A. Project Title: Enabling Technologies for Grape Transformation

B. CDFA Contract Number: 06-0224

C. Time period covered by the progress report: This report covers the period from September 2007 to March 2008. Work discussed here reflects a project funded for the period of June 1, 2006 to May 31, 2008.

D. Principle investigator: Alan B. Bennett, Executive Director, PIPRA, UC Davis
Cooperators: Cecilia Chi-Ham, PIPRA, University of California. David Tricoli, Director, Plant Transformation Facility, UC Davis

E. List of objectives, and description of activities conducted to accomplish each objective

1. Design, develop, and validate a grape-specific transformation system that addresses legal IP, technical and regulatory considerations.

We completed the DNA synthesis and cloning strategy of such recombinase-based marker excision transformation vector. We initiated the physical cloning and assembly of the plant transformation vector which will be necessary for validating the individual components (i.e. selectable markers) and entire recombinase transformation system. Up to now, we have completed about 90% of the assembly. In parallel to synthesizing the recombinase-vector, we have initiated the validation of the plant-based selectable markers, DEF2 and Atwbc19. In this report we present the update of the performance of both of these markers. Based on the legal status of kanamycin, it is increasingly becoming a marker that may be worth considering for application in grapes. Last quarter we started negotiations with Wageningen University and Research Centre to access a recombinase-based vector with an inducible site-specific recombinase that was developed for strawberries (Schaart, Krens et al. 2004). However, these materials had too many intellectual property restrictions for the purpose of the PD research; thus we abandoned pursuing Wageningen's vectors.

2. Develop Agrobacterium and TransBacter™-mediated transformation for California wine grapes and/or cultivars suitable for generating root stocks.

PIPRA completed the design of a recombinase-based transformation vector which should be suitable for Agrobacterium or TransBacter™-mediated transformation protocols. PIPRA engaged in discussions to access the TransBacter™ technology from CAMBIA, Australia. Based on IP limitations of the agreements to obtain TransBacter™, we chose to abandon pursing these materials. Last term, we explored the possibility, from a technical and legal standpoint, of utilizing early 1980's technology based the use of other non-agrobacterial alternatives, i.e. *Rhizobium trifolli*, to transform plants. In this last quarter, we formulated a research strategy in collaboration with Dr. Stan Gelvin, Purdue. The research to develop non-agrobacterial alternatives for plant/grape transformation is outside the scope of this proposal; nevertheless, we have developed an experimental design.

3. Develop strategies to disseminate the biological resources developed under appropriate agreements for the PD community.

On Feb 27th, PIPRA hosted the second formal meeting to propose a Licensing Model to the technology providers of the core components for the transformation system. The proposed licensing terms and structure is being reviewed by the technology providers and UC Davis

Office of Research Staff. These activities will allow the development of strategies to disseminate the plant transformation vectors to the PD research and commercial community.

4. **Explore collaborative opportunities with researchers developing Pierce's disease control strategies that could employ and test the proposed grape transformation system.**

In this reporting period, we met with Dr. Abhaya Dandekar to discuss the possibility of testing the pPIPRA vectors by an independent researcher. To increase the likelihood that the PD research community will adopt this transformation platform, it is important that the vectors are verified by an outside party. In addition, in collaboration with Dr. John Labavitch, we submitted a research proposal that incorporates the pPIPRA transformation platform (Optimizing grape rootstock production and export of inhibitors of *X. fastidiosa* PG activity**).**

F. Research accomplishments and results for each objective

1. Design, develop, and validate a grape-specific transformation system that addresses legal IP, technical and regulatory considerations.

Summarized below is an update of last quarter's progress towards the development of a marker-free plant transformation system for the PD community.

Assembly of Recombinase-Based Plant Transformation Cassettes

Thus far, PIPRA has designed and synthesized about 90% of the recombinase transformation vector (Figure 1). We are currently cloning the last component of the recombinase transformation vector, the recombinase cassette. We expect to complete the entire assembly of the recombinase transformation vector including the recombinase cassette in April 2008.

