A. Interim Progress Report for CDFA Agreement Number 14-0486-SA

B. Expanding the range of grape rootstock and scion genotypes that can be genetically modified for use in research and product development

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D. Time period covered by the report:

January 15, 2015-July 31, 2015

E. Introduction:

This proposal is aimed at applying the progress that has been made in grape cell biology and transformation technology of rootstock genotypes 1103P and 101-14 to additional grape rootstock genotypes in order to expand the range of genotypes amenable to transformation. The research will apply the pre-existing expertise and technical know-how developed for rootstocks 1103P and 101-14 at UC Davis's Plant Transformation Facility to additional rootstocks germplasm important for the California wine industry. For this proposal, we are testing seven additional rootstocks for their amenability to transformation including; 110R, 3309C, Freedom, Harmony, 420A, 140Ru and Salt Creek. This work will expand the range of rootstocks that can be effectively transformed which will allow rootstock-mediated disease resistance technology to be employed across the major wine growing regions in California. Although a rootstock-mediated resistance strategy is the preferred mechanism for achieving resistance to Pierce's Disease in grape, investing in the development of transformation technology for scions will serve an important fallback position should rootstock-mediated resistance fail to confer adequate levels of resistance to the scion and direct transformation of scion varieties be required. Therefore, in addition to testing the utility of our tissue culture and transformation protocols on six additional grape rootstocks, we will also screen six important California scion genotypes for their amenability to transformation including Cabernet Sauvignon (clone 07), Chardonnay (clone 04), Pinot Noir (2A), Merlot (clone 03), Zinfandel (clone 01A), and French Colombard (clone 02). Although it is unlikely that all seven rootstock and six scion genotypes will be amenable to transformation using our established protocols, we believe that a significant number will respond positively. The results of this work will allow for the establishment grape tissue culture and transformation technologies that can be utilized by the PD/GWSS Research Community. It will also establish a germ bank of cell suspension cultures and a repository of somatic embryos for rootstock and scion genotypes used in California, which can be made available to the research community.

F. List of objectives:

1. Develop embryogenic cultures from anther of seven rootstock genotypes and six scion genotypes for use in establishing embryogenic suspension cultures.

2. Develop embryogenic suspension cultures for seven rootstock genotypes and six scion genotypes, which will provide a continuous supply of somatic embryos for use transformation experiments.

3. Establish a germplasm bank of somatic embryos for seven rootstock genotypes and six scion genotypes by plating aliquots of the cell suspension culture on high osmotic medium.

4. Test transformation efficiencies of seven rootstock genotypes and six scion genotypes using our established somatic embryo transformation protocols.

5. Test direct cell suspension transformation technology on seven rootstock genotypes and six scion genotypes.

6. Secure in vitro shoot cultures for seven rootstock genotypes and six scion genotypes using indexed material from Foundation Plant Services and establish bulk meristem cultures for all 13 genotypes for use in transformation.

7. Test Mezzetti et al., 2002 bulk meristem transformation system for seven rootstock genotypes and six scion genotypes as an alternate to somatic embryo transformation.

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

Objective 1. Develop embryogenic cultures from anthers of seven rootstock genotypes and six scion genotypes for use in establishing embryogenic suspension cultures.

Progress:

We collected anthers of rootstock genotypes including 3309C (05), Freedom (01), Richter 110 (01), MGT 420A (04), 140Ru (01), Salt Creek (08) 11-03, 101-14, and scion genotypes Cabernet Sauvignon (clone 07), Chardonnay (clone 04), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard (clone 04) and plated them on four different embryogenic callus inducing media. The media include; 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), MS minimal organics medium supplemented with 20 g/l sucrose 1.0 mg/l 2,4-D and 0.2 mg/l BAP (MSE), MS minimal organics medium supplemented with 30 g/l sucrose 1.0 mg/l 2,4-D and 1.0 mg/l BAP (MS1)or one half strength MS minimal organics medium supplemented with 15 g/l sucrose 1.0 mg/l NOA and 0.2 mg/l BAP (NB). Anthers were collected at two separate weeks in the spring of 2015. The number of anther clusters plated for each genotype is given in table 1, and the number of putative embryogenic calli or embryos developing to date is provided in table 2.

