A. Final Report for CDFA Agreement Number 11-0416-SA

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

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D. Time period covered by the report:

- January 16, 2011 to January 15, 2015

E. 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 homogeneous 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 was made under CDFA Agreement Number 11-0416-SA toward achieving that goal, with concrete benefits already realized by both groups from the creation of this collaboration.

F. 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

G. Description of activities conducted to accomplish each specific objective, and 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

Production of embryogenic callus of 11-03, 101-14, Chardonnay, Cabernet Sauvignon and Richter 110

We have established embryogenic cultures from anthers of 11-03, 101-14, Chardonnay, and Cabernet Sauvignon each year of the grant by plating them onto Nitsch and Nitsch minimal organics medium (1969) 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 2014 we also established embryogenic callus of Richter 110.

Production of embryogenic callus from leaf explants

The National Research Laboratory of Chile (INIA) and UC Davis' Plant Transformation Facility (UCDPTF) explored an alternative method to generating embryogenic callus which utilizes leaf pieces from *in vitro* grown plants. Unlike generating callus from anthers which have a short window of availability in the spring of each year, leaf tissue from *in vitro* plantlets are available year round. In addition, unlike meristem explants which are time consuming and difficult to excise, leaf explants are easy to isolate and can be secured from known pathogen-free tissue culture plantlets. We are maintaining disease free *in vitro* stock plants of 1103, 101-14, Chardonnay and Cabernet Sauvignon that we received from Foundation Plant Services and used these to test the leaf embryogenic culture system. Although we successfully generated embryogenic callus from leaf explants of Thompson Seedless grape, were not been able to replicate this technique for non-table grape genotypes. Based on the advice of Andy Walker and Cecilia Aguero, we are investigating the Mezzetti et al., (2002) protocol which utilizes bulk meristems as the target tissue for transformation. We generated bulk meristems cultures from indexed tissue culture stock tissue for 11-03, 101-14, Chardonnay and Cabernet Sauvignon (figure 1). To date scion genotypes develop bulk meristems more readily than rootstocks. Rootstock genotypes tend to produce excess callus on Mezzetti medium with 3.0 mg/l BAP.

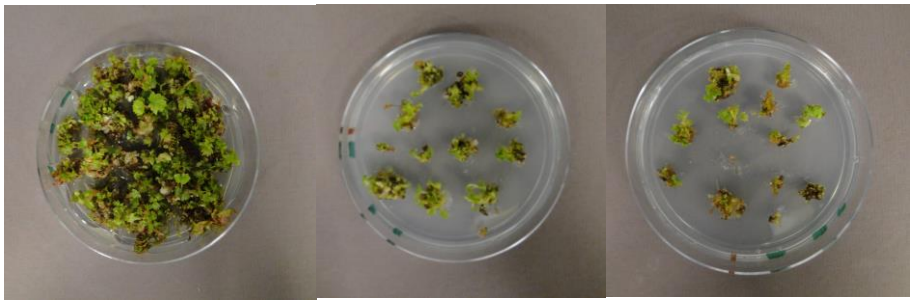

Figure 1 Bulk meristem cultures from left to right, Cabernet Sauvignon, Chardonnay and 1103

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.

Using a modification of INIA's cell suspension protocol, we significantly improved the production of embryogenic grape cultures across a range of genotypes including 1103, 101-14, Cabernet Sauvignon, Chardonnay and Richer 110. We established fresh embryogenic suspension each year and we can 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 2).

