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

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LAYPERSON SUMMARY

We maintain and characterize many populations while breeding Pierce's disease (PD) resistant wine grapes, some of which have been used to develop genetic maps. These maps have been used to identify genetic markers that are tightly linked with PD resistance, which have allowed classical breeding to be greatly expedited through marker-assisted selection. Genetic maps allow the construction of physical maps to identify resistance genes (Riaz et al. 2008; Riaz et al. 2009). The physical map of the *V. arizonica/candicans* b43-17 resistance region allowed us to identify six candidate genes responsible for PD resistance. Sequence comparisons with other plant genomes indicated that multiple tandem repeats of a disease resistance gene family of receptor-like proteins (Leucine Rich Repeats; LRR) were present in the resistance region. This category of genes is involved in the recognition of microbes and in the initiation of a defense response (Bent and Mackey 2007). We completed the cloning of four of the candidate genes: *PdR1b*-1, 2, 5 and 6. We also developed embryogenic callus cultures of the PD susceptible *V. vinifera* Chardonnay and Thompson Seedless and the PD susceptible rootstock *V. rupestris* St. George. These embryogenic callus cultures will be used for the transformation/complementation studies to verify the function of these candidate PD resistance genes. Transformation experiments with *PdR1b-1* and *PdR1b-6* have been initiated. 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 reduce the time required to generate transformed plants by four months. We also initiated total RNA extraction experiments to allow time course examinations of gene function from leaf and stem tissues. These were successfully completed and we are now ready to evaluate gene function over time in inoculated and un-inoculated plants of the *PdR1* containing resistant selections F8909-08 and F8909-17, their resistant parent b43-17, their susceptible parent *V. rupestris* A. de Serres, and the susceptible control Chardonnay. These plants have been established in the greenhouse and will soon be inoculated.

INTRODUCTION

New cultivars bred to resist *Xylella fastidiosa (Xf)* infection and subsequent expression of Pierce's disease (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 and 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 single genes from 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 cisgene approach in grapes is similar to the natural clonal variation that exists in many winegrape cultivars. This linkage-drag-free 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). The physical mapping 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 in the recognition of microbe-associated molecular patterns (MAMP) like compounds, which initiate 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 progress on the cloning and testing of four candidate resistance genes.

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. A refined genetic map of chromosome 14, which contains the PD resistance locus, was generated from three grape mapping populations derived from b43-17. The resistance locus segregates as a single dominant gene and mapped as *PdR1a* in the F1 selection 8909-17 and as *PdR1b* (allelic forms) in its sibling F8909-08. Clone H69J14 from a b43-17 BAC library, containing both markers flanking the *PdR1b* resistance locus, was sequenced using 454 sequencing. Further detailed analysis of the assembled, as well as unassembled sequences, revealed the presence of a high number of transposable elements (TE). Chromosome 14 is known to have the second largest number of TEs in the Pinot noir grape genome sequence (Moisy et al. 2008). TE's play a key role in the diversification of disease resistance genes through a process termed TE-induced gene alteration (Michelmore 1995). Given the complexity of this region due to the large number of TE tandem repeats, a fosmid library was generated with an insert size of 35-40kb from the H69J14 BAC clone. The second round of sequencing was performed using fosmid end-sequencing as well as shotgun reads.

The first assembly generated 10 contigs, with a portion of the sequence remaining unassembled. Analysis of assembled and unassembled sequences revealed the presence of four candidate genes, *PdR1b*.1–4, which appear to be receptor-like proteins, a class of resistance proteins. *PdR1b*.1-2 and 4 were cloned into a pGEM-T easy vector (Promega) and subcloned into vector pDE00.0113 containing the 35S promoter and ocs3' terminator (**Figure 1**). The second assembly allowed the identification of two new candidate genes and showed that *PdR1b*.1 was longer than previously found. As a consequence, we repeated the amplifications from BAC H69J14, using primers that hybridize to regions flanking the open reading frames (ORFs). So far, we have re-amplified and confirmed the sequences of *PdR1b*.1, *PdR1b*.2, *PdR1b*.5 and *PdR1b*.6. We have sublconed *PdR1b*.1 and *PdR1b*.6 into binary vectors pCAMBIA-1303 (www.cambia.org) and pDU99.2215 (**Figure 1**). PdR1b.1 is the largest gene, sharing a high degree of homology with *PdR1b*.2, 3, 4, and 5.. *PdR1b*.6 is significantly different from the rest. It has a kinase domain, which suggests it might be involved in PD resistance in combination with *PdR1b*.1 or one of the other candidates. pCAMBIA-1303 was included in the experiments because it carries a hygromicin resistance gene that improves the selection of transformants (D. Tricoli, pers. comm.). An additional advantage is that it allows the subcloning of the gene in one step, by replacing the gus gene with the gene of interest. The resulting plasmids were used for transformation via *Agrobacterium tumefaciens* of Chardonnay, Thompson Seedless, St. George and tobacco SR1. A similar procedure will be followed with the remaining four genes.

