FINAL 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
CDFA CONTRACT NUMBER: 06-0224

***Project Title**: Enabling Technologies for Grape Transformation*

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***Time period covered by the progress report**: July 1, 2006 - July 31st, 2009*
(Grant funding period July 1st, 2006 - June 30th, 2009 & no cost extension until June 30th, 2009)

OBJECTIVES & ACCOMPLISHMENTS OF PROPOSED RESEARCH

1. Design, develop, and validate a grape-specific transformation system that addresses legal IP, technical and regulatory considerations.
2. Develop Agrobacterium and TransBacter™-mediated transformation for California winegrapes and/or cultivars suitable for generating root stocks.
3. Develop strategies to disseminate the biological resources developed under appropriate agreements for the PD community.
4. Explore collaborative opportunities with researchers developing Pierce's disease control strategies that could employ and test the proposed grape transformation system.

Described below is a description of the activities and accomplishments for each of the objectives completed during the research funding period.

OBJECTIVE 1. DESIGN, DEVELOP, AND VALIDATE A GRAPE-SPECIFIC TRANSFORMATION SYSTEM THAT ADDRESSES LEGAL IP, TECHNICAL AND REGULATORY CONSIDERATION.

PIPRA's team performed an extensive evaluation of the IP surrounding the relevant plant transformation technologies and technical requirement to develop a marker-free system suitable for grape transformation. Based on these analyses, the proposed system includes a Par- A recombinase-based marker removal system under the control of an estradiol-inducible promoter. The original strategy proposed to develop single a plant binary DNA plasmid with the numerous components outlined in Figure 1. Through a recombination event, the final transgenic lines would contain only the gene of interest, the footprint of the recombination recognition site (RRS), and right and left transfer DNA borders (TDNA).

To develop this system, the individual components were tested in grapes and/or tobacco, a model plant system with a faster transformation cycle than grapes. And secondly, we designed and initiated development of plant binary DNA plasmids useful to generate marker-free plants. Based on the grant results, we present recommendation on alternate research strategies to achieve the proposed goal to generate marker-free transformed grape plants.

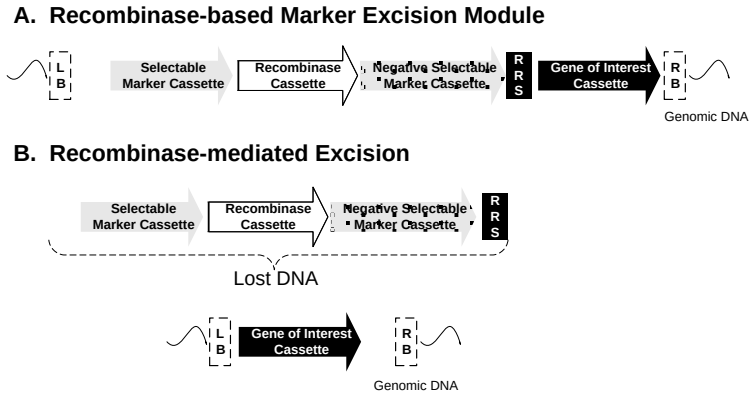


Figure 1. Recombinase-Excision Strategy. Panel A: Recombinase-based transformation construct contains a gene of interest cassette and two selectable marker cassettes flanked by recombinase recognition sequences (RRS). Panel B: Inducible expression of the recombinase excises the selectable marker cassette. Transgenic lines with successful recombination events can be isolated by treatment with a negative selection agent and expression of the gene of interest.

TESTING COMPONENTS OF THE MARKER-FREE TRANSFORMATION SYSTEM

PIPRA and UC's Plant transformation facility individually tested the components of the recombinase system (Figure 1) including plant selectable marker, negative selectable marker, promoters useful in the marker free system, including an inducible promoter to drive expression of the recombinase enzyme and constitutive promoters with freedom-to-operate and suitable for strong expression in grapes. Preliminary testing and transgenic lines were generated to test ParA recombinase activity.

PLANT 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 emphasis of this research was on identifying plant transformation selection genes with freedom to operate and that are suitable for grape genetic engineering.

