Unified Grant Management for Viticulture and Enology
PRELIMINARY ANNUAL REPORT 2022-2023 FUNDING CYCLE

CALIFORNIA GRAPE ROOTSTOCK IMPROVEMENT COMMISSION (CGRIC)
CALIFORNIA GRAPE ROOTSTOCK RESEARCH FOUNDATION (CGRRF)

1. **Summary**:

Project Title: Stacking disease and pest resistance in grapevine rootstocks
Principle Investigator: Abhaya M. Dandekar, Plant Sciences Department, UC Davis

The goal of this project is to develop grapevine rootstocks that combine their existing resistance to pests like Phylloxera and/or to nematodes with RNAi-mediated resistance to bacterial crown gall disease. The project was initiated on March 31, 2020 with input from members of the commission during their meeting on Feb. 4, 2020. Based on this input we will target in addition to the proposed rootstock GRN1 the following additional pest resistant rootstocks: 1103P, 101-14 Mgt, 110R and Freedom. We were successful in developing somatic embryo lines for all five of the target rootstocks. This allowed us to accomplish objective 1. Using these somatic embryo cultures, we initiated the transformation process of introducing the RNAi-mediated resistance into the first 5 including Thompson Seedless (TS) as a control initiating Objective 2. Transformation has been completed with all five genotypes but performance of 110R embryos is slower. We have already started to obtain transgenic plants from the TS, GRN-1, 101-14 Mgt and 1103P and Freedom embryo lines. Obtaining transgenic plants from110 R will take a few months. In objective 2 of this proposal, we will propagate these plants to test their resistance to crown gall. We have successfully developed a rapid method to test crown gall resistance in young plantlets. This rapid method was validated on all 5 rootstock genotypes and showed that all of the wild type plants were all very sensitive to crown gall formation. Genotypes GRN-1, 1103P and 101-14 Mgt transgenic plants were tested in the greenhouse. Transgenic plants of Freedom are planned to test in early Spring 2024. These initial results will be compared to plantlets obtained from embryo lines expressing the RNAi-based resistance.

2. **Final Progress Report**: Oct. 1, 2022 to Sept. 30, 2023 for 2022-2023 funding cycle.

3. **Project Title and UGMVE proposal number**: Stacking disease and pest resistance in grapevine rootstocks – 2022-2023.

4. **Principal Investigator**: Abhaya M. Dandekar, Department of Plant Sciences, University of California, Davis; 1 Shields Ave; Davis CA 95616.

Cooperator(s): Andrew M. Walker - Louise Rossi Endowed Chair, Department of Viticulture and Enology; University of California, Davis; 1 Shields Ave Davis CA 95616.

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5. **Objective(s) and Experiments Conducted to Meet Stated Objective(s)**:

Goal: To develop grapevine rootstocks that combine existing resistance to pests with RNAi-mediated resistance to disease. We propose to accomplish this goal via the following two objectives in a period of three years.

Objective 1: Develop and introduce crown gall resistance into grapevine rootstocks resistant to *Phylloxera* and nematodes

In our proposal we had selected a single rootstock GRN-1 (*V. rupestris* x *M. rotundifolia*) based on its high resistance to *Phylloxera* and nematodes. However, after our discussion with the board on Feb 4, 2020 we have included in this study additional rootstocks based on their recommendation as these additional rootstock genotypes are of interest to California growers. These other rootstocks have a similar profile as they are resistant to different pests and environmental stress but are sensitive to crown gall. These include 1103P (*V. berlandieri* x *V. rupestris*), 101-14 Mgt. (*V. riparia* x *V. rupestris*), Freedom 1613 (*V. solonis* x. *Othello*) x Dog Ridge, 110R (*V. berlandieri* x *V. rupestris*) x Dog Ridge. Thompson Seedless somatic embryos are used as the control. In the spring 2020, we initiated embryogenic callus from anther filaments of all of the above genotypes using methods that we have previously described (Aguero et al., 2006). The genotype 110R showed slower growth compared to other genotypes. The embryogenic callus was induced to make embryos that can be clonally multiplied via repetitive embryogenesis where single embryos produce clones of themselves. We have begun to germinate these embryos to make sure that they are able to be germinated into plantlets. This is where we are currently confirming the ability of all the embryogenic cultures to germinate.