Sequence analysis and alignments to identify introns and exons on the *PdR1b*.1 gene were performed using the GeneQuest module of Lasergene v 8.1, which facilitates the prediction of coding regions using the Borodovsky's Markov method and predicts intron/exon boundaries using species-specific patterns by aligning to known genes. We also utilized the GeneMark (http://exon.biology.gatech.edu/) program using both *Arabidopsis thaliana* and *Medicago truncatula* settings. By using both systems, we identified two small introns from position 1-168 and position 3128-3191 that are 167bp and 361bp, respectively. With *A. thaliana* as the model system, the size of predicted protein was 976 amino acids, and with *M. truncatula* it was 964 amino acids. It is interesting to note that all four genes from the 12X assembly of PN40024 carry large introns. We plan to make sequence comparisons and identify protein domains for other putative candidate genes. Experiments are underway with specific primers to amplify the regions of the genes using total extracted RNA and making make sequence comparisons among Pinot noir and the five different genes[MSOffice1].

Objective 2. To conduct expression studies of the candidate genes, hardwood cuttings were collected in November to generate at least 15 plants of the susceptible *vinifera* control (Chardonnay), resistant and susceptible parents (b43-17 and *V. rupestris* A. de Serres) and two resistant F1 selections (F8909-08 and F8909-17). Stem and leaf tissues were used for the total RNA from both resistant and susceptible genotypes using a cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol as described previously (Iandolino et al., 2004). Two other methods were tested to get better yield and quality of total RNA with less genomic DNA. The best yield of total RNA was obtained by the procedure described by Reid et al. 2006.

We used a time course analysis to evaluate expression and to determine when the resistance gene(s) is activated. Total RNA was extracted from the leaves and stem tissue of un-inoculated plants at 1, 3 and 5 weeks after inoculations with the *Xf*. ELISA screening was carried out after 12 weeks to quantify the amount of *Xf* in tissues. **Figure 2** presents the results of RNA extracted from the young leaves of the four genotypes before the bacterial inoculations and stem tissue two weeks after inoculations. First-strand cDNA synthesis will be performed with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Three different control housekeeping reference genes were tested as controls (Actin, Chitinase and B-actin). The actin gene (F-actgctgaactggaaattgt; R- acggaatctctcagctccaa) as described Vasanthaiah et al. 2008 worked very well in our system. We designed specific primers for quantitative PCR from two of our candidate genes (*PdR1*.1 and *PdR1*.6) and used comparative RT-PCR to check the effectiveness of the primers and the whole setup. We observed differences in the level of expression of the genes indicating that our RT-PCR system is working. Experiments that are more detailed are underway to monitor the gene expression of all six candidate PD resistant genes.

