Renewal Progress Report for CDFA Agreement Number 11-0416-SA

Development of a Grape Tissue Culture and Transformations Platform for the California Grape Research Community

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Introduction:

Tissue culture of grape plants remains an inefficient process for many genotypes. The procedure is labor intensive, limited to specific genotypes, and requires a significant amount of time to establish embryogenic cell cultures and convert cell cultures into whole plants. The efficiency of establishing and regenerating plants for many important grape genotypes remains very low and are not at the level required to allow for the predictable, cost effective and timely recovery of tissue culture plants needed to successfully offer grape tissue culture and transformation through a self-sustaining service-based facility. These challenges include the successful establishment and multiplication of embryogenic cultures, prevention of tissue necrosis caused by oxidation, conversion of embryos into true-to-type plants, transformation of embryogenic callus, and the regeneration of non-chimeric transgenic plants from embryogenic cells. The goal of this agreement is to leverage the expertise of the National Research Laboratory of Chile, (INIA), and the Ralph M. Parsons Foundation Plant Transformation Facility at UC Davis (UCDPTF) to significantly increase the efficiency of tissue culture and transformation technology in grape genotypes important to their respective countries. The proposed collaboration combines pre-existing expertise and technical know-how to expedite the development of efficient tissue culture and transformation protocols for grape varieties of importance to the PD/GWSS research community. Results of this collaboration will accelerate the delivery of PD/GWSS research results in genotypes that are relevant to the research community. This report outlines the progress that has been made to date toward achieving that goal, with concrete benefits already realized by both groups from the creation of this collaboration.

List of objectives

1. To establish an international collaboration between leading laboratories in the US and Chile that share a common goal of accelerating the development of efficient tissue culture and transformation protocols for grape varieties of importance to the viticulture industries in their respective countries.

2. To develop a self-sustaining service facility that will provide grape tissue culture and transformation services for at least one rootstock and one wine grape genotype in support of the PD/GWSS Research Community

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

Objective 1 To establish an international collaboration between leading laboratories in the US and Chile that share a common goal of accelerating the development of efficient tissue culture and transformation protocols for grape varieties of importance to the viticulture industries in their respective countries.

Objective 1a. Adapt tissue culture and transformation methodologies developed by our Chilean partner for grape genotypes of importance to California including; 11-03, 101-14, Cabernet Sauvignon, and Chardonnay

Progress:

Production of embryogenic callus of 11-03, 101-14, Chardonnay and Cabernet Sauvignon.

In spring of 2013, we again harvested anthers from grape genotypes 11-03, 101-14, Chardonnay and Cabernet Sauvignon and plated them onto two different callus induction media; Murashige and Skoog minimal organics medium (Murashige and Skoog, 1962) supplemented with 60 g/l sucrose, 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.0 mg/l benzylaminopurine (BAP) (PIV) or Murashige and Skoog minimal organics medium supplemented with 20 g/l sucrose, 1.0 mg/l naphthoxyacetic acid (NOA) and 0.2 mg/l BAP (NB medium). In addition to establishing new embryogenic callus for 11-03, Chardonnay and Cabernet Sauvignon, this year we were successful in obtaining embryos from anthers of genotype 101-14. Summary of the response of the various genotypes on the two different media is shown in Table 1. Overall, in our hands, grape anthers demonstrated greater development of embryogenic callus on PIV than on NB medium. At the 2013 Pierces Disease Symposium roundtable session on stacking resistant genes, interest was expressed in being able to transform rootstocks Freedom and 110R. Therefore in 2014 we plan on collecting anthers of Freedom and 110R with the goal of generating embryogenic suspension cultures to add to our collection.

Table 1. The number and percentage of anther cultures plated in 2011, 2012 and 2013 that developed into embryogenic cultures on PIV or NB medium.

Genotype	2013		2012		2011	
	PIV	NB	PIV	NB	PIV	NB
Cabernet	1/287 (0.3)	0/217 (0)	0/200 (0)	0/280 (0)	3/400 (0.8)	NT
Chardonnay	22/344 (6.4)	18/344 (5.2)	9/184 (4.9)	2/156 (3.6)	4/400 (1.0)	NT
1103	3/294 (1.0)	0/287 (0)	0/75 (0)	1/196 (0.5)	2/150 (1.3)	NT
101-14	3/322 (0.9)	0/409 (0)	0/140 (0)	0/275 (0)	NT	NT

Production of embryogenic callus from leaf explants

This year we had a visiting scientist from Argentina try to adapt INIA's leaf embryogenic callus induction protocol for wine grapes, but despite several attempts to date, we have only been able to generate embryogenic callus from leaf explants of Thompson Seedless.

Objective 1b. Increase the efficiency of maintaining embryogenic cultures and reduce the time required for *in vitro* regeneration of grape plants from embryogenic cultures by adapting INIA's cell suspension technology and UC Davis Plant Transformation Facility's temporary immersion system (TIS) for use in grape tissue culture and transformation.

