Progress Report for CDFA agreement number 11-0240-SA

Project Title: Engineering multi-components resistance to Pierce's Disease in California Grapevine Rootstocks

Principal Investigator (PI):
Abhaya M. Dandekar, Plant Sciences Department; UC Davis, Davis, CA 95616
Phone: 530-752-7784; fax: 530-752-8502; Email: amdandekar@ucdavis.edu

Cooperators:
Andrew Walker, Viticulture and Enology Department; UC Davis, Davis, CA 95616.
Phone: 530-752-0902; fax: 530-752-0382; Email: awalker@ucdavis.edu

Ana M. Ibáñez, Plant Sciences Department; UC Davis, Davis, CA 95616
Phone: 530-752-5325; fax: 530-752-8502; Email: amibanez@ucdavis.edu

Sandie Uratsu, Plant Sciences Department; UC Davis, Davis, CA 95616
Phone: 530-752-5325; fax: 530-752-8502; Email: sluratsu@ucdavis.edu

Kourosh Vahdati, Plant Sciences Department; UC Davis, Davis, CA 95616
Phone: 530-752-5325; fax: 530-752-8502; Email: kvahdati@ucdavis.edu

David Tricoli, Plant Sciences Department; UC Davis, Davis, CA 95616
Phone: 530-752-5325; fax: 530-752-8502; Email: dmtricoli@ucdavis.edu

Cecilia Aguero, Viticulture and Enology Department; UC Davis, Davis, CA 95616.
Phone: 530-752-0902; fax: 530-752-0382; Email: cbaguero@ucdavis.edu

Reporting Period: October 2011 to February 2012

LAYPERSON SUMMARY
Current strategies for Pierce's Disease (PD) control emphasize deploying transgenic rootstocks that deliver PD control to the untransformed scion. We propose to develop transgenic grapevine rootstocks resistant to Pierce's Disease using new transformation protocols for the commercially relevant grapevine rootstocks '101-14' and '1103-P'. Our genetic approach involves developing transgenic rootstocks that deliver therapeutic proteins like HNE-CecB that can protect an untransformed scion from PD. Our approach is to improve grapevine transformation, exploring the possibility of *in planta* transformation of shoot apical meristems (SAM). We have successfully isolated SAM tissues from grapevine rootstocks '101-14' and '1103-P' and from 'Thompson Seedless' as a control. These SAM tissues are being tested for regeneration potential using different media and hormone concentrations that have worked in other grapevine cultivars. We obtained callus from grape rootstock '1103-P' shoot apical meristems, we also obtained callus and embryos from grape rootstock '101-14' shoot apical meristems. The outcome of this research would be the successful development of a more efficient and rapid transformation system for commercially relevant grapevine rootstock and applicable to scion varieties using SAM.

INTRODUCTION
Several presentations at the 2010 Pierce's Disease Symposium highlighted transgenic strategies using various promising transgenes with potential for conferring resistance to *Xylella fastidiosa* (*Xf*), the causal agent of Pierce's Disease (PD). However, most such projects have not yet demonstrated such control in commercially significant rootstocks (Dandekar, 2010; Gilchrist, 2009; Labavitch, 2010; and Lindow, 2009). This is partly because the current grapevine transformation and regeneration system was developed at UC Davis a decade ago in rootstocks and scion varieties like *Vitis vinifera* 'Thompson Seedless' (Aguero et al., 2005b, 2006). This system is cumbersome and slow because it uses embryogenic callus developed from young anthers, a tissue available for one brief period during each growing season. It takes around ~6-8 months to generate transgenic somatic embryos from callus lines derived from anther tissue. Additionally, somatic embryogenic callus lines are not available for some widely used commercial rootstocks such as '101-14' and '1103-P'. To overcome this hurdle, we are developing a transformation system