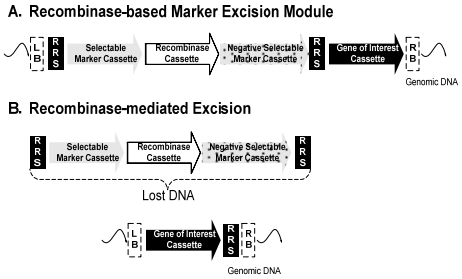

Figure 1. Diagram of the Recombinase-Excision Strategy.
Panel A Recombinase-based transformation construct contains a gene of interest cassette and two selectable marker cassettes flanked by recombinase recognition sequences (RRS). Panel B: Inducible expression of the recombinase excises the selectable marker cassette. Transgenic lines with successful recombination events can be isolated by treatment with a negative selection agent and expression of the gene of interest, i.e. marker gene, GFP.

Validation of Components of the Recombinase-based marker excision System

Determining Efficacy of Inducible Promoter Activity in Tobacco and Grape

The recombinase enzyme in this marker-free system will be regulated by an inducible promoter. This transcription regulatory control is necessary in order to activate excision activity during specific stages of the transformation process. We are considering incorporating either the estrogen (Zuo, Niu et al. 2000) or glucocorticoid (Aoyama and Chua 1997; Ouwerkerk, de Kam et al. 2001)-inducible promoter systems developed by the Chua laboratory at Rockefeller University. Currently, we are prioritizing testing the XVE, estrogen-inducible system in tobacco and grapes because it has been previously used in a cre-lox based marker free system in Arabidopsis (Zuo, Niu et al. 2001). In addition, the glucocorticoid-system utilizes dexamethasone treatment which may compromise regeneration of plant tissue and has been shown to have a relatively high background level (Zuo, Niu et al. 2000).

Currently, an estrogen receptor based transactivator inducible XVE system with GUS as a reporter gene is being tested in the genetic transformation of tobacco and grape. Transgenic XVE:GUS plants have already been transformed and will be used for GUS expression assays. In addition, we are performing an intellectual property analysis of the various components of the

XVE system. If the IP analysis and transcription expression data are favorable, this inducible system will be used for the expression of the recombinase ParA.
In the event the inducible promoters are leaky, we will utilize constitutive promoters such as the FMV34S. In parallel, we are cloning a recombinase expression module under the transcriptional control of the FMV34S.

Testing Negative Selectable Marker for the Recombinase System

As previously explained, a negative selection gene will be used to eliminate plant cells in which a recombinase event failed to occur (Figure 1). The negative selection approach contemplates the use of cytosine deaminase due to the sensitivity that this dominant gene shows in the presence of 5-fluorocytosine. This negative selection system has been used in a recombinase-system for the transformation of plastids (Corneille, Lutz et al. 2001) and strawberry (Schaart, Krens et al. 2004). To avoid potential IP issues associated with the material exchange, we opted to isolate the cytosine deaminase gene from E. coli K12 MG1655. We completed molecular assembly of the negative selectable marker cassette, driven by the constitutive FMV34S promoter (Figure 2). We are currently cloning the negative selection marker cassette into a plant transformation binary vector for testing in tobacco and grape.

Figure 2. Plant Transformation Construct to Test the Negative Selection Marker in Tobacco and Grape.