Progress:

Using a modification of INIA's cell suspension protocol, we have significant improved the production of embryogenic grape cultures across a range of genotypes including 1103, 101-14, Cabernet Sauvignon and Chardonnay. This year we will expand our collection to include Freedom and 110R.We are now routinely maintaining cell suspension cultures on liquid WPM medium (Lloyd and McCown, 1981) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 10.0 mg/l Picloram, 2.0 mg/l metatopolin, 2g/l activated charcoal, 100 mg/l ascorbic acid and 120 mg/l reduced glutathione (Pic/MTag) grown in 125 ml shake flasks on a gyratory shaker at 90 rpms in the dark. Once established, the suspensions are easily maintained by withdrawing 10 ml of the suspension each week from the flask and replacing it with 10 ml of fresh medium (figure 1). We are also maintain Temporary Immersion Bioreactors of these genotypes to serve as backup cultures should anything happen that would compromise the suspension cultures such as mechanical failure of the shakers or contamination of the cultures. In these bioreactors, cultures can be maintained for three months without additional handling (figure 2). We have also developed a methodology to store embryos on agar-solidified medium in a quiescent state (see below). While UCDPTF is exploring temporary immersion bioreactors for growing grape embryogenic cultures, our Chilean colleagues are investigation stir tank bioreactors as a cost effective improvement to their airlift bioreactor system (figure 3).

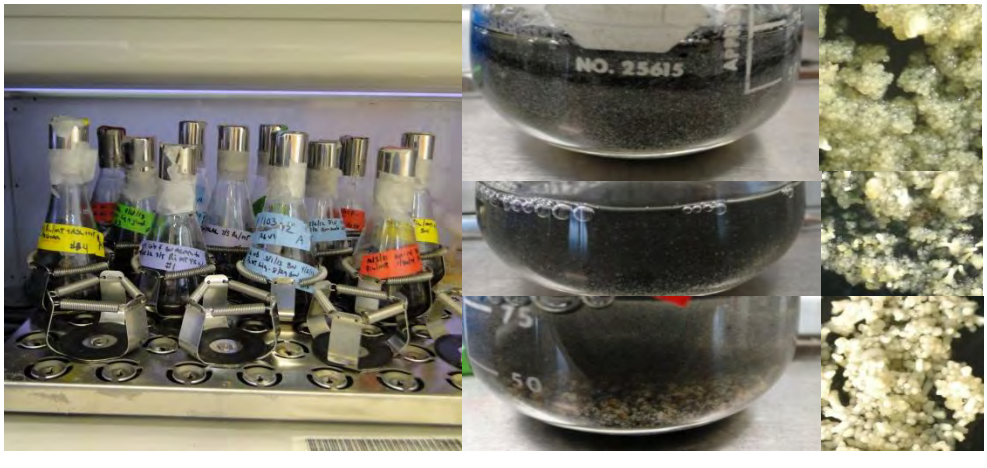

Figure 1. Production of high quality embryogenic callus in shake flasks (left). Close-up image of Chardonnay (top right), 1103 (center right) and 101-14 (bottom right) in cell suspension cultures on WPM medium supplemented with 1g/l casein, 1M MES, 2000 mg/l activated charcoal,10 mg/l picloram, 2 mg/l meta-topolin.

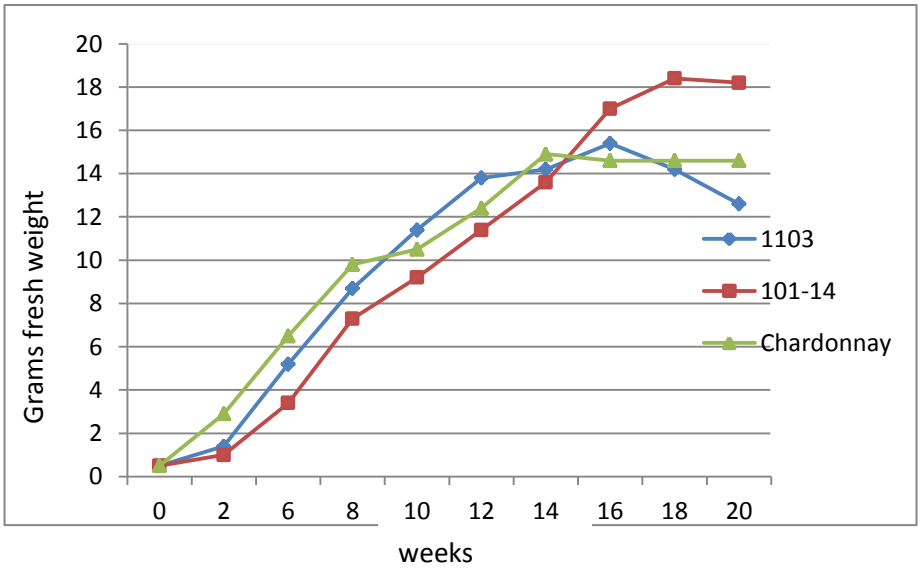

Figure 2. Embryogenic callus growth (grams fresh weight y axis) of 1103, 101-14 and Chardonnay in bioreactor demonstrating that callus can be maintained in bioreactors for sixteen to twenty weeks without medium additions or culture manipulation.

