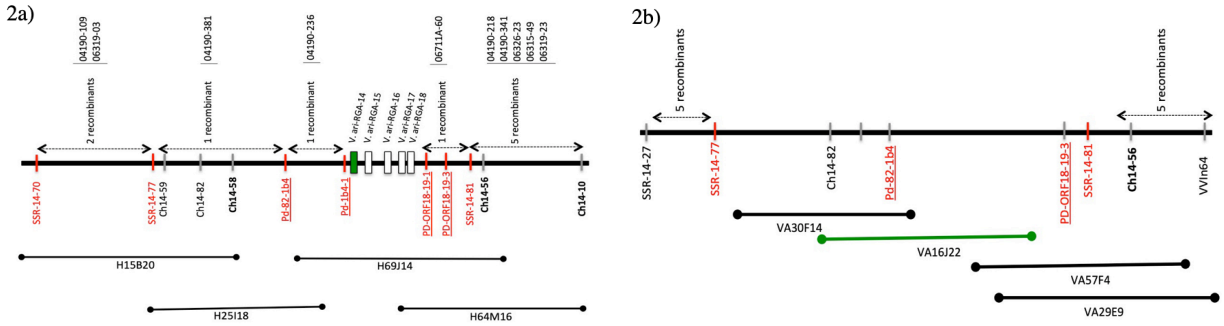
Table 3. Summary of parental framework maps of three resistant backgrounds. R8918-05 was a resistant male seedling with b40-14 father.

	R8918-05 map		b41-13 map		T03-16 map	
Chromosome	Mapped Markers	Map length (cM)	Mapped Markers	Map length	Mapped Markers	Map length (cM)
Chr1	15	55.833	10	59.4		
Chr2	4	49.147	3	37.1		
Chr3	6	33.415	7	31.7		
Chr4	11	69.33	12	68.5		
Chr5	17	52.01	9	54.2		
Chr6	11	38.684	11	70.7		
Chr7	12	69.944	8	56.4		
Chr8	11	50.986	20	63.2		
Chr9	10	58.755	9	55.3		
Chr10	10	54.542	6	50.3		
Chr11	9	57.501	6	41.1		
Chr12	9	62.413	9	27.6		
Chr13	11	55.563	7	35.1		
Chr14	27	64.121	31	85.3	12	79.5
Chr15	8	29.554	3	7.8		
Chr16	9	59.326	2	2.5		
Chr17	12	43.963	6	45.1		
Chr18	12	58.062	14	88		
Chr19	13	40.443	5	27.5		
Total	217	1003.592	178	906.8	12	79.5
Ave marker distance (cM)	4.6		5.1		6.6	
Number of gaps > 20 cM	9		8		0	

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, the physical map of *PdR1b* spans 604 Kb that includes the flanking markers Ch14-77 and Ch14-81 used for marker assisted screening. The physical map of b40-14 (*PdR1c*) covers 426 Kb and consisted of four overlapping BAC clones. BAC clone H43I23 (206 Kb) that contains *PdR1a* was also sequenced and it showed complete homology to the sequence of *PdR1b* haplotype, indicating that the parents of b43-17 must be closely related. Figure 2 shows the physical maps of PdR1b and PdR1c.

Figure. 2. Physical map of PD resistant accessions. 1a) b43-17 map, markers in bold were used as probes to screen library, marker in red are SSR markers. Four underlined markers were developed from b43-17 sequence, other were designed from the PN40024 sequence.



Multiple ORFs of the Leucine-Rich Repeat Receptor Kinase gene family were identified. These genes regulate a wide range of functions in plants including defense and wounding responses for both host as well as non-host specific defense. For the *PdR1b* locus, the genetic window is limited to the 82 Kb distance between markers SSR-1b4-1 and PD-Orf18-19-1; five ORFs in that regions were associated with disease resistance (Fig. 3). A total of 21 ORFs were identified in 604 Kb sequence of *PdR1b* sequence in comparison to the 18 ORFs in *PdR1c* sequence.

The PN40024 sequence was 230 Kb with many gaps; potentially some ORFs are not accounted for. The sequences of Cabernet Sauvignon (CS) within the flanking markers was 527 Kb long. All three sequences show an abundance of transposable elements dispersed within the resistant gene analogs (RGA). Genome sequence comparisons to the CS sequence show sequence divergence in the region of RGA and the sequences show above 90% homology in the genomic region that flanks the resistance. Comparison of *PdR1* region in b43-17 and b40-14 also showed sequence divergence for the region of the resistant genes, and for a number of transposable elements indicating significant differences between two accessions. Currently, we are working to finalize the manuscript for publication.

