Final Report for CDFA Agreement Number 11-0240-SA

Title of project:
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

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

Kim Q. Tran, Plant Sciences Department; UC Davis, Davis, CA 95616
Phone: 530-752-5325; fax: 530-752-8502; Email: qtran@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

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Introduction:
A substantive issue for the grapevine community in California trying to battle Pierce's Disease (PD) with genetic approaches using different transgenes, has been the serious limitation of the transformation of commercially relevant grapevine rootstocks that was never attempted. Whereas we have protocols and methods developed about a decade ago in place to introduce transgenes (via Agrobacterium-mediated transformation) in cultivars like Thompson Seedless and St George and a few scion cultivars (Aguero et al., 2005, 2006) we had never attempted to transform the commercially relevant rootstocks. This system though tractable was cumbersome and slow because it used embryogenic callus developed from young anthers, a tissue available for one brief period during each growing season. It took six to eight months to generate transgenic somatic embryos from callus lines derived from anther tissue. Additionally, somatic embryogenic callus lines were not available for some widely used commercial rootstocks such as '101-14' and '1103-P'. To overcome this hurdle, during the research period of this agreement we developed 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 utilize meristem tissue from different crops. However success using 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 was 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 micropropagated cultures. Tissues from etiolated cultures and meristem wounding using fragmented meristems gave the best results. This latter system had not been tested in many rootstocks. Taken together, these studies indicated that SAM was an interesting tissue worthy of investigation, 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 regeneration from SAM (Galinha et al., 2009).

List of objectives

The goal of this project was to develop a shoot apical meristem-based regeneration system to produce transgenic grapevine rootstocks to control Pierce's Disease. This goal was accomplished with the below listed 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 proficiency at dissecting and excising the meristematic dome tissues that contained the merisematic stem tissues from field-grown '101-14' and '1103-P' rootstocks. A similar technique is used routinely for pathogen elimination at UC Davis Foundation Plant Services (UCD-FPS). We worked with UCD-FPS (in particular Adib Rowhani and his colleagues) to develop optimal protocols to successfully excise viable SAM tissues. Once excised the sterile meristematic explants were cultured to examine their potential for organogenesis or somatic embryogenesis using different media and hormone concentrations and combinations. We investigated various hormone and medium compositions to identify those that lead to proliferation of the SAM and regeneration; some protocols are described below.

Initially, we following published protocols to induce "meristematic bulk" (MB) which is proliferation of merisetmatic stem cells from the dissected SAM tissues (Mezzetti at al. 2002, Dhekney et al 2011). This was accomplished different concentrations of benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), and a specific medium composition. Based on the results of preliminary experiments, we investigated the role of hormone concentrations, combinations and different media formulation to accomplish the regeneration of SAM obtained 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[1] mg/L	**0.1**	**0.2**	**0.5**	**1.0**	**2.0**
2,4-D[2] 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

[1]BA = benzyladenine, [2]2,4-D = 2,4-dichlorophenoxyacetic acid

Activity 1B: Observations and data from activity 1A, allowed us to further optimize regeneration protocols using meristematic tissues 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, to test the ability of 2,4-D to induce somatic embryogenesis, explants were exposed to, 0 µM (N1), 1 µM (N2), 5 µM (N3), 10 µM (N4), or 50 µM (N5) of 2,4-D (Table 2) that was added to the induction media. 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 a total of 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[1] µM	5	5	5	5	5
2,4-D[2] µM	0	1	5	10	50

[1]BA = benzyladenine, [2]2,4-D = 2,4-dichlorophenoxyacetic acid

The resulting callus was transferred every three weeks to MSNB media that contained MS salts and vitamins supplemented with *myo*-inositol, sucrose, BA, and naphthaleneacetic acid (NAA) and maintained at 26ºC in light (65 µmol/s · m^2) for the 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 was transfered 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 divided 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 line was 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 initiated after 4 weeks. We successfully obtained '101-14'and '1103-P' callus, embrygenic callus, embryos and plants when N2 and N3 induction media was used.

Activity 1C: Observations and data from 1B, allowed us to further refine the protocol. 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 or N3, composed of Nitsch and Nitsch (NN) salts and vitamins, myo-inositol, sucrose, 5 µM BA, and 1 µM 2,4-D, or 5 µM

BA and 5 µM 2,4-D, respectively (Table 2). The protocol followed used the same conditions as in Activity 1B, to produce callus, embryos, and plants of both rootstocks. There were 50 meristem explants per treatment, replicated three times, for each medium and each rootstock cultivar. The objective of this activity was to confirm the reproducibility of the regeneration protocol developed in activity 1B and optimizing the time required for the process. We successfully confirmed the reproducibility of the regeneration protocol and obtained '101-14'and '1103-P' callus, embryogenic callus, embryos and plants.

Activity 2: Transform, select, regenerate, and confirm transgenic grapevine plants using the new protocol.
'101-14' and 1103-P embryogenic callus (obtained in activity 1B) were infected with *Agrobacterium* containing the existing HNE-Cecropin B vector (pDU04.6105; Dandekar et al., 2012) to produce six '101-14' independent transgenic grape rootstock lines, that were acclimatized and transferred to the greenhouse. Through the completion of this activity the goal of the present research project "To develop a shoot apical meristem-based regeneration system to produce transgenic grapevine rootstocks to control Pierce's Disease" was successfully achieved.

