A. Interim Progress 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: March 2013-July 2013

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 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.

F. List of objectives:

I. 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.

II. 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 I 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.

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

Progress:

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

We harvested additional anthers from grape genotypes 11-03, 101-14, Chardonnay and Cabernet sauvignon in the spring of 2013 and plated them onto either Murashige and Skoog minimal organics medium supplemented with 60 g/l sucrose, 1.0 mg/l 2, 4-D and 2.0 mg/l BAP (PIV) or Murashige and Skoog minimal organics medium supplemented with 20 g/l sucrose, 1.0 mg/l NOA and 0.2 mg/l BAP (NB medium). We have established robust embryogenic callus for 11-03 and Chardonnay from anther filaments collected in 2012 demonstrating that the results we achieved in 2011 were repeatable in 2012. In addition we have embryogenic callus forming from Chardonnay anther filaments harvested in the spring of 2013.

Next Steps:

- Generate embryos from anthers collected in spring 2013 for 11-03, 101-14, Chardonnay and Cabernet sauvignon.
- Continue to try to develop embryos from anthers of 101-14

B. Production of embryogenic callus from leaf explants

The National Research Laboratory of Chile (INIA) and UC Davis' Plant Transformation Facility (UCDPTF) are exploring 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 that we received from FPS (figure 1). We are still trying to apply INIA's technique in our lab, but to date we have not had much success with rootstock cultures. We continue to consult with INIA to trouble shoot the protocol.

Figure 1 Disease-free cultures of Chardonnay and 101-14 and Cabernet from Foundation Plant Services (FBS). We secured shoot tips from 1103 from newly established index plants from Russell Ranch.

Next Steps:

- Continue to work with our Chilean partners, to generate embryogenic callus from leaf explants of Chardonnay, Cabernet sauvignon, 101-14 and 11-03

C. Increasing embryogenic callus tissue

INIA has developed a method of rapidly increasing embryogenic callus by cycling the callus between agar-solidified medium and liquid media in shake flasks. This technique allows for rapid increase in callus fresh weight while minimizing oxidation and the development of detrimental phenolic compounds in the callus. We have modified this protocol and significant improved the production of embryogenic grape callus across a range of genotypes including 1103, 101-14 Cabernet and Chardonnay by modifying INIA's method of cycling the callus between agar-solidified medium and liquid media in shake flasks. Using anther filament callus generated in 2011, we are now maintaining stock suspension cultures on WPM medium 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). Embryogenic grape callus grown on agar-solidified medium is transferred to 25 mls of Pic/MTag in 125 ml shake flasks and grown on a gyratory shaker at 110 rpms in the dark. After the suspensions are established, 10 ml of the suspension is withdrawn each week from the flask and replaced with 10 mls of fresh medium. One ml of suspension that is removed from the flask is plated onto agar solidified Woody Plant Media (WPM) supplemented with 20 g/liter sucrose, 1g/liter casein hydrolysate, 500 mg/liter activated charcoal, 0.5 mg/liter BAP, 0.1 mg/liter NAA 400 mg/l (BN). This period we have further enhanced this system through the addition of 5% sorbitol to the plating medium which improves the quality of the embryogenic cultures that develop after plating. Embryogenic cells plated on BN medium produces high quality embryogenic cultures at the appropriate stage for use in transformation in approximately 4-8 weeks. Previously we maintained stock callus exclusively on agar-solidified plates which were subcultures every 3-4 weeks. Sub-culturing these plates was labor intensive since often the callus becomes oxidized when maintained on plates for long time intervals requiring non-oxidized callus to be carefully separated from oxidized callus. One of the advantages of our new suspension protocol is that it does not require cycling material between agar and liquid medium. Instead, cells are maintained in suspension by continually sub-culturing them on a weekly basis which results in the continual production of non-phenolic producing embryogenic cells (figure 2). These cultures have now been maintained for a full year without loss of regeneration potential of phenolic oxidation. New cell suspension cultures have been established from 2012 callus demonstrating that the procedure is repeatable.

