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PROJECT TITLE: Molecular breeding support for the development of PD resistant winegrapes.

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INTRODUCTION

Identification, understanding and manipulation of novel sources of resistance are the foundation of a successful breeding program. This project evolved from two previously funded projects: 1) Genetic Mapping – "Genetic mapping of *Xylella fastidiosa* resistance gene(s) in grape germplasm from the southern United States"; and 2) Functional Characterization – "Molecular and functional characterization of the *Xylella fastidiosa* resistance gene(s) from b43-17 (*V. arizonica*)". Both of these projects supported the PD resistance grape breeding project – "Breeding Pierce's disease resistant winegrapes". Genetic markers linked to *X. fastidiosa* resistance from the former two projects were used to perform marker-assisted selection (MAS) to accelerate our PD resistant winegrape and the table and raisin grape breeding of David Ramming in the past. Outcomes from these projects include BAC libraries of the highly resistant *V. arizonica* accessions, b43-17 and b40-14. The b43-17 BAC library was used to physically map the *PdR1* locus and several candidate genes were identified. Five genes were cloned and constructs were developed to transform tobacco, Chardonnay, Thompson Seedless and St George, which are being tested for function.

The new merged project has five key objectives: to identify novel sources of PD resistance for use in broadening the genetic base of resistance; to utilize improved sequencing technology to facilitate and accelerate marker discovery and the identification of new and unique resistance genes; to clone and characterize unique DNA sequences (promoters) that regulate the expression of candidate PD resistance genes cloned from the *V. arizonica* b43-17 *PdR1b* locus; and to evaluate and compare lines with native and 35S promoters. To broaden the genetic base of PD resistance breeding, we surveyed over 250 accessions of *Vitis* species growing in the southern US and Mexico to identify new PD resistant accessions. Analysis using population genetics methods allowed us to better understand gene flow among resistant species and their taxonomic and evolutionary relationships. PD resistance in southeastern *Vitis* spp. seems to be different than the resistance in *Vitis* from the southwest and Mexico. We have already identified new PD resistant accessions that are genetically and phenotypically different, were collected from different geographic locations, and have different maternal inheritance of key marker genes. We are continuously developing and expanding breeding populations from new promising resistant lines. These populations will be tested to study the inheritance of resistance. Then next generation sequencing will be used on the recently identified resistant accessions to expedite marker discovery and confirm that they are unique. Then genetic maps will be developed to identify genomic regions associated with resistance, and genetic markers will be used for the stacking of multiple resistance genes to breed winegrapes with durable PD 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 can be employed in production of cisgenic plants. Cisgenesis is the transformation of a host plant with that host plant's genes and promoters (Holmes et al. 2013). Alternatively, other well characterized *vinifera*-based promoters; either constitutive (Li et al. 2012) or activated by *X. fastidiosa* (Gilchrist et al. 2007), could be utilized. Development of *V. vinifera* plants transformed with grape genes and grape promoters might mitigate concerns about transgenic crops harboring genetic elements derived from different organisms that cannot be crossed by natural means. Proven resistance gene constructs could be transformed into a broad array of elite *V. vinifera* cultivars.

OBJECTIVES

The overall goal of this project is to provide molecular genetic support to the PD resistant winegrape breeding program. These efforts include discovering new sources of PD resistance; identifying functionally unique loci or genes with the help of population genetics and comparative sequence analysis; creating genetic maps with SSR and SNP markers to tag resistance regions; and providing genes and sequences to validate and characterize the function of candidate PD resistance genes. These genes under the control of promoters derived from grape will then be transformed into elite *V. vinifera* cultivars.

The specific objectives of this project are:

1. Provide genetic marker testing for mapping and breeding populations produced and maintained by the PD resistance breeding program, including characterization of novel forms of resistance.
2. Complete a physical map of the *PdR1c* region from the *V. arizonica* b40-14 background and carry out comparative sequence analysis with *V. arizonica/candicans* b43-17 (*PdR1a* and *b*).
3. Employ whole genome (WG) sequencing (50X) of recently identified PD resistant accessions and a susceptible reference accession; use bioinformatics tools to identify resistance genes, perform comparative sequence analysis and develop SNP markers to be used for mapping.
4. Clone *PdR1* 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, including characterization of novel forms of resistance.

