Project Report for CDFA contract number. 08-0451

Project Title: Functional Testing and Delivery of Pierce's Disease-Specific Promoters from Grape
Principle Investigator: David Gilchrist (dggilchrist@ucdavis.edu); (530)-752-6614
Co-Principle Investigator: James Lincoln (jelincoln@ucdavis.edu) (530)752-6587
Department of Plant Pathology, University of California, Davis, CA 95616.
Cooperator: Douglas Cook (drcook@ucdavis.edu) (530)754-6561. Department of Plant Pathology, University of California, Davis, CA 95616

Time period covered by this report: October 2008-March 2009.

Duration of Project: This project is to conclude on June 30, 2009 with characterization and delivery of transgenic plants bearing two *Xylella fastidiosa*-inducible promoters (9353 and 7061),

Keywords: Pierce's Disease, disease responsive promoters, molecular diagnostic tool, Xf-specific expression of heterologous genes, targeted gene expression

Objectives:

1. Evaluation of whole plant transgenics with stem inoculation under greenhouse conditions

This research will conclude with the release of the two *Xylella fastidiosa*-inducible (*Xf*) promoters now in stable transgenic Thompson Seedless lines. Evaluation of the first 10 lines each of promoter G9353 and G7061 has been completed, confirming that they are *Xf*-inducible. The data also showed substantial differences in level of expression among the individual transgenic lines. We are currently completing the investigation of 10 additional independent transgenic lines of each promoter-GFP fusion by inoculation with *Xf*. By the completion of this project in July 2009 we will have evaluated the timing and location of promoter-GFP response to *Xf*. Criteria for selecting the prime transgenic lines to be released to PD researchers are that they express a specific, rapid and strong response to Xf infection with low background response.

2. Confirm the specificity of response of promoter G9353 to Xylella fastidiosa vs Xanthomonas campestris using intact stem inoculation under greenhouse conditions

It is essential that the response to *Xf* be specific. Initial observations are consistent with response to Xf but not to the related bacterium *Xanthomonas campestris* pv. *Vesicatoria*.

3. Promoter distribution to current researchers and long term storage

We will package and release the most responsive transgenic lines of each of the two promoters and binary plasmids containing the validated Xf-inducible promoters, G9353 and G7061 to all interested researchers. These promoter-GFP fusions are currently being used for studying the timing and incidence of *Xf* infection, action of pathogen effector molecules (Bruening) and could be used to determine plant response to mechanical inoculation or sharpshooter feeding.

Discussion of the Project in Relation to the Objectives:. This project was initiated in July 2004 as a priority research area by the Pierce's Disease Research Board (3). The need for *Xf*-inducible promoters was based on the fact that the constitutive promoters, used universally to drive the expression of transgenes, suffer from two disadvantages. Firstly, they are protected by existing patents with the attendant limitation for commercial use, and secondly, the constitutive expression of certain transgenes is widely considered to have serious potential deleterious effects. For example, there are recent reports showing a deleterious effect from constitutive

expression of disease resistance genes, effects that are remedied by expression from an inducible promoter (5, 6). Specifically, the constitutive expression of genes that normally are under control of stress-responsive promoters (infection responsive and resistance genes) is likely to be disadvantageous to the plant if continuously expressed (5). At the outset, an Affymetrix GeneChip was used to characterize the expression of ~15,000 *Vitis vinifera* genes in the presence of *Xf* infection and drought stress (1, 2) (Figure 1).

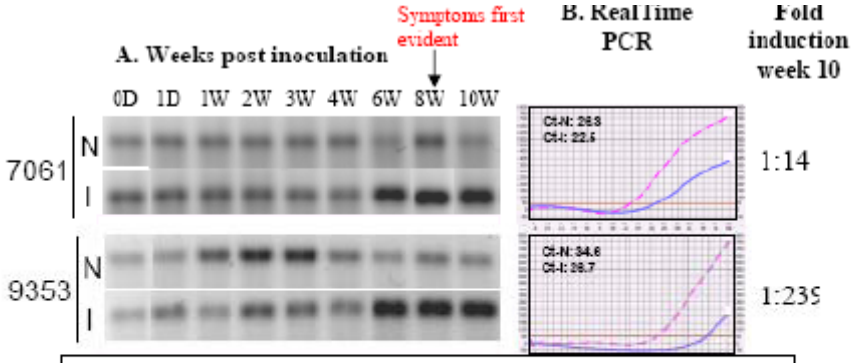

Figure 1. *Xylella fastidiosa* responsive promoters were obtained from genes found to be up-regulated in *X. fastidiosa* infected field grown plants but not up-regulated by water stress alone. Example data used to identify individual genes from which promoters were recovered as illustrated for # 7061 and 9353.