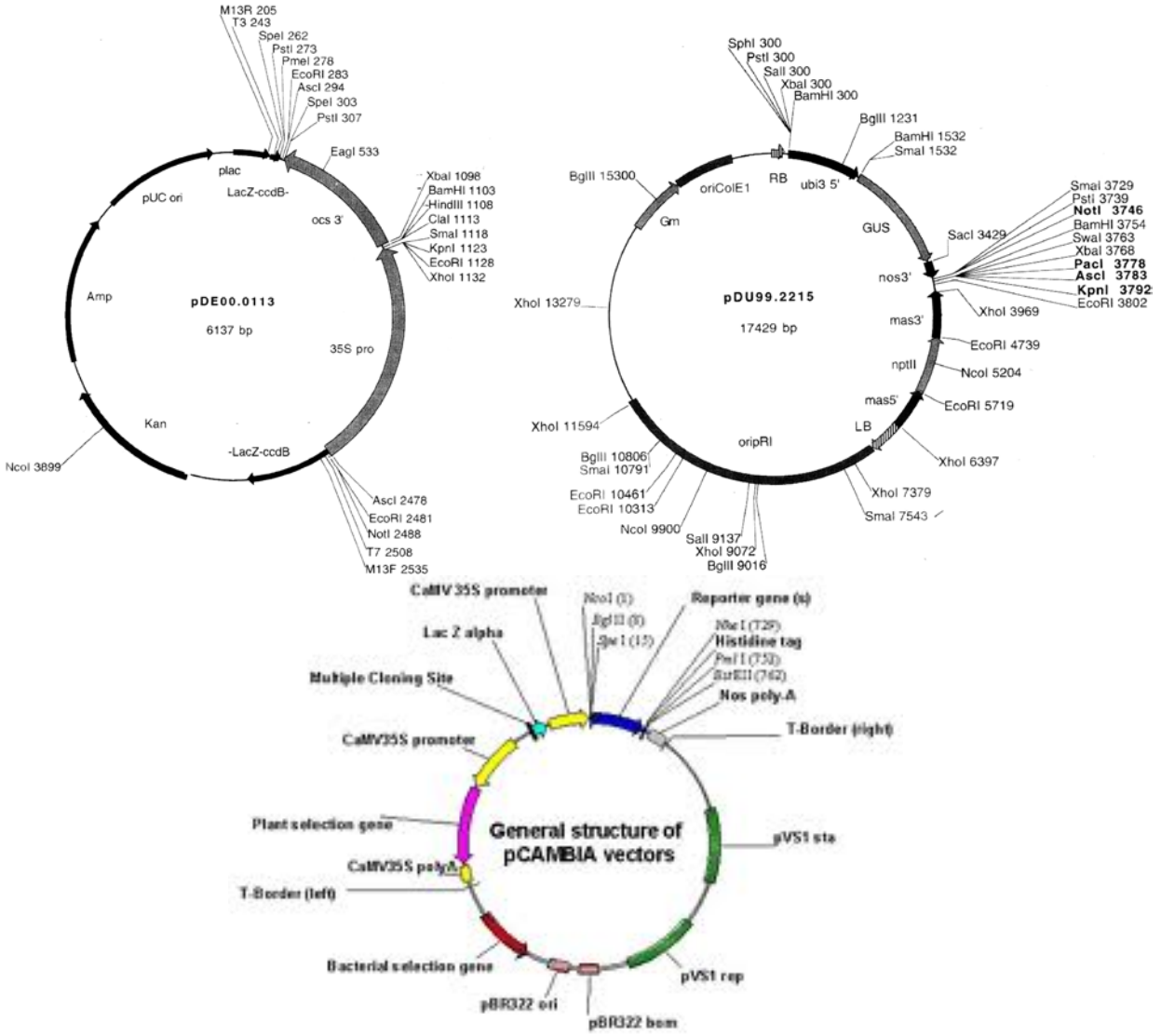

Figure 1. Vectors used in genetic transformation with *PdR1b* candidate genes. *PdR1b*.1 was subcloned into pDE00.0113 and then the expression cassette was moved into the binary plasmid pDU99.2215. *PdR1b*.1 and *PdR1b*.6 were also subcloned directly into the binary plasmid pCAMBIA 1303 in the place of the reporter gene. Because the two binary plasmids have different plant selection genes, it is possible to co-transform tobacco with both genes.

Objective 3. Development of alternative protocols for genetic transformation for the validation of gene constructs. Once the gene constructs are completed, they must be tested to see if they contain the resistance genes. This is done by inserting the gene(s) into a susceptible plant and testing to see if the inserted gene makes it resistant. Currently the most widely used method for the production of transgenic/cisgenic grapes is based on *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 and Chardonnay and the rootstock *V. rupestris* St. George. These cultures of embryogenic calli can be readily used for transformation (Agüero et al. 2006).