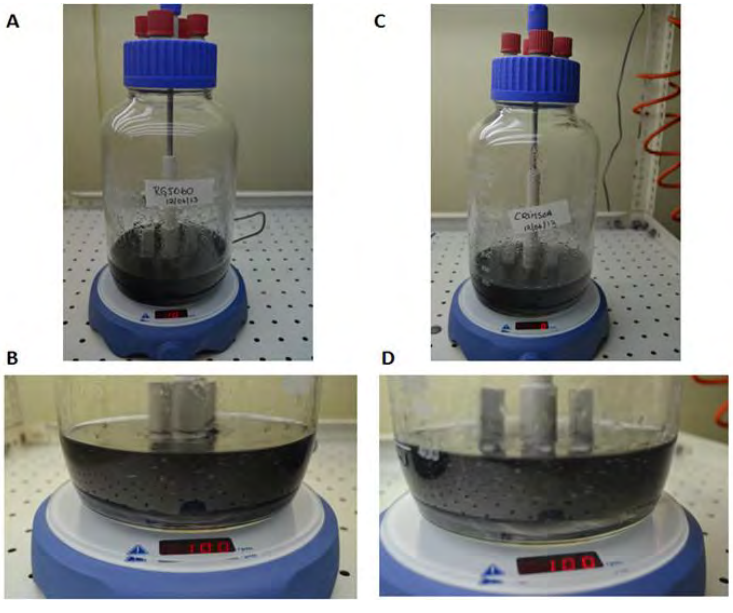

Figure 3. INIA is developing stirred tank reactors for SE genotypes in grapes. General (A and C) and detailed views (B and D) of the Red Globe (A and B) and Crimson (B and C) genotypes under culturing.

Objective 1c. Enhance the efficiency of whole plant regeneration from embryogenic cultures of grape.

In addition to using cell suspension, temporary immersion and stir tank reactors techniques to reduce labor, we investigated methods for storing high quality embryogenic cultures over an extended period of time. Although we initially evaluated storing embryos at four degrees centigrade on agar solidified plates or at minus 80 degrees centigrade using cryopreservation, we have found that increasing the osmotic strength of the medium offers a simple solution for maintaining high quality somatic embryos over an extended period of time. One ml of embryo suspension can be plated onto WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA 50 g/l

sorbitol and 14 g/l agar and cultured in the dark at 26 degrees centigrade. Cells plated onto this medium develop somatic embryos within approximately 4-8 weeks. Embryos do not germinate into plants, but remain as quiescent somatic embryos. They can be maintained in this state for up to six months without loss of viability and upon transferred to WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP lacking sorbitol, they germinate into whole plants (figure 4). These embryos serve as an excellent source of embryos for use in transformation. Given the high efficiency of conversion of the cell suspensions to embryos, these suspensions have utility for use in developing enhanced transformation protocols, gene editing technology, protoplast culture and tilling populations for grape.