We completed a survey of 219 southwestern US and northern Mexico *Vitis* accessions using SSR and chloroplast markers to evaluate their genetic diversity and establish their relationships with known sources of resistance being used in the PD breeding program (Riaz and Walker 2013). In Fall 2014, we added 120 new accessions that were collected from multiple collection trips from States bordering Mexico or that were acquired from Central and South America. A total of 88 of these accessions are currently being greenhouse screened to test for PD resistance. A set of 32 SSR and 14 chloroplast markers were evaluated on the 120 new accessions, making a total of 239 accessions under test for the discovery of new and unique resistant germplasm. Currently we are in the process of finalizing the analysis of the genotyping data and preparing for population studies.
In 2012, 2013 and 2014 crosses were made with additional resistant accessions: b41-13, b43-57, b47-32, SC36, and T03-16. Small seedling populations were marker tested for off-types for up to four markers before they went for the greenhouse screening (Table 1). In Spring 2014, we have developed F1 and BC1 breeding populations using two of the resistant accessions, b46-43 and T03-16, that are geographically unique, have different maternal origin, and are genetically diverse. Seeds were extracted, treated and planted from the 2014 crosses. We plan to complete the greenhouse testing of the F1 and BC populations over the next year. We isolated the genomic DNA and completed the testing for off-types for a total of 578 plants with four markers. All extraneous plants were removed and remaining plants were trained to obtain green cuttings to develop 4-5 replicates of each seedling to be greenhouse tested. Results of the GH screen will be complete in Summer 2015.

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

The accession b40-14, a pure form of *V. arizonica*, was tested and found to be homozygous resistant to PD. Two resistant siblings of this population were used to develop the 07386 (R8917-02 x *V. vinifera*) and 07744 (R8918-05 x *V. vinifera*) populations. Genetic mapping and QTL analysis with the 07744 population identified a major locus for PD resistance on chromosome 14. PD resistance from b40-14 (which we have named *PdR1c*) maps in the same region as *PdR1a* and *PdR1b* between flanking markers VVCh14-77 and VVIN64 and within 1.5 cM. The allelic comparison of SSR markers within the 20cM region including the *PdR1c* locus revealed that *PdR1c* locus is unique and sequences and genomic features would be distinct from b43-17. Figure 1 presents schematic diagram of multiple BC2 and BC3 populations for the breeding program.

All of the seedlings from the breeding populations were tested with markers to identify the recombinant plants, which were then phenotyped with the greenhouse ELISA screen to narrow down the genomic region with PD resistance genes. We also developed new SSR markers using the b43-17 sequence generated in this study for comparative sequence analysis. Two of the SSR markers, SSR82-1b4 and ORF18-19-3 were tested on the combined set of recombinant plants (Table 2) to narrow down the genetic window. We found four recombinants between Ch14-81 and VVIn64 on one side and one recombinant between Ch14-77 and Ch14-27 marker. With the help of these markers we confined the *PdR1c* locus to 325 Kb based on the sequence of b43-17 (Fig. 2).

We developed a BAC library from b40-14 genomic DNA. To complete the physical map of the *PdR1c* locus, we completed the screening of the BAC library. By utilizing the b43-17 sequence, we have designed probes that amplify a single amplicon of 600-650 bp using b40-14 genomic DNA. The complete library of b40-14 is on nylon filters, sequence specific probes were used to pull out BAC clones that represent the *PdR1c* genomic region. We used two probes, Ch14-56 and Ch14-58 and identified 11 and 19 BAC clones, respectively. Six clones were positive with both probes. In next stage, we selected only BAC clones that represent *PdR1c*. The resistant selection b40-14 is homozygous resistant and genomic DNA carry both resistant haplotypes c and d. As the PD resistance is mapped in haplotype c only, we wanted to develop a physical map with BAC clones that represent c. All positive clones were tested with three SSR markers that were polymorphic for b40-14 and allowed us to distinguish between haplotype c and d. At the final stage, we selected two BAC clones VA29E9 and VA57F4 that overlap and are ~ 200Kb in size. The selected BAC clones were cultured to generate large amount DNA that was purified using Qiagen large insert library kit. A total of 20ug of purified DNA of each BAC was sent to UCI genomics high through put facility for PAC BIO RS II sequencing. Currently we are waiting for sequencing results to carry out assembly, annotation and comparison to b43-17 sequence. We have also identified BAC clone H43-I23 from the b43-17 BAC library that represents *PdR1a* haplotype (F8909-17). This clone was also sent for the sequencing and results are pending.