Two alternative transformation techniques via organogenesis have been tested to reduce the time needed to produce transgenic grapes. These methods were developed in Thompson Seedless and are based on the use of meristematic bulks or etiolated meristems as explants for inoculation with *Agrobacterium* (Mezzetti et al. 2002, 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 an effective system. The second method employs genetic transformation of *V. vinifera* via organogenesis (Mezzetti et al 2002). In this method, shoot apical meristem slices are prepared from meristematic bulks for *Agrobacterium*-mediated transformation of grape plants. Using this procedure, transgenic plants can be produced in a much shorter time interval. We have streamlined this procedure and have obtained transgenic shoots using Mezzetti's method in three months (**Figure 3**). With the successful modification and adoption of this technique, we expect that the time required for

transformation will be shortened to approximately six months instead of one year or longer via embryogenic callus. The green fluorescent protein (GFP) was used as a reporter gene for monitoring the occurrence of transformed and chimeric plants. **Figure 3** shows both procedures in comparison with transformation via somatic embryogenesis. No plants were regenerated from etiolated meristems and the procedure was laborious and time consuming. Transformation via meristematic bulks represented a better method to produce transgenic plants in a shorter period of time, although its efficiency was very low (1 plant in 50 sections). GFP expression in transgenic leaves was uniform; indicating a stable non-chimeric transformation of shoots regenerating in selection medium.

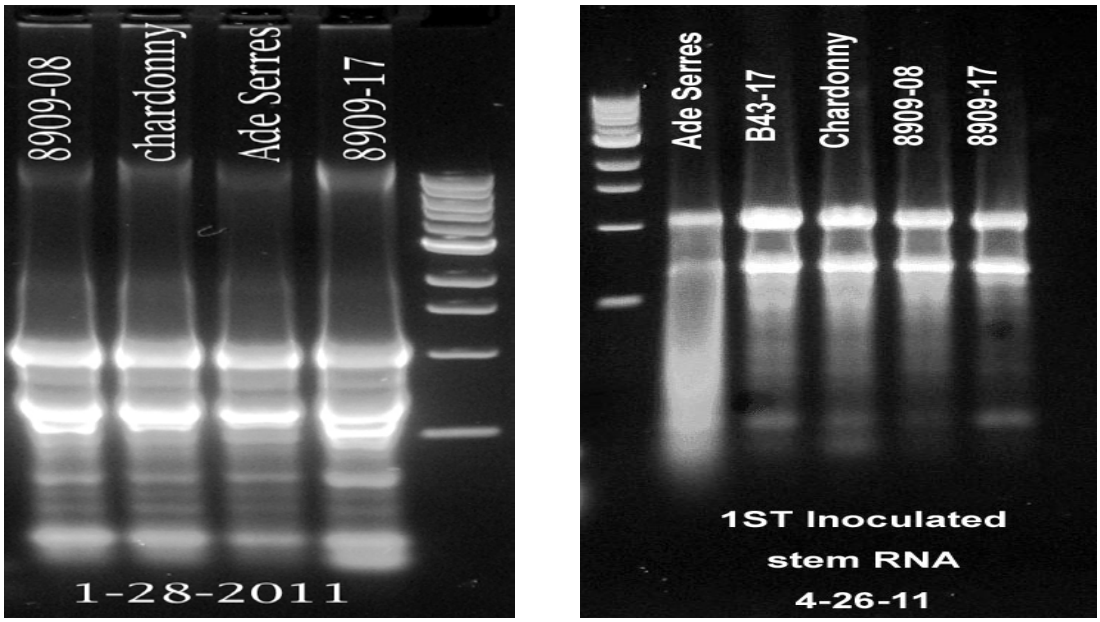

Figure 2. Total RNA extracted from the leaves and stem tissue of susceptible control and resistant plants.

We have inoculated pre-embryogenic cultures of Thompson Seedless, Chardonnay and *V. rupestris* St George with *A. tumefaciens* EHA105 pCH32, carrying binary plasmids with *PdR1b*.1 and *PdR1b*.6 coding sequences. Overnight cultures of the bacteria in LB medium + antibiotics were diluted to 10^8 cells·ml^{-1} using liquid co-cultivation medium. Pre-embryogenic calli were placed on a sterile glassfiber filter (GFF) overlaid on co-cultivation medium. The *Agrobacterium* culture was poured over the callus and excess was blotted with sterile filter paper after five min. The callus on GFF was then transferred onto fresh co- cultivation medium. After 48 hr in the dark, the callus pieces were sub-divided into small clumps, about 2 mm in diameter, and cultured on selection medium. Currently, calli are being selected with 100 ug/ml kanamycin or 15 ug/ml hygromicin. We have also transformed meristematic bulks of Thompson Seedless following a similar procedure except that meristematic bulk slices were submerged in the *Agrobacterium* solution. We are producing meristematic bulks from Chardonnay and St. George (**Figure 4**) that will be tested as soon as they are ready for inoculation.