using meristematic stem cells present in the shoot apical meristem (SAM). In plants like grape, all aboveground plant parts are generated from a cluster of stem cells present in the central dome of the SAM (Sablowski, 2007, Gordon et al., 2009). Genetic factors regulated through cytokinin signaling determine and control the number of stem cells (Gordon et al., 2009). Several research- and commercial-scale transformations use meristem tissue from different crops. Use of SAM for transformation has occurred with a limited number of grapevine varieties. Mullins et al. (1990) co-cultivated adventitious buds of *Vitis rupestris* 'St. George' rootstock with *Agrobacterium* and produced transgenic plants. However, the methodology was never repeated. Mezzetti et al. (2002) transformed *V. vinifera* 'Silcora' and 'Thompson Seedless', cultivars with a strong capacity to differentiate adventitious shoots, using a meristematic tissue culture system. The culture type was unique and the overall application to other cultivars is unclear. Levenko and Rubtsova (2000) used *in vitro* internode explants to transform three *V. vinifera* scions and a rootstock, but did not provide sufficient details for the technique to be repeated. Dutt et al. (2007) described a simple transformation system for 'Thompson Seedless' using explants from readily obtainable micropropagation cultures. Tissues from etiolated cultures and meristem wounding using fragmented meristems gave the best results. This latter system has not been tested in many rootstocks. Taken together, these studies indicate that SAM is an interesting tissue to investigate, particularly since it is available all year. Additionally, much is now known about various developmentally regulated genes in plants like *Arabidopsis* that suggests how hormone input can be used to manipulate the developmental patterns of SAM (Galinha et al., 2009).

OBJECTIVES

The goal of this project is to develop a shoot apical meristem-based regeneration system to produce transgenic grapevine rootstocks to control Pierce's Disease. This goal will be accomplished by two activities.

Activity 1: Develop a SAM-based regeneration system for important rootstocks.
Activity 2: Transform, select, regenerate, and confirm transgenic grapevine plants using this new system.

We have been focused on activity one, the development of a regeneration system using stem cells present in the shoot apical meristem.

RESULTS AND DISCUSSION

Activity 1: Develop a shoot apical meristem regeneration system for grapevines that provides faster, more efficient production of transgenic plants.

Our first step has involved developing expertise and proficiency at dissecting and excising the meristematic dome from field-grown '101-14' and '1103-P' rootstocks, using 'Thompson Seedless' as a control. A similar technique is used routinely for pathogen elimination at UC Davis Foundation Plant Services. We have worked closely with Adib Rowhani and his colleagues to learn the best technique for excising a SAM. A pictorial outline of the process with '101-14' is shown (Figure 1). The sterile meristematic tissues are then cultured to examine their potential for organogenesis or somatic embryogenesis using different hormone concentrations and combinations. We are currently investigating various hormone and media compositions to identify those that lead to proliferation of the SAM; some are described below.

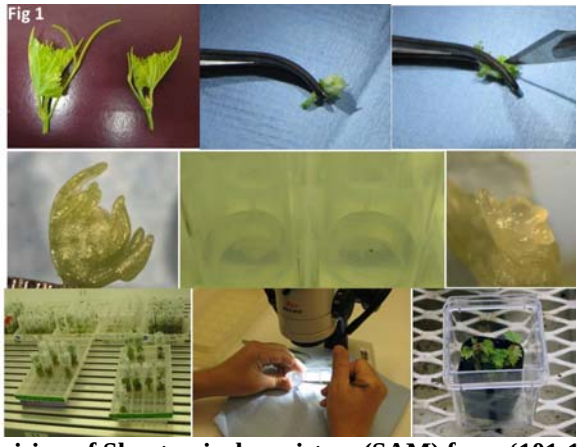

Fig. 1 Process for the excision of Shoot apical meristem (SAM) from '101-14' grapevine shoot tips.

We have been following published protocols that use different hormone concentrations and combinations to proliferate SAMs and induce a "meristematic bulk" (MB) (Mezzetti at al. 2002, Dhekney et al 2011). This is done using benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthaleneacetic acid (NAA).