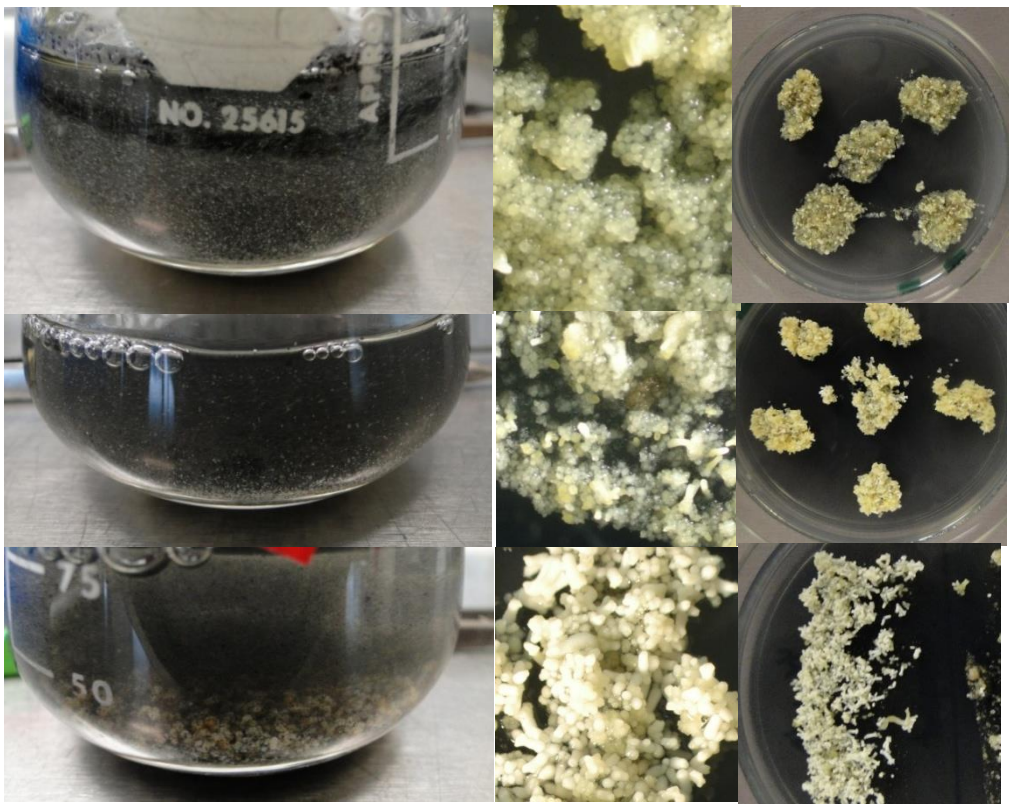

Figure 2. Production of high quality embryogenic callus from cell suspensions. Chardonnay (top), 1103 (middle) and 101-14 (bottom) in cell suspension cultures on WPM medium supplemented with 1g/l casein, 1M MES,1000 mg/l activated charcoal,10 mg/l picloram, 2 mg/l meta-topolin (left), close up of cell suspension cultures (middle) and embryogenic callus developing 4 weeks after plating 1 ml of suspensions on WPM medium supplemented with 1g/l casein, 1M MES, mg/l activated charcoal,0.5 mg/l BAP and 0.1 mg/l NAA (right).

In addition to evaluating INIA's liquid shake flasks methodology on grape genotypes 101-14, 1103, Chardonnay and Cabernet sauvignon, we explored UCDPTF's temporary immersion system (TIS) for use in rapidly increasing embryogenic callus. This method of increasing embryogenic callus has proven very efficient, and we have found that by adding ascorbic acid to the culture medium and growing the cultures in the dark we can significantly reduce phenolic development in the callus. Robust growth rates of 1103, 101-14 and Chardonnay callus cultures were achieved for a minimum of 3 months without addition of fresh medium to the bioreactor (figure 3). This system is advantageous from a labor management perspective, since it allows

one to maintain stock embryogenic cultures indefinitely in temporary immersion with medium exchanges occurring only once every three months.