Objective 3. Employ whole genome sequencing (WGS) (50X) of recently identified PD resistant accessions and a susceptible reference accession; use bioinformatics tools to identify resistance genes, perform comparative sequence analysis and develop SNP markers to be used for mapping.

We identified multiple new PD resistant accessions that were used to develop small breeding populations in 2012-2013. More crosses were made in 2013 and 2014 to expand existing, and make new, breeding populations (see final report). Our focus is on two new resistant accessions, b46-43 and T03-16. Both of which have shown very low bacterial levels in repeated greenhouse screens. Resistant accession b46-43 is homozygous resistant to PD. Crosses to develop BC1 populations were made in 2014. We have extracted DNA of the F1 population to test it with markers to validate that all progeny were true to type. Our approach of traditional bi-parental mapping populations has played an important role in gene discovery and understanding of PD resistance in North American *Vitis* species, and both bi-parental and multi-parental breeding populations remain the foundation of our breeding program. In this project, we want to combine traditional SSR marker system and next generation sequencing to carry out SNP discovery and potential SNP markers will be developed. We will pursue the WGS (whole genome sequencing) approach only on those resistant lines for which we have strong greenhouse screen results, information on the heritability of the PD resistance, and results from pre-screening of the population using the limited mapping strategy. The BC1 populations in the b46-43 and T03-16 background are under testing and will be ready for WGS approach in Summer/Fall 2015.

Objective 4. Cloning of *PdR1* genes with native promoters.

In 2014, we employed the PAC BIO RSII sequencing approach to sequence H69J14 and three other overlapping BAC clones. The assembled sequence data generated a 604Kb long fragment without gaps (see previous reports). We identified multiple open reading frames of the Leucine-Rich Repeat Receptor Kinase gene family that regulates a wide variety of functions in plants including stem cell maintenance, hormone perception, and defense and wounding response for both host as well as non-host specific defense. With the help of molecular markers, we have limited the genetic region that carries the five open reading frames (ORF) to 82 Kb – these ORFs are associated with disease resistance and other plant functions described above. There are multiple ORF's that are outside this genetic region and have 99% sequence similarity to the candidate genes. Currently the major challenge is to isolate and clone the specific ORF within the region, verify the ORF sequence, as well as the untranslated 5' (UTR) sequence and promoter region, so that decisions can be made regarding the length of the sequence that will be used to make construct. We have acquired binary vectors pCLB1301NH and pCLB2301NK (Feechan et al. 2013) that have been optimized to carry large DNA sequences, thus allowing us to insert candidate genes plus surrounding sequences. Two ORFs, V.ari-RGA14 and V.ari-RGA18, are 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 TE (Fig. 3).

We have designed a set of 11primer pairs that generate overlapping amplified products to verify the genomic sequence of RGA-18. Figure 4 details the location of first 7 primer pairs on the sequence of RGA-18. BAC DNA was amplified with primers and amplified products were run on an agarose gel to verify the size of the amplicon. After size verification, bands were cut from the gel and cloned using pGEM-T easy vector cloning kit. Multiple colonies were selected, cultured and DNA was extracted for sequencing. So far we have completed the procedure for RGA18. Similar procedures will be adopted for the surrounding region of the RGA-14 that is in progress.

Both RGA14 and 18 are very similar in the sequence profile with the exception that RGA 18 is 2946bp in size and lacks the first 252 bp sequence that is part of RGA14. Functional analysis of the protein sequence of both RGA revealed that RGA-14 lacks a signal peptide in the initial part of the sequence. This was further verified by using 3'RACE to specifically amplify RNA from grapevines transformed with V.ari-RGA14 under 35s promoter. The results showed that mature mRNA does not contain a signal peptide, necessary for proper membrane localization, at the beginning of the sequence. We are verifying the upstream and downstream sequences that could be involved in post-transcriptional regulation. These sequences will be included, along with native promoter in the new constructs.