Table 1. Summary of the number of plates of grape anthers explanted to four different media formulations

GRAPE ANTHERS	MSI	MSE	PIV	NB
Zinfandel 01A R5V12	4	1	3	4
Colombard 04 R1V13	3	1	4	3
Pinot Noir 02A R6V17	2	1	4	3
MGT 420A R2V3	4	2	3	4
140Ru 01 R2V7	5	2	5	4
Freedom 01 R4V6	5	2	6	5
3309C 05 R1V3	4	1	4	4
CAB 07 R2V12	4	3	3	4
Salt Creek 08 R2V3	3	0	4	3
TS PIA-9 (transgenic)	6	1	2	3
TS 02A C1V21	2	2	2	2
110R R3V3	1	0	1	1
1103 A6V2	1	1	1	0
101-14 A5V5	1	0	1	1
Total	45	17	43	41

Table 2. Number (percentage) of anther clusters forming putative embryogenic callus or embryos to date.

Genotype	PIV	MSE	MS1	NB
3309C (05)	1/196 (0.5)	0/196 (0)	0/196 (0)	0/196 (0)
Freedom (01)	0/294 (0)	0/49 (0)	0/147 (0)	0/245 (0)
Richter 110 (01)	0/49 (0)		0/49 (0)	2/49 (4)
MGT 420A (04)	1/147 (0.7)	1/98 (1.0)	5/196 (2.5)	1/196 (0.5)
140Ru (01)	0/49 (0)		0/49 (0)	0/49 (0)
Salt Creek (08)	5/196 (2.5)		4/147 (2.7)	1/147 (0.7)
11-03	0/49 (0)	1/49 (2.0)		8/49 (16)
101-14	0/49 (0)		0/49 (0)	0/49 (0)
Cabernet Sauvignon (07)	1/98 (1.0)	4/147 (2.7)	1/147 (0.7)	1/196 (0.5)
Cabernet Sauvignon (08)	5/539 (0.9)			
Pinot Noir (2A)	4/196 (2.0)	0/49 (0)	0/96 (0)	6/147 (4.0)
Zinfandel (01A)	2/147 (1.7)	0/49 (0)	11/196 (5.6)	0/196 (0)
Colombard (04)	7/172 (4.1)	0/49 (0)	16/123 (13.0)	2/123 (1.6)

Next Steps:

Increase developing callus/embryos on agar-solidified medium prior to initiating suspension cultures.

Objective 2. Develop embryogenic suspension cultures for seven rootstock genotypes and six scion genotypes, which will provide a continuous supply of somatic embryos for use transformation experiments.

Progress:

We have established suspension cultures for Richter 110, 1103, 101-14 and Chardonnay from callus initiated in 2014. Once we have initiated adequate amounts of callus from anther of the other genotypes which we excised in spring 2015, we will initiate suspension cultures for rootstock genotypes 3309C (05), Freedom (01), MGT 420A (04), 140Ru (01), Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard

Next Steps:

Initiate suspension cultures for rootstock genotypes 3309C (05), Freedom (01), MGT 420A (04), 140Ru (01), Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard once adequate amounts of embryogenic tissue has developed.

Objective 3. Establish a germplasm bank of somatic embryos for seven rootstock genotypes and six scion genotypes by plating aliquots of the cell suspension culture on high osmotic medium.

Progress:

We have established a germplasm bank of somatic embryos on 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, 5% sorbitol and 14 g/l phytoagar (BN-sorb) for Richter 110, 1103, 101-14 and Chardonnay which provide a reliable source of embryos for use in transformation studies.

Next Steps:

Once embryogenic suspension cultures are initiated, establish a germplasm bank of stored somatic embryos for rootstock genotypes 3309C (05), Freedom (01), MGT 420A (04), 140Ru (01), Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard by plating aliquots of suspension cultures on sorbitol containing medium

Objective 4. Test transformation efficiencies of seven rootstock genotypes and six scion genotypes using our established somatic embryo transformation protocols.

Progress:

Transformation experiments have been initiated using known amounts for somatic embryos as determined by fresh weight for Richter 110, 1103, 101-14 and Chardonnay using a construct containing the DsRed florescent scorable marker gene which will allow us to monitor the progress of transformation in real time without sacrificing any tissue (table 3). Thompson Seedless is being included as a positive control. DsRed expression will be evaluated at 1, 2 and 3 months post inoculation.