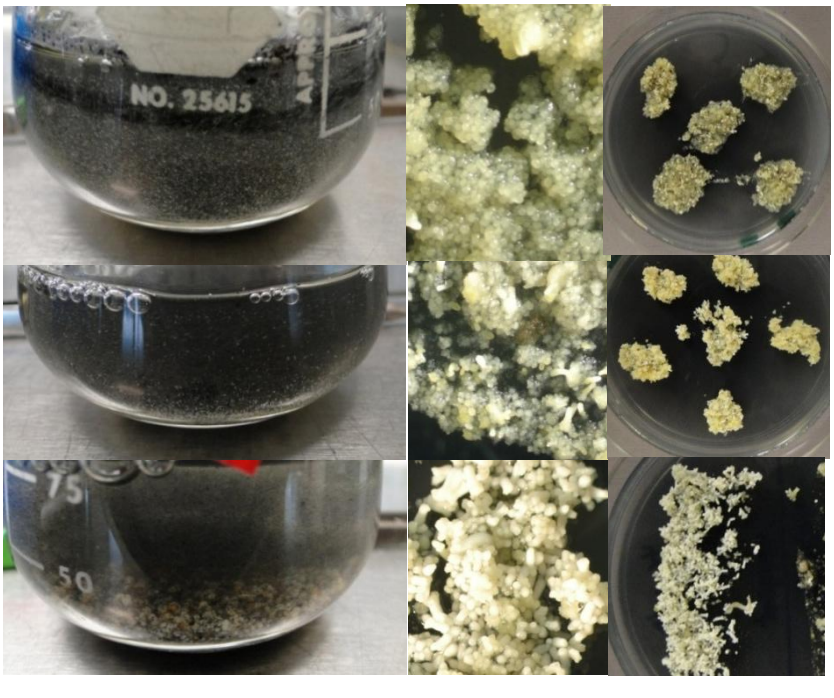

Figure 2. Production of high quality embryogenic callus from cell suspensions. Chardonnay (top), 1103 (middle) and 101-14 (bottom) in cell suspension cultures on WPM medium supplemented with 1g/l casein, 1M MES, 1000 mg/l activated charcoal,10 mg/l picloram, 2 mg/l meta-topolin, 100 mg/liter ascorbic acid and 120mg/l reduced glutothione (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.

2. Increase the efficiency and reduce the time required for *in vitro* regeneration of grape plants from embryogenic callus by adapting INIA's grape bioreactor technology and UC Davis Plant Transformation Facility's temporary immersion system (TIS) for use in grape tissue culture and transformation.

Progress:

In addition to evaluating INIA's liquid shake flasks methodology on grape genotypes 101-14, 1103, Chardonnay and Cabernet sauvignon, we are exploring UCDPTF's temporary immersion system (TIS) for use in rapidly increasing embryogenic callus. As reported previously, although this method of increasing embryogenic callus has proven very efficient, we were concerned because the callus produced was highly oxidized exhibiting excessive accumulation of phenolic compounds. However this period, 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 (figure 3). Growth rates of 1103 101-14 and Chardonnay callus cultures were repeated this period and we again demonstrated that robust cell growth can be achieved for a minimum of 3 months without addition of fresh medium to the bioreactor (figure 4). Based on these results, we are now using this temporary immersion system to increase 1103, 101-14, Chardonnay, Cabernet and Thompson Seedless. This system is advantageous from a labor management perspective, since it may allow one to maintain stock embryogenic cultures indefinitely in temporary immersion with medium exchanges occurring only once every three months. When needed sample of callus can be removed and transferred to agar-solidified medium a few weeks prior to initiating transformations.