We also initiated a large experiment of resistant and susceptible plants with multiple repeats, and time points for control (mock or un-inoculated) and inoculated plants. Table 3 details the time points and number of genotypes in the study set. We made hardwood cuttings of all experimental plants in November 2014, and the experiment is in process. Plants were inoculated with bacteria and water (mock control) and first batch of samples for RNA extractions were collected. This time course experiment will allow us to study: 1) the expression profile of the candidate resistant genes at different intervals; 2) compare the expression of the two candidate RGA to determine which one is involved in the mitigating resistance to PD; 3) compare the cDNA sequence to determine the transcription start site and to determine if introns are involved; and 4) determine if there is any alternative splice variation. The results of this experiment will also act as a guide for the expression analysis in the transformed plants resulting from the native promoter.

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

Once the gene constructs are completed, they must be tested to see if they confer resistance. 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 uses *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 (TS) and Chardonnay (CH) and the rootstock *V. rupestris* St. George (SG) (Agüero et al. 2006). We have 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 the test of a set of 5 candidate genes. Transgenic plants did not show PD tolerance. However, expression of V.ari-RGA19 and 20 produced some lines with dwarf

phenotypes. Results are in accordance with the latest assembly obtained using PAC BIO SRII system and 3 additional overlapping BAC clones. They show that only one of the sequences tested, V.ari-RGA14, lays within the narrower resistance region of 141 kb defined by two recombinants obtained recently. We used 3'RACE to specifically amplify RNA from grapevines transformed with V.ari-RGA14. The results showed that mature mRNA does not contain a signal peptide, necessary for proper membrane localization, at the beginning of the sequence. We are in the process of construct development with binary vectors that are modified to carry larger fragments of the DNA for the next phase of the project.

CONCLUSIONS AND LAYPERSON SUMMARY

The development of breeding and mapping populations with the two new PD resistance sources, b46-43 and T03-16, is proceeding. These two accessions support the lowest levels of bacteria of any we have tested. They are geographically isolated from b43-17 and genetically different based on a recent genetic diversity study of over 250 accessions from the southern US and northern Mexico. We have started the screening of F1 and BC1 populations created from these two backgrounds. Marker testing and a limited mapping strategy will proceed in Spring 2014. The results from this work will allow us to use markers to facilitate stacking of these resistance sources with *PdR1* from b43-17 – the multiple resistance genes should make resistance more durable. We have completed the genetic mapping of PD resistance from b40-14 and named it *PdR1c*. This resistance source maps within the *PdR1b* locus, and may be an alternative gene within this complex replicated locus. We are physically mapping this gene to improve our understanding of the locus. We are using whole genome sequencing to generate single nucleotide polymorphism (SNP) markers to accelerate the genetic mapping in b46-43, which has shown to have exceptional resistance in multiple greenhouse trials. The use of SNP markers in combination with our SSR based mapping will accelerate the identification of closely linked markers for breeding and should also allow more rapid characterization of b46-43's resistance. Finally, we have been sequencing the *PdR1* locus to better define the 5 candidate genes and prepare them for complementation tests. This effort is also identifying their promoters so that we can avoid the use of constitutive non-grape promoters like CaMV 35S. We have tested versions of the *PdR1* candidate genes transformed with 35S and they have not worked. We hope that the sequencing efforts we have employed recently to fine-tune these gene candidates and the addition of *PdR1*'s native promoter will allow one of more of the 5 gene candidates to confer resistance in transformed Chardonnay.

PUBLICATIONS AND PRESENTATIONS

Riaz S, Krivanek AF, Xu K and Walker MA. 2006. Refined mapping of the Pierce's disease resistance locus, *PdR1*, and *Sex* on an extended genetic linkage map of *Vitis rupestris* x *V. arizonica*. Theoretical and Applied Genetics 113:1317-1329.

Riaz S, Tenscher AC, Rubin J, Graziani R, Pao SS and Walker MA. 2008. Fine-scale genetic mapping of two Pierce's disease resistance loci and a major segregation distortion region on chromosome 14 of grape. Theoretical and Applied Genetics 117:671-681.

Riaz S, Tenscher AC, Graziani R, Krivanek AF, Ramming DW and Walker MA. 2009. Using marker-assisted selection to breed Pierce's disease resistant grapes. American Journal of Enology and Viticulture 60:199-207.

Riaz, S and M.A. Walker. 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 4.