Efficacy of the Par-A Enzyme in the Recombinase System

To test the efficiency of Par-A dependent recombinase-excision activity, we are developing a gene construct with DsRed and GUS reporter gene markers (Figure 3). In this experiment, transformation of the entire recombinase module will be monitored under hygromycin selection and expression of the GUS as the GOI and DSRed markers. Expression of the recombinase enzyme will be controlled by either an estrogen-inducible or constitutive, FMV34S promoter. Efficiency of the recombination activity will be assessed by comparing the DsRed vs GUS marker. Successful recombination of the RRS-flanked DNA should result in the deletion of the DsRed Marker, and at this point, only the GUS marker in the GOI cassette should be present in the grape calli (Figure 3, panel B).
As of now, we have cloned about 90% of the DsRed/GUS ParA expression module shown in Figure 3. We are completing amplification of the ParA recombinase enzyme. We anticipate finishing assembly of the entire plant transformation vector in April, 2008. Upon completion, the transformation construct will be used to test recombinase-mediated excision activity in tobacco and grape. If the Par A enzyme does not function as expected, we may consider increasing efficacy by utilizing a plant codon-optimized gene construct. In preparation for this, we have amplified the Par A codon optimized enzyme and are ready to proceed cloning depending on the outcome of the Par A native enzyme experiments. In parallel, we are testing the expression of the DsRed gene in this module in transient expression assays in Arabidopsis. These preliminary experiments are being performed to ensure the DsRed gene is functional.

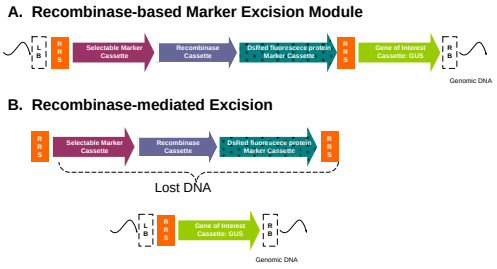

Figure 3. Plant Transformation Construct to Test Efficacy of Par-A mediated Recombinase-Excision Activity in Tobacco and Grape.

Plant Selectable Marker with Greater FTO for Grape Transformation

Update on NPTII Patent Landscape

The most common marker used for research and commercial production is the bacterial neomycin phosphotransferase II (NptII) gene that grants resistance to several antibiotics (Miki and McHugh 2004). Broad issued patents and new patent application claims covering the use of antibiotic resistance genes use for selection of transformed plant cells are in place in the U.S. and not generally available for license. One of the broader patents in this family, US6174724, will expire in the summer of 2008. Though we initially expected the FTO to increase after this patent's expiration, there is concern that a pending patent application may prolong the limited freedom-to-operate of Mosanto's kanamycin IP as a plant selectable marker. PIPRA is following its prosecution and considering the effects it may have on our current IP/FTO strategy for the grape transformation platform system.

In addition, PIPRA has now tested two recently described plant-derived markers (Dirk, Williams et al. 2001; Dirk, Williams et al. 2002; Miki and McHugh 2004; Mentewab and Stewart 2005). The plant peptide deformylase (DEF) from Arabidopsis confers tolerance, when overexpressed, to DEF-specific inhibitors which are otherwise lethal to plants. The Arabidopsis ABC transporter, Atwbc19, provides kanamycin resistance levels comparable to the bacterial-NptII gene when overexpressed. In contrast to the bacterial-NptII gene and bacterial homolog of Atwbc19, which provide tolerance to a broader spectrum of antibiotics, the plant transporter appears to provide tolerance only to kanamycin. These two markers have the advantage that, because they are plant-derived genes, risk of horizontal gene transfer resulting in bacterial chemical resistance is greatly reduced. PIPRA has engaged in productive licensing discussions to include these technologies in the transformation vector system. In addition, we initiated the experiments to test the plant-based, DEF2 and Atwbc19, and more routinely used hygromycin and glufosinate selectable markers. In the latter experiments, we are comparing Monsanto's CaMV35S vs. Purdue's MAS promoter, which would be available for licensing.

