ENABLING TECHNOLOGIES FOR GRAPE TRANSFORMATION

Project Leader:
Alan B. Bennett
PIPRA
University of California
Davis, CA 95616
abbennett@ucdavis.edu

Cooperators:
Ana Karina Ramijan
PIPRA
University of California
Davis, CA 95616
anaramijan@ucdavis.edu

David Tricoli
Plant Transformation Facil.ty
University of California
Davis, CA 95616
dmtricoli@ucdavis.edu

Ralph M. Parson
Plant Transformation Facility
University of California
Davis, CA 95616

Cecilia Chi-Ham
PIPRA
University of California
Davis, CA 95616
clchiham@ucdavis.edu

Reporting Period: The results reported here are from work conducted September 2006 to September 2007.

ABSTRACT
Navigating the intellectual property (IP) rights of commonly used research tools is essential to prevent downstream legal or regulatory obstacles for deployment of new technologies. This is particularly true in the area of agricultural biotechnology and specifically as related to plant transformation technologies. In light of the complex patent landscape and recognizing the robust intellectual property portfolio among universities and public research institutions, this project seeks to leverage the public portfolio of technologies to support the development of a patent pool of technologies available for plant transformation. This research project will develop and test a grape-specific transformation system for the generation of genetically engineered *Vitis* that addresses legal IP issues, meets high technical standards and is designed with attention to the emerging regulatory framework. The proposed plant transformation system can serve as a platform tool for the practical deployment of transgenic Pierce's disease (PD) control strategies.

INTRODUCTION
PIPRA, the Public Intellectual Property Resource for Agriculture, is a public sector multi-institutional program designed to provide the framework to manage IP and develop tools that will facilitate humanitarian or commercial development of promising agricultural innovations. In research to control PD, several transgenic strategies have been tested and show long-term promise. However, the gene transfer tools utilized for research are, in general, proprietary and do not provide features that are likely to be compatible with evolving regulatory frameworks. As a consequence, promising research conducted today may need to be replicated with different tools and technologies if transgenic plants are ever to be deployed for commercial field production. The objective of the research project is to design and test a plant transformation system that addresses IP and regulatory issues and that could be used for research and commercial deployment of transgenic PD control strategies in grapes.

OBJECTIVES
1. Design, develop, and validate a grape-specific transformation system that addresses legal IP, technical and regulatory considerations.
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
Transformation vector system
Although excision and removal of selectable markers has been accomplished in many plant species that can be subjected to subsequent rounds of breeding, this approach is not feasible in grape cultivars because of the inability to engage in subsequent rounds of breeding. Here we proposed a strategy that has been demonstrated in several model systems and uses recombinase-mediated gene excision to remove the selectable marker from the genome, after selection of transformed plants, by a mechanism which does not support re-integration (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-based transformation cassette is designed to incorporate three distinct functionalities: selection for cells that are initially transformed, an inducible recombinase gene that can be transiently activated to excise the selectable marker cassette and a second negative selectable marker (Perera et al. 1993, Gleave et al. 1999) to eliminate cells in which recombinase-mediated excision does not occur.

This approach can achieve removal of the selectable marker during the first generation plant tissue culture stage. PIPRA's legal and scientific staff performed a review of the intellectual property landscape surrounding recombinase-based plant transformation systems and this scientific and legal information was subsequently used to design a Par A recombinase- based marker removal transformation vector suitable for asexually propagated crops, such as grapes. Although recombinase-mediated gene excision systems have been filed for patent protection (Moller et al. 2004), preliminary evaluation and licensing discussion indicate that the specific technologies incorporated in this vector are available for non-exclusive licensing. Thus far, PIPRA designed and initiated synthesis of the recombinase transformation vector (Figure 1).