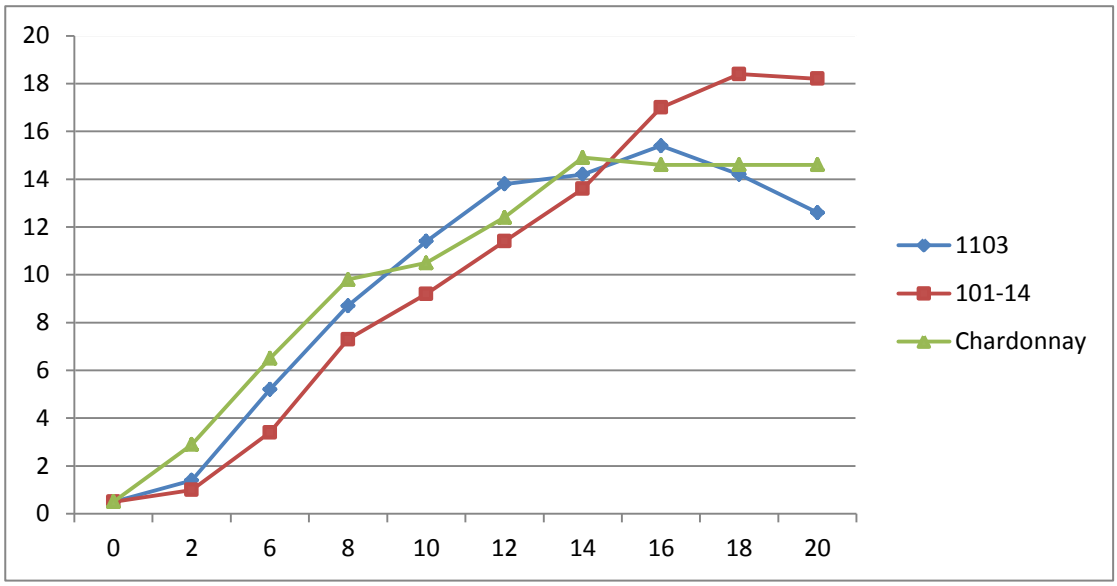

Figure 3. Embryogenic callus growth of 1103, 101-14 and Chardonnay in bioreactor demonstrating that embryogenic callus can be maintained in bioreactors for three months with no medium additions of culture manipulations.

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 associated with growing embryogenic cultures, we investigated methods for storing high quality embryogenic cultures over an extended period of time. We 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 as described above, 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. These embryos serve as an excellent source of embryos for use in transformation (figure 4). 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.