Promoters for two of the induced genes that appeared in only the PD expressing plants and localized to vascular tissue, referred to hereafter as G9353 and G7061, were sequenced from bacterial artificial chromosome (BAC) clones. Regions 5' to the coding sequence were isolated by PCR and used to produce promoter-GFP-reporter fusions (Figure 2) for transformation into the *Xf* susceptible Thompson Seedless grape background. We received 42 transformants (Table 1) from UC Plant Transformation Facility in 2006 from constructs of the promoters G9353 and G7061 fused to GFP. A line bearing each promoter will be selected from the 42 independent transformants for maximum specificity and sensitivity to the presence of *X. fastidioasa.*

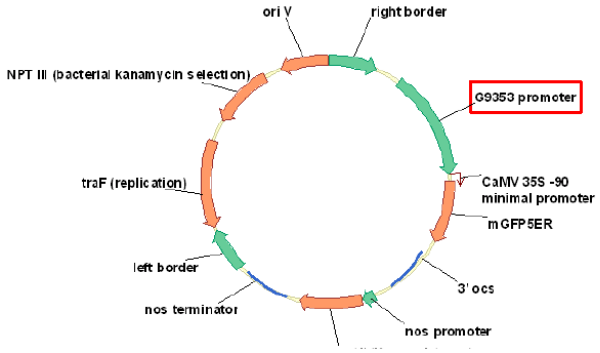

Figure 2. Binary plasmid containing the G9353 promoter sequence fused to GFP, which will be available to Pierce's Disease researchers upon request.

In addition to their utility for engineering PD resistance in grape, the availability of *Xf*-induced reporter gene expression can provide an extremely powerful tool to study and characterize host responses in their intact cellular and tissue context. With such tools, it should be possible to examine the chemical and/or physical

Table 1. Transgenic plants containing promoters G7061 and G9353

Promoter ID	Putative function of the microarray transcript induced by Xf	total # of transformants with the promoter-GFP fusions	# of transformants tested to date	Results Xf uptake into detached leaf;	# transformants petiole inoculated and analyzed	# transformants to be stem inoculated analyzed
G7061	unknown	22	12	9 of 12 show GFP expression	4 of 10 show GFP expression	22
G9353	Alpha-tubulin	20	10	3 of 10 show GFP expression	3 of 10 show GFP expression	20

Table 1. Results and current needs of inoculation experiments on transgenic Thompson Seedless plants bearing the first two promoters G7061 and G9353 fused to GFP. Transgenic plants were inoculated with 10^7 cfu/ml of *X. fastidiosa*, into leaf petiole, maintained in the greenhouse and assayed for GFP expression with confocal microscopy at 14 days. Similar stem inoculations are in progress.

cues from the insect or pathogen that trigger host gene expression and the deleterious effect of the disease. Lastly, an immediate and direct application has been identified through our collaboration with the Bruening laboratory wherein, promoter G9353 expression is activated by injection into the grapevine petiole of *Escherichia coli* cells expressing a single *Xf* protein, the protein synthesis elongation factor EF-Tu

We currently have clonal representatives of 42 transgenic lines for direct analysis by inoculation with *Xf*. Initial testing of the first 50% of these plants as reported earlier revealed two important characteristics. First, both G9353 and G7061 promoter-GFP fusions were activated following inoculation with *Xf* in most, but not all of the lines tested, and, as expected, considerable variation was seen in the level of response among independently transformed plant lines to *Xf*. (Table 1). Even though all lines tested were shown by PCR to contain the respective sequences, not all lines expressed GFP in the presence of *Xf* to the same degree and several expressed none. This was not unexpected in working with uncharacterized transgenic material but it does confirm the need to assess all lines quantitatively for specificity, timing, and sensitivity of response to *Xf* or Xf effector molecules, which we are now doing. The goal is to identify one transgenic line each of G9353-GFP and G7061-GFP that meets the dual need to effectively drive potential anti-PCD transgenes and to serve as a diagnostic tool for researchers studying the activity of bacterial genes or chemicals that lead to Pierce's Disease (Figure 3 as an example).

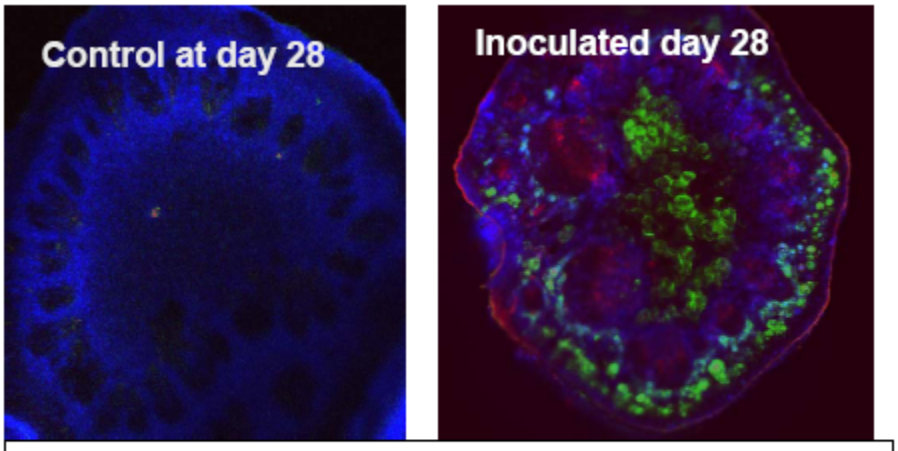

Figure 3. Example of GFP expression driven by Promoter 9353 in the presence of *Xylella fastidiosa*, 28 days after inoculation with 20 ul of the bacteria at 10^5 cfu per ml.