The initial proposal aimed at testing two plant-derived markers that were developed and patented by PIPRA member institutions [1-4]. We tested the Arabidopsis genes **plant peptide deformylase (DEF)** and **ABC transporter (Atwbc19)** as potential plant selection genes in grapes. *DEF* confers tolerance, when overexpressed, to DEF-specific inhibitors (actinonin) 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, which provides tolerance to a broader spectrum of antibiotics, the plant transporter *Atwbc19* 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 engaged in productive licensing discussions to include these technologies in the marker-free transformation system. Despite the availability of freedom-to-operate with these markers via licensing opportunities, the transformation efficiencies using DEF or Atwbc19 as selectable markers in grapes were not suitable and thus, neither gene can be considered for grape transformation.

During the course of the research contract, PIPRA followed the legal status of *the* most commonly used plant selection gene in plant research and crop development, ***neomycin phosphotransferase II*** **(NPTII)** that grants tolerance to the antibiotic kanamycin. At the beginning of the research proposal the freedom-to-operate was limited due to broad patent rights owned by Monsanto Company. The patent family covered the use of any antibiotic, and more specifically NPTII, for plant selection. Included in the family, was a pending application (US Patent Application No. 08/127,100), which included very broad claims which, if granted, could have limited FTO well into the timeframe in which this technology may have been used in grape research and development. By the end of 2008, Monsanto's threatening patent application and one of the broadest patents (US Patent No. 6,174,724) was abandoned. Given the recent shift in legal status of Monsanto's patent portfolio NPTII, the use of this marker in plant selection is now a viable option. UC's transformation facility tested the NPTII marker under the transcriptional control of Purdue's MAS promoter and Monsanto's CaMV35S promoter. The research showed both promoters are suitable to drive expression of the NPTII gene in grape transformation.

The **hygromycin phosphotransferase (HPT)** gene is another commonly used marker in plant selection, particularly in grape transformation services by UC's Plant Transformation Facility. PIPRA's information on the IP status reveals that the selectable marker is no longer under the umbrella of Monsanto's expired IP assets on the use of *any* antibiotic gene for plant selection. However, the hygromycin plant selection system is currently protected by patents owned by Novartis Ag (formerly owned by Eli Lilly & Company). Novartis patents continue to be active and the expiration dates are still a few years away. UC's transformation facility tested the HPT marker under the transcriptional control of Purdue's MAS promoter and Monsanto's CaMV35S promoter. The research showed either promoter is suitable to drive expression of the hptII marker in grapes. The MAS::HPTII construct is effective to use as a grape selection gene.

The two plant selectable markers, AtDEF and Atwbc19, are not good candidates for grape transformation. Given the increase freedom-to-operate on NPTII selection gene and its efficiency in grape transformation, we strongly recommend using NPTII in grape transformation. Finally, HPT is technically a good candidate; however, freedom-to-operate needs to be considered.

NEGATIVE SELECTION GENE: The plant transformation, marker-free system, will incorporate a negative selection marker to eliminate transformants in which a recombinase event failed to occur (Figure 1*). We demonstrated the cytosine deaminase (codA) gene is an effective negative selection gene in tobacco plants, tobacco callus and grape callus.* To do this we transformed grape and tobacco with a cytosine deaminase gene under the regulatory control of FMV34S promoter and tested the concentration of 5-flurocytosine (5-FC) necessary to eliminate (kill) transgenic tissues.

In tobacco, data from the wild type explants shows that 5-FC is not toxic to wild-type plants. It appears that concentrations of 5-FC between 200-250 mg/L have the most significant toxic effect on transgenic explants. In grape callus, data shows that the range of toxicity lies between 150 and 300 mg/L. Conditions may need to be further fine tuned between this concentration ranges.

INDUCIBLE AND CONSITUTIVE PROMOTERS FOR GRAPE RESEARCH: *In this research, we identified and tested an inducible promoter to drive expression of the recombinase enzyme and strong, constitutive promoters with freedom-to-operate in grapes.*

The proposed recombinase marker free system includes an inducible promoter for precise control of expression of the recombinase gene. Inducible control is preferred over constitutive expression 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 [5]. This system was preferred over another inducible promoter, the glucocorticoid-system [6, 7], which requires the use of dexamethasone treatment that can often inhibit plant tissue regeneration as well as contain high background levels [8].

