Progress report for CDFA Contract Number: 06-0224

Progress Report To: The Pierce's Disease & Glassy-winged Sharpshooter Board of the California Department of Food & Agriculture and the University of California Pierce's Disease Research Grants Program

Project Title: Enabling Technologies for Grape Transformation

Time period covered by the progress report: This report covers the period from November 2008 to March 2009. The grant funding period ends April, 2009.

Principal investigator: Alan B. Bennett, PIPRA abbennett@ucdavis.edu
Cooperators: Sara Bird, PIPRA swbird@ucdavis.edu; Cecilia Chi-Ham, PIPRA clchiham@ucdavis.edu; David Tricoli, Ralph M. Parson Plant Transformation Facility dmtricoli@ucdavis.edu

List of Objectives and Description of Activities Conducted to Accomplish Objectives

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

After PIPRA's team performed an extensive evaluation of the IP surrounding the relevant plant transformation technologies, a grape transformation system was designed. The system was designed to implement a Par A recombinase-based marker removal system under the control of an estradiol-inducible promoter. With regards to the status of the proposed transformation vector, cloning is approximately 95% complete. Difficulties in standard enzyme based DNA cloning have caused us to seek the help of Blue Heron Biotechnology for de novo synthesis of the final components in our plant transformation vector. The anticipated completion of the vector is early March to April, 2009. While waiting for completion of the plant transformation vector, we have separately tested individual components of the overall system in grape as well as tobacco to validate their functionality.

2. **Develop alternatives to *Agrobacterium*-mediated transformation for California wine grapes and/or cultivars suitable for generating root stocks.**

The recombinase-mediated plant transformation vector that PIPRA has designed is well suited for use in Agrobacterium transformation protocols. Previously, we have investigated alternatives to Agrobacterium-mediated plant transformation such as *Rhizobium trifolli* but have not continued to pursue this because this work would be outside the scope of this proposal.

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

The deliverable for this objective is crafting a license that consolidates biological and patent rights from the numerous technology providers. 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 UC Davis Office of Research Staff and Alan B. Bennett at PIPRA was completed and the draft is under review by the other technology providers.

4. **Explore collaborative opportunities with researchers developing PD control strategies to link the developed transformation technologies with specific PD resistance technologies.**

There have been several members of the PD research community requesting PIPRA's vectors for grape transformation. As soon as the vectors are completed, we will provide all the necessary materials and information to those interested.

Summary Of Major Research Accomplishments And Results For Each Objective

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

Below is a summary of last quarter's progress towards the completion of a marker-free plant transformation system in grape for the PD community.

Assembly of Recombinase-Mediated Plant Transformation Vector

As mentioned in last quarter's update, difficulties in cloning prevented completion of the recombinase-mediated transformation vector. In December, we sent the incomplete vector to Blue Heron Biotechnology, a DNA synthesis company. They are currently working on synthesizing the recombinase cassette, the final piece, into the transformation vector backbone. The anticipated completion of the vector is March-April, 2009.

Validation of Components of the Recombinase-based marker excision System

Determining the Efficacy of the Inducible Promoter Activity in Tobacco and Grape

Prior to using the XVE promoter in our plant transformation system, we checked the IP status of the XVE system as well as tested its functionality in grape and tobacco. As mentioned previously, an IP analysis revealed that a license from the Rockefeller University is necessary and they have agreed to provide such a license under reasonable terms. In addition, we transformed grape and tobacco with an XVE::GUS construct to test the efficacy of the promoter upon estradiol induction. The results in grape are promising and support continued use of this inducible promoter for future work.