Walker, A. and S. Riaz. 2010. Map-based identification and positional cloning of Xylella fastidiosa resistance genes from known sources of Pierce's disease resistance in grapes. Proceedings of the CDFA Pierce's Disease/Glassy-winged Sharpshooter Board, San Diego, CA, December 16-17 Pp. 261-266.

Walker, A., S. Riaz and C. Agüero. 2010. Molecular characterization of the putative *Xylella fastidiosa* resistance gene(s) from b43-17 (*V. arizonica/candicans*). Proceedings of the CDFA Pierce's Disease/Glassy-winged Sharpshooter Board, San Diego, CA, December 16-17 Pp. 267-271.

Walker, A. and A. Tenscher. 2010. Breeding Pierce's disease resistant winegrapes. Proceedings of the CDFA Pierce's Disease/Glassy-winged Sharpshooter Board, San Diego, CA, December 16-17 Pp. 255-260.

Walker, A. and A. Tenscher. 2011. Breeding Pierce's disease resistant winegrapes. Proceedings of the CDFA Pierce's Disease/Glassy-winged Sharpshooter Board, Sacramento Pp. 204-209.

Walker, A. and A. Tenscher. 2012. Breeding Pierce's disease resistant winegrapes. Proceedings of the CDFA Pierce's Disease/Glassy-winged Sharpshooter Board, Sacramento Pp. 233-240.

Walker, A. and A. Tenscher. 2013. Breeding Pierce's disease resistant winegrapes. Proceedings of the CDFA

RESEARCH RELEVANCE

The goal of this research is to understand the genetics of PD resistance and provide genetic support to our PD resistance breeding of wine, table and raisin grapes. We successfully mapped the resistance genes from a form of *V. arizonica* and used the linked markers to greatly expedite our breeding program. We are now searching for additional forms of PD resistance in other species from a variety of geographic locations across the southern US and Mexico, with the goal of combining resistance from several species together to ensure durable resistance

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

INTELLECTUAL PROPERTY: PD resistant varieties will be released through the Office of Technology Transfer (Patent Office) of the University of California, Davis.

Table 1. 2014 crosses to incorporate new PD resistance sources.

Cross ID	Female Name	Male Name	Seedlings tested
14-360	F2-35	DVIT 2236.2 (*V. nesbittiana*)	90
14-367	F2-35	12340-13	50
14-321	Rosa Minna	12305-55	28
14-308	Rosa Minna	12305-55	19
14-364	Rosa Minna	A28	19
14-347	Rosa Minna	A28	23
14-322	Rosa Minna	12305-56	15
14-313	A14	French Colombard	53
14-324	F2-35	12305-56	47
14-340	ANU71	Grenache blanc	38
14-303	C23-94	Nero d'Avola	64
14-362	F2-35	ANU67	31
14-363	F2-35	SAZ 7	52
14-368	F2-35	12340-14	35
14-336	F2-35	12305-83	14
Total			**578**

Table 2. Using two new SSR markers, SSR82-1b4 and ORF18-19-3, to compress the genetic window for a new PD resistance locus, *PdR1c.*

Genotype	14-29	14-27	VVCh14-77	SSR82-1b4	ELISA Results	ORF18-19-3	14-81	VVIn64	UCV025	VVIp26
09367-35	+	+	+	+	R	+	+	+	+	+
09367-37	+	+	+	+	R	+	+	+	+	+
09367-38	+	+	+	+	R	+	+	+	+	-
09367-41	-	-	-	-	S	-	-	-	+	
12325-78	+	+	+	+	R	+	+	+	-	-
12326-18	+	+	+	+	R	+	+	-	-	-
12327-54	-	-	-	-	S	-	-	+	+	+
09367-26	-	-	-	-	S	-	-	+	+	
09367-30	-	-	-	-	S	-	-	+	+	+
09367-07	-	-	+	+	R	+	+	+	+	+
09367-12	+	-	-	-	S	-	-	-	-	-
09367-40	+	+	+	+	R	+	+	+	+	+
09367-43	+	+	+	+	R	+	+	+	+	+

Table 3. List of genotypes that are part of the study for controlled experiment. Stem samples will be collected at 7 time intervals from both mock and Xf inoculated plants for RNA extractions. All plants are maintained in the same greenhouse