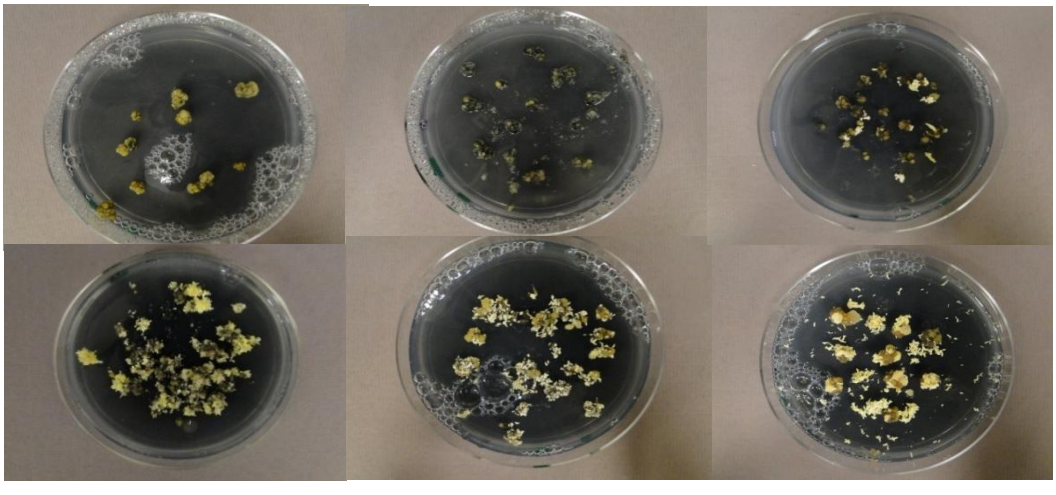

Figure 3. Callus growth of 1103, Chardonnay and 101-14 in temporary immersion system (TIS) on Pic/MT (top panels) and subsequent callus development after transfer to agar-solidified BN medium supplemented with 5 g/l sorbitol (lower panels).

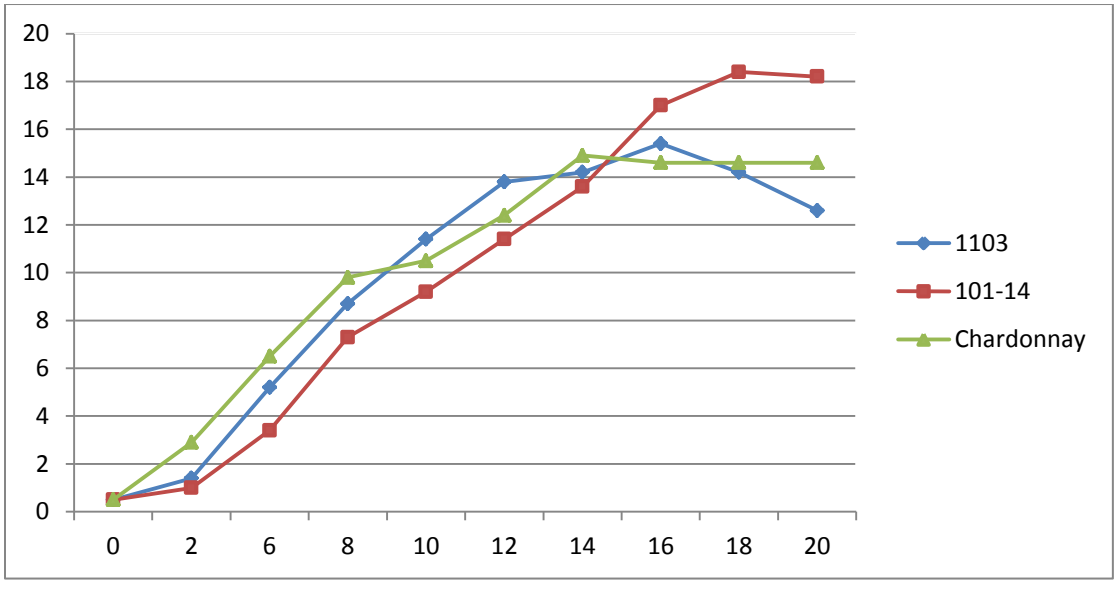

Figure 4. Embryogenic callus growth of 1103, 101-14 and Chardonnay in bioreactor demonstrating that callus can be maintained in bioreactors for three months with no medium additions of culture manipulations.