Experiment 1. '101-14' shoot tips were harvested and immediately dissected to produce explants composed of the apical meristem plus microscopic leaf primordia. Explants were immediately placed in Petri dishes containing induction media composed of Murashige and Skoog (MS) salts and vitamins, 0.1 g L^{-1} myo-inositol, 30 g L^{-1}sucrose and hormones 2,4-D and BA at four and five different concentrations, respectively (Table 1). There were three explants per treatment, replicated three times (nine explants total per treatment). The explants were transferred to fresh medium every three weeks and incubated in the dark at 25ºC.

Table 1. Effect of BA and 2,4-D levels on somatic embryogenesis of '101-14' grape rootstock					
BA mg L^{-1}	**0.1**	**0.2**	**0.5**	**1.0**	**2.0**
2,4-D mg L^{-1}					
0	B1	B2	B3	B4	B5
0.5	B6	B7	B8	B9	B10
1	B11	B12	B13	B14	B15
2	B16	B17	B18	B19	B20

At this point, the domes have neither swelled nor proliferated in treatments B1 to B5, B7 to B10, B12 to B15, and B18 to B20. We obtained callus and embryos from '101-14' meristems in treatments B6, B11, B16, and B17.

Experiment 2. We have also investigated altering the media composition by using different media, employing 'Thompson Seedless' (TS) as a control with which we have previous experience in culture and '101-14' and '1103-P' rootstocks. '101-14', '1103-P' and 'Thompson Seedless' shoot tips were harvested from field-grown material and immediately dissected to produce explants composed of the apical meristem plus microscopic leaf primordia. Explants were immediately placed in Petri dishes containing five different induction media, N1 to N5, composed of Nitsch and Nitsch (NN) salts and vitamins, 0.1 g L^{-1} myo-inositol, 20 g L^{-1} sucrose, 0.5 µM BA, and 0 µM (N1), 1 µM (N2), 5 µM (N3), 10 µM (N4), or 50 µM (N5) 2,4-D (Table 2). Here we were testing the ability of 2,4-D to induce somatic embryogenesis. There were 15 explants per treatment, replicated three times, for 45 explants per treatment. The explants were been transferred to fresh medium twice every three weeks and incubated in the dark at 25ºC.

Table 2. Effect of BA and 2,4-D levels on somatic embryogenesis of TS, '101-14'and '1103-P' grape rootstock					
Medium	**N1**	**N2**	**N3**	**N4**	**N5**
BA µM	5	5	5	5	5
2,4-D µM	0	1	5	10	50

Resulting callus were transferred twice every three weeks to MS salts and vitamins medium supplemented with 0.1 g l^{-1} myo-inositol, 20 g l^{-1} sucrose, 1 µM BA and 11 µM Naphthaleneacetic acid (NAA) and maintained at 26ºC in light (65 µmol s^{-1} m^{-2}) for development of somatic embryos. Proliferating embryogenic material were then transferred three times every three weeks to X6 medium supplemented with 3.033 g l^{-1} KNO_3, 0.364 g l^{-1} NH_4Cl, 60.0 g l^{-1} sucrose, 1.0 g l^{-1} myo-inositol, 7.0 g l^{-1} TC agar, 0.5 g l^{-1} activated charcoal and maintained at 26ºC in light (65 µmol s^{-1} m^{-2}) for development and proliferation of somatic embryos. In this second experiment we obtained '101-14'callus, '101-14' embryos and '1103-P' callus from meristems in treatments N2 (**Fig. 2**), which contains 5 µM BA and 1 µM 2,4-D.