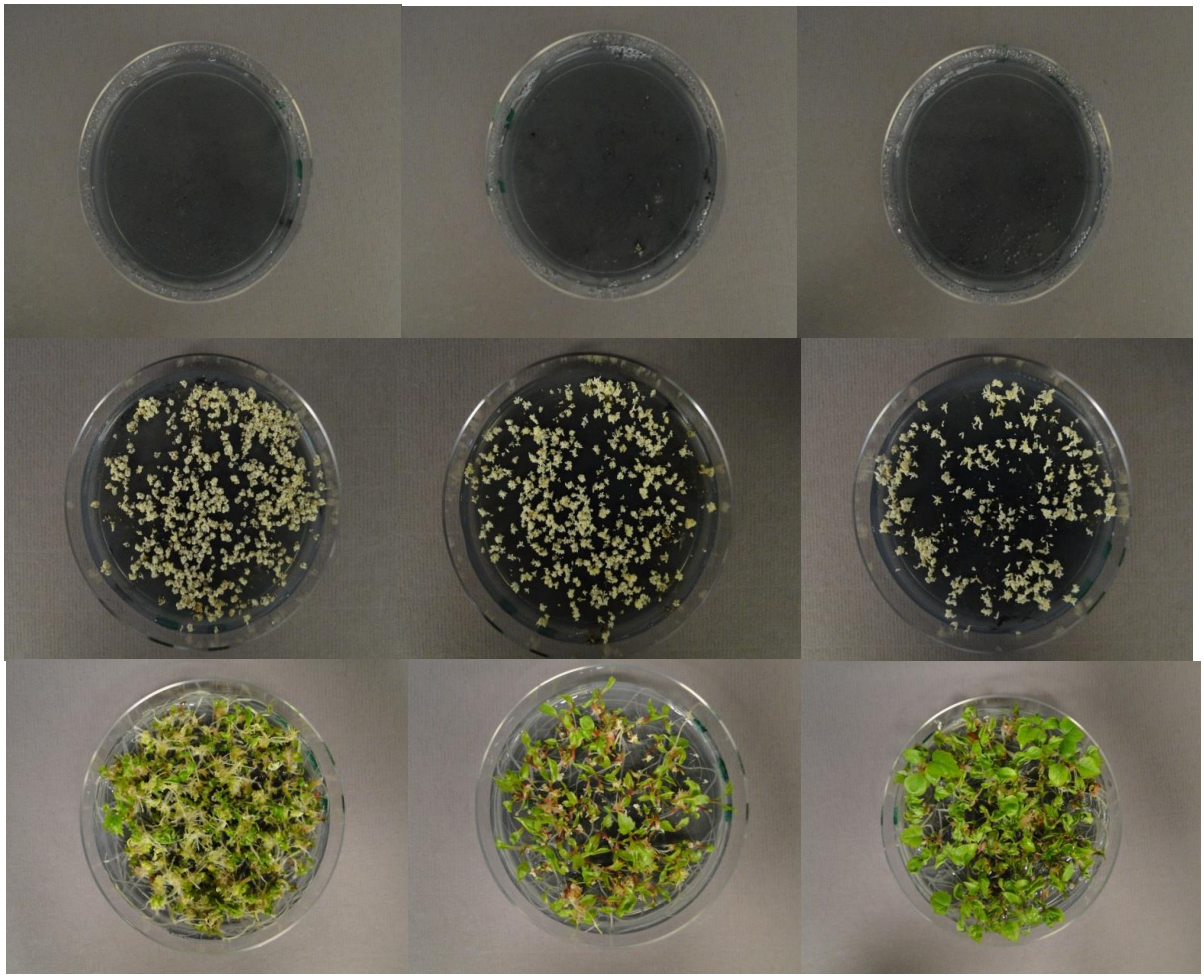

Figure 4. Long-term storage of somatic embryos form left to right of Cabernet Sauvignon, 1103 and Chardonnay, after plating 1 ml of cell suspensions onto WPM supplemented with 20 g/l sucrose, 1g/l casein,1M MES, 500 mg/l activated charcoal, 0.5 mg/l BAP, 0.1 mg/l NAA, 50 g/l sorbitol and 14 g/l agar (top row). Quiescent embryos of Cabernet Sauvignon, 1103 and Chardonnay five months after storage in the dark without sub-culturing (middle row). Cabernet Sauvignon, 1103 and Chardonnay 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 (lower row).

Regeneration of whole plants from embryos of 1103 and 101-14 is the rate limiting step in the production of transgenic plants. Transformation frequencies are now relatively high for these rootstocks, but regeneration of whole plant from transgenic tissue still requires many months in culture. In other species, the basal salt formulation of the tissue culture medium can have a dramatic effect on the regeneration efficiency. Therefore, we evaluated eight different salt formulations in an attempt to improve the efficiency of whole plant regeneration from embryos of 1103 and 101-14. Salt formulations test included Andersons, Chee and Pool, DKW, Gamborg's B5, MS, WPM, SH and X6. All media were supplemented with 1.0 g/l casein and 500 mg/l activated charcoal and 0.1 mg/l BAP. Significant differences were seen between the various salt mixtures with the best regeneration occurring on DKW, SH and WPM. However, rootstock regeneration remains less efficient than regeneration from table grapes or wine grapes. During the period of the grant we determined that the best regeneration media for grape rootstocks was Preece's salt formulation with is comprised of 50 % DKW salts and 50% WPM salts supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP or 0.1 mg/l Zeatin and 8 g/l agar (figure 5).