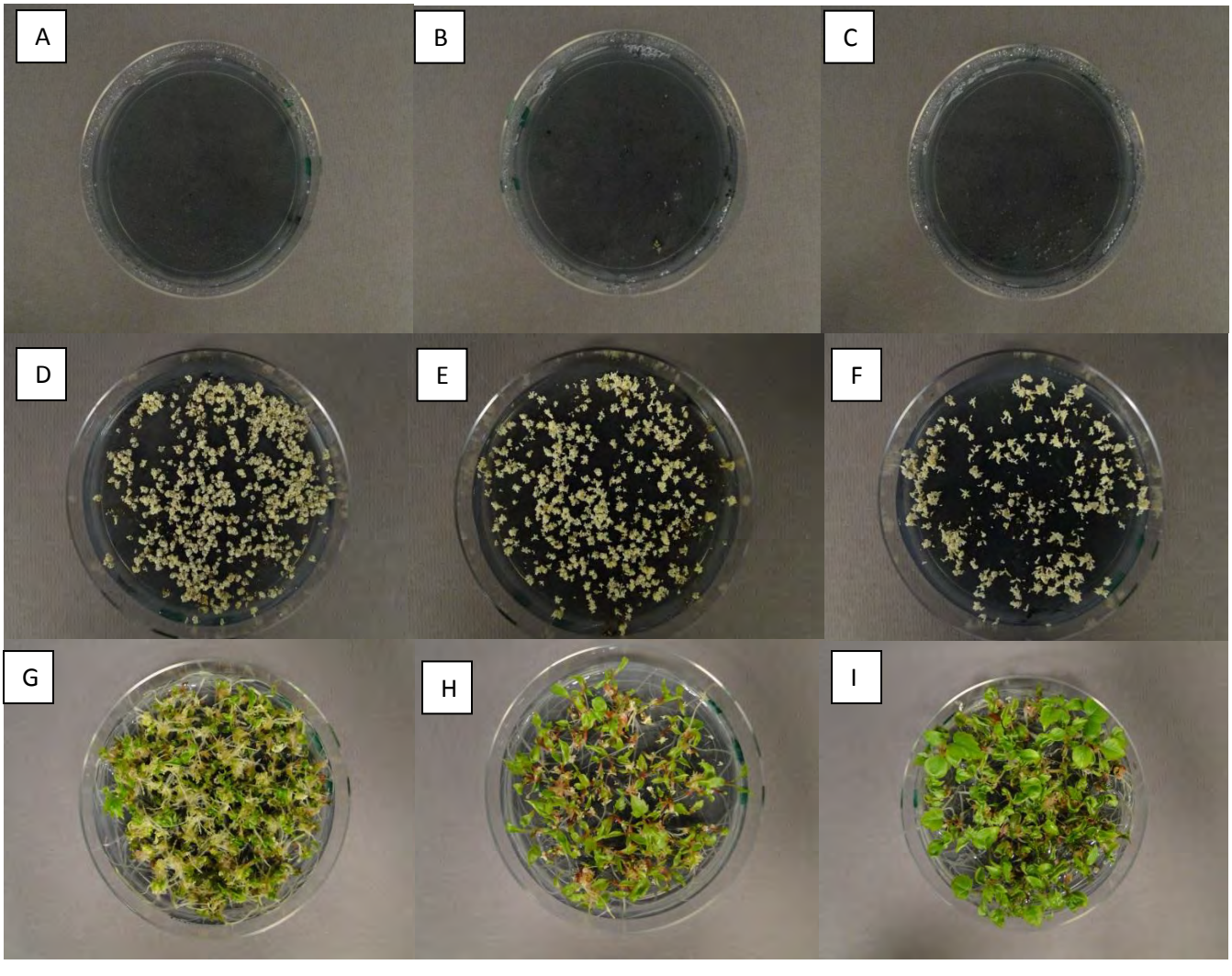

Figure 4. Long-term storage of somatic embryos. Cabernet Sauvignon (A), 1103 (B) and Chardonnay (C), after plating 1 ml of cell suspensions onto WPM supplemented with 20 g/l sucrose, 1g/l casein,1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 50 g/l sorbitol and 14 g/l agar. Quiescent embryos of Cabernet Sauvignon (D), 1103 (E) and Chardonnay (F) five months after storage in the dark without sub-culturing. Cabernet Sauvignon (G), 1103 (H) and Chardonnay (I) fifteen days after transferring embryos stored for five months onto WPM supplemented with 20 g/l sucrose, 1g/l casein,1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP and 8 g/l agar and cultured in the light.

We evaluated numerous media components in an attempt to improve the efficiency of whole plant regeneration from embryos of 1103 and 101-14. As reported last period, preliminary results indicate that our standard grape regeneration medium is not optimal for genotype 1103. Reductions in the levels of the cytokinin, BAP and the elimination of the auxin NAA from the regeneration medium significantly enhanced regeneration of non-transformed 1103 and 101-14 embryos and decrease the time required to regenerate whole plants (figure 4).

Objective 2. Develop a cost effective grape tissue culture and transformation platform for at least one priority California wine grape, and one California grape rootstock which will provide PD/GWSS Research Community with a predictable supply of experimental plant material while reducing labor and maximizing tissue culture and transformation efficiency.

Although regeneration from non-transformed grape embryos is very efficient, plant regeneration from transformed embryos is still time consuming. This may be due to prolonged periods on counter selection medium containing antibiotics to eliminate *Agrobacterium*. However, using our new medium formulation, we have successfully generated whole transgenic grapevines of 1103 expressing the scorable marker gene dsred (figure 5). We have also successfully produced transgenic plants of 101-14 and 1103 containing Abhaya Dandekar's 35S HNE-CecB gene construct (figures 6). We have observed that embryo density appears to impact plant regeneration with high embryo density inhibiting whole plant regeneration.

Figure 5. Transgenic 1103 grape plants 129019-009 and 129032-029 transformed with the scorable marker gene dsred and the plant selectable marker gene nptii. Top panels bright field, lower panel fluorescence. Plants were regenerated on WPM supplemented with 20 g/l sucrose, 1g/l casein,1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP 400 mg/l carbenicillin, 150 mg/l timentin, 100 mg/l kanamycin and 8 g/l agar.

