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

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

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Time period cover by the report: 5 Months (October 2012 and February 2013).

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., 2005, 2006). This system though tractable 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 hormone input can be used to manipulate the developmental patterns of SAM (Galinha et al., 2009).

List of 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.

Description of activities conducted to accomplish each objective, and summary of accomplishments and results for each objective

Activity 1: Develop a SAM 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. The sterile meristematic explants are then cultured to examine their potential for organogenesis or somatic embryogenesis using different hormone concentrations and combinations and different medium composition. We are currently investigating various hormone and medium compositions to identify those that lead to proliferation of the SAM; some are described below.

We started our research following 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. Based on the results of the initial research, we investigated the role of hormone concentrations, combinations and different media formulation to accomplish the regeneration of SAM from '101-14' and '1103-P' rootstocks.

Activity 1A: '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, *myo*-inositol, sucrose and the hormones 2, 4-D and BA at four and five different concentrations, respectively (Table 1). There were 20 combinations of hormones, three explants per treatment, and 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. We obtained callus, embryos, and plants from '101-14' meristems in treatments B8 and B9.

Table 1. BA and 2,4-D concentrations used for specific '101-14' SAM treatments.					
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

Activity 1B: Observations and data from activity 1A, allowed us to design a new experiments using meristematic explants from '101-14' and '1103-P'rootstocks. Grapevine shoot tips were harvested in 2011 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, *myo*-inositol, 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). To test the ability of 2,4-D to induce somatic embryogenesis explants, the explants were transferred to fresh medium twice every three weeks and incubated in dark at 26ºC for 6 weeks. There were 15 explants per treatment, replicated three times, for 45 explants per treatment.

Table 2. Concentrations of BA and 2,4-D used to investigate somatic embryogenesis of TS, '101-14'and '1103-P' grape rootstocks					
Medium	**N1**	**N2**	**N3**	**N4**	**N5**
BA µM	5	5	5	5	5
2,4-D µM	0	1	5	10	50

The resulting callus were transferred every three weeks to MSNB media consisted of MS salts and vitamins medium supplemented with *myo*-inositol, sucrose, BA, and naphthaleneacetic acid (NAA) and maintained at 26ºC in light (65 µmol/s · m^2) for development of embryogenic callus for a total of six weeks. Proliferating embryogenic material was then transferred from MSNB to X6 medium supplemented with KNO_3, NH_4Cl, sucrose, *myo*-inositol, TC agar, and activated charcoal. The embryogenic callus were transfer every three weeks to fresh X6 medium and maintained at 26ºC in light (65 µmol s · m^2) for development and proliferation of somatic embryos for 6-9 weeks. Emrbyogenic callus or somatic embryos produced in X6 were divide into two halves, half was maintained in X6 and the other half was transferred to WPM salt and vitamins containing sucrose, casein hydrolyzate and activated charcoal, then cultured at 26ºC in light for 12 weeks. Somatic embryos at the late cotyledonary stage from each lines were transferred to MS1B media with MS salt and vitamins supplemented with sucrose, myo-inositol and BA and placed in light at 26ºC for 2-4 weeks. Germinated somatic embryos were then transferred to ½ MS medium containing sucrose, calcium gluconate and NAA, plants regeneration started after 4 weeks. We successfully obtained '101-14'and '1103-P' callus, embryos and plants

The objective of this activity was to confirming the reproducibility of the regeneration system developed in activity 1B and optimizing the time required for the process. There were 50 meristem explants per treatment, replicated three times, for each medium and each cultivar.

Activity 1C: Observations and data 1B, allowed us to refine this experiment. 101-14' and '1103-P' shoot tips were harvested in 2012 from field-grown material and immediately dissected to produce explants composed of the apical meristem plus microscopic leaf primordial. Explants were immediately placed in Petri dishes containing two different induction media, N2 to N3, composed of Nitsch and Nitsch (NN)

salts and vitamins, myo-inositol, sucrose, 5 µM BA, and 1 µM and 5 µM 2,4-D, respectively (Table 2). The experiment was done following the same conditions as in Activity 1B, to produce callus, embryos, and plants of both rootstocks.. The objective of this activity was to confirming the reproducibility of the regeneration system developed in activity 1B and optimizing the time required for the process. There were 50 meristem explants per treatment, replicated three times, for each medium and each cultivar.

Activity 2: Transform, select, regenerate, and confirm transgenic grapevine plants using this new system. '101-14' and 1103-P embryogenic callus (obtained in activity 1B) has been infected with *Agrobacterium* containing the existing HNE-Cecropin B vector (pDU04.6105; Dandekar et al., 2012) to produce transgenic grape rootstocks.

Publications produced and pending, and presentations made that relate to the funded project

Dandekar, A.M., H. Gouran, A.M. Ibáñez, S.L. Uratsu, C.B. Aguero, S. McFarland, Y. Borhani, P.A. Feldstein, G. Bruening, R. Nascimento, L.R. Goulart, P.E. Pardington, A. Chaudhary, M. Norvell, E. Civerelo and G. Gupta. 2012. An Engineered innate defense protects grapevines from Pierce's disease. Proc. Nat. Acad, Sci. USA 109 (10): 3721-3725.

Dandekar, A.M., A. Walker, A.M. Ibáñez, K.Q. Tran, G. Dio, S.L. Uratsu, K. Vahdati, D. Tricoli, C.A. Aguero. 2012. Engineering multi-components resistance to Pierce's Disease in California Grapevine Rootstocks. Pierce's Disease Research Progress Report. Dec 2012. pp. 104-108.

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. Sacramento, CA. pp. 107-110.

Research relevance statement, indicating how this research will contribute towards finding solutions to Pierce's disease in California

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.

