ENABLING TECHNOLOGIES FOR GRAPE TRANSFORMATION

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

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

Sara Boettiger
PIPRA
University of California
Davis, CA 95616
(sboettiger@ucdavis.edu)

David Tricoli
PIPRA
University of California
Davis, CA 95616
(dmtricoli@ucdavis.edu)

Tamara Holst PIPRA
University of California
Davis, CA 95616
(|tlpholst@ucdavis.edu)

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ABSTRACT
Patenting of agricultural biotechnologies has expanded dramatically over the last 25 years and can represent a significant barrier to new crop development. Thus, navigating the intellectual property (IP) rights of commonly used research tools is essential to prevent downstream legal or regulatory obstacles for deployment of promising new technologies. The research proposed here seeks to develop and test a grape-specific transformation system for developing 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 the 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 proposed here 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
PIPRA has proposed to identify a suite of complimentary technologies that are scientifically functional and legally deployable for public research and potential commercial uses. Described below are technologies believed to meet these needs.

Plant Transformation
Of a limited number of high efficiency plant transformation methods, the method of choice for essentially all researchers is *Agrobacterium tumefaciens*-mediated transformation. In this process, genes are delivered to plant cells via contact with *Agrobacterium* that harbor plant transformation vectors containing a DNA cassette flanked by *Agrobacterium* T-DNA borders. The T-DNA sequences facilitate transfer and integration of the desired transgene into the plant genome. 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 will thus seek to identify alternate strategies to the use of both *Agrobacterium* and T-DNA borders as components of the gene transfer vehicle.

Agrobacterium Alternatives

Rhizobium trifolii, Rhizobium, *Sinorhizobium meliloti*, and *Mesorhizobium loti* species have all been demonstrated to introduce new genetic material into plants (Schilperoort et al. 1986, Broothaerts et al. 2005). 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). PIPRA is currently assessing the legal landscape surrounding the use of these strains for plant transformation.

P-DNA Technology

PIPRA proposes 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 patented P-DNA sequences that are functionally comparable to those from *Agrobacterium* (Rommens 2004, Rommens et al. 2004, Rommens et al. 2005). While P-DNA borders from *Vitis* have not been reported, we propose to use degenerate primers to isolate putative functionally equivalent sequences from grape. Additionally, we have made arrangements to search for P-DNA border sequences in a Pinot noir cultivar that has recently had its genome sequenced through a collaboration between the Italian Istituto Agrario di San Michele and Myriads Genetics Inc. P-DNA borders are attractive as they allow the creation of transformation vectors in which the entire transferred DNA is plant-derived.

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. A number of new selectable markers have recently been described (Miki and McHugh 2004) and notably, two plant-derived markers have been reported (Dirk et al. 2001, 2002, 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 (Dirk et al. 2001, 2002). The *Arabidopsis* ABC transporter, *Atwbc19*, provides kanamycin resistance levels comparable to the bacterial-*NptII* gene when overexpressed (Mentewab and Stewart 2005). 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 discussions to include these technologies in the transformation vector system.

Marker-free technology

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 kill 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. Although recombinase-mediated gene excision systems have been filed for patent protection (Moller et al. 2004), preliminary evaluation indicates these technologies are available for non-exclusive licensing.

Promoters

Regulatory elements that control the expression of desirable traits or selectable markers in specific plant or tissue organs and developmental stages are desirable when developing biotechnology products. PIPRA has created a database of promoters with technical and legal information. This database is populated with over 700 promoters and has been valuable in analyzing the IP availability of regulatory elements. A wide array of these promoters is patented; however PIPRA staff and a team of patent attorneys have worked to identify a subset of promoters that are either freely available as public domain resources or owned by PIPRA members. Of particular interest to grape research are the PD responsive grape promoters identified by Dr. Cook and colleagues at the University of California, Davis. A selection of these promoters will be included in the vector system and will accommodate varied expression pattern needs (i.e. constitutive, PD responsive, root specific).

Testing and Validation of Transformation System
Due to the time period covered by this proposal and the recalcitrant nature of *Vitis vinifera* transformation, PIPRA and The Ralph M. Parsons Foundation Plant Transformation Facility will use the Thompson Seedless grape variety. Once we generate a sufficient number of independent transgenic events to obtain statistically meaningful data, we can apply the findings to a more targeted effort on select wine grape cultivars and root stocks. Additionally, system components will be tested separately before integrating all pieces into a single system. Experimental characterization of these vectors will rely on a gene of interest cassette comprised of a marker gene. The final system will offer an alternative to *Agrobacterium*-mediated transformation, P-DNA borders, alternative plant selection markers, recombinase-mediated marker gene excision and a variety of promoter options.

Explore collaborative opportunities with transgenic PD Control Strategies
The outcome of this research lends itself for collaborative projects. An important aspect of this project is adoption and improvement of the transformation system by researchers utilizing transgenic approaches for PD management. PIPRA is actively exploring collaborations within the PD consortia.