Next Steps:

Once germplasm banks of somatic embryos are established, begin testing our transformation system on somatic embryos of rootstock genotypes 3309C (05), Freedom (01), MGT 420A (04), 140Ru (01), Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard using the scorable marker gene DsRed.

Table 3. Transformation experiments to access the amenability of transformation of stored grape embryos for a range of rootstock and scion genotypes using the scorable fluorescent marker gene DsRed.

Variety	Date	Experiment #	Tissue Weight
TS-14 (Control)	6/26/2015	159050	0.53
	7/24/2015	159070	0.52
Chardonnay	6/26/2015	159048	2.72
	7/10/2015	159064	1.12
	7/17/2015	159068	1.12
	7/24/2015	159071	0.57
Richter	6/26/2015	159049	0.49
	7/10/2015	159065	1.65
	7/17/2015	159069	1.83
	7/24/2015	159072	0.42
1103	7/24/2015	159073	1.11
101-14	7/24/2015	159074	0.86

Objective 5. Test direct cell suspension transformation technology on seven rootstock genotypes and six scion genotypes.

Progress:

We are leveraging the progress we have made in developing high quality cell suspensions that have the ability to rapidly regenerate whole plants when plated onto agar-solidified medium by exploring direct transforming our grape cell suspension cultures with the scorable marker gene DsRed. One to two ml of a grape cell suspensions grown in liquid Pic/MT 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 cells are subjected to heat shock by placing the conical tube in a 45 degree water bath for 5 minutes. After heat shock the supernatant is removed and replaced with 5 ml liquid BN medium containing 200 uM acetosyringone and the *Agrobacterium* strain and appropriate 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 ml of the supernatant is removed. The grape and *Agrobacterium* cells are then re-suspended and transferred to sterile Whatman filter paper in an empty 100 x 20 mm petri dish. Any excess fluid was carefully bloated up with a second sterile filter paper. The plates 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, 200 mg/l kanamycin, 50 g/ sorbitol and 14 g/l agar. The filter paper is transferred to fresh medium every 2 weeks. Within eight weeks resistant embryos develop. 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, 400 mg/l carbenicillin, 150 mg/l timentin, 200 mg/l kanamycin, 0 g/ sorbitol and 8 g/l agar for germination. The time from inoculation to the recovery of germinating embryos is only 10 weeks (figure 1).

Figure 1. Transformation of cell suspension cultures of 1103 and development of transgenic embryos on 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 transfer to WPM supplemented with 20 g/l sucrose, 1g/l casein, 1M MES, 500 mg/l activated charcoal, 0.1 mg/l BAP, 8 g/l agar for plant regeneration (bright field top panel, fluorescence, lower panel)

We are testing the direct cell suspension transformation on Richter 110, 1103, 101-14 and Chardonnay and using the DsRed transgene. However we continue to observe highly variable transformation frequencies from experiment to experiments. Some experiments result in very high numbers of resistant embryos while other experiments fail to produce any embryos. The more critical issue is that transgenic embryos that form, although normal in appearance, have been very recalcitrant to regenerate into whole plants. Some continue to develop multiple embryo clusters while others fail to germinate. We will continue to test additional genotypes using this

system. We need to achieve more consistent transformation frequencies of suspension cultures and more consistent regeneration of whole plants from the transgenic embryos if this transformation method is to serve as a viable alternative to the transformation of stored embryos. A summary of the experiments and the transformation frequency is given in table 4.

Table 4. Number of embryogenic colonies forming after inoculating approximately one ml of cell suspension with Agrobacterium and plating onto selection medium.

Genotype	Experiment	# 0f putative transgenic embryos/ml of plated suspension
101-14	151068	19
	151111	16
	151119	2
	151139	1
	151050	0
	151042	0
	151077	0
1103	151069	0
	151030	0
	151029	0
	151026	0
	151025	0
	151115	3
	151140	0
	151043	0
	151078	0
Chardonnay	151119	2
	151080	0
Richter 110	151070	1
	151117	callus
	151141	0
	151040	0
	151079	0
Thompson Seedless	151049	0
	151113	0
	151076	0

Next Steps:

Once embryogenic suspension cultures are initiated for 3309C (05), Freedom (01), MGT 420A (04), 140Ru (01), Salt Creek (08), and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone

01A), and Colombard, test direct transformation of suspension cultures using the scorable marker gene DsRed.