The next step was to transform these embryos with the binary vector pDE00.0201 (Fig 1A) to successfully express the RNAi-mediated resistance to crown gall as we have previously

described (Escobar et al., 2001, 2002, 2003). We transformed all 6 embryo lines, GRN-1, 1103P, 101-14 Mgt, 110R, Freedom and Thompson Seedless as a control. Freedom transgenic plants (Fig 2) were recently obtained and are multiplying in the greenhouse. 110R embryos were recently transformed as that genotype took longer than others to generate cultures of embryos at the correct stage to transform. Transformed 110R recovery work will take about 6 more months to complete. We have already started obtaining transgenic tissue culture plants from the TS, GRN-1, 1103P and 101-14 Mgt. and Freedom embryo lines. They are currently multiplying in the lab and greenhouse. Transgenic plants from other genotypes will be obtained in the future (Tables 1 and 2).

Wild type plants of all the six genotypes used in our experimentation were obtained from Foundation Plant Services (FPS) at UC Davis are being maintained in the greenhouse to use as controls. Currently, transgenic plants from TS, GRN-1, 1103P and 101-14, Mgt are propagated to evaluate for crown gall resistance by challenging with different *Agrobacterium* strains. The second round of inoculation will be carried out in Spring 2024. Transgenic plants from Freedom will be tested with these.

Objective 2: Evaluate the efficacy of the combined resistance to disease and pests.

Here we have initiated two activities; the first is to develop an efficient micropropagation system to multiply plantlets obtained from individual embryo lines that will be used for testing the RNAi-mediated resistance to bacterial crown gall delivered by the binary vector pDE00.0201 shown in Fig 1A. The second activity that we have initiated is to develop efficient root systems that can be used for testing not only crown gall but also to confirm the pest resistance status of the combined resistance present in the individual rootstocks. To successfully accomplish this activity, we have cloned the two-root inducing (RolB and RolC) genes from *Agrobacterium rhizogenes* strain A4 (ArA4) (Britton et al., 2008). Using the known DNA sequence of this strain we were able to design appropriate primers to successfully PCR amplify these two genes on a single fragment of DNA. This fragment of DNA also contains the natural regulatory regions that are competent to express these two genes simultaneously in plant tissues. This piece of DNA that contains these two genes was incorporated to create two binary vectors shown in Fig 1B and C. The first (Fig 1B) also contains a gene that gives a red florescence and will be useful to identify the induced roots. The second binary (Fig 1C) contains a gene that improves the resilience of the root system. We plan to initially test these on wild type plants germinated from the different embryo lines that we now have in culture to validate their sensitivity to crown gall. First trial was initiated transforming leaf petioles with the vector 1B, caring red florescence and RolB and RolC. Some leaves and leaf petioles of WT and transgenic plants were transformed with a vector carrying RUBY gene. Red color gall formation was observed in infected leaves and leaf petioles and this was not observed in leaves of transgenic plants.

To evaluate the degree of resistance to crown gall, we developed a protocol using wild type tissue culture plants that were obtained from the somatic embryos and their leaf petioles. The assay we developed is very sensitive and quick, giving results within 3 weeks after challenging with *Agrobacterium* strains. For this experiment we used three different *Agrobacterium* strains; C58, 20W-5A and A281. Liquid *Agrobacterium* cultures were prepared with a cell density of 10^8 cells/mL by culturing bacteria from a fresh plate. Eight plants or leaf petioles were inoculated from each genotype. When inoculating the shoots, a scalpel was dipped in bacteria solution and stabbed in three places on the stem starting from the bottom. In the leaf petiole assay, petioles were dipped in *Agrobacterium* solution for 20 minutes. Then the inoculated leaves were transferred to co-cultivation media for 48 hours and transferred to a media supplemented with Timentin, an antibiotic to control the overgrowth of the infecting bacteria. Tumor formation was observed over three weeks after inoculation. For this analysis the size of the gall was considered and a scale of 1-4 was used. No galls = 0, 25% = 1, 50% = 2, 75% = 3 and 100% = 4. Average score from eight replicates were considered when deciding the degree of resistance. Galls were observed after 2 ½ to 3 weeks in inoculated shoots and leaf petioles. Results from these initial tests were included in the previous reports. Protocols that were developed using stems and leaf petioles were used to evaluate the degree of resistance to crown gall, in transgenic tissue culture plants that are available in the lab. Parallel experiments were conducted with wild type plants from all the genotypes. This was helpful to evaluate the crown gall resistance compared to wild type.