While we are 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. Embryos were generated from apical (leaf) buds in NB2 plates ('Red Globe' and 'Crimson') and transferred into X6 solid medium. Once they reach pro-embryo stage (usually 2 months), biomass is used as inoculum for the reactors. The bioreactors are filled with 400 mL of X6 medium. Considerable biomass production (still not quantified) is seen after 10-15 days of stirring (100 rpm) which can be filtered to re-inoculate the reactors or for return into regular solid media (figure 5).

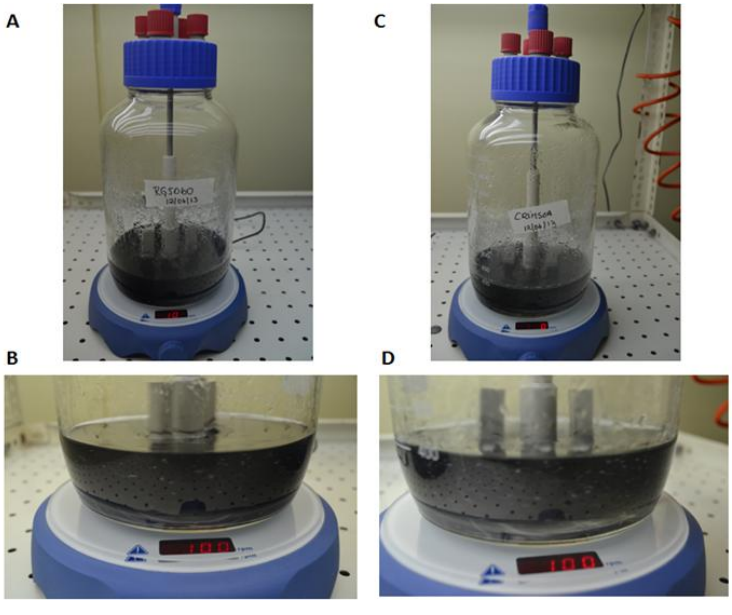

Figure 5. INIA is developing stirred tank reactors for SE genotypes in grapes. General (A and C) and detailed views (B and D) of the Red Globe (A and B) and Crimson (B and C) genotypes under culturing.

- **Next Steps:** Once INIA has verified the utility of their stir bioreactor, we will secure the design and test the system in Davis

3. Enhance the efficiency of whole plant regeneration from embryogenic callus of grape cultures.

As reported last period, preliminary results indicate that our standard grape regeneration medium is not optimal for genotype 1103. We found that although genotype 1103 benefits significantly from a reduction in the BAP concentrations further improvements were still required. Studies this period demonstrated that further reductions on hormone concentrations enhanced regeneration of non-transformed 1103 embryos and decrease the time required to regenerate whole plants (figure 6). This regeneration response in now approaching that seen in our model system, Thompson Seedless.

Germination Ratings for 1130

	NAA mg/l			
	0.1	0.5	1.0	5.0
BA mg/l				
0.1	**3**	3	2	1
0.5	3	2	2	1
1	2	2	2	1
5	1	1	1	1

Germination Ratings for 1130

	NAA mg/l			
	0	0.01	0.05	0.1
BA mg/l				
0.0	3	3	4	5
0.01	5	4	4	4
0.05	3	2	2	2
0.1	**5**	4	3	3

Figure 6. Rating for regeneration of non-transgenic 1103 embryos (5 best to 1 worst) on WPM + 1g/l casein + 1M MES + 500 mg/l activated charcoal + various concentrations of BAP and NAA. Photos to the right of the tables are of plants growing on 0.1 mg/l BAP + 0.1 mg/l NAA) (top right) and 0.1 mg/l BAP + 0.0 mg/l NAA (lower right).

Next Steps:

- Continue to develop media formulations that will allow maintenance rapid regeneration of shoots from embryogenic callus of 1103, 101-14.

Objective II. 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.