IP Strategy
Effectively accomplishing the goals of this project will require parallel approaches addressing an IP strategy as well as a technology development strategy. The IP strategy will evaluate patent landscapes related to each element in the grape transformation system. It will also refine and implement a plan to access IP rights to selected technologies that are necessary to develop and utilize the transformation technologies embodied in a series of grape-specific transformation vectors. This will require substantial bilateral and multi-lateral negotiations and the development of agreements that can be implemented across many technology users and projects. This system is envisioned to be made available under a packaged licensing agreement that will encompass all system components and be pre-negotiated on a non-profit research royalty-free and a commercial fee-per-use basis.

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 2-3 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, represents 41 non-profit universities and research institutions in 12 countries which account for at least 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 Pierce's disease and Glassy-winged Sharpshooter applications.

REFERENCES

Aguero, C. B., S. L. Uratsu, C. Greve, A. L. T. Powell, J. M. Labavtich, 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 U S A 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.
Rommens, C. M. 2004. All-native DNA transformation: a new approach to plant genetic engineering. Trends Plant Sci 9: 457-64.
Rommens, C. M., O. Bougri, H. Yan, J. M. Humara, J. Owen, K. Swords, and J. Ye. 2005. Plant-derived transfer DNAs. Plant Physiol 139: 1338-49.
Rommens, C. M., J. M. Humara, J. Ye, H. Yan, C. Richael, L. Zhang, R. Perry, and K. Swords. 2004. Crop improvement through modification of the plant's own genome. Plant Physiol 135: 421-31.
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.

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SUPPORT FOR THE MANAGEMENT OF INTELLECTUAL PROPERTY WITHIN THE PIERCE'S DISEASE RESEARCH INITIATIVE AND RESEARCH COMMUNITY

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

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

Sara Boettiger
PIPRA
University of California
Davis, CA 95616
(sboettiger@ucdavis.edu)

Josef N. Geoola
PIPRA
University of California
Davis, CA 95616
((jngeoola@ucdavis.edu))

Reporting Period:

ABSTRACT
The Public Intellectual Property Resource for Agriculture (PIPRA) and the California Department of Food and Agriculture Pierce's Disease/Sharpshooter Board (Board) began collaborations in 2005 with the goal of instituting an intellectual property (IP) management strategy inline with the Pierce's disease (PD) research consortium's mission. Within the last year, a number of information resources have been made available by PIPRA specifically tailored for the PD research community. These resources include a publicly accessible, live and comprehensive database of all PD related IP and scientific literature, an analysis of the IP and scientific literature surrounding PD research, and an IP landscape surrounding a promising PD specific technology. Collectively, these resources allow scientists to have an integrated view of the technical and legal aspects involved in their projects.

INTRODUCTION
The Public Intellectual Property Resource for Agriculture (PIPRA) is a not-for-profit research organization hosted by the University of California, Davis. PIPRA currently represents 41 public sector organizations from twelve different countries and its mission is to enable access to agricultural intellectual property (IP). PIPRA offers a range of services to address legal issues that arise during research and deployment of bio-technologies. PIPRA and the California Department of Food and Agriculture Pierce's Disease/Glassy-winged Sharpshooter Board (Board) began collaboration in 2005 to address IP issues surrounding Pierce's disease (PD) research and development. In particular, the threat PD poses to California's $16.5 billion wine industry requires foresight to seek and secure commercial deployment of feasible technologies resulting from funded research. In terms of IP, the Board would like to ensure that technologies with the potential to control PD could be promptly deployed without becoming tangled in a legal web of licenses, rights, and lawsuits.

Technologies resulting from research funded by issue-focused consortia and conducted at multiple institutions, as in the case of the PD consortium, can face three basic IP problems during research and development. First, the researchers themselves may not be aware of their obligations or opportunities with regard to patenting research discoveries. Second, once patented, new discoveries are rightfully the property of the funded research institution or university, which may have internal policies regarding licensing that may be inconsistent with the objectives of the consortia. And third, the new technologies may be blocked by already existing patented technologies. These kinds of IP issues are not uncommon in industry consortia. They are, however, often resolved up front by contractual relationships or formal joint ventures that take into account the participants' IP management strategies. Consortia of universities and other public research entities, however, typically do not have developed IP management strategies in place, in part due to the fact that public sector researchers often pay little heed to the proprietary nature of their research inputs and outputs.

PIPRA recognizes that an IP management strategy for the PD consortium needs to take a multilateral approach toward maximizing the effectiveness of the consortium's intellectual assets. Rather than focusing solely on IP protection, IP management for the PD consortia should also set milestones for technology development, assess marketing opportunities, and seek a better negotiating position during IP exchange. In essence, PIPRA seeks to aid the Board in coordinating IP to allow for access and protection, both of which are essential to the productivity of research across multiple institutions, while creating opportunities and incentives for further commercial development.

The first step toward effective IP management is the availability of information resources specifically tailored to Board funded PD researchers. Such resources provide scientists with technical and legal information critical for the deployment of marketable products with maximum security over IP rights. This report discusses the information resources specific to the PD research consortium developed by PIPRA. Included will be detailed descriptions of the IP and scientific literature database