Def9-Actinonin Transformations in Grape

We co-cultured embryogenic grape callus with LBA4404 containing pKM24-def2-D. After co-cultivation, inoculated callus and non-inoculated controls were transferred to callus induction medium containing 250 mg/l actinonin. Material is being subcultured every 21 days to fresh medium. To date we are still not seeing any actinonin resistant callus developing from the 125 callus pieces we inoculated (Figure 4). Therefore, we have discontinued this experiment. On March 5, we initiated another experiment, to examine reduced actinonin levels in the selection medium. Inoculated Thompson seedless callus was plated on callus induction medium containing 125 mg/l actinonin, and we will follow the material over six months

Atwbc19- Kanamycin Selection in Grape

We initiated replicated experiments comparing 35s::pAtwbc19 to 35s:: nptII, and mas::pAtwbc19 to mas::nptii in Thompson Seedless grape. There can be significant run-to-run variability in grape transformations presumably do to the physiological stage of the callus. Therefore, for experiments initiated on the same date, callus was harvested, pooled, and separated into two groups; one group was inoculated with 35s::pAtwbc19 the other with 35s:nptii. We are monitoring the transformation frequency as determined by the number of inoculated callus pieces developing kanamycin resistant colonies. We are seeing normal kanamycin resistant callus development from material inoculated with 35s::nptii and mas::nptii but no resistant callus formation from material inoculated with 35s::pAtwbc19 or mas::Atwbc. (Table 1 and figure 4).

Hygromycin Selection in Grape Under Transcriptional Control of the CaMV35S and MAS Promoters

We initiated multiple grape experiments to explore the efficiency of mas::hpt versus 35s::hpt in grape transformation. Preliminary results are given in Table 2. We anticipate additional hygromycin resistant colonies appearing over the next few months. To date, it appears that both Mosanto's 35s and Purdue's MAS promoters can be used to drive the hpt plant selectable marker gene for use in grape transformations.

Table 1 Development of kanamycin resistant embryogenic grape callus and embryo germination on medium supplemented with 200 mg/l kanamycin sulfate, post inoculation with an Agrobacterium carrying the 35s::nptii, 35s::pAtwbc19 gene, mas :: nptii or mas pAtwbc19 genes.

Experiment	Date initiated	PIPRA #	Construct	# (%) Transgenic embryogenic callus
071025	3/7/2007		35s::wbc19	0/50 (0)
071026	3/7/2007		35s::nptii	7/25 (28)
071037	3/9/2007		35s::wbc19	0/50 (0)
071038	3/9/2007		35s::nptii	25/50 (50)
071044	3/16/2007		35s::wbc19	0/50 (0)
071045	3/16/2007		35s:nptii	27/50 (54)
071170	9/19/2007	PIPRA 445	mas:: wbc19	0/60 (0)
071171	9/19/2007	PIPRA 120	mas::nptii	5/60 (8)
071174	9/28/2007	PIPRA 445	mas:: wbc19	0/60 (0)
071175	9/28/2007	PIPRA 120	mas::nptii	37/60 (62)

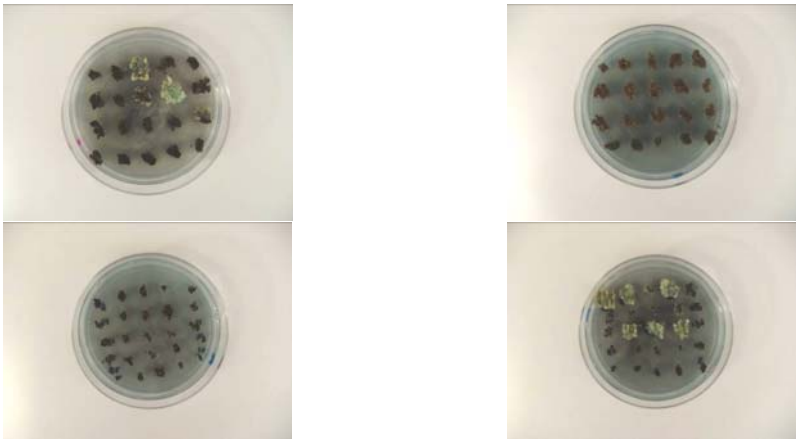

Figure 4. Development of kanamycin resistant embryogenic grape callus on callus induction medium supplemented with 200 mg/l kanamycin sulfate, post inoculation with an Agrobacterium carrying the mas:: nptii (top, left) and 35s:nptii (bottom, left). Colonies have failed to develop from callus inoculated with Agrobacterium containing mas::atwbc19 (top, right) or 35s::pAtwbc19 gene (bottom, right).

