ENABLING TECHNOLOGIES FOR GRAPE TRANSFORMATION

Principal Investigator:
Alan B. Bennett
PIPRA
abbennett@ucdavis.edu

Cooperators:
Sara Bird
PIPRA
swbird@ucdavis.edu

Cecilia Chi-Ham
PIPRA
clchiham@ucdavis.edu

Ana Karina Ramijan
PIPRA
anaramijan@ucdavis.edu

David Tricoli
Plant Transformation Facility
dmtricoli@ucdavis.edu

Ralph M. Parson
Plant Transformation Facility
dmtricoli@ucdavis.edu

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ABSTRACT
The release of new technologies is often hampered by downstream legal and regulatory roadblocks. A thorough analysis of the current intellectual property (IP) rights of commonly used research tools is crucial to avoiding these obstacles, especially in regards to agricultural biotechnology, specifically plant transformation-related technologies. Keeping in mind the intricate patent landscape as well as the strong IP technology portfolio in the public sector, this project looks to combine these available technologies for plant transformation. The goal of this research project is to develop a grape-specific recombinase-based marker excision system for the generation of genetically engineered *Vitis* that is marker free and can be more amenable to market entry. The anticipated construct will provide a convenient means of instituting the various Pierce's disease control strategies. In this reporting period, we present our results in validating key components of the transformation platform.

INTRODUCTION
PIPRA, Public Intellectual Property Resource for Agriculture, is a nonprofit, public sector organization comprising a multitude of universities and institutions designed to manage the complex Intellectual Property (IP) landscape as well as develop the tools for the deployment of commercial and humanitarian agricultural technologies. Pierce's disease (PD) research has already generated promising long-term transgenic control strategies. Unfortunately, the proprietary nature of these gene transfer tools is unlikely to incorporate features that are compatible with evolving regulatory frameworks. Thus, research output with commercial potential but developed using technologies with limited freedom-to-operate (FTO) may need to be reengineered with legal and regulatory considerations. This research project aims to develop and test a transformation system not only for research, but for commercial development of PD control strategies in grape that address IP and regulatory issues.

OBJECTIVES
1. Design, develop, and validate a grape-specific transformation system that addresses legal IP, technical and regulatory consideration.
2. Develop alternatives to *Agrobacterium*-mediated transformation for California wine grapes and/or cultivars suitable for generating root stocks.
3. Develop strategies to disseminate biological resources under appropriate licensing agreements for the PD community.
4. Explore collaborative opportunities with researchers developing PD control strategies to link the developed transformation technologies with specific PD resistance technologies.

RESULTS
Tranformation vector system: There are several marker removal strategies that have been employed in various plants species. However, because of the long generation time of grapes, those strategies which depend on multiple plant generations are not feasible for grape cultivars. Our strategy, which has been tested in several model plant systems, utilizes a recombinase-based excision for removal of the selectable marker, after transgenic lines have been selected for, without the possibility for re-integration of the marker (Dale and Ow 1991, Russell et al. 1992, Gleave et al. 1999, Sugita et al. 1999, Sugita et al. 2000, Hohn et al. 2001, Zuo et al. 2001, Schaart et al. 2004). The recombinase-mediated transformation vector is designed with a three-part system: a plant marker to select for early transformants, a recombinase gene driven by an inducible promoter that can be transiently activated to remove the plant selectable marker, and a negative selectable marker (Perera et al. 1993, Gleave at al. 1999) to kill the cells in which poor or incomplete recombinase-mediated excision has occurred. With this strategy, we will be able to eliminate the selectable marker during the first generation of the plant tissue. Currently, PIPRA's recombinase transformation vector is in its final stages of cloning.

Selectable markers: Previous research focused on testing other plant selectable markers such as DEF and Atwbc19; however, neither of these markers is suitable for grape transformation. Thus far, NPTII and hygromycin have shown to be more efficient plant selectable markers. Though there is a pending application, it appears the NPTII marker may have greater FTO in the near future.

Negative Selection System: In addition, PIPRA has proposed using a negative marker selection which will provide a means of eliminating transformants in which the recombinase gene has failed to properly excise the unwanted DNA. Our negative selection system makes use of cytosine deaminase which shows impressive sensitivity when exposed to 5-fluorocytosine. This negative selection system has already been tested with the recombinase-mediated excision strategy for plastid transformation (Corneille, Lutz et al. 2001) and strawberry (Schaart, Krens et al. 2004). IP analysis revealed that we had appropriate freedom to operate (FTO) to isolate the cytosine deaminase gene from *E. coli* K12 MG1655. Cloning of this cassette is complete, with the cytosine deaminase gene being driven by the FMV34S constitutive promoter. The cassette has been successfully cloned into a binary transformation vector and transformed into grape and tobacco callus. The transformed tissue will be treated with increasing amounts of 5-fluorocytosine to identify the concentration necessary to eliminate the tissue. Those experiments are currently ongoing.

A parallel experiment aims to test the efficacy of the Par-A excision activity prior to commercial and humanitarian development of our vector. We will employ another type of negative selection involving DsRed and GUS reporter genes (**Figure 1, panel A**). The recombinase efficiency can be evaluated by comparing expression levels of the two visual markers. A successful recombination event will result in the deletion of the RRS-flanked DNA, which contains the hygromycin plant selection marker as well as the DsRed marker. Therefore, only the GUS marker, placed in the GOI cassette should be expressing in the plant **(Figure 1, panel B)**. We are currently in the final stage of the cloning of this construct. The last step is the insertion of the completed Par-A recombinase cassette module.