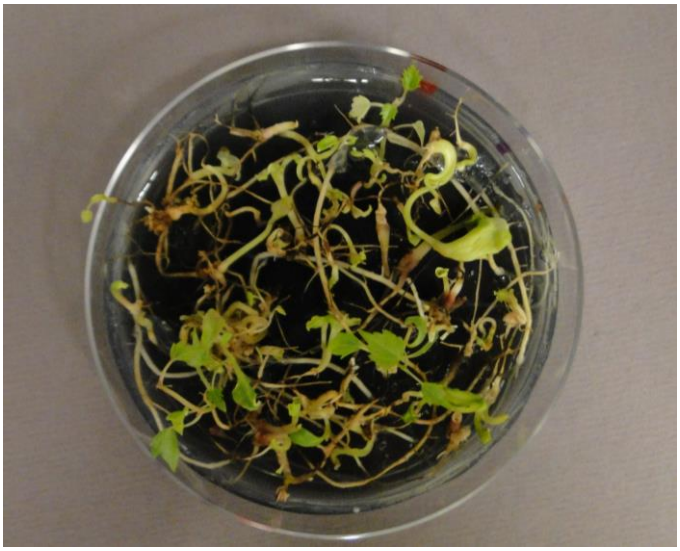

Figure 5 Regenerating transgenic shoots of 1103 developing from embryos plates on Preece medium supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP and 25 mg/l hygromycin.

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.

Objective 2a. Improve grape rootstock transformation efficiency for 1103 and 101-14 using embryos harvested from robust growing cell suspension cultures.

We evaluated the use of a heat shock treatment on somatic embryos prior to inoculating with *Agrobacterium tumefaciens.* Preliminary results indicate that a 10 minute heat shock treatment at 45 degrees centigrade increased the transformation frequency in Thompson Seedless, 1103 (table 1) and 101-14. While transformation frequencies for 101-14 and 1103 are relatively high, transformation frequencies of Cabernet Sauvignon and Chardonnay remain low and further improvements are needed.

Table 1. Transformation experiments with 1103 embryos comparing transformation efficiencies after exposure to 10 minutes of heat shock at 45 degrees centigrade verses no application of heat shock prior inoculation with *Agrobacterium tumefaciens.*

Number of Experiment	Heat Shock	Genotype	# (%) transgenic colonies
3	-	1103	17/73 (23.3)
5	+	1103	72/140 (51.4)

We developed a robust suspension system for 1103 and 101-14 which provide a continuous source of somatic embryos for transformation. Embryogenic cell suspensions are harvested from cell suspension culture on a weekly basis as part of the process required for feeding the suspension cultures. As described above, one milliliter of the suspension can be plated on sorbitol containing medium for regeneration of somatic embryos which enter a quiescent state and can be stored for later use for over six months without any additional manipulation. Large quantities of embryos can then be collected from the plates and transformed with *Agrobacterium* when transformations are requested. Secondary transgenic embryo arises from the epidermis of

the inoculated embryos while the remainder of the inoculated embryo turns necrotic due to the selective agent, kanamycin or hygromycin (figure 6). The surviving secondary embryo can be harvested and transferred to regeneration medium for plant production. Using this system, we have been able to generate transgenic embryos for both 1103 and 101-14. We are currently utilizing this technique for transformation request for PD/GWSS Researchers (Table 2).

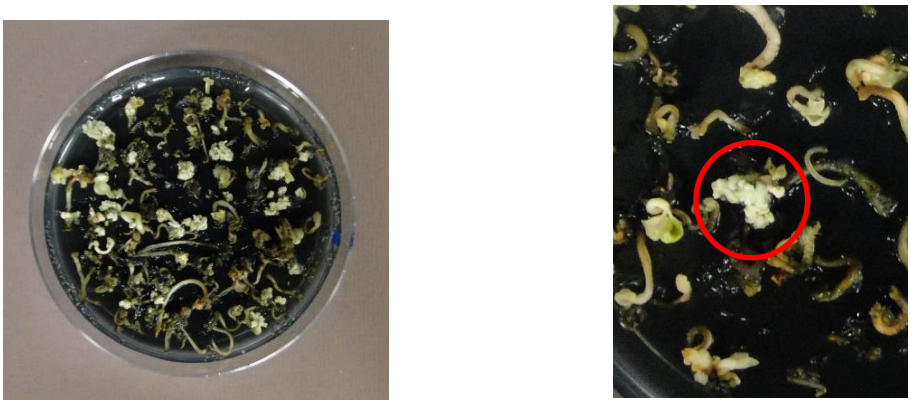

Figure 6. Clusters of transgenic secondary embryos developing from *Agrobacterium* inoculated 1103 somatic embryo plated on 200 mg/liter kanamycin sulfate (left) and close up of one secondary embryo cluster (right).