Objective 6. Secure in vitro shoot cultures for seven rootstock genotypes and six scion genotypes using material from Foundation Plant Services fields and establish bulk meristem cultures for all 13 genotypes for use in transformation.

Progress:

We are maintaining disease free *in vitro* stock plants of 101-14, Chardonnay and Cabernet that we received as *in vitro* cultures from Foundation Plant Services (FPS). For material that was not available through FPS we have collected shoot tips from field material grown at FPS. This includes genotypes 3309C (05), Freedom (01), Richter 110 (01), MGT 420A (04), 140Ru (01), Salt Creek (08) 11-03, and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), and Colombard (clone 04). Four inch shoot tips were collected from the FPS collection, cut into 3 inch sections, transferred to 50 ml centrifuge tubes and surface sterilized in 0.526% sodium hypochlorite for 15 minutes followed by three rinses in sterile distilled water. The shoot tip was cut into nodal sections and any tissue damaged by sterilization was removed. The nodal sections were transferred onto agar solidified Chee and Poole C2d Vitis medium containing 5mg/l chlorophenol red or agar solidified MS minimal organics medium supplemented with 3.0 mg/l BAP, 0.1 mg/l IBA, 0.1 mg/l GA3 and 5 mg/l chlorophenol red . The addition of chlorophenol red to the medium allows us to identify any contaminated shoots before the bacteria or fungus is visible based on the pH change of the medium. Once aseptic shoot cultures are established, we will transfer shoot meristems to establish bulk meristem cultures as described by Mezzetti et al, 2002.

We have generated the bulk meristems 11-03, 101-14, Chardonnay and Cabernet sauvignon. To generate bulk meristem cultures, shoot tips are excised and transferred to Mezzetti salts modified with 1.0 mg/l BAP. After 4 weeks tissue was transferred to Mezzetti medium with 2.0 mg/l BAP and finally after an additional 4 weeks tissue was transferred to Mezzetti medium with 3.0 mg/l BAP. Bulk meristems developed readily for Thompson Seedless, Chardonnay and Cabernet. We initially had established bulk meristem cultures for 1103 and 101-14, but these do not appear to be sustainable over long periods of time on the medium we are using. Repeated sub-culturing of this tissue on 3 mg/l BAP resulted in rapid callus growth. We also tried substituting Tridiazuron (TDZ) for BAP at 3 mg/l but this did not solve the re-callusing issue.

Next Steps:

Generate bulk meristem cultures for rootstock genotypes 3309C (05), Freedom (01), Richter 110 (01), MGT 420A (04), 140Ru (01), Salt Creek (08) 11-03, and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A).

Objective 7. Test Mezzetti et al., 2002 bulk meristem transformation system for seven rootstock genotypes and six scion genotypes as an alternate to somatic embryo transformation.

Progress:

Bulk meristems of Thompson Seedless, Chardonnay and Cabernet Sauvignon were sliced into thin, 2mm slices and inoculated with agrobacterium strain EHA105 and co-cultures on Mezzetti medium supplemented with 3 mg/l BAP at 23 degrees centigrade. After three days, the thin slices were transferred to Mezzetti medium supplemented with 3 mg/l BAP , 400 mg/l carbenicillin, 150 mg/l timentin and 25 mg/l kanamycin sulfate. After three weeks tissue was transferred to the same medium formulation, but the kanamycin level was increased to 50 mg/l. After an additional three weeks the tissue was transferred to medium of the same formulation but the kanamycin level was increase to 75 mg/liter. Subsequently tissue was subcultures every three weeks on medium containing 75 mg/l kanamycin. Since the construct used to transform the bulk meristems contained DsRed we were able to monitor transformation efficiencies in real time. To date, we have only been successful producing transgenic shoots from bulk meristems of Thompson Seedless. Twenty four of the 75 thin slices sections of Thompson seedless produced deRed sectors and three of these regenerated into shoots. We were able to produce DsRed expressing callus on Cabernet sauvignon and Chardonnay, but none of this tissue regenerated into shoots (Table 5). In our hands the use of kanamycin at 75mg/l appears to be suboptimal for selection. Although we did identify a limited number of DsRed shoots for Thompson Seedless, many additional shoots which developed on selection

medium containing 75 mg/l kanamycin were non-transgenic based on DsRed expression. If it were not for the use of the scorable marker DsRed, we would not be able to distinguish the true transgenic shoots from the non-transgenic escape shoots until they were transferred to rooting medium with kanamycin. We will consider using 100 mg/l kanamycin or more in the future.