Progress:

Increasing the osmoticum of the medium significantly improves the quality of the somatic embryo cultures of rootstock genotypes

Last period we investigated how the medium on which the stock callus was maintained influences the transgenic response. Using Thompson Seedless as a model system , we found that after only nine weeks post inoculation, a much higher percentage of transgenic colonies were generated from callus maintained on WPM + 1g/l casein + 1M MES + 500 mg/l activated charcoal + 0.5 mg/l BAP + 0.1 mg/l NAA compared to our standard Pic/MT medium. Furthermore transgenic embryos developed much faster on callus from the BN treatment that the Pic MT treatment. This period we have applied these techniques to the rootstock genotypes 101-14 and 1103. However one of the issues we have observed with maintaining callus of 101-14 and 1103 on BN medium is that embryos germinate prematurely. Due to the risk of generating chimeric transgenic plants, these large embryos make poor targets tissue for transformation. Based on our work with rice, we have investigated the additional of the sugar alcohol sorbitol into the medium to increase the osmoticum of the medium. We evaluated sorbitol at 0, 3, 6 and 12%. Increasing sorbitol significantly inhibits germination of grape embryos allowing them to be maintained at the heart and small torpedo stage for extended periods of time resulting in better stage embryos for use in transformation than on PIC/MT medium (figure 7). We have now begun to routinely maintain callus stock cultures on medium containing 5% sorbitol.

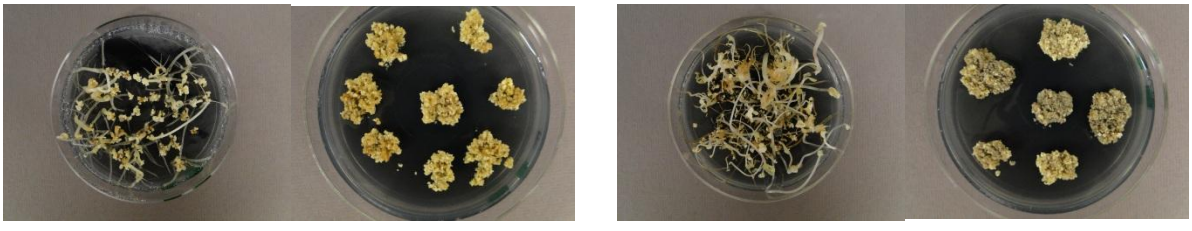

Figure 7. Influence of sorbitol on maintenance of embryogenic cultures of 1103 (left) and Chardonnay (right). Cells plated on BN medium without sorbitol develop large embryos whereas cells plated on BN medium supplemented with 5% sorbitol remain as compact globular, heart and torpedo shaped embryos.

We are transforming 101-14 and 1103 embryo cultures maintained on BN medium supplemented with 3% sucrose and 5% sorbitol and are developing transgenic putative transgenic cultures. These embryos are being transferred to different regeneration medium in an attempt to identify a medium that allows for rapid regeneration of transgenic embryos. We are currently in the process of determining transformation frequencies for these genotypes and improving regeneration of transgenic colonies into whole plants.

Heat shock treatment has been used in some species to enhance transformation frequencies. Using Thompson Seedless as a model species, we investigated the use of a 10 minute heat shock at 45 degrees centigrade on transformation of embryogenic grape callus. In paired experiments, we found that subjecting callus to heat shock prior to agrobacterium inoculation significantly increased the transformation efficiency (table 1 and figure 8). Based on these results in Thompson Seedless we are applying heat shock technology to 101-14 and 1103 transformations (figure 9).

Table 1. Thompson Seedless callus transformation experiments comparing transformation efficiencies after subjecting callus to 10 minutes of heat shock at 45 degrees centigrade verses no application of heat shock prior inoculation with Agrobacterium.