We have developed an assay for greenhouse grown grape plants to evaluate resistance to crown gall. Cuttings from the initial plants were rooted using an aeroponic system (mist bed) in the greenhouse. Plants developing enough healthy roots were transferred to pots and grown to reach proper height of strength to be inoculated. Six replicates were included for each genotype (Fig 3A). When plants were about 6-8 inches height with proper stem diameter (pencil thickness), they were inoculated with *Agrobacterium strain* A281 stabbing in three places on the one side of the stem, starting from the crown. Other side of the stem was inoculated using a needle in three sites using 10ul/site from a bacterial solution of 10^8 cells/mL (Fig 3B). Areas where stems were inoculated were wrapped with parafilm to protect the wounded area (Fig 3C). Results were compared with wild type genotypes and among different transgenic events. According to the results best two lines were chosen for the next round of testing to confirm the best line from each genotype. These lines will be the crown gall resistant elite lines.

6. **Summary of Major Research Accomplishments and Results by Objective**

The current roster of grapevine rootstocks used in California are typically resistant to an individual disease or pest. Therefore, combining resistance traits in a single rootstock could make for a more sustainable and durable solution. Objective 1 of this project is to stack

resistance traits in grapevine rootstocks and develop a single rootstock that has resistance to multiple pathogens. To achieve this objective, first we selected the rootstocks of interest to CA growers that are naturally resistant to *Phylloxera* and nematodes. In spring 2020, we initiated embryogenic callus from anther filaments of the genotypes of interest. The embryogenic callus was induced to make embryos that can be clonally multiplied via repetitive embryogenesis where single embryos produce clones of themselves. For accomplishments within Objective 2 we have begun germinating these embryos to develop plantlets that will be multiplied by micropropagation. Thompson Seedless somatic embryos and plants were generated to serve as the control. Table 1 summarizes all the events taken place from obtaining somatic embryos to transformation. We have also successfully cloned the root-inducing RolB and RolC genes from *Agrobacterium rhizogenes* A4 and build two binary vectors (Fig 1B, C) that will be used to develop root systems for testing the RNAi-mediated resistance to crown gall more efficiently. Wild type Thompson Seedless leaf petioles were transformed with the vector 1B, carrying red florescence and RolB and RolC genes.

Wild type grape shoots and leaf petioles of genotypes GRN-1, 1103P, 01-14 Mgt., Freedom, 110R and Thompson Seedless were challenged with *Agrobacterium* strains C58, 20W-5A and A281 to evaluate the degree of suppression of crown gall disease. Wild type plants of all the genotypes showed susceptibility to crown gall when challenged with above *Agrobacterium* strains. Several transgenic events of above genotypes show resistance to crown gall disease in the rapid *in vitro* petiole assay. Those results were presented in previous reports. These transgenic events are currently being propagated for testing in the greenhouse to confirm the resistance development in mature plants. Second round testing will be carried out in Spring 2024 and Freedom transgenic plants will be included in testing. Transgenic plants of 110 R will be included when they are ready. Transformed 110R embryos are showing slower growth in vitro. As the initial step, those shoots were multiplied and challenged with *Agrobacterium* strains to evaluate the resistance to crown gall. They were first tested in the lab and then in the greenhouse to confirm the resistance. Results were compared with the wild type plants to determine the degree of resistance to crown gall disease.