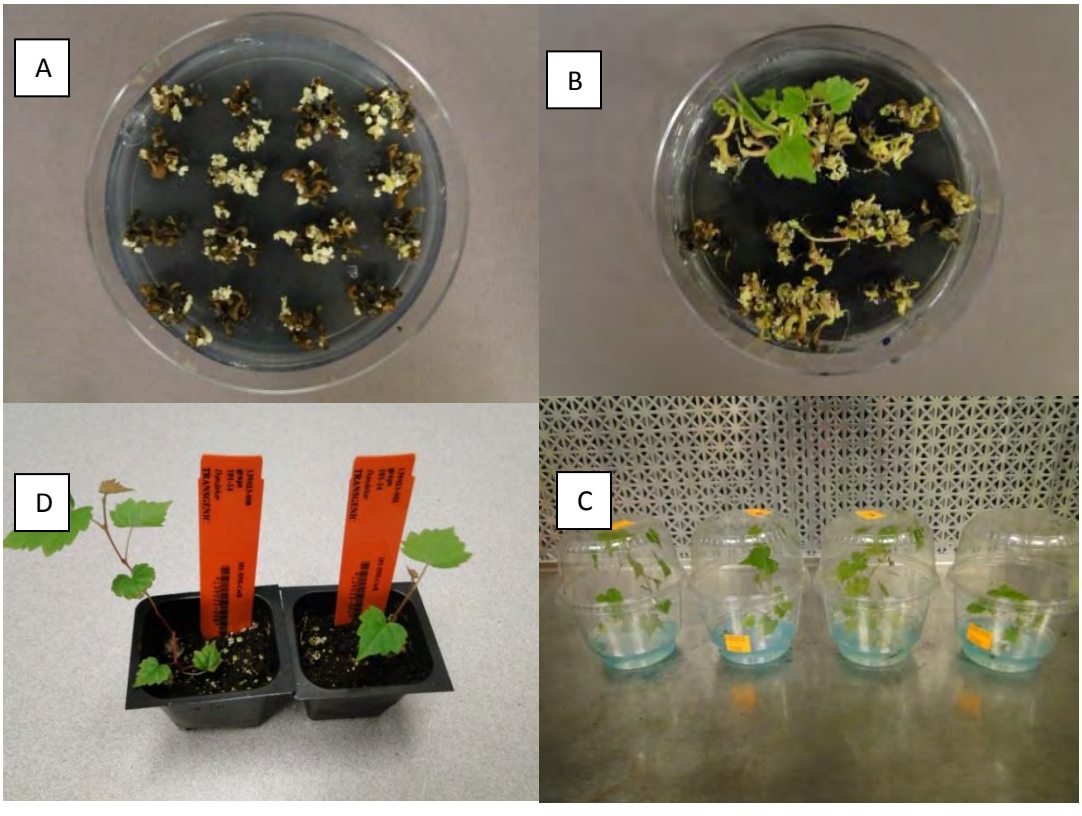

Figure 6 Transgenic 101-14 embryos (A), shoots (B), plantlets (C) and plants acclimated to soil (D) transformed with 35S HNE-CecB

PD/GWSS researchers have expressed an interest in stacking PD resistance genes. Therefore, we are conducting a proof of concept study on stacking genes using sequential transformation of embryos. We are testing this by transforming 101-14 with an *Agrobacterium* culture containing Dr. Dandekar's 35s OM/Ramy/Flag H construct and selecting for the hygromycin plant selectable marker gene. Hygromycin resistant colonies were isolated and increase and when sufficient quantities were available, they were re-inoculated with an *Agrobacterium* containing crown gall resistance genes and the kanamycin plant selectable marker. Cultures are being grown on medium containing both hygromycin and kanamycin to select for cells and embryos that contain both selectable marker genes which should contain both the 35s HNE- CecB and grown gall resistant genes (figure 7).

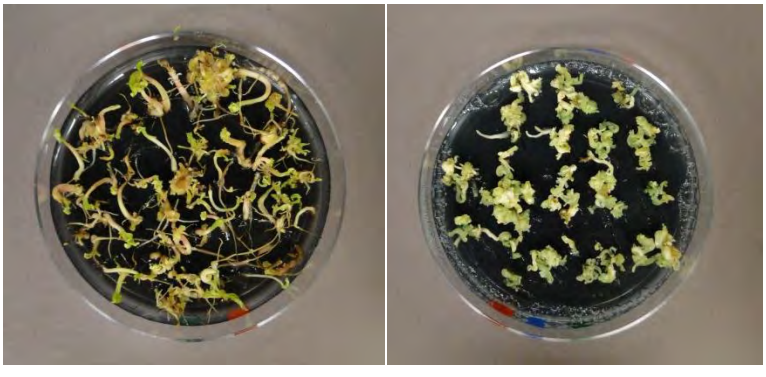

Figure 7 Transformation of 101-14 with Dandekar's 35s OM/Ramy/Flag H construct, and selected for hygromycin resistance (left) and samples of callus transformed with the 35s OM/Ramy/Flag H construct re-transformed with Dandekar's lab's crown gall resistant construct and selected for both hygromycin and kanamycin resistant (right).

