Virginia Wine Board
Project #15-1662-01
Annual Report - July 2016

Identifying genomic markers for fungicide resistance in grape powdery and downy mildew

Principal Investigators

David Haak, Assistant Professor, Dept. of Plant Pathology, Physiology and Weed Science, Virginia Tech, Blacksburg, VA 24061-0390. Tel. 540-231-1186. Fax (departmental) 540-231-7477. Email: dhaak@vt.edu.

Anton Baudoin, Associate Professor, Dept. of Plant Pathology, Physiology and Weed Science, Virginia Tech, Blacksburg, VA 24061-0331. Tel. 540-231-5757. Fax (departmental) 540-231-7477. Email: abaudoin@vt.edu.

Graduate student **Xuewen Feng**

Objectives

1. Develop genomic markers associated with quinoxyfen (Quintec) resistance (QR) of grape powdery mildew.
2. Identify genetic targets of quinoxyfen as well as genes associated with resistance in grape powdery mildew.
3. Develop genomic markers to identify species and phosphite resistant isolates in grape downy mildew.

Powdery Mildew

Quinoxyfen: Since the mode of action of quinoxyfen is unknown, we cannot detect resistance by screening for a mutated fungicide target site directly. In order to identify markers most closely associated with the fungicide's action (and/or resistance), we carried out genetic sequencing at both the genomic (DNA) level and transcriptomic (gene expression or RNA) level. The aim was to identify genomic markers associated with resistance and structural gene changes in response to quinoxyfen treatment in both sensitive and resistant isolates. Pairing these approaches provides a window on the mechanism(s) involved in resistance *and* a refined set of genomic markers directly associated with fungicide resistance that can be used to screen field-collected isolates for resistance.

To identify genomic markers associated with resistance, we collected single spores from 10 resistant isolates and 10 sensitive isolates. These spores were then grown in culture until there was sufficient growth for isolation of a large quantity (2 µg) of high-quality DNA. We pooled the samples of DNA into two pools (R and S) for high-throughput sequencing on the Illumina Hi_Seq 2500. This technique, commonly called 'pool-seq' is used to reduce the number of individual genomic differences (markers) by only identifying markers that exist

between the pools and excluding variations within the pools (effectively reducing the number of informative markers from tens of thousands to thousands). To ensure we were calling true differences between pools and not sequencing errors we obtained ca. 30X coverage of the *Erysiphe necator* genome (greater than the recommended 20X). From these data we identified several thousand markers. To refine this set further we conducted an outlier test (Fisher's exact test) to identify genetic differences that were highly differentiated (therefore more likely to be reliable and robust markers across vineyards). This resulted in a set of 100 highly differentiated markers. These isolated markers can be further refined by identifying the set of markers directly connected with genes that differ between R and S isolates exposed to quinoxyfen.

To identify differentially expressed genes we devised a factorial experiment wherein sensitive and resistant spores were germinated on quinoxyfen-treated and untreated leaves for 24 hours. Spores were harvested and RNA was extracted for high-throughput sequencing to generate 'transcriptomes'. To generate sufficient RNA for sequencing we had to conduct multiple replicates over a period of 3 months. We have generated 50X coverage which is the standard depth of coverage required in such 'RNA-seq' studies to detect important but rarely expressed variants. These samples were delayed at the sequencing facility for 2 months but the data are now in house and analysis is underway. The gene markers that are finally selected to identify genetic targets of quinoxyfen should co-exist between these 'gene-expression' level data and the differentiated markers at the genomic level (above). The selected markers will be used in the current season to increase the efficiency of screening isolates from the field for quinoxyfen resistance.

Strobilurin: Similar to quinoxyfen isolates, we have conducted a 'pool-seq' analysis using two pools of 10 isolates each. Because we could leverage our existing high-coverage data for error correction of sequencing reads we were able to generate these sequence data using a reduced quantity of DNA and on the Illumina Mi-Seq. Not only was this a cost saving measure but the Mi-Seq generates longer read lengths which make it easier to assemble highly repetitive genomes (such as, *Erysiphe necator*). Even with the reduced number of reads from the different platform, we obtained ca. 15X coverage and (following the protocols above) we have identified a refined set of (150) genomic markers, which are present in one group but not the other. These isolated markers can be used in high-throughput screening of isolates from field collected samples.

Community tools: The quality of the reference genome is essential to the modern molecular genetic studies on *Erysiphe necator* and serves as a resource for more quickly identifying markers associated with additional resistance phenotypes. Using the high depth sequencing data we have obtained, we are generating draft genome assemblies guided by the available reference genome for both resistant and sensitive isolates. We will use the transcriptomic data to annotate these draft assemblies and these assemblies will be used to improve the existing powdery mildew reference genomes. After annotation, these genomes, genomic markers, and raw data will be made publicly available.

Downy Mildew

Genome sequencing: To date, no publicly available *Plasmopara viticola* reference genome exists, therefore we could not take advantage of the tools used in identifying markers for powdery mildew. To enable the use of these tools and generate markers we are developing *de novo* draft genomes. To do this we generated a large amount of high-quality DNA from two isolates and have sequenced them using the Illumina Hi-Seq 2500. The industry standard is to generate short read length (125 bases) and long read length (6000 bases) data. As mentioned above (powdery mildew) the short read length data are used for error correction and the longer read length data can act as a scaffold to bridge across highly repetitive regions of the genome. Generating these *de novo* assemblies is important for determining structural variation (such as copy number variants, insertions/deletions, inversions and translocations) among populations. This is an important update to our methods as recent work in plant pathogenic Oomycetes (the group to which downy mildews belong) have identified structural genomic variation as important sources of fungicide resistance.

The sequencing center had not worked with Oomycete data previously and required additional time for library construction prior to sequencing. Currently we are generating the genome assemblies that will allow us to take advantage of the sequence data and identify several hundred markers (as above for powdery mildew), which will be refined to population-specific markers in order to sort through the population structure of grape downy mildew in Virginia and identify markers associated with phosphite resistance.

Sample collecting: We have collected a substantial number of downy mildew isolates for the follow up genetic screening. We are in the process of generating sufficient DNA material for the screening of the markers. We also have planned to take ~30-50 more field samples in at least 10 various locations to achieve a wider geographic sampling range. The markers selected above will be used in the current season to sort through these downy mildew samples to investigate the genetic relationship between different populations.

Summary: In this project we have identified robust genetic markers associated with powdery mildew resistance to quinoxyfen and strobilurin and we are beginning to identify the genes associated with resistance to quinoxyfen. We are also generating diagnostic markers for downy mildew for both population identification and possible phosphite resistance. In the upcoming year, we will begin testing the genetic markers across known populations in the state to define a core diagnostic set that can be freely used across vineyards. Together these tools will allow us to better assess levels of resistance among vineyards in Virginia and provide a set of community tools that, in the future, will allow more rapid identification of markers associated with novel resistance types.