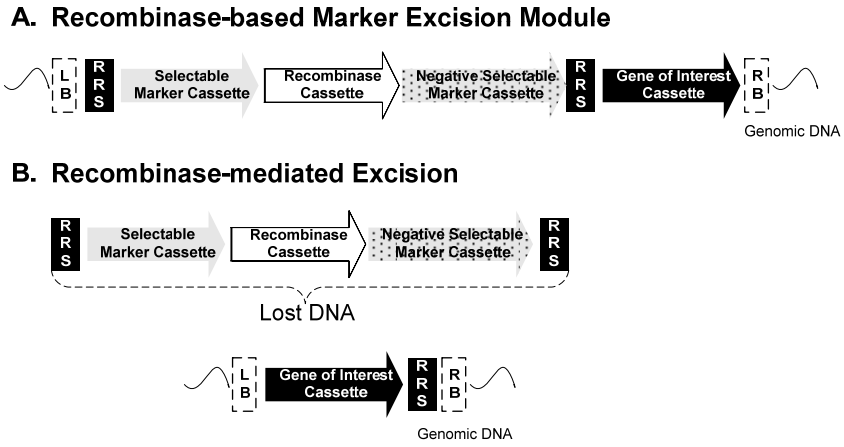

Figure 1. Diagram of the Recombinase-Excision Strategy. Panel A: Recombinase-based transformation construct contains a gene of interest cassette and two selectable marker cassettes flanked by recombinase recognition sequences (RRS). Panel B: Inducible expression of the recombinase excises the selectable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

Selectable markers

Genetic engineering of plants typically requires the co-integration of trait-conferring genes with genes that confer positive or negative selection to facilitate identification of genetically modified cells. 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). However, in spite of the fact that *NptII* has been determined to be safe by numerous regulatory agencies, consumers express concern over residual non-plant antibiotic resistance genes in genetically modified crops. Furthermore, broad issued patents and new patent claims covering the use of antibiotic resistance genes for plant transformant selection are in place in the U.S. and not generally available for license. PIPRA has now tested two recently described plant-derived markers (Dirk et al. 2001, 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. For these experiments, young embryogenic grape (Thompson Seedless) callus were plated on callus induction medium containing increasing levels of inhibitors (Figure 2). Callus was plated into 24 well plates containing increasing concentrations of kanamycin, hygromycin, and glufosinate. To test the efficacy of the *DEF2* inhibitor in grapes, increasing concentrations of actinonin (0, 15, 30, 60, and 120 mg/L) were added to the media. Callus was subcultured every two weeks onto fresh inhibitor containing media. Plates were evaluated for callus development at eight weeks. Thus far, we are observing some reduction in callus growth with increasing levels of all the inhibitors, especially hygromycin (Figure 2). The preliminary results indicate that

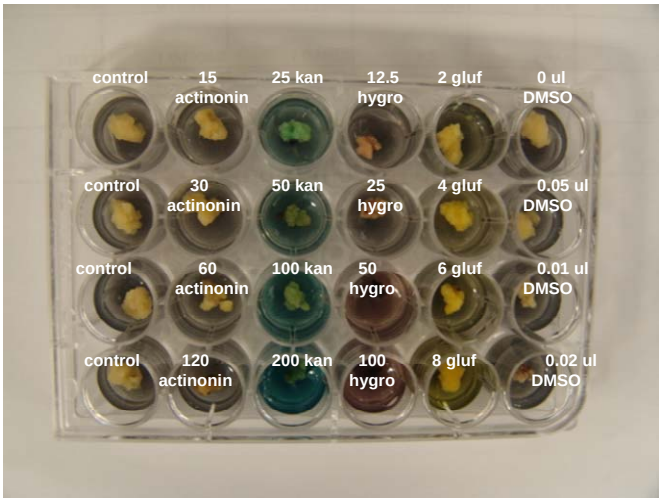

Figure 2. Grape callus growth on various selective agents (actinonin, kanamycin, hygromycin, and glufosinate) (mg/L). Control samples contain no inhibitor (control) or a solvent used for the actinonin inhibitor, DMSO.

higher concentrations of actinonin are required to achieve better selection in grapes. While the growth reduction with actinonin is encouraging, we also observed some reduction in growth with increasing DMSO concentrations, a chemical utilized in the actinonin buffer.