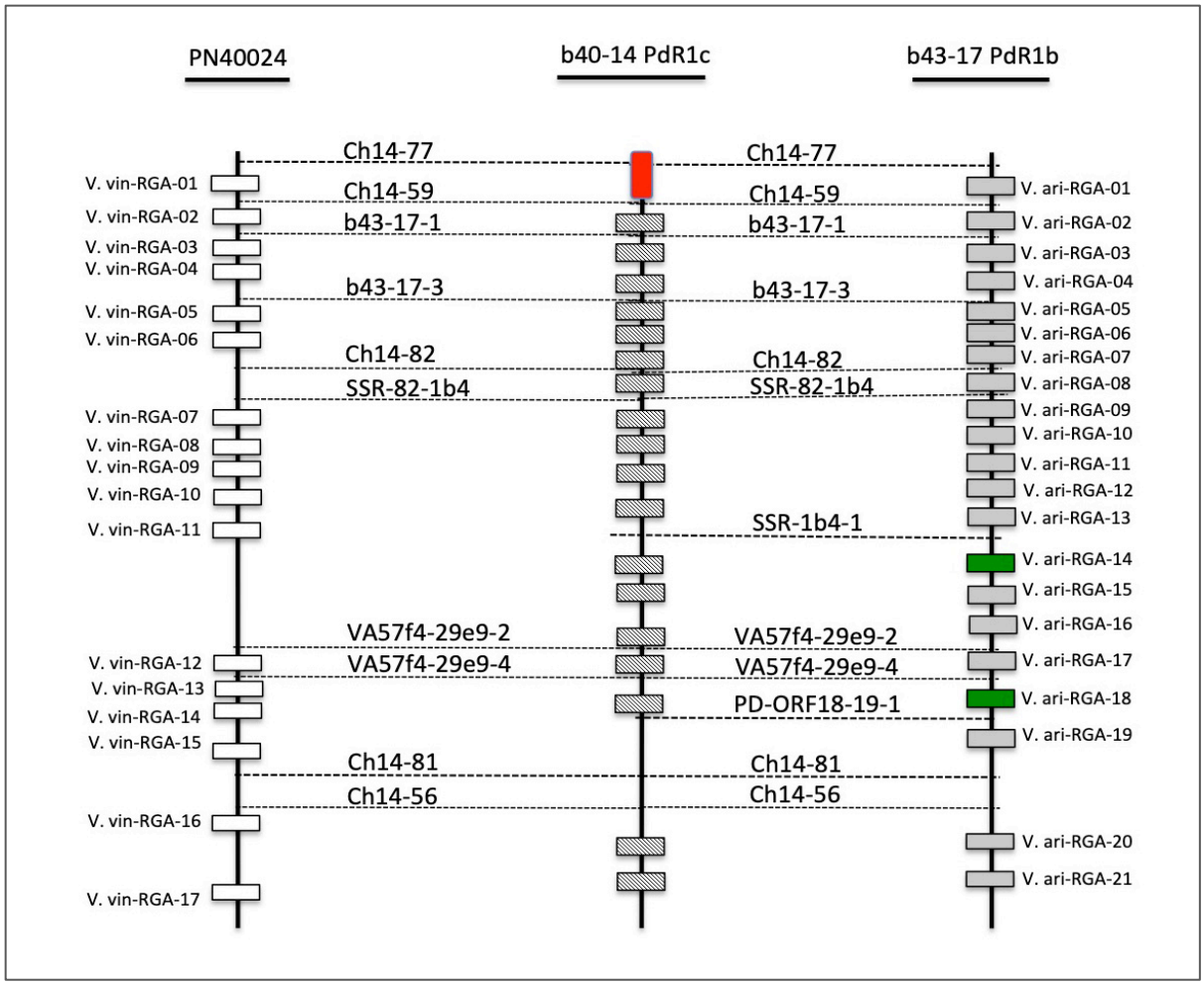


Figure 3. Comparison of open reading frames (ORFs) in three different backgrounds. PN40024 is a susceptible reference genome, dashed lines show the placement of markers to provide alignment for comparison among sequences. The red regions represent the gap between the Ch1459 and Ch14-77 markers in the assembly. Green blocks in *PdR1b* sequence are two candidate resistance genes for which constructs were developed for transformation experiments.

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 with 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*) were used. 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 and weekly RNA extractions were performed. Samples were also collected from 30cM above point of inoculation for ELISA screening. We have completed RNA extractions of 496 samples.

Objective 4. Cloning PD resistance genes with native promoters.

With the help of molecular markers, we limited the genetic region that contains the *PdR1b* resistance locus to 82 Kb. Five ORFs of the Leucine-Rich Repeat Receptor Kinase gene family, associated with disease resistance,

were identified within the resistance region boundaries. Two ORFs V.ari-RGA14 and V.ari-RGA18 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). Fragments that contain the entire coding region of V.ari-RGA14 and V.ari-RGA18 plus ~3 Kb upstream and ~1 Kb downstream sequences were synthesized and cloned into pCLB2301NK (Feechan et al. 2013) at Genewiz Inc to produce plasmids pCLB2301NK-14 and pCLB2301NK-18. (See FINAL REPORT FOR CDFA AGREEMENT NUMBER 14-0137-SA and 17-0427-000-SA for details).