Table 2. Development of hygromycin resistant embryogenic grape callus and embryo germination on medium supplemented with 25 or 50 mg/l hygromycin, post inoculation with an Agrobacterium carrying the 35s::hpt, or mas :: hpt genes.

Experiment	Date Initiated	PIPRA #	Construct	Hygromycin Concentration mg/liter	# (%) Transgenic resistant callus
071186	10/23/2007	PIPRA 443	mas::hpt	50	6/50 (12)
071187	10/23/2007	PIPRA 449	35s::hpt	50	0/50 (0)
071189	10/25/2007	PIPRA 443	mas::hpt	25	5/50 (10)
071190	10/25/2007	PIPRA 449	35s::hpt	25	0/50 (0)
071191	10/31/2007	PIPRA 443	mas::hpt	25	1/50 (2)
071192	10/31/2007	PIPRA 449	35s::hpt	25	4/50 (8)
071193	11/2/2007	PIPRA 443	mas::hpt	50	0/50 (0)
071194	11/2/2007	PIPRA 449	35s::hpt	50	0/50 (0)

IP Strategies Around Agrobacteria-Mediated Plant Transformation

Non-Agrobacteria Strains

We primarily explored the possibility of utilizing early 1980's technology based the use of *Rhizobium trifolli* to transform plants. *Rhizobium trifolli*, *Rhizobium*, *Sinorhizobium meliloti*, and *Mesorhizobium loti* species have all been demonstrated to introduce new genetic material into plants. Although transformation rates are reduced, experimental data indicates these bacterial species can provide an alternative to *Agrobacterium*-mediated transformation (Schilperoort, Hooykaas et al. 1986; Broothaerts, Mitchell et al. 2005; Jefferson 2005). We assessed the legal landscape surrounding the use of these non-agro strains for plant transformation and used the advice of a patent attorney to evaluate the use of *Rhizobium* strains as a legal alternative to *Agrobacterium* strains for plant modification application. In brief, the legal information suggests that the *Rhizobium trifolli* strain, originally disclosed and patented in the early 1980's, are in the public domain because the patents have expired. However, in spite of the likely viability of this gene delivery strategy, attempts to access the original *Rhizobium* strains deposited by Dr. Hooykaas and colleagues at the Centraal Bureau voor Schimmel cultures (CBS) have been unsuccessful to this point.

Identification of Putative Grape P-DNA Transfer Borders Using Bioinformatic Approaches

It appears likely that TransBacter or non-agro strains alone may be an insufficient work-around to Agrobacterium-mediated gene transfer and that a comprehensive strategy will also require alternatives to T-DNA borders which mediate gene transfer into the plant genome. Thus, PIPRA proposed, as one strategy, to employ plant-derived "P-DNA" borders that can functionally substitute for Agrobacterium-derived T-DNA border sequences. The J. R. Simplot Company discovered and filed for patent protection P-DNA sequences that are functionally comparable to those from Agrobacterium (Rommens 2004; Rommens, Humara et al. 2004; Rommens, Bougri et al. 2005). Transformation competent P-DNA border sequences have been identified from the dicots: potato, tomato, pepper, alfalfa, and Arabidopsis and monocots: rice, barley and wheat (Rommens, Humara et al. 2004; Rommens, Bougri et al. 2005). While P-DNA borders from Vitis have not been reported, we propose employ bioinformatics approaches to identify and test putative P-DNA sequences in grape. Alternatively, we propose to design degenerate primers to isolate putative functionally equivalent sequences from grape. P-DNA is an attractive technology as it allows creating transformation vectors in which the entire transferred DNA is plant-derived. It appears likely that the combination of TransBacter and P-DNA provides an effective work-around strategy for Agrobacterium-mediated plant transformation.

