Interim Progress Report for CDFA agreement number 11-0240-SA

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

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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 includes improving 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 various media and hormone additions that have worked in other grapevine cultivars. We have successfully obtained '101-14' and '1103-P' callus, embryos and plants from shoot apical meristems, using solid medium, the cytokinin benzyladenine (BA), and the auxin 2,4-dichlorophenoxyacetic acid (2,4-D). We are currently confirming the reproducibility of a shoot apical meristem-based grapevine regeneration system for more efficient production of transgenic plants. The outcome of this research would be the successful development of a more efficient transformation system for commercially relevant grapevine rootstock and scion varieties using SAM tissue.

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, developed at UC Davis a decade ago, used 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 six to eight 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 transformation systems 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 plant hormones can be used to manipulate the developmental patterns of the 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.

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 involved developing expertise and proficiency at dissecting and excising the meristematic dome from field-grown '101-14' and '1103-P' rootstocks. A similar technique is used routinely for pathogen elimination at UC Davis Foundation Plant Services. We 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 were 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.

For experiment one, we followed published protocols that use different hormone concentrations and combinations to proliferate SAMs to induce "meristematic bulk" (MB) (Mezzetti at al. 2002, Dhekney et al 2011). This was done using benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), and a specific medium composition. For experiments 2 and 3, we investigated the role of hormone concentration, hormone combination, and different medium compositions to produce embryogenesis in '101-14' and '1103-P' SAMs.

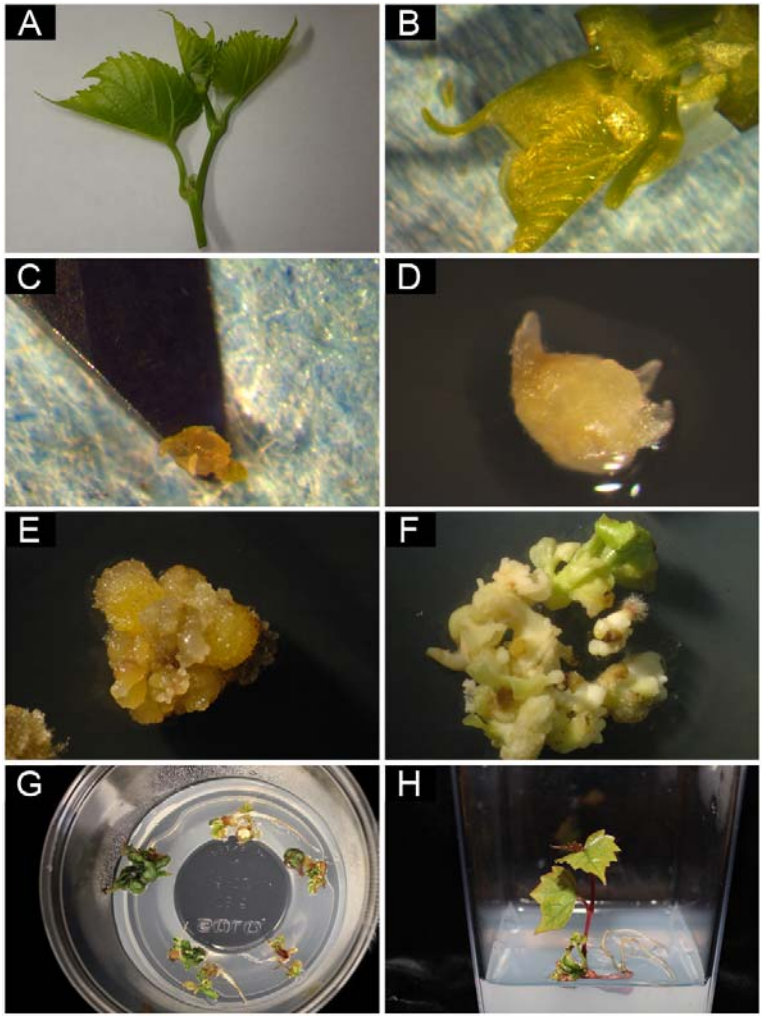

Fig. 1 Regeneration system for '101-14' grape rootstock shoot apical meristem

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 medium composed of Murashige and Skoog (MS) salts and vitamins, 0.1 g/L myo-inositol, 30 g/L 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 hormone combination). 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 concentration on somatic embryogenesis of '101-14' grape rootstock					
BA mg/L	**0.1**	**0.2**	**0.5**	**1.0**	**2.0**
2,4-D mg/L					
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