Methodology to Accomplish Objectives from the Original Proposal:

Objective 1 Whole plant inoculation to determine strength, timing and location of promoter-GFP fusion induction as the final determinant of promoter response.

Promoter-GFP fusions for G9353 and G7061 were introduced into the *V. vinifera* cultivar Thompson Seedless by the UCD Transformation Facility. As indicated earlier, a total of 42 plants were received (Table 1). The transgenic grape plantlets are grown in the greenhouse and each primary transgenic was assayed for the presence of the transgenes by genomic PCR prior to any further experimentation. While all plants tested to date contain the transgene not all appear to be activated to the level of detectable fluorescence, even though analysis by qPCR confirmed that the promoter was active; just not sufficiently active for GFP detection. Hence, there is sufficient variation in the strength of the GFP fluorescence response to require that all independent transformants must be tested to identify the plants with the most rapid and strongest response with minimal background. For example, quantitative data indicates that the activation must be greater than 4-fold to be visualized as differential by confocal fluorescence microscopy. This value represents the first cutoff point to retain a particular clone. The remaining plants will be subjected to final testing with whole plant inoculations.

Objective 2: ***Confirm the specificity of response of promoter G9353 to Xylella fastidiosa vs Xanthomonas campestris using intact stem inoculation under greenhouse conditions***

An important question is: are these promoters specific to *Xf* or do they also respond to closely related bacteria such as *Xanthomonas campestris* pv. *vesicatoria*? Inoculations of both Xylella and Xanthomonas were conducted on cloned copies of G9353 plant #2 in the greenhouse by petiole puncture with 10^7 cfu/ml. GFP fluorescence, indicative of induced expression of G9353, was detected in leaves inoculated with *Xf* but not in cloned G9353 plants inoculated with *X. campestris* pv *vesicatoria* (4). The most responsive promoter transgenics will be tested for response to X. c. vesicatoria to be certain that specificity is maintained.

Objective 3: Package and release the validated Xf-inducible promoters, G9353 and G7061 as promoter-GFP fusions in transgenic Thompson Seedless grape lines.

We will prepare and distribute G9353 and G7061 as binary plasmids to anyone wishing to introduce these *Xf*-responsive elements into additional grape cultivars. Clones of the transgenic Thompson Seedless plants will be available to other grape researchers or the industry. There will no testing of additional promoters as originally proposed. These promoters will be useful to investigators studying the timing and incidence of Xf infection, effect of potential pathogenicity dependent molecules, and effect of anti-PD or PD resistance genes. We speculate that these transgenic plants also may have utility in monitoring inoculation by sharpshooter feeding over time. We will establish ramets of each of the most responsive lines and maintain stock cultures of the two promoters in Thompson Seedless in the Gilchrist lab by the *in vitro* propagation/maintenance procedure developed and used by David Tricoli in the Parson's Transformation Facility on campus. We anticipate that regenerative clones of the two most effective GFP-linked transgenic lines can be maintained in this fashion for at least 2-4 years without regeneration.

Intellectual Property (IP): This project will generate intellectual property; specifically promoters that drive expression of grape genes due to the presence of *X. fastidiosa*. The issue of IP will be handled in the following manner: 1) all sequences derived will be placed in the public domain through a collaboration with PIPRA, 2) IP protection will be sought for functionally verified promoters sequences, 3) all materials will be made available to qualified researchers, including all those associated with the PD projects through a standard material transfer agreement.

Contribution of this work to solving the PD problem in California:

This effort will, at its conclusion, have developed enabling technology for PD researchers to address two basic research issues. First the goal of this research was to clone and characterize unique DNA sequences from grape that specifically regulate the expression of grape genes in tissues that are infected with *X. fastidiosa* or are receiving systemic signals of pathogen presence. In addition, these promoters fused to GFP are specific tools to monitor the presence and movement of the bacteria in relation to grape plant response. The promoters, G9353 and G7061 each will be available as GFP fusion constructs in transgenic Thompson Seedless clonally propagated lines. The line bearing each promoter will have been selected from the 42 independent transformants for specificity and sensitivity to the presence of *X. fastidiosa* and Xf EF-Tu. Currently, Dr. Elizabeth Rogers (USDA, Parlier, CA) has requested and has received the 9353 promoter for use in Pierce's Disease research. Additional users include John Labavitch (PGIP) and Steve Lindow (DSF) In addition, the G9353 and G7061 sequences will be available for distribution as binary constructs to grape researchers. The goal of this research was to clone and characterize unique DNA sequences from grape that specifically regulate the expression of grape genes in tissues that are infected with *X. fastidiosa (Xf)* or are receiving systemic signals of pathogen presence. In addition, these promoters, when fused to GFP, are specific tools to non-destructively study the presence and movement of the bacteria in infected grape canes or petioles

Publications resulting from this work:

One paper on the detection, cloning, expression, and analysis of the selected GFP-promoter fusions, including the specificity of their response to *Xf* will be submitted to a peer reviewed journal at the end of this project.

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