The XVE system [5] with a GUS reporter gene was tested in transformed grape callus. The GUS enzyme assay was performed directly on the tissue and a colorimetric change was observed. XVE system showed tight GUS expression and regulation at 50 uM levels of the XVE inducer, estradiol. No background expression was detected in transformed plant cells without the XVE inducer. Thus, from an experimental point of view, the XVE inducible system seems a suitable system to regulate gene expression, i.e. recombinase enzyme, in grape plant cells.

With the support from legal counsel, PIPRA performed an IP analysis on the three parts of the XVE fusion protein. To conduct the IP evaluation of the XVE system, we divided this system into three parts. Part A considered the 3 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 2). 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. There is a verbal understanding of a potential commercial use of Rockefeller's promoter for the PD/GWSS Board funded research.

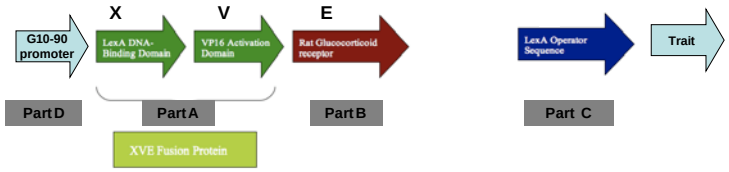


Figure 2. XVE Inducible Promoter System Estradiol Activated. NCBI Accession AF309825.

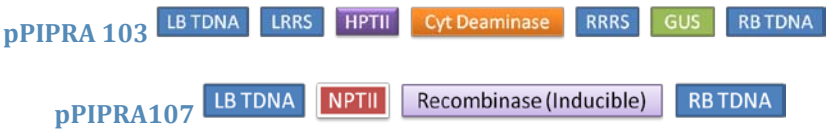
PIPRA tested three constitutive promoters for expression in grapes: Purdue's MAS, University of California's UC FMV34S, and G10-90 from the XVE system. Based on initial conversations with the respective technology owners, the promoters are available for licensing. The MAS promoter was successfully used to drive the *nptII* and *hpt* plant selectable markers. The FMV promoter was used to drive expression of GUS in grapes callus. And finally, the constitutive G10-90 from the XVE system (Figure 2, part D) successfully drove expression of the GUS gene in grape callus.

We showed, from scientific and IP standpoints, the estrogen-inducible XVE promoter system is a suitable candidate for the tight control of transcription activity in plants. However, as described in the section below, the XVE promoter may have leaky expression in bacteria, which may cause low levels of unregulated expression of the recombinase enzyme and cause premature excision of the DNA flanked by the recombinase recognition sites in the plant binary DNA plasmid shown in Figure 1. Leaky bacterial expression is presenting significant obstacles for developing a single plant binary DNA plasmid for the recombinase-based marker-free system –as shown in Figure 1. Alternatives on circumventing this issue are also described below (Alternative suggestions for marker-free system in grapes).

EFFICACY OF THE PAR-A ENZYME IN THE RECOMBINASE SYSTEM: To test the efficacy of the ParA recombinase enzyme we developed two plant binary DNA plasmids, pPIPRA103 and pPIPRA107 (Figure 3). The plasmid pPIPRA103 contains the cytosine deaminase negative selection gene flanked by the left and right recombinase recognition sites (LRRS and RRRS). This plasmid also contains the GUS reporter gene outside the recombinase excision sites. Plasmid pPIPRA107 contains the recombinase enzyme under the inducible XVE promoter to be transformed into the plant cells (tobacco and grape).

In cells with induced-recombination activity, we expect for the cytosine deaminase construct to be excised and lost and, as a result, the substrate 5-FC would not be toxic to the cells. Co-transformed plant cells which failed to have recombinase activity should express the cytosine deaminase and 5-FC should be lethal. All co-transformed plant cells should express GUS regardless of positive or negative induced-recombination activity.

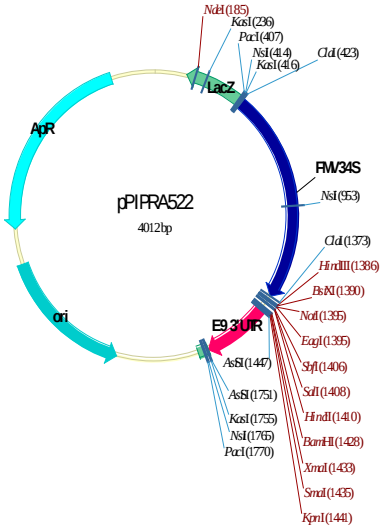
Figure 3. Plant Binary DNA plasmids to test Par A activity in tobacco and grape



Tobacco seedlings were transformed with pPIPRA103 and 107 and selected in hygromycin and kanamycin; thus, cells are expected to contain both plasmids. This month, we harvested T1 seeds in which recombinase activity can be induced and analyzed. Until date the grape callus was transformed with pPIPRA103 but has not been subsequently transformed with pPIPRA107.