Table 2. Inventory of transgenic 1103 and 101-14 plants generated with various genes of interest

Genotype	Transgene	Number of Plants Produced
1103	**35s HNE-CecB**	**15**
101-14	**35s HNE-CecB**	**25**
101-14	**pDU10.1818**	**10**
101-14	**HNE-CecR**	**6**

Objective 2b. Leverage the progress we have made in developing high quality suspension cultures that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium by testing direct transformation of cell suspension cultures.

We leveraged the progress we have made in developing high quality grape suspension cultures that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium by developing a method for direct transformation of our grape suspension cultures. Eight to 10 ml of a grape cell suspensions grown in liquid Pic/MTag medium and containing pre-embryogenic masses or small globular embryos are collected in a 15 ml conical centrifuge tube and pelleted by centrifugation at 1000 x G for 3 minutes. The supernatant is removed and the cells are washed by re-suspending them in WPM medium without charcoal. Cells are pelleted by centrifugation at 1000 x G for 3 minutes and are washed two additional times in WPM medium. After the last wash, the cells are subjected to heat shock by placing the 15 ml conical tube in a 45 degree water bath for 5 minutes. After heat shock the supernatant is removed and the cells are re-suspended in 5 ml liquid BN medium containing 200 uM acetosyringone and the *Agrobacterium* strain EHA105 carrying the desired vector at an OD 600 of 01.-0.2. The suspension is centrifuged at 1000 x G for 5 minutes and allowed to incubate for 25 minutes at room temperature. After 25 minutes, all but 0.5-1.0 ml of the supernatant is removed. The grape and *Agrobacterium* cells are then re-suspended and transferred onto sterile Whatman filter paper in an empty 100 x 20 mm petri dish. Any excess fluid is allowed to evaporate by leaving the petri dishes in the hood for a few hours. The cells are 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, 4 ml PPM, 50 g/ sorbitol, 14 g/l agar and 200 mg/l kanamycin, or 25 mg/l hygromycin. Sub-culturing of the plated cells is achieved by simply transferring the filter paper with the cells onto fresh medium on a biweekly basis. Within eight to 12 weeks transgenic embryos develop (figure 7). Developing embryos are transferred to WPM

supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP or 0.1 mg/l zeatin, 400 mg/l carbenicillin, 150 mg/l timentin, 0 g/ sorbitol, 8 g/l agar and 200 mg/l kanamycin or 25 mg/l hygromycin for germination. The time from inoculation to the recovery of transgenic embryos can be as short as 10 weeks. We have successfully used this technique to produce transgenic embryos of 1103 and 101-14 (figure 7). The system has been employed successfully using both kanamycin and hygromycin selection. We see significant amount of experiment to experiment variability in the number of transgenic embryos developing, with numerous experiments yielding no transgenic colonies and other experiments generating variable number of colonies which are listed in (Table 3). However, if this protocol can be made more consistent, it represents a significant advance in our transformation system since it greatly increases transformation efficiencies while minimizing labor inputs. It appears that the plating density of the cells (too high or too low) and the quality to the suspension impacts transformation efficiency. As with our embryo-based transformation system, the limiting step in this protocol is also the regeneration of whole plants from transgenic embryos and we continue to explore media modification to enhance regeneration potential.