Table 5. Preliminary results of bulk meristem transformation using the scorable marker gene DsRed

Genotype	Number (%) explants generated DsRed callus	Number (%) explants generated DsRed shoots
Cabernet sauvignon	1/36 (3)	0/36 (0)
Chardonnay	2/38 (5)	0/38 (0)
Thompson Seedless	24/75 (32)	3/75 (4)

Next Steps:

Once bulk meristem cultures have been established for genotypes 3309C (05), Freedom (01), Richter 110 (01), MGT 420A (04), 140Ru (01), Salt Creek (08) 11-03, and scion genotypes Cabernet Sauvignon (clone 07), Pinot Noir (clone 2A), Zinfandel (clone 01A), test thin sliced transformation using the scorable marker gene DsRed using higher concentration of kanamycin in the selection medium.

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

No presentations or publications have been made to date under this agreement

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

UC Davis Plant Transformation Facility has previously developed grape transformation technology for 101-14 and 1103P; two important grape rootstocks for the California grape industry for use in rootstock-mediated Pierce's disease resistance strategy. However, if rootstock-mediated resistant strategies are to be successful deployed, additional rootstock genotypes will need to be transformed in order to adequately cover the major wine growing regions in California. We therefore are testing our grape rootstock transformation technology on seven additional rootstock genotypes including 110R, 3309C, Harmony, Freedom, 420A, 140Ru and Salt Creek. Additionally, it is not yet known if a rootstock-mediated Pierce's disease resistance strategy will prove to confer durable, commercially viable levels of resistance to the grafted scion. If rootstock-mediates resistance proves unsuccessful, direct transformation of scion clones with disease resistant transgenes may be required. Therefore as a fallback strategy, in addition to testing the range of rootstocks that we can transform, we are also testing our existing transformation technology on a select group of scions. For this proposal we are testing our transformation protocol to six scion varieties including Cabernet Sauvignon (clone 07), Chardonnay (clone 04), Pinot Noir (clone 2A,), Zinfandel (clone 01), Merlot (clone 03) and French Colombard (clone 02).

J. Layperson summary of project accomplishments.

UC Davis Plant Transformation Facility has previously developed a method for genetically modifying 101-14 and 1103P; two important grape rootstocks for the California grape industry. This technology will allow us to introduce genes useful in combating Pierce's disease into the rootstocks of grape and allow us to test whether a modified rootstock is capable of conferring resistance to the grafted scion. This strategy is commonly referred to as rootstock-mediated resistance. If rootstock-mediated resistant strategies are to be successful deployed

throughout California, additional rootstock genotypes besides 1103P and 101-14 will need to be modified in order to adequately address the rootstock requirements of the diverse wine growing regions in the State of California. We therefore are currently testing if our method for genetically modifying grape rootstocks can be used successfully on seven additional rootstock genotypes used in California wine grape production. These include; 110R, 3309C, Harmony, Freedom, 420A, 140Ru and Salt Creek. Since it is not yet known if a rootstock-mediated disease resistance strategy will prove to confer durable, commercially viable levels of resistance to the grafted scion we are also testing our method for modifying grapes on a select group of scions including; Cabernet Sauvignon (clone 07), Chardonnay (clone 04), Merlot (clone 03), Pinot Noir (clone 2A,), Zinfandel (clone 01), and French Colombard (clone 02). The results of this work will allow for the establishment of a self-sustaining grape tissue culture and transformation service that can be utilized by the PD/GWSS Research Community. It will also establish a germ bank of cell suspension cultures and a repository of somatic embryos for rootstock and scion genotypes used in California, which can be made available to the research community.

K. Status of funds.

We anticipate that all funds allocated for fiscal year FY2015- will be expended by the end of the fiscal year.

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

Methods developed under this proposal will be employed as part of a, cost-effective grape tissue culture and transformation service that can be utilized by the PD/GWSS Research Community.

References

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