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 April to August 2008. Work discussed here reflects a project funded for the period of June 1, 2006 to May 31, 2008 and extended for one year under a no-cost extension request.

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 95% of the assembly. During the last months, we have concentrated our efforts on completing the cloning.

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.

No further work on this objective was performed since it is outside the scope of the funded work.

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

The deliverable for this objective is crafting a license that consolidates biological and patent rights from the numerous technology provider. To complete this objective we have been working with UC Davis legal counsel to draft a license acceptable to PIPRA's host institution. Final review of the model license by Davis Office of Research Staff and Alan B. Bennett at PIPRA was completed. The draft will be reviewed by other technology providers.

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

PIPRA has received requests for the grape vectors from the PD research community. As soon as the vectors are ready for distribution, we will provide the materials and information to the PD researchers. As proposed by Labavitch and colleagues in Optimizing grape rootstock production

and export of inhibitors of *X. fastidiosa* PG activity. Vectors will be tailored and build to meet this proposals objectives.

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 95% of the recombinase transformation vector (Figure 1). We are currently cloning the last component of the recombinase transformation vector, the recombinase cassette. We had anticipated to complete the entire assembly of the recombinase transformation vector including the recombinase cassette in April 2008. However, we had technical problems in cloning. We are working on this last cassette.

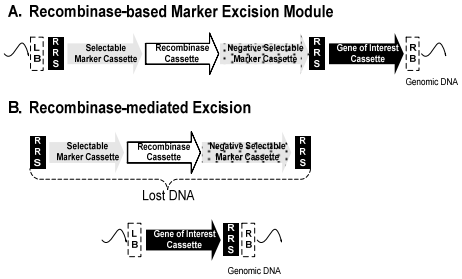

Figure 1. Diagram of the Recombinase-Excision Strategy.
Panel A Recombinase-based transformation construct contains a gene of interest cassette and two selectable marker cassettes flanked by recombinase recognition sequences (RRS). Panel B: Inducible expression of the recombinase excises the selectable 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

We are continuing to pursue the use of XVE as an inducible promoter in grapes and tobacco. The major advance in this area was completing an IP audit of the XVE promoter. The work was performed in collaboration with the Baker & McKenzie LLP. The information gathered suggests that the a license from Rockefeller University is necessary. Other IP was identified and for the proposed use in the PD proposal there is significant FTO.

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 finalized cloning the negative selection marker cassette into a plant transformation binary vector for testing in tobacco and grape. The construct is being sent for transformation into plants.

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).

We completed amplification of the ParA recombinase enzyme; the XVE inducbile promoter was recently cloned and is being sequenced. Once the sequence is verified the entire construct will be ready for transformation. 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.

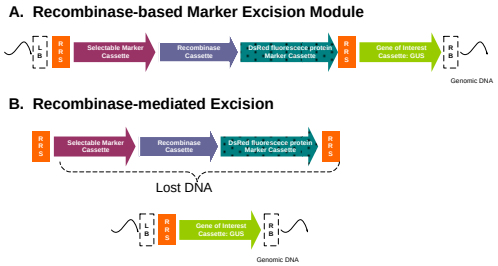

Figure 3. Plant Transformation Construct to Test Efficacy of Par-A mediated Recombinase-Excision Activity in Tobacco and Grape.

Plant Selectable Marker with Greater FTO for Grape Transformation

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

After last quarters report, no further advancement is necessary for this objective.

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

UC Davis Office of Research and PIPRA are finalizing the model license. 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.

The vectors will be employed by Dr. John Labavitch and colleagues in the research proposal that incorporates the pPIPRA transformration platform (Optimizing grape rootstock production and export of inhibitors of *X. fastidiosa* PG activity). PIPRA has also received requests for the vector, we will provide materials when ready.

G. Publications, reports, and presentations where the information generated from the research was presented: None this last quarter

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. We continue to develop the vectors and test the individual components.

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 are finalizing a model license that can be used to distribute the materials. Once approved by UC Davis, the license draft will be reviewed by prospective technology providers.

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

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

REFERENCES CITED

Aguero, C. B., S. L. Uratsu, et al. (2005). "Evaluation of tolerance to Pierce's Disease and Botrytis in transgenic plant of Vitis vinifera L. expressing pear PGIP gene." Mol Plant Pathology **6**(1): 43-51.

Aoyama, T. and N. H. Chua (1997). "A glucocorticoid-mediated transcriptional induction system in transgenic plants." Plant J **11**(3): 605-12.

Broothaerts, W., H. J. Mitchell, et al. (2005). "Gene transfer to plants by diverse species of bacteria." Nature **433**(7026): 629-33.

Corneille, S., K. Lutz, et al. (2001). "Efficient elimination of selectable marker genes from the plastid genome by the CRE-lox site-specific recombination system." Plant J **27**(2): 171-8.

Dirk, L. M., M. A. Williams, et al. (2001). "Eukaryotic peptide deformylases. Nuclear-encoded and chloroplast-targeted enzymes in Arabidopsis." Plant Physiol **127**(1): 97-107.

Dirk, L. M., M. A. Williams, et al. (2002). "Specificity of chloroplast-localized peptide deformylases as determined with peptide analogs of chloroplast-translated proteins." Arch Biochem Biophys **406**(1): 135-41.

Guevara, J. (2008). IDENTIFICATION OF PLANT-DERIVED TRANFER-DNA SEQUENCES, P-DNAS, AND THEIR FLANKING REGIONS IN THE VITIS VINIFERA GENOME USING HIDDEN MARKOV MODELS. Dpt Plant Sciences. Davis, University of California. **B Sc.:** 113.

Jaillon, O., J. M. Aury, et al. (2007). "The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla." Nature **449**(7161): 463-7.

Jefferson, R. A. (2005). Biological gene transfer system for eukaryotic cells (patent application). US20050289667A1, CAMBIA.

Mentewab, A. and C. N. Stewart (2005). "Overexpression of an Arabidopsis thaliana ABC transporter confers kanamycin resistance to transgenic plants." Nat Biotechnol.

Miki, B. and S. McHugh (2004). "Selectable marker genes in transgenic plants: applications, alternatives and biosafety." J Biotechnol **107**(3): 193-232.

Ouwerkerk, P. B., R. J. de Kam, et al. (2001). "Glucocorticoid-inducible gene expression in rice." Planta **213**(3): 370-8.

Reisch, B. and J. Kikkert (2005). "Testing transgenic grapevines for resistance to Pierce's Disease." 2005 Pierce's Disease Research Symposium: 58-61.

Rommens, C. M. (2004). "All-native DNA transformation: a new approach to plant genetic engineering." Trends Plant Sci **9**(9): 457-64.

Rommens, C. M., O. Bougri, et al. (2005). "Plant-Derived Transfer DNAs." Plant Physiol **139**(3): 1338-49.

Rommens, C. M., J. M. Humara, et al. (2004). "Crop improvement through modification of the plant's own genome." Plant Physiol **135**(1): 421-31.

Schaart, J. G., F. A. Krens, et al. (2004). "Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene." Plant Biotechnol J **2**(3): 233-40.

Schilperoort, R. A., P. J. J. Hooykaas, et al. (1986). A process for the incorporation of foreign DNA into the genome of dicotyledonous plants. E. P. Office. Netherlands, Leiden University. **A1:** 11.

Zuo, J., Q.-W. Niu, et al. (2000). "An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants." The Plant Journal **24**(2): 265-273.

Zuo, J., Q. W. Niu, et al. (2001). "Chemical-regulated, site-specific DNA excision in transgenic plants." Nat Biotechnol **19**(2): 157-61.

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.