Promoters

PIPRA identified constitutive promoters (Purdue's MAS, University of California's UC FMV34S) suitable for grape transformation. In addition, we identified three 3'UTRs that are in the public domain and that could be used for the proposed vectors; 3'UTRs from pea rubisco E9, nos, CaMV35S.

***Agrobacterium* alternatives**

Of a limited number of high efficiency plant transformation methods, the method of choice for essentially all researchers is *Agrobacterium tumefaciens*-mediated transformation. Patent coverage for *Agrobacterium*-mediated transformation in the U.S. is uncertain because of a long interference which has delayed issuance of the primary patent for over 20 years. By comparison to its European counterpart we can reasonably conclude that when the US patent issues, it will contain methods claims to the use of *Agrobacterium* and T-DNA border sequences (Fraley et al. 1991). PIPRA's transformation strategy has been to identify alternate strategies to the use of both *Agrobacterium* and T-DNA borders as components of the gene transfer vehicle.

We have primarily explored the possibility of utilizing early 1980's technology based on the use of *Rhizobium trifolli* to transform plants. *Rhizobium trifolii, 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 et al. 1986, Broothaerts et al. 2005, Jefferson 2005). We have 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.

CONCLUSIONS

Several promising transgenic approaches have addressed the PD threat to California's wine grape industry (Aguero et al. 2005, Reisch and Kikkert 2005). Of the projects that tested transgenic strategies for PD resistance, each used proprietary technologies that could not be deployed commercially due to IP issues and would likely not survive regulatory scrutiny. Moving forward, it is important to develop a transgenic technology platform in grape with accompanying IP analysis that will allow transfer of control strategies from the laboratory to commercial fields. Anticipating potential IP roadblocks is particularly important in *Vitis* research because it has a high market value, is recalcitrant to routine transformation protocols and has a long tissue regeneration timeframe. Grapes may take two to three years per generation and decades to breed industry-acceptable cultivars and it is impractical to employ research strategies that ultimately need to be repeated for commercial deployment due to IP issues that were not addressed at the start of the project. PIPRA, as a clearinghouse of patented technologies, accesses an IP portfolio that represents ~45% of the proprietary agricultural innovations developed in the public sector. Thus, PIPRA is well positioned to develop technology packages that provide a clear legal pathway for research that is targeted towards practical PD and Glassy-winged sharpshooter applications.

REFERENCES

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.

Broothaerts, W., H. J. Mitchell, B. Weir, S. Kaines, L. M. Smith, W. Yang, J. E. Mayer, C. Roa-Rodriguez, and R. A. Jefferson. 2005. Gene transfer to plants by diverse species of bacteria. Nature 433: 629-33.

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 USA 88: 10558-62.

Dirk, L. M., M. A. Williams, and R. L. Houtz. 2001. Eukaryotic peptide deformylases. Nuclear-encoded and chloroplast-targeted enzymes in Arabidopsis. Plant Physiol 127: 97-107.

Dirk, L. M., M. A. Williams, and R. L. Houtz. 2002. Specificity of chloroplast-localized peptide deformylases as determined with peptide analogs of chloroplast-translated proteins. Arch Biochem Biophys 406: 135-41.

Fraley, R. T., R. B. Horsch, and S. G. Rogers. 1991. Genetically Transformed Plants. *In* EP0131620 [ed.]. Monsanto Company.

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.
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: 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.
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 Pierce'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 Biotechnology Journal 2: 233-240.
Schilperoort, R. A., P. J. J. Hooykaas, A. Hoekema, R. J. M. van Veen, and H. den Dulk-Ras. 1986. A process for the incorporation of foreign DNA into the genome of dicotyledonous plants, pp. 11. *In* E. P. Office [ed.]. Leiden University, Netherlands.
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, S. G. Moller, and N. H. Chua. 2001. Chemical-regulated, site-specific DNA excision in transgenic plants. Nat Biotechnol 19: 157-61.

FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierce’s Disease and Glassy-winged Sharpshooter Board, and the University of California Pierce’s Disease Grant Program.