MOLECULAR CHARACTERIZATION OF THE PUTATIVE *XYLELLA FASTIDIOSA* RESISTANCE GENE(S) FROM B43-17 (*VITIS ARIZONICA / CANDICANS*)

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ABSTRACT
Significant advances in the classical and molecular breeding of Pierce's disease (PD) resistance have been made by exploiting resistance from North American *Vitis* species. The resistant species, *Vitis arizonica/candicans* b43-17, was used to study the inheritance of resistance to *Xylella fastidiosa (Xf)*, generate mapping populations and to position loci on genetic maps linked to this homozygous dominant resistance gene termed *PdR1*. BAC clones were identified with markers linked to the PD resistance locus, and they were separated into *PdR1a* and *PdR1b* categories using a polymorphic marker for b43-17 and sequencing was completed for a clone carrying the *PdR1b* locus. Preliminary data analysis found that the resistance region is enriched with repetitive transposable elements, making the sequence assembly process very challenging. From the partial assembly of the region, we identified four tandem repeats of Serine Threonine Protein Kinase with a Leucine-rich Repeat domain gene family in the resistance region. The availability of the 12X genome assembly of Pinot noir (PN40024) provided a sequence without any gaps and allowed us to make comparisons. The 12X assembly of the PD susceptible PN40024 also carried four tandem repeats of the Serine-Threonine Protein Kinase gene family and all of them carried introns. We initiated cloning of one candidate gene *PdR1b.1* for further sequence verification and to develop constructs for use in complementation experiments. We also initiated cultures of embryogenic callus of two *V. vinifera* cultivars, Chardonnay and Thompson Seedless, and one rootstock, St. George. These cultures are now embryogenic and will be used for transformation/complementation studies with *PdR1* gene candidates. 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 to cut down the time period needed to generate transformed plants to four to six months.

LAYPERSON SUMMARY
Our companion project "Map-based identification and positional cloning of *Xylella fastidiosa* resistance genes from known sources of Pierce's disease (PD) resistance in grapes" has identified four candidate genes coming from *V. arizonica / candicans* b43-17 that may be responsible for PD resistance. The next step in the process is to test these candidate genes by transforming them into a PD susceptible grapevine to see if one or more of the gene candidates are responsible for resistance. To do this we have to more completely sequence the PD resistance region (*PdR1*) since it contains complicating genetic factors called transposable elements. We have started this "clean-up" process. We have also developed callus tissue that is capable of developing into new plants (embryogenic) from flower tissue of Chardonnay, Thompson Seedless and St. George. The gene candidates can be inserted into these embryogenic callus tissues and if these genes are responsible for resistance the plantlets regenerated from these tissues will be PD resistant. Development of embyrogenic callus is difficult and slow and this spurred the development of an alterative technique based on meristem tissue from shoots. We now have embryogenic tissue developed from this meristematic tissue that will allow the *PdR1* gene candidates to be tested in a wider range of winegrapes and more rapidly.

INTRODUCTION
New cultivars bred to resist *Xylella fastidiosa (Xf)* 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 very limited amounts of the *Vitis* species genome would be added to the elite parent, thus limiting the impact on its fruit and wine quality while making it PD resistant. The linkage-drag-free cisgenic approach is attractive, and also allows the opportunity to stack additional resistance genes from other *Vitis* sources, even if these genes originate from the same chromosomal position in different species or accessions (Jacobsen and Hutten 2006). We have been breeding PD resistant wine grapes, and it has been possible for us to maintain and characterize genetic populations that were by-products of the breeding program. These populations have allowed: the construction of genetic maps; identification of genomic regions associated with PD resistance and other traits of interest; the selection of markers that are tightly linked to PD resistance to greatly expedite breeding through marker-assisted selection (MAS); and the use of genetic maps to lay the foundation for the construction of physical maps to identify resistance genes (Riaz et al. 2008; Riaz et al. 2009). The physical map of the resistance region from *V. arizonica/candicans* b43-17, *PdR1*, allowed the identification of potential

candidate resistance gene(s). Preliminary comparison indicated that the *PdR1* region contains multiple tandem repeats of Serine Threonine Protein Kinase with a LRR domain (STPK-LRR) gene family. This category of genes belongs to a group involved in plant resistance. Their defense mechanism is based on compounds involved 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 the cloning progress of one of the candidate resistance genes, *PdR1b.1*.