Last quarter, we used bioinformatic approaches to search for putative PDNA borders in the *Vitis vinifera* sequence data available to date (Guevara 2008). Currently, there are three main efforts dedicated to the sequencing and mapping of the vitis genome. The first is a collaboration effort between University of Nevada, Reno, Commonwealth Scientific and Industrial Research Organization/CAES Genome Facility and UC Davis, whose goal is to develop an EST map. The second effort is being undertaken by the IASMA (IASMA - Instituto Agrario San Michele all'Adige, Italy), and their goal is to sequence the *Vitis vinifera* cultivar Pinot noir. The third effort involves France's National Center for Sequencing (Genoscope) and National Institute for Agricultural Research (INRA) and the Italian Institute of Applied Genomics (IGA) and Vitis Genome Analysis (VIGNA). The Vitis sequencing effort that best fits the needs for *in silico* P-DNA mining is the latter cooperation between the French and Italian sequencing projects, known as the International Grape Genome Program. Vitis genome sequence data is updated regularly and available for download through their Genoscope Database located (http://www.genoscope.cns.fr/externe/English/Projets/Projet_ML/projet.html). This database

offers the most current genomic assembly and chromosomal annotations, as well as a BLAST-like tool specific for searching queries in the grape genome. Genoscope also uploads their information to the NCBI website (http://www.ncbi.nlm.nih.gov), so data mining can be done from either server. This international effort, unlike others, offers the full genome sequence and not only EST's. It has been reported that P-DNAs are usually found in intron-exon junctions as well as in introns, so a full genomic map is the best resource for finding putative P-DNA sequences (Jaillon, Aury et al. 2007).

To search for putative P-DNA borders in grape, we first applied Simplot's BLAST bioinformatics parameters utilized to identify functional TDNA borders in several plant species (Rommens 2004; Rommens, Humara et al. 2004; Rommens, Bougri et al. 2005). However, this approach did not yield significant results when searching the Vitis genome. Therefore, we developed an alternate bioinformatics strategy. This strategy relies on Simplot's characterization of PDNAs which revealed conserved motifs functionally necessary for the transfer and incorporation of foreign DNA into plants. In our bioinformatics approach, we first identified DNA conserved motifs for the various domains of the right and left plant DNA transfer borders; including the upstream region, border alternative, and downstream region. Since there appears to be no correlation between the homology of PDNA sequences and the taxonomical degree of proximity, we chose to generate conserved motifs from PDNA with the highest transformation efficiencies (Rommens, Bougri et al. 2005). PDNAs from rice, tomato, potato, Arabidopsis and pepper were aligned using the T-COFFEE application. The conserved motifs identified for the right and left TDNA borders were identified (results not shown).

Having identified conserved DNA motifs in the left and right borders of PDNAs (Figure 6), we used the Hidden Markov Model (HMM) to search for regions in the Vitis genome that shared the conserved PDNA motifs. In contrast to BLAST which identifies homology within a string of DNA sequences, HMM searches for sequences which share a common DNA pattern. As a result, HMM may yield more significant results than BLAST. The HMM results were subsequently analyzed with a local alignment application (WATER), which was used to identify the similarity between the conserved DNA motif sequences and grape putative PDNA border alternatives. These results were filtered based on the similarity shared between the motif and the output sequence, as well as the resulting alignment score. A number of vitis DNA matches that fulfilled the requirements, data not shown. Finally a transformation efficiency range was calculated for each of the identified border and flanking alternatives. This value was calculated based on a local alignment between the putative *Vitis* sequences and the alternatives reported by J.R. Simplot (Rommens, Bougri et al. 2005). The similarity value between sequences obtained from the alignment was divided by the transformation efficiency reported by J.R. Simplot and multiplied by 100. The obtained percentile score indicated a probable transformation efficiency of between 15% and 80%. The next step is to determine if these putative grape PDNA regions are functionally comparable to agrobacteria TDNA borders and determine their *in vivo* transformation efficiency.