We have been evaluating the use of a heat shock treatment on somatic embryos prior to inoculating with *Agrobacterium tumefaciens.* We have preliminary results that indicate that a 5 minute heat shock treatment at 45 degrees centigrade increased the transformation frequency in Thompson Seedless figures 8). We are now testing heat shock pretreatment in 1103 preliminary results also show an increase in transformation frequency.

Figure 8. GUS expression in Thompson Seedless embryos 28 days after inoculation with *Agrobacertium tumefaciens*. Left panel contain embryos subjected to 5 min heat shock at 45 degrees centigrade prior to Agrobacterium inoculation, center panel contains embryos not exposed to heat shock prior to agrobacterium inoculation and right panel contains non-inoculated control embryos

By inoculating globular and torpedo embryos from stored embryos (figure 4), rather than embryogenic callus, we have been able to significantly reduce the time from initiation of transformation to the delivery of transgenic plants in Thompson Seedless without the creation of chimeric plants. Using this starting material we can deliver transgenic Thompson seedless plants in 6-8 months as opposed to an average of 12-14 months required using embryogenic callus as the starting tissue (figure 9). We are in the process of applying these techniques to rootstock transformations of 101-14 and 1103.

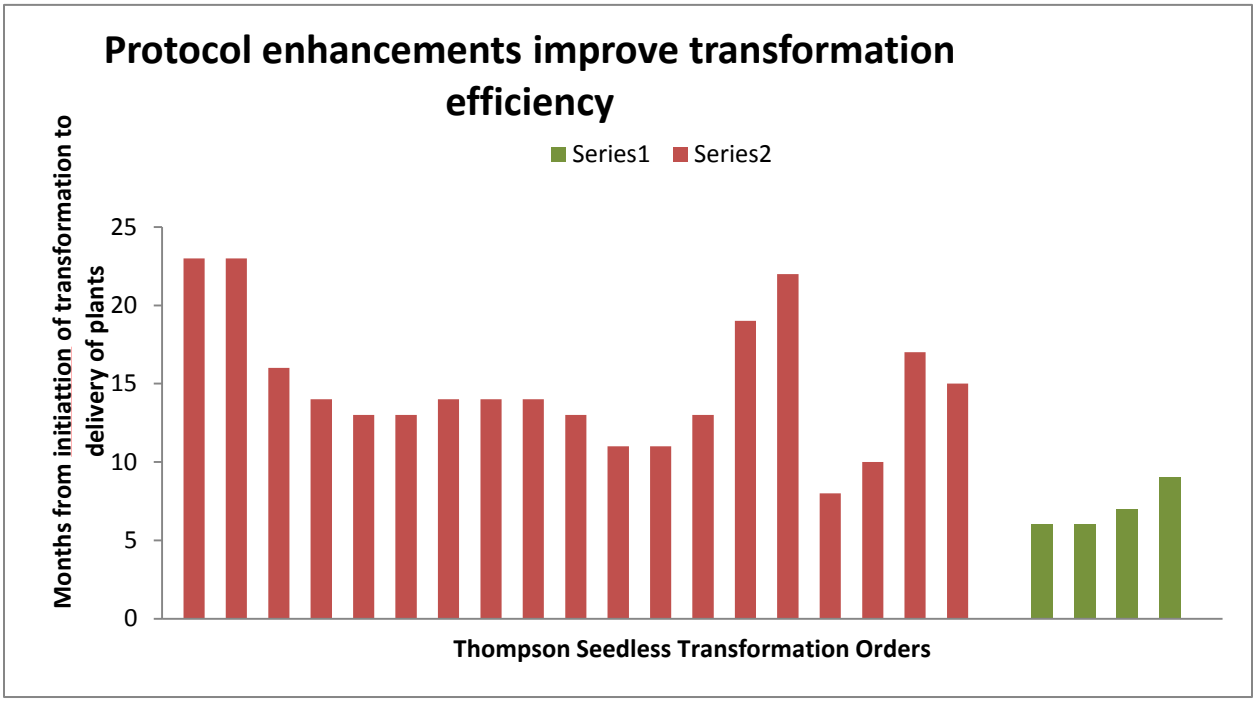

Figure 9 Improvement in the time required from co-cultivation to transgenic plant regeneration using embryogenic callus as the starting tissue for transformation (series 2) verses using embryos stored on sorbitol containing medium (series 1)