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. Development of alternative protocols for genetic transformation for the validation of gene constructs
   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. 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: The preliminary assembly of the BAC clone sequence generated 8-10 contigs of significant size, but a large portion of the sequence remained unassembled. Further detailed analysis of the assembled, as well as the unassembled, sequences revealed the presence of a high number of transposable elements (TE). In fact chromosome 14 is the second largest carrier of transposable elements in the sequenced Pinot noir genome (Moisy et al. 2008). Transposable elements play key role in the diversification of disease resistance genes by allowing rapid adaptive change due to their ability to insert into regions of the genome and alter gene function; so called TE-induced gene alteration (Michelmore 1995). Considering the complexity of the *PdR1b* region, we are in the process of developing a fosmid library that would help overcome the problem of many short fragment sizes resulting from the 454 and Sanger shot gun sequencing reads. The fosmid library should produce much longer fragments and work towards a complete assembly of the region (see the companion report "Map-based identification and positional cloning of *Xf* resistance genes from known sources of PD resistance in grapes" for additional information). Meanwhile, we identified four candidate genes from the assembled contigs and have initiated cloning work with one of the candidate genes (*PdR1b.1*), which is 3.1 Kb in size.

Two pairs of primers were designed to clone the first candidate gene into a pCR4-Topo vector. The first set of primers was designed using the sequence builder program, and the second set utilized the Vector NTI program.

PD1-1F TTCTCTTTCATCCGTGAATGTAG
PD1-1R AAAAAATTCYTGGAGAGATGCT

PD1-2F GTAGGCATGATTGGGCCA
PD1-2R AAAATTCYTGGAGAGAGATGCTTATTTT

The PCR reaction was done using AccuPrime Taq Polymerase, which has improved fidelity (PCR Selection Kit- High Specificity, Invitrogen). The PCR reaction was performed at 60ºC annealing temperature and only the second set of primers amplified a fragment the size of the gene. The product of the PCR reaction was inserted into a pCR4-Topo vector. The vector was used to transform chemically competent cells of E. coli DHά5. In next step, we will sequence the DNA to verify it and the construction of binary vector will be initiated.

Objective 2. Expression studies of candidate genes by nested RT-PCR: To conduct the expression studies of the candidate genes, hardwood cuttings will be collected in November to generate plants. The stem and leaf tissue of these plants will be used for the total RNA from both resistant (b43-17, F8909-08) and susceptible genotypes (A de Serres, Chardonnay) using a cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol as described by Iandolino et al. (2004) with minor modifications. Results of this experiment will be available in the next report.

Objective 3. Development of alternative protocols for genetic transformation for the validation of gene constructs and in-vivo complementation of the candidate genes. Currently the most commonly used method for the production of transgenic or cisgenic grapes is based on *Agrobacterium* transformation followed by regeneration of plants from the embryogenic callus. We have established cultures of pre-embryogenic callus derived from anthers of *V. vinifera* 'Thompson Seedless' and 'Chardonnay' and the *V. rupestris* rootstock 'St. George'. These cultures of embryogenic calli have been used for transformation (Aguero et al, 2006).

In addition to embryogenic callus, we are testing two additional transformation methods. The first one is based on development of transgenic plants from shoot apical meristems via *Agrobacterium*-mediated transformation (Dutt et al. 2007). The ease of producing and maintaining in vitro micro-propagation cultures from a large number of cultivars makes shoot tip

based transformation a very effective system. The second method employs genetic transformation of *V. vinifera* via organogenesis (Mezzetti et al 2002). This method utilizes shoot apical meristem slices prepared from the entire meristematic bulk for *Agrobacterium*-mediated transformation of grape plants with the gene *DeH9-iaaM*. With this procedure, they were able to generate transgenic plants in much shorter time interval. We have streamlined this procedure and have already obtained transgenic shoots using Mezzetti's method in three months expressing GFP (**Figure 1**). So we expect that the time required for transformation will be shortened to approximately six months instead of one year via embryogenic callus. In later steps, we will use transformation with green fluorescent protein (GFP) to test the uniformity and effectiveness of this procedure as well as its utility with a range of cultivars; currently it is only being used with Thompson Seedless.

Figure 1. Regeneration and selection of meristematic transformed tissue with GFP protein (the green tissue is transformed).

CONCLUSIONS

The last step in the characterization of a resistance gene is to verify that the isolated gene functions in a host plant. This process requires that the gene be transformed into susceptible host and challenged by the disease agent. *Agrobacterium*-based transformation can be used with grape but initiating transformable and regenerable tissue is often a problem with grape. We have obtained regenerating callus of Chardonnay, Thompson Seedless and St. George for use in testing the four *PdR1* region gene candidates. We have also utilized another technique to speed the development of embryogenic tissue in this case from meristems 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.

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