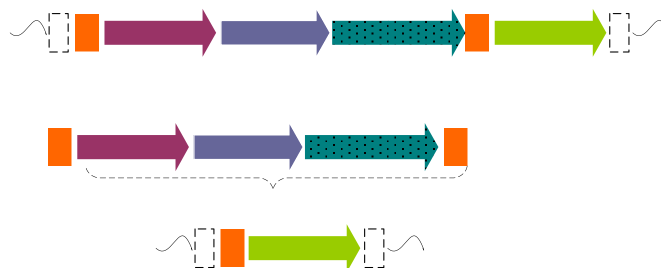

Figure 1. *Negative selection construct to test efficacy Par-A mediated recombinase activity in tobacco and grape.*

Promoters: This transformation platform will include a number of constitutive promoters for expression in grapes (Purdue's MAS, University of California's UC FMV34S, G10-90 from Zuo, Niu et al. 2000). In addition, we have incorporated an estrogen-inducible promoter system for tight control of transcription activity. This promoter will be used to regulate the expression of the recombinase gene. Precise control of expression of the recombinase gene is preferred to avoid premature excision which may occur due to the leaky expression of constitutive promoters. The estrogen-induced XVE system has been previously used in a cre-lox-mediated marker free system in Arabidopsis (Zuo, Niu et al. 2001). This system was preferred over another inducible promoter, the glucocorticoid-system (Aoyama and Chua 1997; Ouwerkerk, de Kam et al. 2001), which requires the use of dexamethasone treatment that can often inhibit plant tissue regeneration as well as contain high background levels (Zuo, Niu et al. 2000). The XVE system (Zuo, Niu et al. 2001) with a GUS reporter gene was tested in tobacco and grape transformants with promising results **(Figure 2)**.

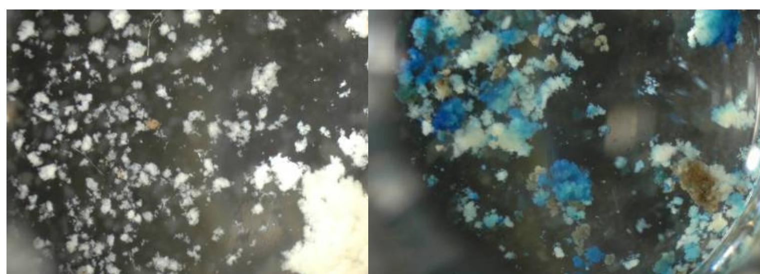

Figure 2. *GUS expression in grape callus transformed with pER8:GUS grown on medium without estradiol (left) and grape callus grown for 48 hours on medium containing 50 uM estradiol (right)*

In addition, with support from legal counsel, we recently concluded the IP analysis of the XVE system. To conduct the IP evaluation of the XVE system, we divided this system into three parts. Part A considered the three components of the XVE fusion protein: LexA Binding Domain (X), VP16 Transcription-Activation Domain (V), and Estrogen Receptor (E). Part B considered IP related to the LexA Operator sequence. The LexA Operator sequence would be situated before the gene it is regulating, in this case the recombinase Par-A gene. Finally, part C reviewed the legal landscape around the constitutive G10-90 promoter which drives the XVE fusion protein **(Figure 3)**. The results from this review show the technology would require licensing from Rockefeller University. PIPRA originally obtained the XVE system from Rockefeller University under a research only material transfer agreement. To consider commercial use of Rockefeller's promoter, we have initiated conversations in order to include this critical component as part of the patent pool that would be made available to the PD community.

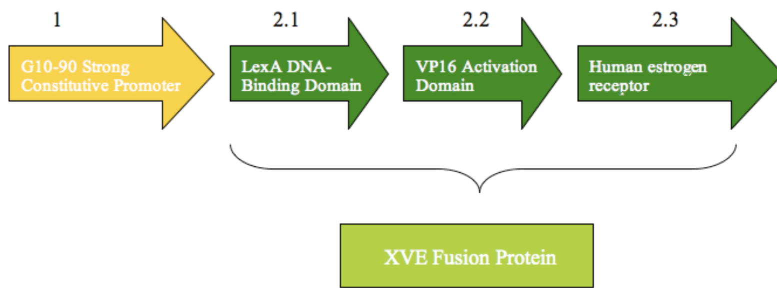

Figure 3. *The XVE fusion protein driven by the constitutive G10-90 promoter. Completed IP analysis of this piece now provided us complete FTO.*

CONCLUSIONS

Research to combat the threat of PD on California's wine grape industry has led to the development of several promising transgenic approaches (Aguero et al. 2005, Reisch and Kikkert 2005). Regardless of the success of these projects, they encompassed proprietary technologies that would hinder their downstream commercial production due to IP restrictions. In order to advance the transgenic grape technology, it is critical that thorough IP analysis be conducted in conjunction with the research such that new control strategies in the lab can be adopted by the commercial sector without unnecessary delays or need to reengineer transgenic plants. Because of the incredibly long generation time of grapes, up to two-three years, it is not feasible to develop technologies that must be repeated during the commercial phase of development because of IP restrictions that could have been avoided at the beginning of the project. For example, the thorough IP analysis on the recombinase-mediated plant transformation system for grape provides a clear legal pathway for commercial applications of these technologies. PIPRA's approach to form a patent pool of the technologies necessary or the PD community has paved the way for the development of technologies with maximum FTO for research on PD and glassy-winged sharpshooter applications.

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