Testing the Negative Selectable Marker for the Recombinase System

As previously explained, our plant transformation vector will incorporate a negative selection marker to eliminate transformants in which a recombinase event failed to occur (Figure 1). We will utilize a cytosine deaminase codA gene which is lethal when exposed to 5-fluorocytosine (5-FC). Last quarter, we reported that a vector containing the cytosine deaminase cassette had been cloned into grape and tobacco to determine the appropriate concentration of 5-FC necessary to kill the transgenic tissue. We now report the results from this experiment (Table 1). Leaf explants from 11 independent tobacco lines were transferred to regeneration medium containing varying levels of 5-FC with or without hygromycin selection. The explants were subcultured on three different occasions every three weeks. Data collection scored each explant in forming callus, with increasing concentrations of 5-FC. As expected, data from the wild type explants

Figure 1. Map of negative selection system for tobacco and grape.

shows that 5-FC is not toxic to plants without the cytosine deaminase gene. It appears that concentrations of 5-FC between 200-250 mg/L have the most significant toxic effect on transgenic explants. At these concentrations, it appears that some of explants plated without hygromycin were able to regenerate into plants. This result indicates that the explants were originally chimeric and thus the calli that formed are a result of non-transgenic cells lacking the cytosine deaminase gene. The same experiments will be performed on grape when the transgenic calli mature. These results are promising and suggest we will be able to fine tune the concentrations of 5-FC necessary to eliminate the cells that contain the cytosine deaminase gene.

Table 1

5-FC mg/L	0	0	50	50	100	100	150	150	200	200	250	250
Hygromycin mg/L	25	0	25	0	25	0	25	0	25	0	25	0
wild type	dead	plant	dead	plant	dead	plant	dead	plant	dead	plant	dead	plant
Transgenic line												
089001-001	plant	plant	plant	plant	plant	plant	callus	callus	callus	plant	dead	plant
089001-003	plant	plant	plant	plant	plant	plant	plant	callus	callus	callus	callus	callus
089001-004	plant	plant	plant	plant	plant	callus	callus	dead	dead	dead	dead	dead
089001-005	plant	plant	plant	plant	callus	callus	callus	callus	callus	callus	dead	plant
089001-006	plant	plant	plant	plant	callus	callus	callus	callus	callus	callus	dead	callus
089001-011	plant	plant	plant	plant	callus	plant	callus	plant	callus	callus	callus	callus
089001-012	callus	callus	callus	callus	callus	callus	callus	callus	callus	callus	dead	dead
089001-015	plant	plant	plant	plant	callus	callus	callus	plant	dead	dead	dead	dead
089001-016	plant	plant	callus	callus	plant	callus	dead	callus	dead	plant	dead	dead
089001-017	plant	plant	plant	plant	callus	callus	callus	callus	callus	callus	dead	dead
089001-019	callus	plant	plant	plant	callus	plant	callus	callus	callus	callus	dead	dead

Efficacy of the Par-A Enzyme in the Recombinase System

As soon as we receive the completed vector from Blue Heron, we will begin transformation so that the entire system can be tested in transgenic grape and tobacco. Recombinase efficiency will be tested by utilizing the hygromycin selection, as well as the GUS marker gene and the cytosine deaminase negative selection. The hygromycin selection will select for callus that are true transformants while the cytosine deaminase will act as a negative selection to kill any transformants that have not had a successful recombination event. The plants that survive the exposure to 5-FC should test positive for GUS as the marker is set outside the recombinase recognition sites and will not be excised.

While we wait for the final construct to be finished, we have initiated steps to test the efficacy of the Par-A recombinase gene. To do this, we will subsequently transform transgenic tobacco and grape that already harbor the cytosine deaminase gene within the recombinase recognition sites (Figure 1) with the ParA recombinase under control of the XVE inducible promoter (Figure 2). We anticipate that the addition of estradiol (inducer) will activate the production of the Par-A enzyme which would in turn excise the part of the DNA

Figure 2. pPIPRA 107: Map of Inducible Promoter driving the ParA recombinase enzyme with the NPTII plant selection marker.

containing the cytosine deaminase and hygromycin cassettes, leaving only the GOI cassette harboring GUS. Selection using 5-flourocytosine should eliminate those cells in which a successful recombination event failed to occur. If the recombinase works, we expect to be able to recover cells which only contain the GUS gene. As of now, we are sending the pPIPRA 107 recombinase plasmid (Figure 3) to the transformation facility to perform the transformation experiments; we anticipate results in tobacco within a month. If this experiments indicates the efficacy of the ParA recombinase, we expect the final Recombinase Vector will be fully functional as all the components have been individually tested.