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

We have established *Agrobacterium* mediated transformation systems followed by regeneration of plants from embryogenic callus and meristematic bulks (Agüero et al. 2006, Xie et al. 2016). *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 (CH), Thompson Seedless (TS) and the rootstock *V. rupestris* St. George (SG). The evaluation of 44 transgenic of CH and TS (10-11 lines per genotype x 2 constructs) showed that all transgenic lines displayed disease symptoms with different degrees of intensity; TS being considerably more susceptible than CH. Although some lines exhibited fewer symptoms or lower bacteria concentrations, none reached the levels of the resistant biocontrols (See FINAL REPORT FOR CDFA AGREEMENT NUMBER 14-0137-SA and 17-0427-000-SA for details).

Plant regeneration of transgenic SG has been more challenging (Table 4), however promising results were obtained with one RGA14 line that was inoculated in August 2018. Cane Maturation Index Means of untransformed and transgenic were 4.9 and 1.7 respectively, while Leaf Scorching Index Means were 4.5 and 3. Figure 4 shows re-growth in transgenics after cutting back of plants for sampling 12 wks after inoculation (right). None of the untransformed resumed their growth (left). Shoot lignification 30 cm above POI is shown at the bottom of the figure. ELISA tests also produced significant differences between untransformed (410,000 cfu/ml) vs transgenic (120,000 cfu/ml). This finding agrees with results from sequencing of cDNA from b43-17, the original source of resistance, inoculated with *X. fastidiosa*, showing the amplification of fragments that comprise sequences identical to RGA14 but different from RGA18. Currently, we are testing one SG RGA18 line, which was inoculated on January 18, 2019. The rest of the SG lines are dwarf and grow very slowly, consequently more *in vitro* transformations have been initiated. Co-transformations with both pCLB2301NK-14 and pCLB2301NK-18 are also underway.

Table 4. Regeneration and transformation of St. George with *PdR1* candidate genes.

Type	No. lines	No lines PCR positive
pCLB2301NK-18		
St. George	4	4 (1 inoculated Jan 2019)
29-42	3	not tested
pCLB2301NK-14		
St. George	1	1 (1 inoculated Aug 2018)
29-07	6	not tested

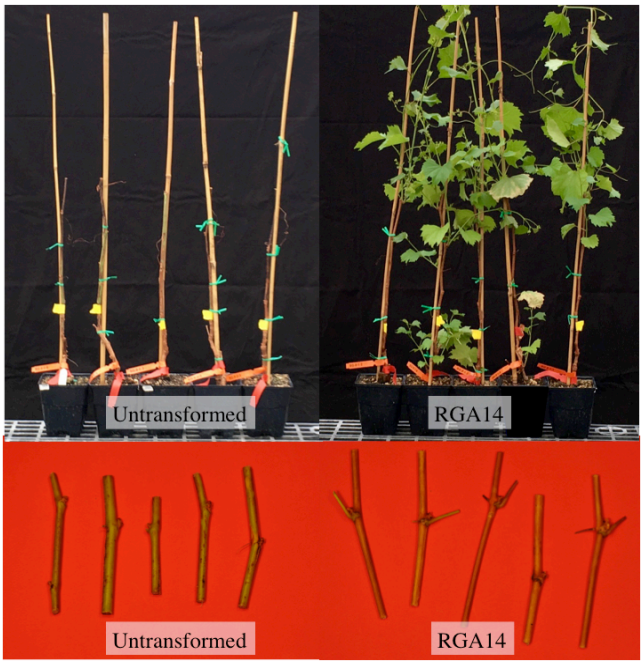


Figure 4. Appearance of inoculated plants transformed with candidate *PdR1* resistance gene (RGA14)

Transformation of meristematic bulk of genotypes selected from the 04-191 population, which are 50% *vinifera*, 25% b43-17 and 25% *V. rupestris* A. de Serres are also being pursued. Two of these genotypes, designated 29-07 and 29-42 have produced plants that show GFP fluorescence (Table 4) and will be acclimated to greenhouse conditions for further testing. Evaluating these lines plus additional transgenic SG could help to clarify the role of genetic background in *PdR1b* resistance.

In addition, we are generating meristematic bulks of b43-17, and U0505-01, which is 87.5% vinifera, positive for *PdR1b* molecular markers and highly resistant to PD, for reverse genetic analysis using CRISPR-Cas systems.

CONCLUSIONS

We completed greenhouse screening, marker testing and QTL analysis of breeding populations from 13 new resistance sources. We identified T03-16 and b41-13 as candidates to expand population sizes. Crosses were made to expand these breeding populations for framework map development in order to identify other genomic regions of resistance. Genetic mapping and QTL analysis was completed for both accessions. Results show that PD resistance resides on chromosome 14 at the same genomic position of *PdR1*. This brings the total to 13 accessions with *PdR1* locus. We have 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 and b43-17. We completed greenhouse screening, of CH and TS lines transformed with RGA18 and RGA14. Although some transgenic lines responded better than untransformed plants to *Xylella* infection, none reached the level of resistant bio controls. Promising results have been obtained with one line of SG RGA14. Testing of RGA14 and 18 in SG and other genetic backgrounds, 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

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