We have developed an assay for greenhouse grown grape plants to evaluate resistance to crown gall. Cuttings from the initial plants were rooted using an aeroponic system (mist bed) in the greenhouse. Plants developing enough healthy roots were transferred to pots and grown to reach proper height and strength to be inoculated. Six replicates were included for each genotype (Fig 3A). When plants were about 6-8 inches height with proper stem diameter (pencil thickness), they were inoculated with *Agrobacterium strain* A281 stabbing in three places on the stem, starting from the crown. Other side of the stem was inoculated using a needle in three sites using 10ul/site from a bacterial solution of 10^8 cells/mL. Areas where stems were inoculated were wrapped with parafilm to protect the wounded area (Fig 3C). Degree of gall formation was

observed after 1, 2 and 3 months after inoculation. In previous report the results of first round testing were shown. For this analysis the size of the gall was considered and a scale of 1-4 was used. No galls = 0, 25% = 1, 50% = 2, 75% = 3 and 100% = 4. Average score from six replicates were considered when deciding the degree of resistance. According to the results best two lines were chosen for the next round of testing to confirm the best line from each genotype. These lines will be the crown gall resistant elite lines.

7. **Outside Presentations of Research**:

There has been no opportunity to present this work. We look forward to communicating our research results to end-users and stakeholders given an appropriate opportunity.

8. **Research Success Statements**:

This project in 3 years is establishing a pathway to stack disease and pest resistance in grapevine rootstocks. We are currently at last year of this 3-year project. The project output will be elite rootstock lines that are resistant to both crown gall and *Phylloxera*. The elite rootstocks developed by this project will be candidates for commercialization after field-testing to evaluate horticultural attributes and further validation of disease and pest resistance under field conditions. The validation of the stacking strategy developed by this project can be used to stack additional sources of resistance using RNAi against other pathogens.

9. **Funds Status**: Include a general summary of how funds were spent.

We have expended 100% of the funds in 2023.

Table 1: Availability of somatic embryos and regenerated plants in the lab and greenhouse

Rootstock	Somatic embryos generated	Germinated to obtain wildtype plants	Embryos transformed to stack crown gall resistance	Transgenic plants in tissue culture	Wildtype plants in the green house	Transgenic plants in the green house
TS	Yes	Yes	Yes	Yes	Yes	Yes
GRN-1	Yes	Yes	Yes	Yes	Yes	Yes
1103 P	Yes	Yes	Yes	Yes	Yes	Yes
101-14 Mgt	Yes	Yes	Yes	Yes	Yes	Yes
110 R	Yes	Yes	Yes	In Progress	Yes	No
Freedom	Yes	Yes	Yes	Yes	Yes	Yes

Table 2: Transgenic events of desired rootstock genotypes available in the lab and greenhouse
*Indicates no galls formed in *in vitro* rapid petiole test compared to wild type. Some of the other transgenic events that were recently received are still being tested in the lab and greenhouse.

Transgenic Events				
Thompson Seedless	GRN-1	101-14 Mgt.	1103P	Freedom
19121906 (TS-06)	*20106601 (GRN-1-01)	20106701 (101-14-01)	*20106801 (1103 P-01)	29946801 (Fredom-01)
*19121907 (TS-07)	20106602 (GRN-1-02)	*20106702 (101-14-02)	20106802 (1103 P-02)	29946802 (Fredom-02)
*19121908 (TS-08)	*20106604 (GRN-1-04)	20106703 (101-14-03)	20106803 (1103 P-03)	29946803 (Fredom-03)
*19121909 (TS-09)	20106606 (GRN-1-06)	*20106704 (101-14-04	20106804 (1103 P-04)	
191219010 (TS-10)			*20106805 (1103 P-05)	
191219012 (TS-12)				