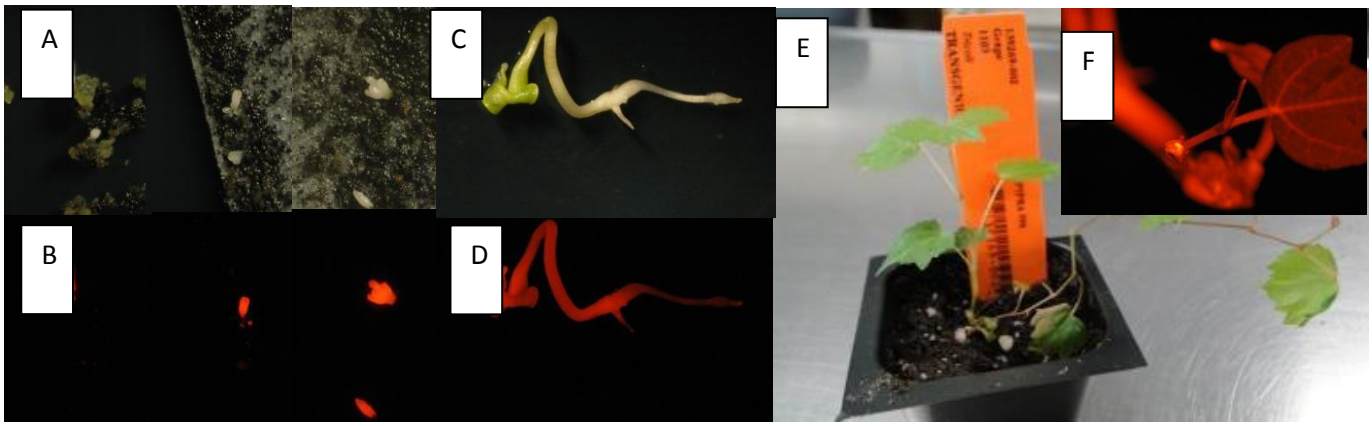

Figure 7. Transformation of suspension cultures of 1103 with the fluorescent DsRed gene plated on BN sorbitol medium supplemented with 400 mg/l carbenicillin, 150 mg/l timentin, 4 ml PPM and 25 mg/l hygromycin. (A) Grape embryo expressing the DsRed gene confirming the transgenic status of the developing embryos. (B) germination of transgenic embryos after transferring to WPM medium supplemented with 20 g/l sucrose, 1 g/l casein, 1mM MES, 500 mg/l charcoal,0.1 mg/l BAP and 8 g/l agar; bright field(C) and fluorescence (D) Regeneration of whole plant expressing DsRed (F).

Table 3. The number of transgenic embryogenic colonies forming after inoculating grape suspension cultures with *Agrobacterium* and plating onto selection medium

Number of Experiment	germplasm	Selection	Average number of Transgenic colonies generated	Range of transgenic colonies generated per experiment
1	101-14	hygromycin	6	
4	1103	kanamycin	5	1-7
11	1103	hygromycin	10.7	1-50
4	101-14	kanamycin	11.75	3-24

Objective 2c. Develop methods for transforming multiple trait genes into grape through sequential transformation using two different plant selectable marker genes.

Since researchers expressed an interest in stacking multiple resistant strategies in a single transgenic grape line, we performed sequential transformations in which we transformed grape embryos with the first construct containing a gene of interest and the hpt plant selectable marker gene and select for hygromycin resistant

transgenic secondary embryos on medium containing hygromycin. Once hygromycin secondary embryos developed, they were increased. Once sufficient numbers of embryos were produced, they were re-inoculated with an *Agrobacterium* culture containing a second construct with a gene of interested and the kanamycin plant selectable marker gene (nptii) and cultured on medium containing both kanamycin and hygromycin. Developing embryos should contain both selectable marker genes and both genes of interest. We have produced putatively sequentially transformed embryos for 101-14 and 1103 (figure 8). Once plants are recovered they can be tested for the presence of both genes. Although this technique can be used to stack genes it is very inefficient and labor intensive and stacking traits genes in a single T-DNA is recommended.