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 is transformed into a 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 six *PdR1* region gene candidates. We have also tested another technique to speed the development of transgenic tissue 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.

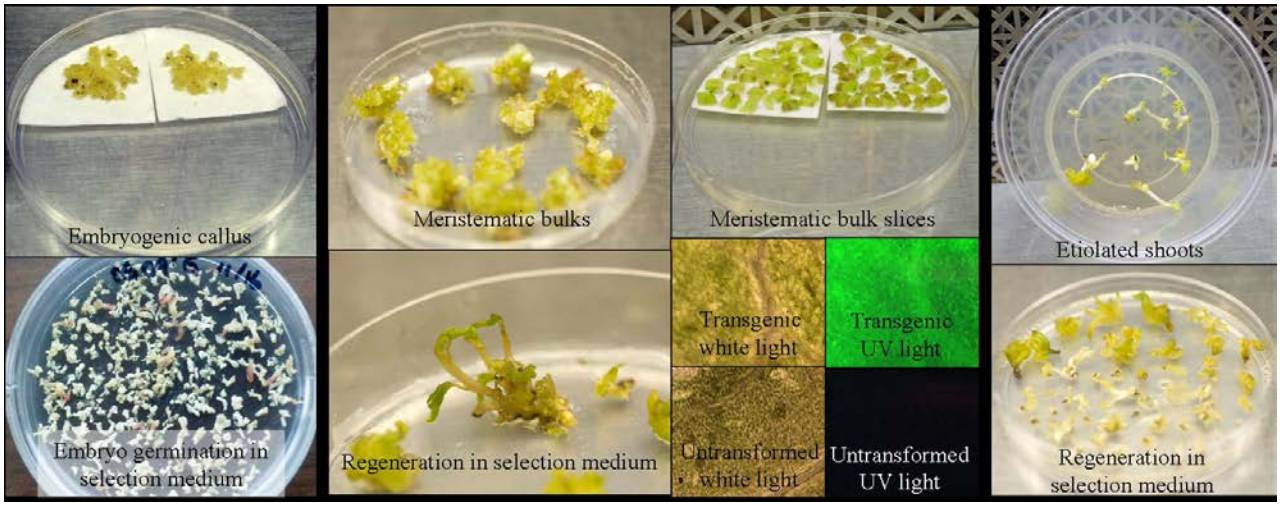

Figure 3. (A) Embryogenic callus developed from anthers are inoculated with *A. tumefaciens*. Transformed embryogenic callus is selected after about four months. An additional 4-6 months are needed for embryo germination and plant development. (B) Meristematic bulks, developed from shoot apical meristems, are sliced and inoculated with *A. tumefaciens*. Transformed bulks are selected after about 3 months. An additional four months are needed for shoot elongation and rooting. Pictures on the bottom right show green fluorescence protein (GFP) expression in transgenic leaflets. (C) Fragmented shoot apices from etiolated shoots are inoculated with A. tumefaciens. Transformed meristems are selected after 2-3 months. An additional 2-3 months are needed for plant regeneration and rooting.

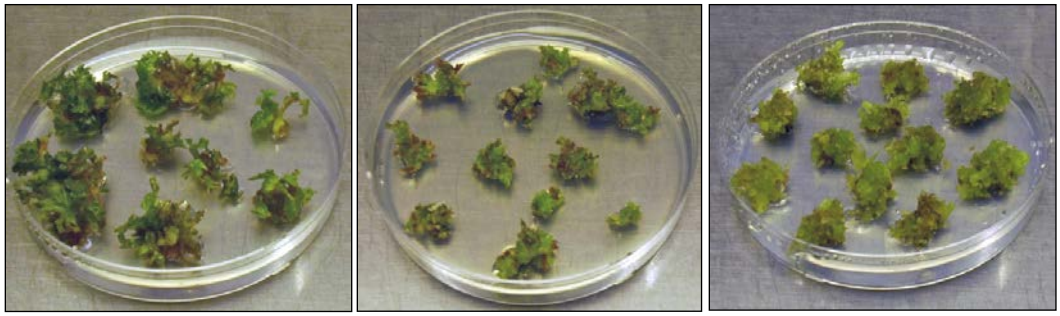

Figure 4. Meristematic bulks of Chardonnay (left), St. George (middle) and Thompson Seedless (right).

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