2. Develop Agrobacterium and TransBacter™-mediated transformation for California wine grapes and/or cultivars suitable for generating root stocks.

PIPRA engaged in discussions to access the TransBacter™ technology from CAMBIA, Australia. However, based on IP limitations of the agreements to obtain TransBacter™ , we chose to abandon pursing these materials. Last term, we explored the possibility, from a technical and legal standpoint, of utilizing early 1980's technology based the use of other

non-agrobacterial alternatives, i.e. *Rhizobium trifolli*, to transform plants. In this last quarter, in collaboration with Dr. Stan Gelvin in Purdue we formulated a research strategy to use non-agro strains for plant transformation. The research to develop non-agrobacterial alternatives for plant/grape transformation is outside the scope of this proposal; nevertheless, we have developed an experimental design.

3. Develop strategies to disseminate the biological resources developed under appropriate agreements for the PD community.

PIPRA has done a significant amount of progress in this objective, On Feb 27th, PIPRA hosted the second formal meeting held in San Diego to propose a Licensing Model to the technology providers of the core components for the transformation system. The proposed licensing terms and structure is being reviewed by the technology providers and UC Davis Office of Research Staff. These activities will allow the development of strategies to disseminate the plant transformation vectors to the PD research and commercial community.

4. Explore collaborative opportunities with researchers developing Pierce's disease control strategies that could employ and test the proposed grape transformation system.

Now that the vectors are closer to being synthesized, in this reporting period, we met with Dr. Abhaya Dandekar to discuss the possibility of testing the pPIPRA vectors by an independent researcher. To increase the likelihood that the PD research community will adopt this transformation platform, it is important that the vectors are verified by an outside party. In collaboration with Dr. John Labavitch, we submitted a research proposal that incorporates the pPIPRA transformration platform (Optimizing grape rootstock production and export of inhibitors of *X. fastidiosa* PG activity**).**

G. Publications, reports, and presentations where the information generated from the research was presented: Enabling Technologies for Grape Transformation. Pierce's Disease Control Program Research Symposium, December 12-14, San Diego, California.

H. Research relevance statement, describing how this research will contribute towards solving the PD/GWSS problem in California

PIPRA's grape transformation enabling technologies will provide cutting edge marker-removal strategies under pre-negotiated terms of access. The technology packages will provide a clear legal pathway for research that is targeted towards practical Pierce's disease and Glassy-winged Sharpshooter applications.

I. Summary in lay terms of the specific accomplishments of the research project

PIPRA's **research track** has designed a plant genetic engineering (transformation) system that will allow delivering a trait (PD control gene) gene into the grape DNA. The transformation system is unique in that it allows removing selectable markers and other components which are necessary during the initial stages of plant genetic engineering but become unnecessary in subsequent steps. This feature is particularly important when the research plan anticipates commercial deployment as is the case in the field of Pierce's Disease in Grape. PIPRA has completed the DNA synthesis and molecular cloning strategy for this transformation system. We initiated testing the individual components of the transformation platform. In addition, we continued testing the feasibility of using the new plant-derived selectable markers in grapes. The results of this plant selection work are not

very favorable. However, we continue to follow the prosecution of kanamycin patents; as of now, we are encouraged that NPTII marker may have greater FTO after the summer.
The proposed transformation vector requires a robust **licensing strategy** that will allow dissemination of the transformation system to the PD community for research and commercial use. For these objectives, we held the second meeting of technology providers and continue drafting the license language.

J. Summary and status of intellectual property produced during this research project

No new IP has been thus far generated in this project.

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FUNDING AGENCIES: Funding for this project was provided by The Pierce's Disease and Glassy winged Sharpshooter Board of the California Department of Food and Agriculture and the University of California Pierce's Disease Research Grants Program.