		No. of plants	Day 1	Day 4	Day 8	Day 16	Day 23	Day 30	Day 44
					Week1	Week2	Week3	Week4	Week6
Inoculated	A de Serres	28	4	4	4	4	4	4	4
	b43-17	28	4	4	4	4	4	4	4
	F8909-08	28	4	4	4	4	4	4	4
	F8909-17	28	4	4	4	4	4	4	4
	U505-01	28	4	4	4	4	4	4	4
	U505-22	28	4	4	4	4	4	4	4
	U505-35	28	4	4	4	4	4	4	4
	Chardonnay	28	4	4	4	4	4	4	4
Mock	A de Serres	28	4	4	4	4	4	4	4
	b43-17	28	4	4	4	4	4	4	4
	F8909-08	28	4	4	4	4	4	4	4
	F8909-17	28	4	4	4	4	4	4	4
	U505-01	28	4	4	4	4	4	4	4
	U505-22	28	4	4	4	4	4	4	4
	U505-35	28	4	4	4	4	4	4	4
	Chardonnay	28	4	4	4	4	4	4	4

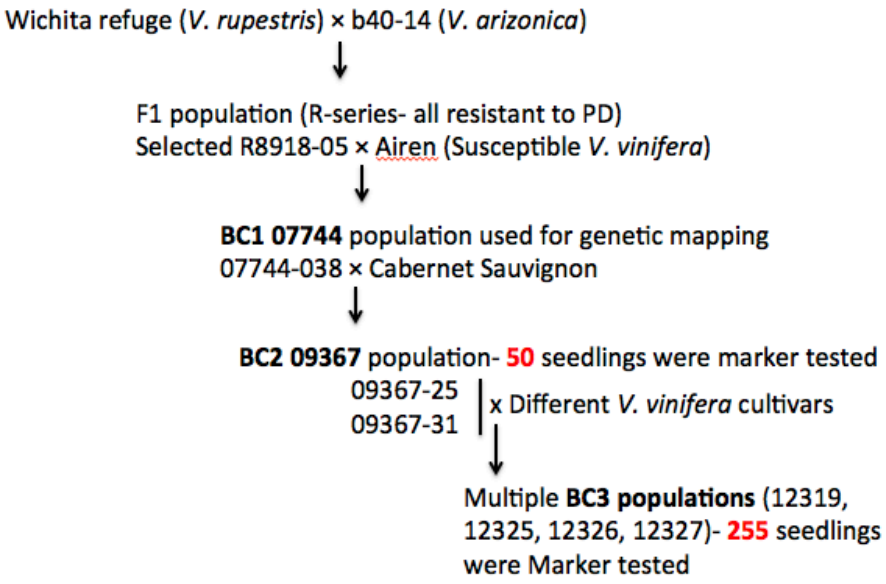

Figure 1. Schematic diagram of multiple BC2 and BC3 populations for the breeding program.

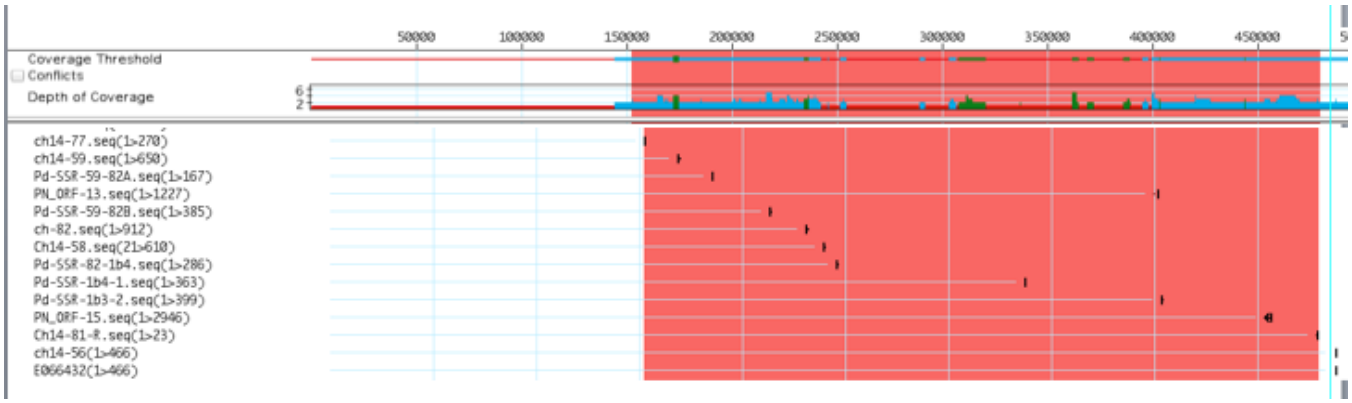

Figure 2. Localization of PdR1c to a 325 bp sequence.