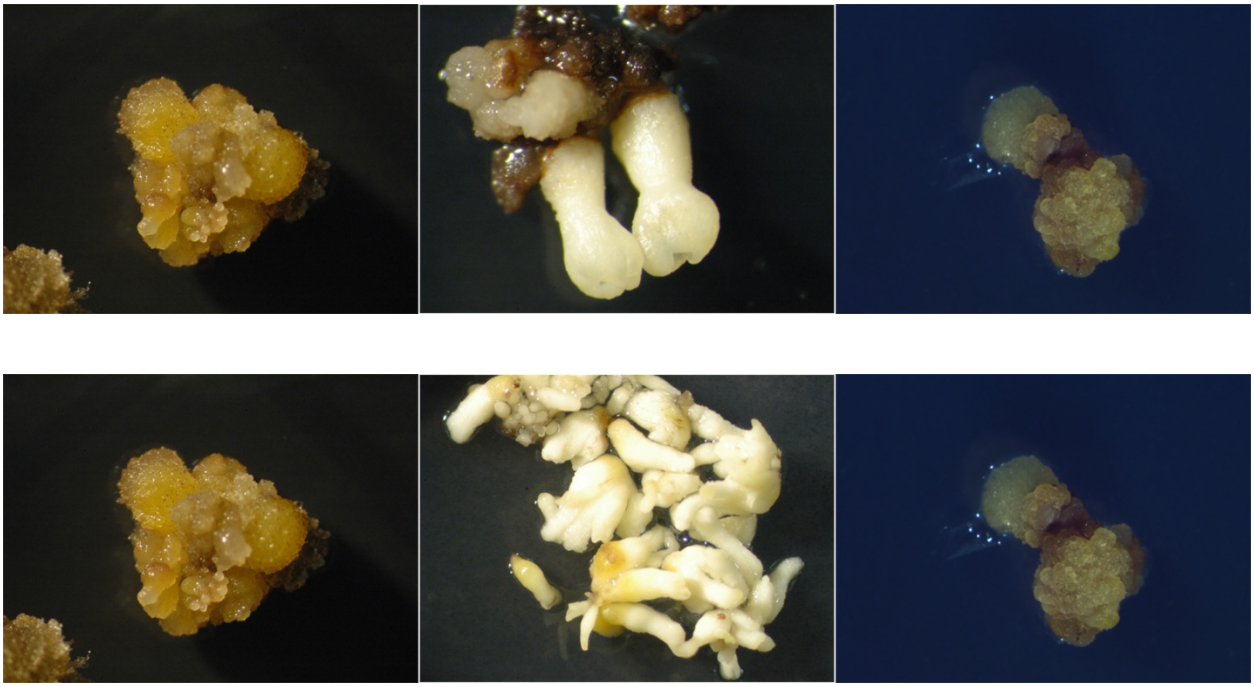

Figure 2. From left to right, callus cultures of '101-14', somatic embryo cultures of '101-14' and callus cultures of '1103-P' from cultured shoot apical meristems.

Observations and data for experiments 1 and 2, allowed us to refine the new experiments with '101-14' and '1103-P' meristems for the Spring of 2012. In addition to solid medium, we will investigate the development of shoot apical meristem regeneration using liquid medium to stimulate growth and differentiation. This has proved useful in both regeneration and transformation (Humberto Prieto's personal communication, La Platina Research Station, National Agriculture Institute, Santiago de Chile). For the liquid phase, we will use "temporary immersion" (TI) in medium that is identical to the solid medium except for the absence of solidifying agent. This phase will be done in collaboration with Dr. David Tricoli at the UC Davis Parsons Plant Transformation Facility.

SUMMARY OF MAJOR RESEARCH ACOMPLISHMENTS AND RESULTS FOR EACH OBJECTIVE

Transgenic rootstocks have been proposed as the best strategy to develop PD-resistant grapevines. However, current transformation protocols use embryogenic callus lines developed from anther culture for transformation and available cultures do not include rootstock genotypes currently used by growers in California. Also, current callus lines have been in culture for a long time and fresh new cultures must be selected, a task limited to a single season each year. This research seeks to overcome this seasonal limitation by developing a transformation system using SAM, as initial explant material that is available year-round. We are currently developing a shoot apical meristem-based grapevine regeneration system for faster, more efficient production of transgenic grapevine plants. We have initiated experiments with solid medium to study the effect of the cytokinin benzyladenine (BA) and the auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) on somatic embryogenesis of TS and '101-14', and '1103-P' rootstocks. We have obtained '101-14'callus, '101-14' embryos and "1103-P" callus from soot apical meristems..