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 protocols for the development of embryogenic callus for grapevine rootstocks from meristematic tissues can be implemented by the UC Davis Plant Transformation Facility to provide transgenic rootstocks for the research community. The objective accomplished in this project directly address 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 outlined the "Top 5 to 10 Project Objectives to Accelerate Research to Practice" and updates the priority research 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.

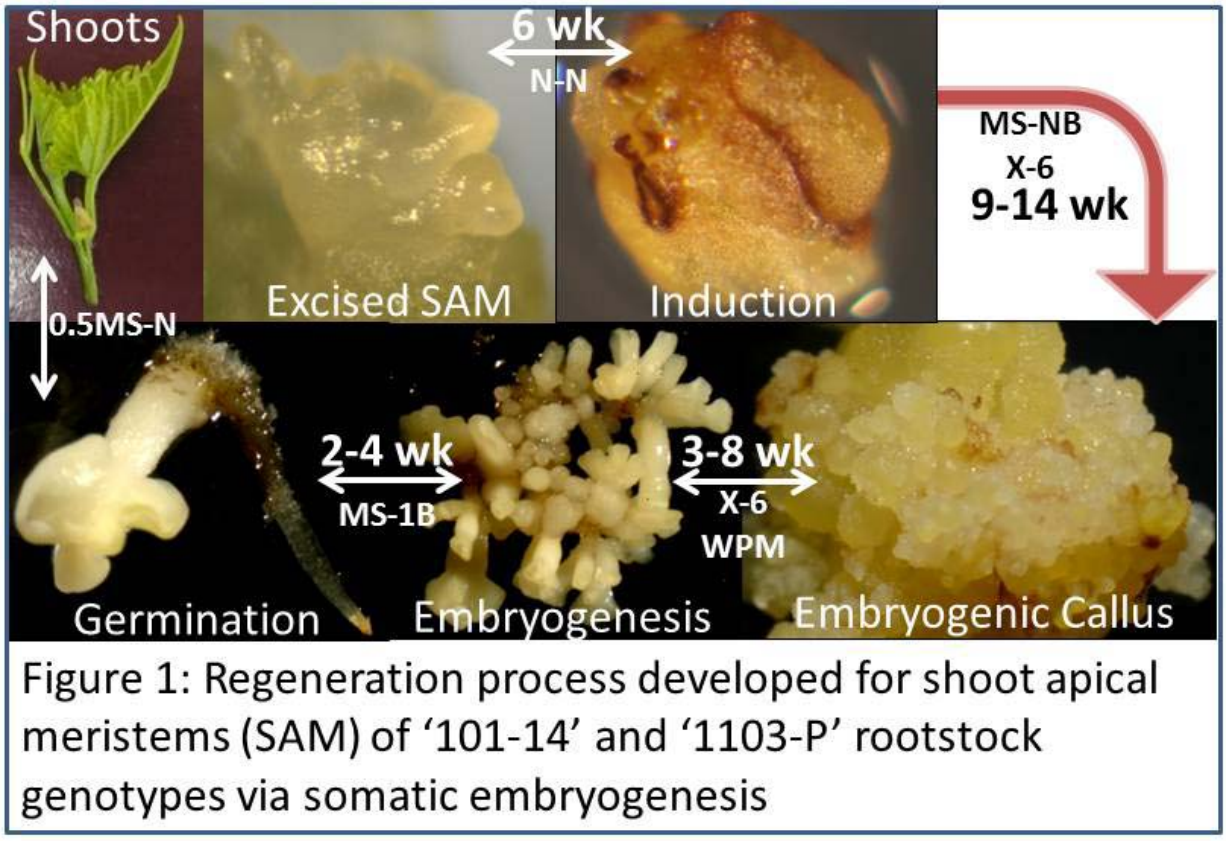

Figure 1: Regeneration process developed for shoot apical meristems (SAM) of '101-14' and '1103-P' rootstock genotypes via somatic embryogenesis

Layperson summary of project accomplishments

This project has developed improved protocols and methods to introduce transgenes (via Agrobacterium-mediated transformation) of commercially relevant rootstocks '101-14' and '1103-P'. During the two year duration of this project we successfully developed grapevine plant regeneration protocols based on excised shoot apical meristems of the two of the prominent rootstocks. Shoot apical meristems (SAM) were dissected from field grown shoots using the protocols developed ware able to undergo morphogenesis in culture to develop embryogenic callus that could be induced to form embryos and then germinated like seed 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 were 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 were transferred to MS-NB medium for a period of 9-12 weeks for differentiation into embryogenic callus. To obtain embryos we used 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 in our protocol to accomplish the regeneration of SAM from these rootstocks (Fig 1). We have also accomplished Agrobacterium-mediated transformation to confirm the reproducibility of a SAM-based grapevine regeneration system for more efficient production of transgenic plants.

Status of funds: The funds for this project have been spent (100%).

Summary and status of intellectual property associated with this project

An invention disclosure will be made for the protocol developed to successfully regenerate and transform the grapevine '110-14' and '1103-P' rootstock before we submit the publication. 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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