Publications Or Reports Resulting From The Project

Enabling Technologies for Grape Transformation. Proceedings, 2008 Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

Enabling Technologies for Grape Transformation. Proceedings, 2007 Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

International Conference, Plant Transformation Technologies. February 4-7th, 2007 Vienna, Austria.

Research Relevance Statement

PIPRA's proposed grape transformation vector and its encompassing technologies will provide a useful marker-removal system with a clear legal pathway for commercial use. PIPRA's approach to form a patent pool of the technologies necessary for the PD community has paved the way for the development of technologies with maximum FTO for research on Pierce's Disease and Glassy-winged sharpshooter applications.

Lay Summary of Current Year's Results

PIPRA is in the final stage of constructing a plant genetic engineering system (transformation vector) aimed at delivering a gene with a desired trait (i.e. control of PD) into grape cells. This transformation system will allow the researcher to remove unwanted DNA that is only necessary during the first step of the transformation but that is subsequently obsolete. This has important consequences, especially when the vector will be deployed for commercial research such as Pierce's Disease research in grape. We were able to test various components of the system, all with promising results. We anticipate finishing the system in March-April of this year, after which time, we will be able to validate the entire system prior to commercial deployment.

Status of Funds

The grant period ends in April of 2009; we expect to have spent all of the funds.

Summary and Status of Intellectual Property Produced During This Research Project

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

Relevant Publications

Aguero, C. B., S. L. Uratsu, C. Greve, A. L. T. Powell, J. M. Labavitch, C. P. Meredith, and A. M. Dandekar. 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: 43-51

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

Corneille, S., K. Lutz, Z. Svab, P. Maliga. 2001. Efficient elimination of selectable marker genes from the plastid genome by the CRE-lox site-specific recombination system. Plant J 27: 171-8.
Dale, E. C., and D. W. Ow. 1991. Gene transfer with subsequent removal of the selection gene from the host genome. Proc Natl Acad Sci U S A 88: 10558-62.
Gleave, A. P., D. S. Mitra, S. R. Mudge, and B. A. Morris. 1999. Selectable marker-free transgenic plants without sexual crossing: transient expression of cre recombinase and use of a conditional lethal dominant gene. Plant Mol Biol 40: 223-35.
Hohn, B., A. A. Levy, and H. Puchta. 2001. Elimination of selection markers from transgenic plants. Curr Opin Biotechnol 12: 139-43.
Miki, B., **and S. McHugh. 2004.** Selectable marker genes in transgenic plants: applications, alternatives and biosafety. J Biotechnol 107: 193-232.
Moller, S. G., J. Zuo, and N. H. Chua. 2004. Inducible site-specific recombination for the activation and removal of transgenes in transgenic plants. The Rockefeller University.
Ouwerkerk, P. B., R. J. de Kam, J. H. Hoge, and A. H. Meijer. 2001. Glucocorticoid-inducible gene expression in rice. Planta 213: 370-8.
Perera, R. J., C. G. Linard, and E. R. Signer. 1993. Cytosine deaminase as a negative selective marker for Arabidopsis. Plant Mol Biol 23: 793-9.
Reisch, B., **and J. Kikkert. 2005.** Testing transgenic grapevines for resistance to Pierce's Disease. 2005 Piearce's Disease Research Symposium: 58-61.
Russell, S. H., J. L. Hoopes, and J. T. Odell. 1992. Directed excision of a transgene from the plant genome. Mol Gen Genet 234: 49-59.
Schaart, J. G., F. A. Krens, K. T. B. Pelgrom, O. Mendes, and G. J. A. Rouwendal. 2004. Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol J 2: 233-240.
Sugita, K., E. Matsunaga, and H. Ebinuma. 1999. Effective selection system for generating marker-free transgenic plants independent of sexual crossing. Plant Cell Reports 18: 941-7.
Sugita, K., T. Kasahara, E. Matsunaga, and H. Ebinuma. 2000. A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. Plant J 22: 461-9.
Zuo, J., **Q.-W. Niu, and N. H. Chua. 2000.** An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J 24: 265-73.
Zuo, J., Q. W. Niu, S. G. Moller, and N. H. Chua. 2001. Chemical-regulated, site-specific DNA excision in transgenic plants. Nat Biotechnol 19: 157-61.