PUBLICATIONS

Dandekar, A.M., A. Walker, A.M. Ibáñez, S.L. Uratsu, K. Vahdati, D. Tricoli, C.A. Aguero. 2011. Engineering multi-components resistance to Pierce's Disease in California Grapevine Rootstocks. Proceedings of the Pierce's Disease Research Symposium. Dec 13-15. Sacramentoo, CA. pp. 107-110.

RESEARCH RELEVANCE STATEMENT

The results of this research will benefit other research groups working on transgenic strategies to control PD and has the potential to benefit research in other crops where transgenic approaches are sought to create pathogen resistance in rootstocks. The objective described in this proposal directly addresses the research priorities outlined in

'attachment A' of the 2010 PD/GWSS proposal RFA. It also addresses the top RSAP priority in the "Enabling tools-Development of grape regeneration and transformation systems for commercially important rootstocks" handout released in December 2009. This document outlines the "Top 5 to 10 Project Objectives to Accelerate Research to Practice" and updates the priority research recommendations provided in the report "PD/GWSS Research Scientific Review: Final Report" released in August 2007 by the CDFA's Pierce's Disease Research Scientific Advisory Panel.

STATUS OF FUNDS: We anticipate that all funds allocated for fiscal year FY2011-2012 will be expended by June 30, 2012.

REFERENCES CITED

Aguero, C.B., S.L. Uratsu , C. Greve, A.L.T. Powell, J.M. Labavitch, C.P. Meredith and A.M. Dandekar. 2005. Evaluation of tolerance to Pierce's disease and Botrytis in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene. Mol. Plant Path. 6:34-51.

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 1-8.

Dandekar, A.M. 2010. *In planta* testing of signal peptides and anti-microbial proteins for rapid clearance of *Xylella*. Proceedings of the 2010 Pierce's Disease Research Symposium.

Dhekney, S.A, Z.T. Li and D.J. Gray. 2011. Factors influencing induction and maintenance of *Vitis rotundifolia* Michx. embryogenic culture. Plant Cell Tiss Organ Cult 105:175-180.

Dutt, M., Z.T. Li, S.A. Dhekney and D.J. Gray. 2007. Transgenic plants from shoot apical meristems of *Vitis vinifera* L. 'Thompson Seedless' via Agrobacterium-mediated transformation. Plant Cell Reports 26:2101-2110.

Galinha, C., G. Bilsborough and M. Tsiantis. 2009. Hormonal input in plant meristems: a balancing act. Sem. Cell. Dev. Biol. 20: 1149-1156.

Gilchrist, D. 2009. Systematic resistance to Pierces's disease by transgenic expressiob of plant-derived abti-apototic genes. Proceedings of the 2009 Pierce's Disease Research Symposium.

Gordon, S.P., V.S. Chickarmane, C. Ohno and E.M. Meyerowitz. 2009. Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem. Proc. Natl. Acad. Sci. USA 106(38): 16529-16534.

Labavitch, J.M. 2010. Optimizing grape rootstock production and export of inhibitors of *Xylella fastidiosa* polygalacturonase activity. Proceedings of the 2010 Pierce's Disease Research Symposium.

Levenko BA, Rubtsova MA (2000) Herbicide resistant transgenic plants of grapevine. Acta Hortic 528: 337–339.

Lindow, S.E. 2009. Control of Pierce's disease by methods involving pathogen confusion. Proceedings of the 2009 Pierce's Disease Research Symposium.

Mezzetti, B., T. Pandolfini, O. Navacchi and L. Landi. 2002. Genetic transformation of *Vitis vinifera* via organogenesis. BMC Biotechnology 2: 1-10.

Mullins, M.G., Archie Tang, F.C., Facciotti, D. 1990. *Agrobacterium*- mediated genetic transformation of grapevines: Transgenic plants of *Vitis rupestris* SCHEELE and buds of *Vitis vinifera*. Nature Biotechnology 8:1041–1045.

Sablowski, R. 2007. The dynamic plant stem cell niches. Curr. Opin. Plt. Biol. 10: 639-644.