Layperson summary of project accomplishments

Whereas we have protocols and methods in place to introduce transgenes (via Agrobacterium-mediated transformation) in cultivars like Thompson Seedless and St George and a few scion cultivars we have never attempted to transform the commercially relevant rootstocks. Eighteen months ago we began this project to develop a regeneration system based on excised shoot apical meristems on two of the prominent rootstocks '101-14' and '1103-P'. We have been successful in developing a process where shoot apical meristems (SAM) dissected from field grown shoots are able to undergo morphogenesis in culture to develop into an embryogenic callus that can be induced to form embryos and then germinated to make plants as outlined in Fig 1. The shoots are obtained from field grown plants that are surface sterilized and then dissected to excise the apical meristem along with a few primordial leaves. These are placed in culture on N-N medium for induction for about 6 weeks. The induction initiates the developmental process of somatic embryogenesis. The induced meristems are transferred to MS-NB medium for a period of 9-12 weeks for differentiation into embryogenic callus. To obtain embryos we can use either X-6 or WPM media or both in succession to induce the formation of embryos (Fig 1) a process which takes

about 3-8 weeks. Transferring embryos to MS-1B allows them to germinate in 2-4 weeks' time and then transferring to 0.5MS-N allows shoot formation and maintains them as plants in culture that can be acclimatized and transferred to the greenhouse and/or the field. It took quite a bit of experimentation to come up with the formulation of the 6 media used to accomplish the regeneration of SAM from these rootstocks (Fig 1). We are currently doing Agrobacterium-mediated transformation to confirm the reproducibility of a SAM-based grapevine regeneration system for more efficient production of transgenic plants.

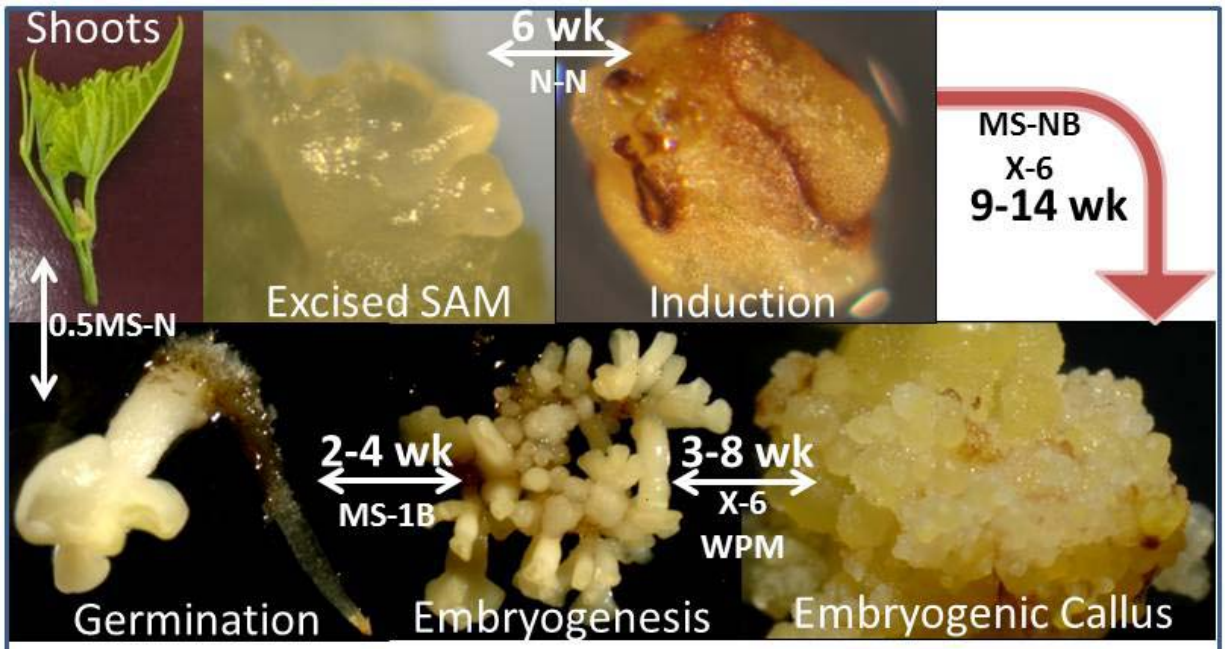

Figure 1: Regeneration process developed for shoot apical meristems (SAM) of '101-14' and '1103-P' rootstock genotypes via somatic embryogenesis

Status of funds: All funds allocated for fiscal year FY2012-2013 will be expended by June 30, 2013.

Summary and status of intellectual property associated with this project

We propose to develop transgenic grapevine rootstocks resistant to Pierce's Disease using new transformation protocols for the commercially important grapevine rootstocks '101-14' and '1103-P'. Our genetic approach involves developing transgenic rootstocks that deliver therapeutic proteins, like the chimeric antimicrobial protein HNE-CecB, which can protect an untransformed scion from PD. Our approach is to improve grapevine transformation by 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'. SAM explants are being tested for regeneration potential using different media and hormone concentrations 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 supplemented with the cytokinin, benzyladenine (BA) and the auxin 2,4-dichlorophenoxyacetic acid (2,4-D). We are currently confirming the reproducibility of a SAM-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.

An invention disclosure will be made for the method developed to successfully regenerate and transform the grapevine '110-14' and '1103-P' rootstock. A fairly broad patent US Patent#5,164,310 of Smith et al., for a method for transforming plants via shoot apex expired on Feb 5, 2011. This means that there is an opportunity to protect a new method provided it has the unique and non-obvious features necessary for patent protection. The transgenic '110-14' and '1103-P'rootstock plants developed as a consequence of this proposal would protected by disclosures made to the UC office of technology transfer, which could develop these further as a US plant patent.

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