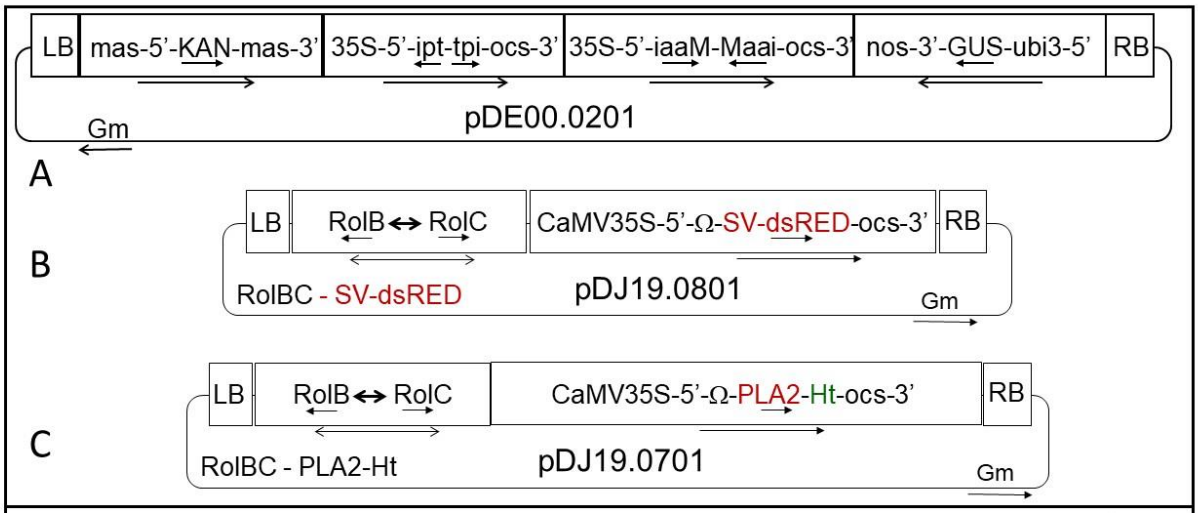

Figure 1: Binary vectors constructed to confer and to test for resistance to crown gall. A) Binary vector pDE00.0201 will be used to develop RNAi-mediated resistance to crown gall. Binary vector pDJ19.0801 (B) and pDJ19.0701 will be used to induce test for efficacy of RNAi-mediated resistance to crown gall.

Figure 2: Freedom tissue culture plants available in the lab. Transgenic plants are grown in the green house to challenge with Agrobacterium strains 20W-5A and A281.

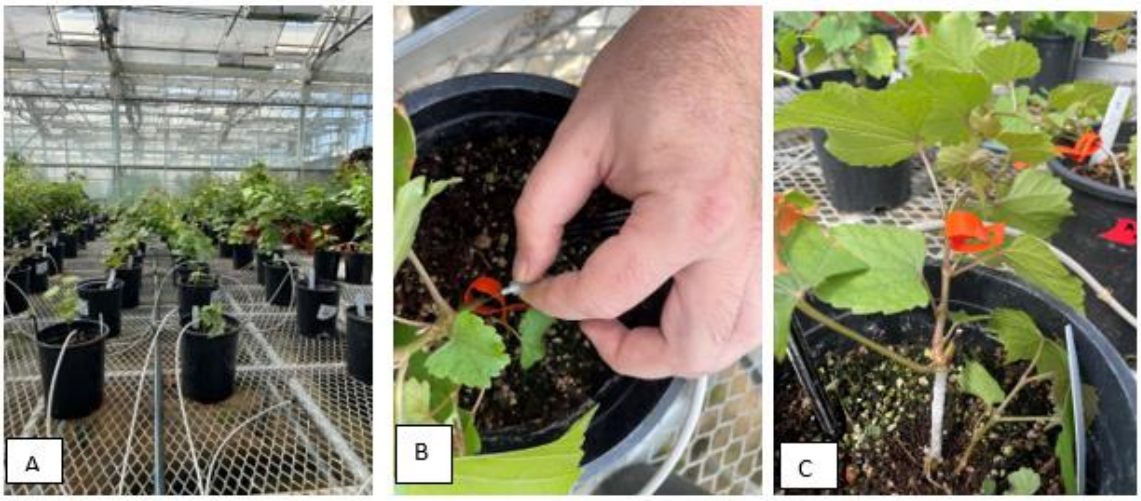

Figure 3: (A) Experimental setup in the greenhouse to evaluate the crown gall resistance in transgenic plants (B) Inoculating the plants using *Agrobacterium* strains 20W-5A. (C) Wrapped stems with parafilm after inoculating.

10. **Literature Cited**:

Aguero, C.B., C.P. Meredith and A.M. Dandekar. 2006. Genetic transformation of *Vitis vinifera* L. cvs. 'Thompson Seedless' and 'Chardonnay' with the pear PGIP and GFP encoding genes. Vitis 45(1): 1-8.

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