If the PD/GWSS Research Community decides to explore gene stacking technology in grapevines, a significant number of transgenic events will need to be generated to ensure that some events are created with the appropriate level of expression of each of the stacked genes. To this regard, we are leveraging the progress we have made in developing high quality cell suspension that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium as shown in figure 4 above. This period we began exploring transforming our grape cell suspension cultures with the scorable marker gene dsred. Two ml of a 1103 grape cell suspensions grown in liquid Pic/MT medium was collected in a 15 ml conical centrifuge tube and pelleted by centrifugation at 1000 x G for 3 minutes. The cells were subjected to heat shock by placing the conical tube in a 45 degree water bath for 5 minutes. After heat shock the supernatant was removed and replaced with 5 ml liquid BN medium containing 200 uM acetosyringone and the *Agrobacterium* strain EHA105 carrying the ubi:dsred vector at an OD_{600} of 01.-0.2. The suspension was centrifuged at 1000 x G for 5 minutes and allowed to incubate for 25 minutes at room temperature. After 25 minutes all but 0.5 ml of the supernatant was removed. The grape and *Agrobacterium* cells were then re-suspended and transferred to sterile Whatman filter paper in an empty 100 x20 mm petri dish. Any excess fluid was carefully bloated up with a second sterile filter paper. The plates were co-cultured for 2-3 days at 23 degrees and then transferred to selection medium consisting of WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin, 50 g/ sorbitol and 14 g/l agar. The filter paper was transferred to fresh medium every 2 weeks. Within eight weeks dsred embryos developed (figure 10). We transferred these embryos to WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin, 0 g/ sorbitol and 7 g/l agar and they have begun germinate. The time from inoculation to the recovery of germinating embryos is only 10 weeks. We have begun testing this technique in 101-14 and dsred embryos are also developing (figure 12). If this protocol is repeatable and can be extended to other genotypes, it represents a significant advance in our transformation system since it greatly increases transformation efficiencies while minimizing labor inputs.

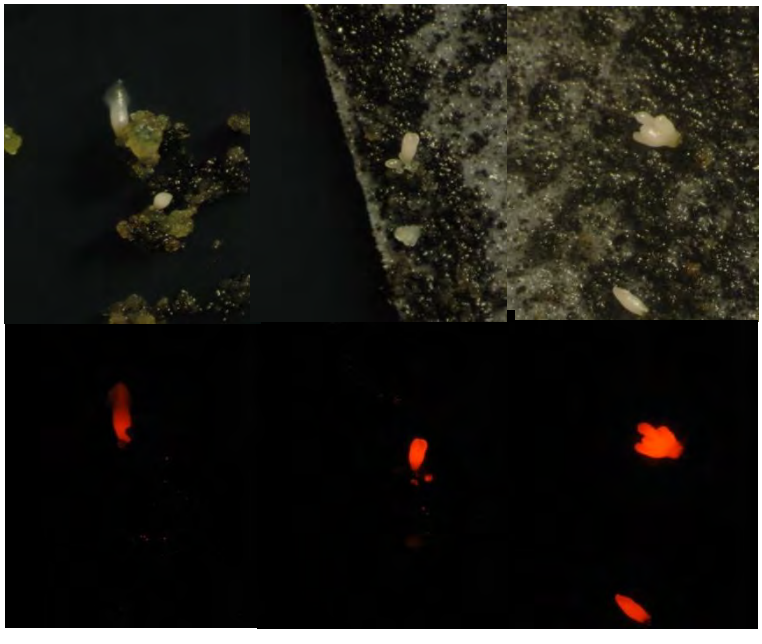

Figure 10. Transgenic dsred expressing 1103 embryos developing from cell suspension cultures six weeks after inoculation with *Agrobacterium* and plates onto WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin, 50 g/ sorbitol and 14 g/l agar (bright field top panel, fluorescence, lower panel).

Figure 11. Germinating, transgenic dsred expressing 1103 embryos developing from cell suspension cultures twelve weeks after inoculation with *Agrobacterium*. Embryos shown in figure 10 above were transferred onto WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, 400 mg/l carbenicillin, 150 mg/l timentin,100 mg/l kanamycin (bright field top panel, fluorescence, lower panel).

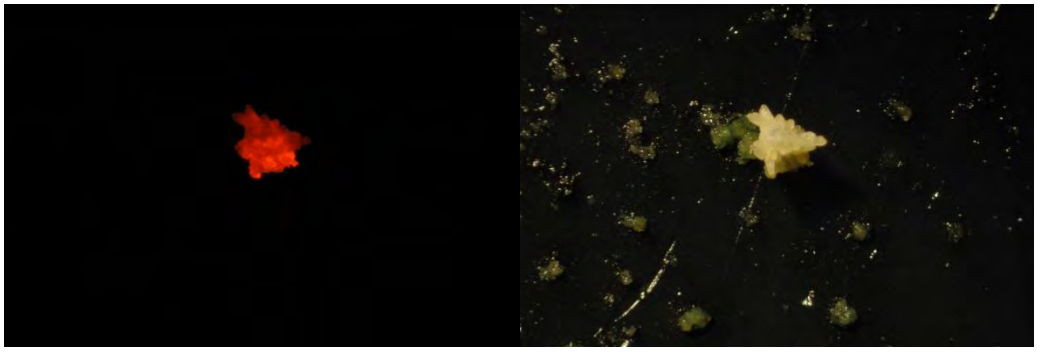

Figure 12. Transgenic dsred expressing 101-14 embryos developing from cell suspension cultures eight weeks after inoculation with Agrobacterium and plates onto WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin, 50 g/ sorbitol and 14 g/l agar (bright field right panel, fluorescence, left panel).