Experiment	Heat Shock prior to inoculation	Selection	# transgenic colonies	% transgenic colonies
121092	+	Hygro	13/16	81%
121090	-	Hygro	1/16	6%
121029	+	Kan	17/32	53%
121028	-	Kan	3/30	10%

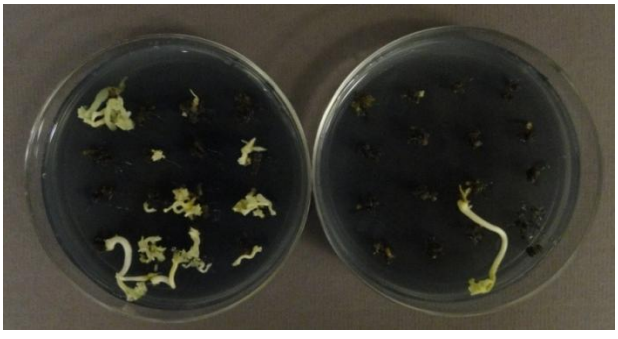

Figure 8. Heat shock enhancement of transformation frequencies as demonstrated in the model system Thompsons seedless. Callus was exposed to 10 min HS at 45 degrees centigrade (left) verses non-treated (right) prior to inoculation with agrobacterium.

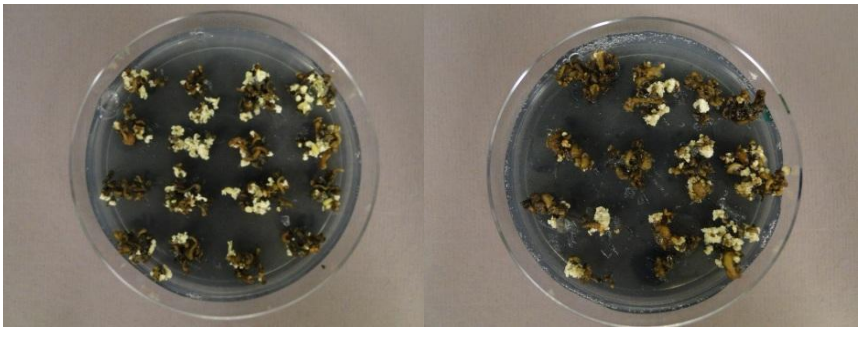

Figure 9. Heat shock enhancement of transformation frequencies applied to 101-14 (left) and 1103 (right)l. Callus was exposed to 10 min HS at 45 degrees centigrade prior to inoculation with Agrobacterium (white calli are putative transgenic colonies).

When using heart and torpedo staged embryos as target tissue for transformation there is always the concern that chimeric plants will be generated. To evaluate this, we have transformed heart and torpedo staged embryos of 1103 using the scorable marker dsred and tracked the development of the transgenic embryos using a fluorescent stereoscope. If maintained on high levels for selection the production of chimeric plants does not appear to be a significant issue (figure 10).

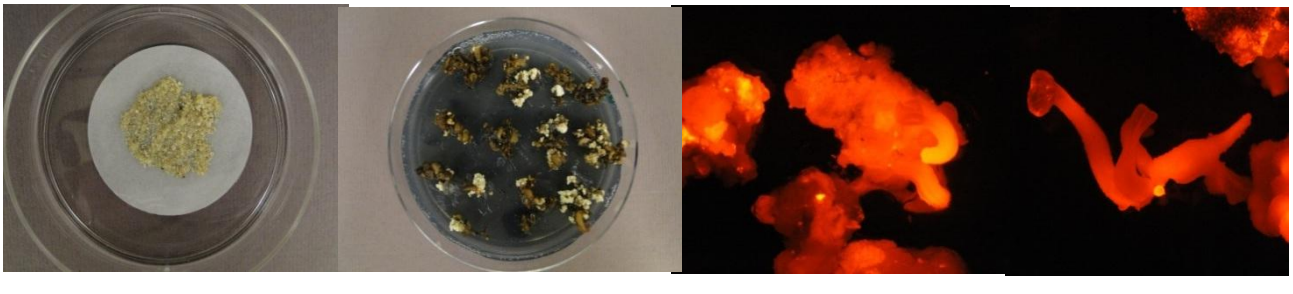

Figure 10. Transformation of 1103 embryos from suspensions grow in shake flasks then plated onto BN medium plus 5% sorbitol prior to inoculating with a dsred containing construct. Dsred expressing callus and non-chimeric embryos are clearly visible.