To date, 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. '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 myo-inositol, 20 g/L sucrose, 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 cultivar. The explants were transferred to fresh medium every 1.5 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 was transferred every 1.5 weeks to MS salts and vitamins medium supplemented with 0.1 g/L myo-inositol, 20 g/L sucrose, 1 µM BA, and 11 µM naphthaleneacetic acid (NAA) and maintained at 26ºC in light (65 µmol/s m^2) for development of somatic embryos. Proliferating embryogenic material was then transferred every 1.5 weeks to X6 medium supplemented with 3.033 g/L KNO_3, 0.364 g/L NH_4Cl, 60.0 g/L sucrose, 1.0 g/L myo-inositol, 7.0 g/L TC agar, and 0.5 g/L activated charcoal and maintained at 26ºC in light (65 µmol/s m^2) for development and proliferation of somatic embryos. In this second experiment, we successfully obtained '101-14'and '1103-P' callus, embryos (Figure 2), and plants

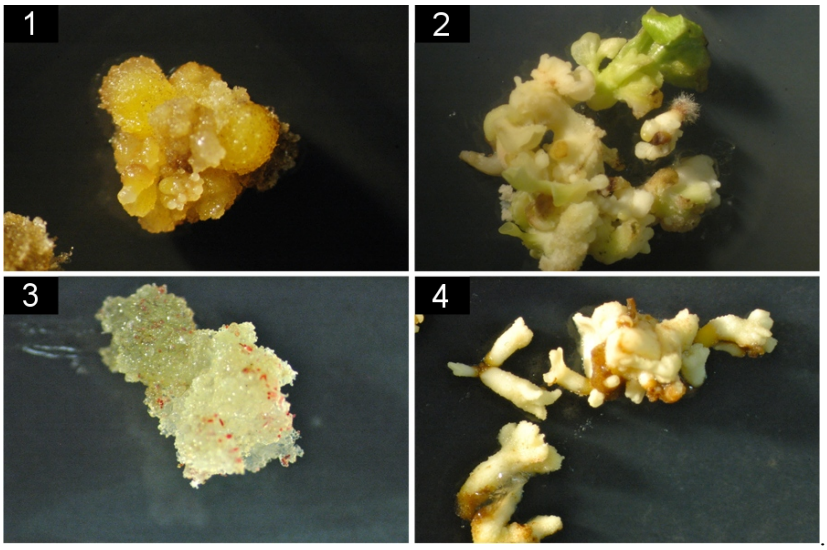

Fig. 2 Grape callus and embryos from '101-14' (1-2) and '1103-P' (3-5) rootstocks regenerated from shoot apical meristems

Experiment 3. Observations and data from experiments 1 and 2, allowed us to refine a third experiment for '101-14' and '1103-P' meristems starting in spring 2012. '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 two different induction media, N2 and N3, composed of Nitsch and Nitsch (NN) salts and vitamins, 0.1 g/L myo-inositol, 20 g/L sucrose, 5 µM BA, and 1 µM or 5 µM 2,4-D, respectively (Table 3). Here, we are replicating the conditions that, in experiment 2, produced callus, embryos, and plants of both rootstocks. We are also confirming the reproducibility of the regeneration system and optimizing the time required for the process. There were 30 explants per treatment, replicated three times, for each medium and cultivar.

Activity 2: Transform, select, regenerate, and confirm transgenic grapevine plants using this new system.

'101-14' and '1103-P' embryogenic callus produced in Activity 1 will be infected with *Agrobacterium* containing the existing HNE-cecropin B vector, pDU04.6105, to produce transgenic grapevines.

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 the SAM as an initial explant material that is available year-round. We have successfully obtained '101-14' and '1103-P' callus, embryos, and plants from shoot apical meristems using solid medium, the cytokinin benzyladenine (BA), and the auxin 2,4-dichlorophenoxyacetic acid (2,4-D). We are currently confirming the reproducibility of a shoot apical meristem-based grapevine regeneration system for more efficient production of transgenic plants.

PUBLICATIONS

Dandekar, A.M**.**, H. Gouran, A.M. Ibanez, S.L. Uratsu, C.B. Aguero, S.McFarland, Y. Borhani, P.A. Fieldstein, G.E. Bruening, R. Nascimento, L. Goulart, P.E. Pardington, A. Choudhary, M. Norvell, R. Civerolo and Goutam Gupta. 2012. An engineered innate immune defense protects grapevines from Pierce's Disease. Proc. Nat. Acad. Sci. USA 109(10): 3721-3725.

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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: All funds allocated for fiscal year FY2011-2012 were expended by June 30, 2012.

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