PIPRA proposed to develop cloning strategy with two DNA plasmids to deliver trait genes in a recombinase-based plant binary system. A shuttle DNA plasmid was first developed in order to clone the researcher's promoter and/or trait gene (Figure 4: pPIPRA522—with the FMV34S promoter). The shuttle DNA plasmid contains a multiple cloning site with unique restriction enzymes to clone any particular gene and promoter.

In the cloning strategy, the entire Gene of Interest cassette in the shuttle DNA plasmid can be excised with a unique restriction enzyme (either *Pac*I, *Kas*I, or *Nsi*I) and cloned into the plant binary DNA plasmid containing the rest of the recombinase based marker system (Figure 3, pPIPRAXXX). We encountered problems in assembling the recombinase-binary DNA plasmid (shown in Figure 3) when introducing the last component, the recombinase enzyme. We employed the de novo DNA synthesis services from Blue Heron Biotechnologies Company to assemble the recombinase-based DNA plasmids. The company believes, based on PCR data, they were able to ligate the vector. However, there is supporting data the DNA flanked by the recombinase recognition sites is excised. Excision presumably is the result of premature recombinase activity in the bacterial cell. To eliminate premature recombinase activity of the plant transformation vector, we considered employing a recombinase enzyme with an intron, which should not express a functional enzyme in bacteria since the coding sequence is interrupted by the intron. However, our USDA collaborators which developed the ParA system report a ParA enzyme with an Arabidopsis intron is not functional in plants. Concerned that the enzyme activity may be impaired with the introduction of an intron and based on the basal expression of recombination activity observed in bacteria, we present alternate considerations for a marker free system for grapes.



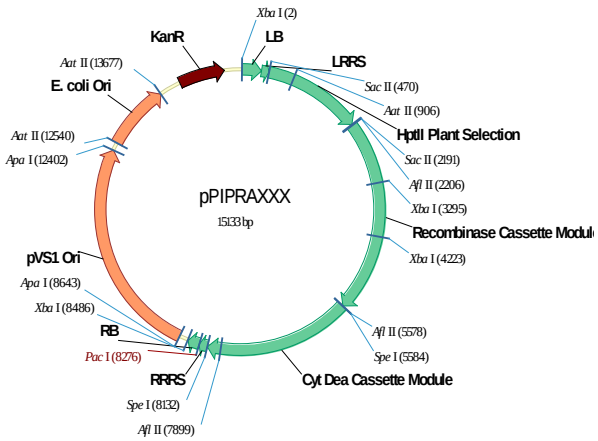


Figure 4: Recombinase-based Marker-free Plant Binary Vector System. Including shuttle vector t introduce Gene of Interest, pPIPRA522—with the FMV34S promoter and a theoretical diagram of the plant binary vector pPIPRAXXX.

ALTERNATIVES FOR A MARKER-FREE SYSTEM IN GRAPES

The first alternate strategy separates the recombinase and DNA with recombinase recognition sites into two separate plant binary vectors (Figure 5). Sequential transformation with these binary vectors was started to be employed in grapes to test the ParA activity with pPIPRA103 and pPIPRA107 (Figure 3). We consider a more effective vector is adding a cytosine deaminase to PIPRA 107 to allow elimination of this DNA after recombination. This strategy circumvents the problem of excision of DNA in the binary vector as the DNA is amplified in the bacterial host.

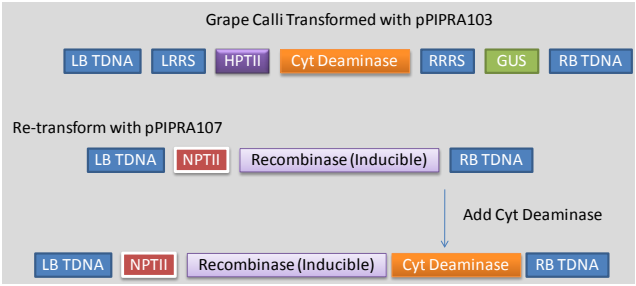


Figure 5. Marker-free strategy employing Recombinase Sequential Transformation Strategy.