Next Steps:

- Increase transformation efficiencies for Chardonnay and Cabernet Sauvignon and regeneration efficiencies for 11-03, 101-14

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

- Presented an update to CDFA Board and Task Force meeting on June 21 2013

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 (PTF) 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. 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. This period we have made significant advances in our ability to increase transformation efficiencies in Thompson Seedless, 1103 and 101-14 by employing a heat shock pretreatment to the tissue prior to inoculating with Agrobacterium. By maintaining stock cells on an improved medium, we have significantly reduced the time required to generate transgenic Thompson Seedless plants from 12 to 6 months. These parameters are now being tested in genotypes 1103 and 101-14 to see if they are applicable for these genotypes. We have also begun maintaining stock cultures using bioreactor technology. By maintaining our bioreactors in the dark, we have also been able to produce high quality embryogenic cultures for 1103 and 101-14 using our temporary Immersion System (TIS) for over three months without manipulation of the cultures resulting in a significant reduction of labor. When transferred from bioreactors to agar solidified medium, this callus converts to embryogenic cultures at a stage ideal for transformation within 4-8 weeks. Lastly, we have made improvements in the medium needed to regenerate 1103 from callus to whole plants. See Table 2 for a summary of the achievements to date.

K. Status of funds.

We anticipate that all funds allocated for fiscal year FY2013-2014 will be expended by the end of the fiscal year.

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.

Table 2. Advancements toward the Development of a Grape Tissue Culture and Transformations Platform for the California Grape Research Community

Goal	UCD PTF Pre-Grant Grape transformation Expertise	UCD PTF Grape transformation Expertise Developed with grant funding	Status	Technology Provider
Number of Genotypes for which we can generate embryogenic cultures from anther filaments	Prior to the grant we only worked with Thompson Seedless	We now have established embryogenic cultures of 1103, Cabernet, Chardonnay, and Thompson Seedless	Ongoing - We are still working on generating embryos from anther filaments for 101-14	UCD
Number of Genotypes for which we can generate embryogenic cultures from in vitro leaves	Not available	We are developing this technology using indexed in vitro plants provided by Foundation Plant Services	Ongoing - INIA is working with UCD to transfer the technology to genotypes important to California	INIA
Reduce labor of maintaining embryo cultures to allow PTF to handle more genotypes-Cell Suspension Technology	Prior to the grant we could only handle Thompson Seedless callus which we grew on agar medium required 3-4 hours labor every 3 weeks and resulted in poor quality callus	We have developed high quality embryo shake flask cultures for 1103, 101-14, Cabernet, Chardonnay and Thompson Seedless requiring labor of only 20 minutes per week to maintain.	Complete	INIA and UCD
Reduce labor of maintaining embryo cultures to allow PTF to handle more genotypes-Bioreactor Technology	Prior to the grant this expertise was not available	We have develop Temporary Immersion bioreactors for 1103, 101-14, Cabernet, Chardonnay, Thompson Seedless callus culture requiring labor of 1 hour every 3 months to maintain	Complete	UCD
Reduce labor of maintaining embryo cultures to allow PTF to handle more genotypes-Spin Flask Technology	Prior to the grant this expertise was not available	Developing Spin flask bioreactors cultures	In progress	INIA
Increase Transformation Efficiency	Prior to the grant Thompson Seedless transformations required 12-14 months to generate transgenic grape vines	Thompson Seedless transformations now require only 6 months to generate transgenic grape vines	Complete	INIA and UCD

Increase range of genotypes that can be transformed	Prior to the grant PTF could only transform Thompson Seedless	Although improvements in transformation frequencies are still required, PTF can now transform Thompson Seedless, 1103, 101-14 and Chardonnay	In progress frequency of transformation and regeneration still need to be improved	INIA and UCD
Increase regeneration frequencies for transgenic embryos of wine and rootstock genotypes	Prior to the grant regeneration of transgenic Thompson Seedless embryos required 8-10 months	Regeneration of transgenic Thompson Seedless embryos now required only 3 months	We are In the process of applying these new techniques to rootstock and wine genotypes	INIA and UCD
Prevent chimeric plant production	Prior to the grant Chimeric plants common	Based on dsred expression, chimeric plants appear to be rare	Complete	UCD