H. Publications produced and presentations made that relate to the funded project.

Tricoli D. M. 2012. Development of a Grape Tissue Culture and Transformation Platform for the California. Pierce's Disease Research Progress Reports, Grape Research Community pp. 225-232

Tricoli D. M. 2013 Development of a Grape Tissue Culture and Transformation Platform for the California. Pierce's Disease Research Progress Reports, Grape Research Community pp. 184-191.

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

This strategic and mutually beneficial partnership leverages the expertise of the National Research Laboratory of Chile, (INIA), and the Ralph M. Parsons Foundation Plant Transformation Facility (UCDPTF) at UC Davis and will accelerate the delivery of PD/GWSS research results. The proposed collaboration combines pre-existing expertise and technical know-how that will expedite the development of efficient tissue culture and transformation protocols for grape varieties of importance to the PD/GWSS research community. The development of a highly efficient service-based tissue culture and transformation platform for grape varieties of relevance to the PD/GWSS Research Community and the wine grape industry will have major benefits for the grape research community, by accelerating the gene function/validation process of identifying PD/GWSS practical control strategies. Development of efficient cell suspension and regeneration methodologies will have utility for use in developing enhanced transformation protocols, gene editing technology, protoplast culture and tilling populations for grape. To ensure broad utility of this technology platform within the research community, once established, we propose offering the enabling technologies through UC's existing core service facility on a time efficient and cost-effective fee-for service basis.

J. Layperson summary of project accomplishments.

This proposal is aimed at establishing an international collaboration between leading laboratories in the US and Chile to reduce the time and cost of tissue culture and transformation for grape varieties of importance to the viticulture industries in their respective countries. The collaboration leverages pre-existing expertise and technical know-how to expedite the development of efficient tissue culture and transformation protocols for grape varieties of importance to the PD/GWSS research community. The two labs have exchanged their latest media formulations and protocols for increasing embryogenic callus and both labs are comparing the effectiveness of each other's techniques using germplasm important to their particular country.

The availability of a reliable supply of highly embryogenic grape cultures is crucial for the development of cost effective service-based grape tissue culture and transformation systems. We have made significant advances in our ability to establish and increase embryogenic cultures for use in tissue culture and transformation experiments. We have developed a high quality cell suspension culture system for genotypes 1103, 101-14, Cabernet Sauvignon and Chardonnay which allows rapid regeneration of whole plants with minimal labor. We have also developed bioreactor technology which allows cultures to be maintained for three months without additional handling. This year we have developed a long term storage culture technique which allows grape somatic embryos to be stored for six months without the use of cryopreservation. These embryos provide a steady supply of material which we are using to develop tissue culture and transformation system for genotypes important to California's PD/GWSS Research Community. These techniques allow for easy maintenance of numerous grape genotypes with minimal labor and will allow us to maintain additional rootstock genotype in our collection for the PD/GWSS Research Community such as Freedom and 110R. These cell culture systems provide a source of high quality embryogenic material which displays a very high frequency of plant regeneration and could be used to develop high throughput transformation technology, gene editing technology, protoplast culture and tilling populations for grape. Using these high quality embryogenic cultures in our transformation experiments, we have demonstrated that we can successfully regenerate transgenic plants of 101-14 and 1103.

Lastly, this year we demonstrated proof of concept in direct transformation of 1103 and 101-14 cell suspensions using a fluorescence marker gene. When perfected, this technique could allow for very rapid production of numerous transgenic lines with minimal labor. This technique could prove vital if the PD/GWSS Research Community decides to pursue a strategy to stack multiple PD resistance strategies in a single transgenic rootstock.

K. Status of funds.

We anticipate that all funds allocated for fiscal year FY2013-2014 will be expended by June 30, 2014

L. Summary and status of intellectual property associated with the project

This collaboration will continue to work closely with PIPRA to address access to intellectual property and know-how for this work in order to make new services widely accessible to the research community. Individual protocols are already being shared between the two groups. Any protocol improvements developed through this collaborative grant will be shared between INIA and UC Davis and will be integrated into an effective grape tissue culture and transformation recharge based service. PIPRA will serve as an interface with INIA to develop strategies to access the products of this research collaboration. The two labs have already benefited from the exchange of information on media formulations and techniques which can be applied to Chilean grape varieties including Salcrik, Freedom and Harmony and California genotypes 11-03, 101-14, Cabernet Sauvignon and Chardonnay which are the targets of the US effort.