A second approach is considering a co-transformation of gene of interest and selection genes in separate plant binary DNA plasmids (Figure 6). This approach requires generating seedlings with both gene of interest and selection genes. Followed by a breeding or cross in which the two separate TDNA are segregated. While this is not ideal for grapes, given their long generation cycle from seed-flower, strategies to accelerate breeding may make this possible (i.e. breeding efforts from Andre Walker lab at UC Davis).

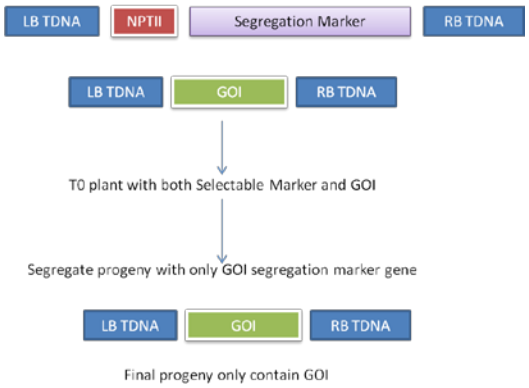


Figure 6. Co-transformation Strategy to generate marker-free grapes.

OBJECTIVE 2. DEVELOP ALTERNATIVES TO *AGROBACTERIUM*-MEDIATED TRANSFORMATION FOR CALIFORNIA WINE GRAPES AND/OR CULTIVARS SUITABLE FOR GENERATING ROOT STOCKS.

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

OBJECTIVE 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 PIPRA worked with UC Davis legal counsel and the technology providers to complete a draft a license acceptable to PIPRA's host institution and supported by the potential technology providers.

OBJECTIVE 4. EXPLORE COLLABORATIVE OPPORTUNITIES WITH RESEARCHERS DEVELOPING PD CONTROL STRATEGIES TO LINK THE DEVELOPED TRANSFORMATION TECHNOLOGIES WITH SPECIFIC PD RESISTANCE TECHNOLOGIES.

The technical hurdles encountered to clone the recombinase plant binary vector have prevented us from generating the DNA plasmids. We remain hopeful that a marker-free system for grapes is possible. Consideration of the two alternate strategies may be alternatives for a marker-free system in grapes.

RESEARCH RELEVANCE STATEMENT

PIPRA's proposed grape transformation 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.

SUMMARY AND STATUS OF INTELLECTUAL PROPERTY PRODUCED DURING THIS RESEARCH PROJECT

No new IP was generated in this project.

RELEVANT PUBLICATIONS

1. Miki, B. and S. McHugh, *Selectable marker genes in transgenic plants: applications, alternatives and biosafety.* J Biotechnol, 2004. **107**(3): p. 193-232.
2. Dirk, L.M., M.A. Williams, and R.L. Houtz, *Specificity of chloroplast-localized peptide deformylases as determined with peptide analogs of chloroplast-translated proteins.* Arch Biochem Biophys, 2002. **406**(1): p. 135-41.
3. Dirk, L.M., M.A. Williams, and R.L. Houtz, *Eukaryotic peptide deformylases. Nuclear-encoded and chloroplast-targeted enzymes in Arabidopsis.* Plant Physiol, 2001. **127**(1): p. 97-107.
4. Mentewab, A. and C.N. Stewart, *Overexpression of an Arabidopsis thaliana ABC transporter confers kanamycin resistance to transgenic plants.* Nat Biotechnol, 2005.
5. Zuo, J., et al., *Chemical-regulated, site-specific DNA excision in transgenic plants.* Nat Biotechnol, 2001. **19**(2): p. 157-61.
6. Aoyama, T. and N.H. Chua, *A glucocorticoid-mediated transcriptional induction system in transgenic plants.* Plant J, 1997. **11**(3): p. 605-12.
7. Ouwerkerk, P.B., et al., *Glucocorticoid-inducible gene expression in rice.* Planta, 2001. **213**(3): p. 370-8.
8. Zuo, J., Q.-W. Niu, and N.-H. Chua, *An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants.* The Plant Journal, 2000. **24**(2): p. 265-273.