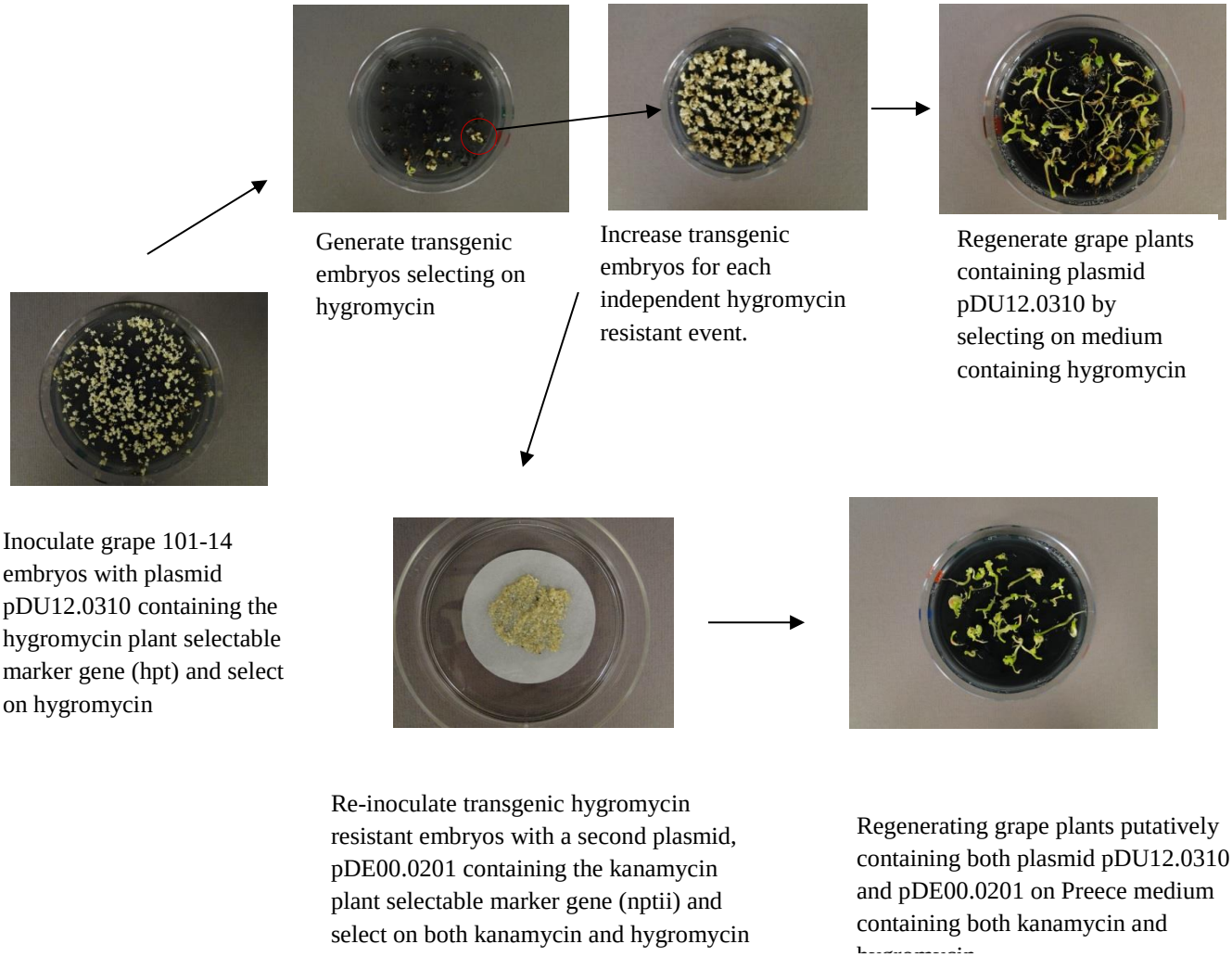

Figure 8. Sequential transformation of 101-14 somatic embryos inoculated initially with plasmid pDU12.0310 and selected on hygromycin. Some hygromycin resistant embryos were induced to regenerate plants while a subset of embryos were re-inoculated with plasmid pDE00.0201 and double selected on hygromycin and kanamycin.

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

Presented an update at the 2012-2014 Pierce's Disease Research Symposium

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

Since the advances made under this project consist of process developments no intellectual protection

was sought since process patents are difficult to protect and enforce.

K. How this work and its results will contribute to solving the Pierce's disease problem in California

This work and its results contribute to solving the Pierce's disease problem in California by providing efficient tissue culture and transformation protocols for grape varieties of importance to the PD/GWSS research community which allows them to test resistance strategies in rootstock genotypes important to California wine grape production in particular 1103P and 101-14. We are currently using these protocols to transform eight new Pierces disease constructs into 1103 or 101-14. The protocols developed here may also be amenable to other rootstock and scion varieties important for California grape production.

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