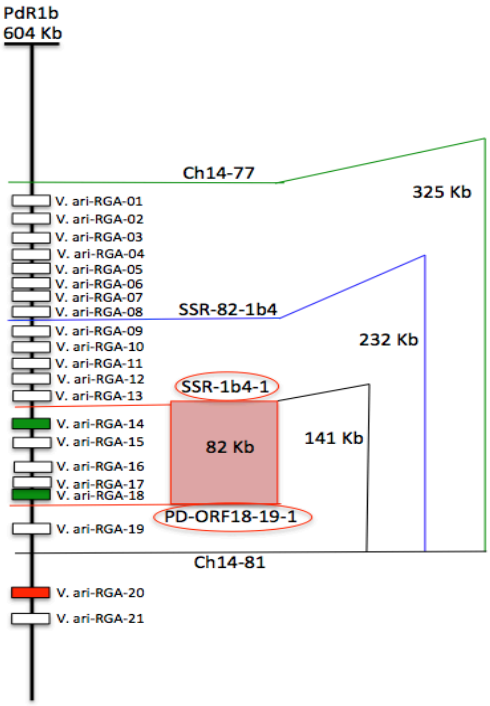

Fig. 3. The physical map of the *PdR1b* locus, identifying 21 resistance gene analogs and 82 Kb (pink section) sequence with the help of recombinant plants. RGA14 and RGA18 are two main candidates.

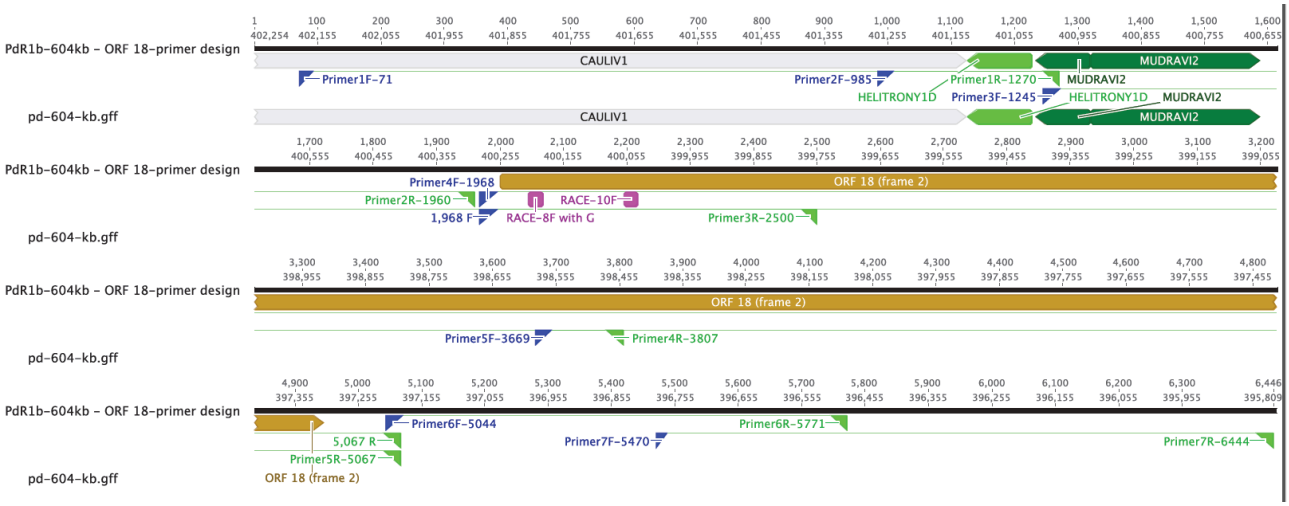

Figure 4. Location of the first seven primer pairs on the sequence of RGA-18.