ISOLATION AND FUNCTIONAL TESTING OF PIERCE'S DISEASE-SPECIFIC PROMOTERS FROM GRAPE

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Reporting Period: This two-year project was initiated on October 1, 2004. Obviously, there are few results to report at this time. Only a discussion of the justification, objectives, and timetable will be presented per request by the Pierce's Disease Symposium organizers.

ABSTRACT
Among the potential solutions to Pierce's disease in grapes are approaches based on gene transfer technology that focus on understanding the underlying biochemical and molecular mechanisms regulating PD. One of the research priorities identified by the 2003 PD/GWSS project reviews and as indicated in the 2004 RFP was the need to identify, clone and characterize unique DNA sequences that specifically regulate the expression of grape genes in tissues that are infected with *Xf*. Emphasis was placed on the urgency and practical utility of isolating promoters of PD responsive genes. One of the major bottlenecks in using transgenes, either expressed as proteins or as inhibiting RNAs in grape (or any plant) is the lack of suitable promoters to specifically drive the expression of a transgene on a specific trait (susceptibility to PD) in particular tissues (e.g., vascular tissue) or in response to particular situations (e.g., sharpshooter feeding or *Xylella* infection). In the absence of tissue or response-specific promoters, transgenic strategies to either understand or control PD one can use only so-called constitutive promoters. The basic problem associated with the use of constitutive promoters is that the transgene is expressed in all cells all the time, not just in the tissue or cells where the gene is needed. Highly controlled induction is needed if the interest is in altering gene expression to avoid a cellular change (disease) that is initiated in one or a few isolated cells. The isolation and characterization of *Xf*-responsive promoters has immediate and direct application to several current PD projects that are studying the biochemical or molecular genetic basis of PD at the cellular and tissue levels in grape. It also is of practical importance that these promoters will be useful in either the up- or down-regulation of the expression of a specific gene-of-interest. The difference in presence or absence of the target gene product is determined by whether the promoter is used to drive a sense or an anti-sense construct of the gene of interest.

INTRODUCTION
The objective of promoter analysis is to identify and characterize cis-acting DNA (adjacent) sequences that, when induced, regulate PD-associated gene expression in grapes. Although regulatory sequences frequently occur just upstream of the transcription start site, they can also be found much further upstream. Transcript abundance can also be controlled post-transcriptionally, often by cis-acting sequences in the 3' untranslated region of a gene. Thus, the challenge in our studies is to demonstrate that the cis-acting sequences have a unique functional role in PD symptom development. It is not the goal of this proposal to understand mechanisms of transcriptional regulation, but rather to isolate and confirm sequences that are active in the regulation of gene expression when *Xf* is present as an inducer of a select set of genes.

To test whether a particular DNA sequence, that lies adjacent to a gene of interest, is involved in the regulation of that gene, it is necessary to introduce such putative regulatory sequences into a cell and then determine if they are activated when the inducer (in our case, *Xf*) is introduced into the system. This is done by combining a regulatory sequence with a reporter sequence (in our case, GFP) that can be used to monitor the effect of the regulatory (promoter) sequences in the presence of *Xf*.

We have identified a set of plant genes whose expression is correlated with infection by *Xylella fastidiosa* as part of a recent study of expressed sequence tags from *Xf*-infected and healthy *V. vinifera* plants in the Napa Valley. The genes are essentially off (silent) in plants that have not been exposed to the pathogen, but strongly induced in both natural field infections and greenhouse inoculated plants. Three of these genes are induced early during disease development, prior to the occurrence of symptoms, while the fourth gene is induced in symptomatic tissues only.

In addition to their utility for engineering PD resistance in grape, the advent of *Xf*-induced reporter gene expression would provide an extremely powerful tool to examine other host responses in their intact cellular and tissue context. With such tools, it should be possible to examine the chemical and/or physical cues from the insect or pathogen that trigger host gene

expression and the deleterious effect of the disease. Moreover, the recent development of *Xf*-GFP strains by Dr. Steven Lindow at UC Berkeley offers the possibility of dual labeling to simultaneously monitor pathogen spatial distribution and host gene expression. Such dual labeling experiments are made possible by the availability of multiple forms of GFP protein engineered to fluoresce with distinct spectral characteristics. It is conceivable, for example, that host genes might be induced specifically in live cells, adjacent to sites of pathogen colonization of xylem elements, and this technology would provide the means to test such hypotheses.

OBJECTIVES

1. Identify and determine sequence of promoters driving genes specifically transcribed in grape tissue or cells of plants infected with *Xf*.
2. Construct transformation-ready vectors containing *Vitis* promoter-GFP reporter gene fusions that will be used for the functional assay of putative promoters. (GFP=green fluorescent protein) identified in (1)
3. Conduct transient functional assays of the promoter-GFP fusions in stems, leaves and roots infected with *Xf*.
4. Produce stable transgenic grape plants with promoters that functioned effectively in the transient assays and characterize the strength of the selected promoters using the GFP-reporter
5. Distribute promoters to Pierce's Disease research community to facilitate characterization of cloned grape genes suspected to be involved in PD susceptibility or resistant to *Xf*. These promoters will have application in situations where the goal is to either up- or down-regulate expression of a specific gene-of-interest; the latter by localized expression of anti-sense gene constructs.

RESULTS

Since this project just began October 1, 2004, there are few results to report. We have employed a postdoctoral researcher and are currently sequencing the BAC clones indicated in the objectives.

Experimental Procedures to Accomplish Objectives

I. DNA Sequencing and promoter identification:

A. Isolation and characterization BAC clones containing the Xylella-induced genes.

Bacterial Artificial Chromosome (BAC) libraries of *V. vinifera* are available as high density filters for gene identification in grapes through the UC Davis CA&ES Genomics Facility (http://cgf.ucdavis.edu/). High-density filter sets of the library were used for hybridization with ^{32}P-labeled probes corresponding to four *Xylella*-induced transcripts. A combination of restriction enzyme fingerprinting and DNA sequencing of BAC-derived PCR products was used to determine that each probe hybridized to a single genomic locus containing the gene of interest. One BAC clone was selected for each transcript and used to prepare a sheared BAC sublibrary, which is currently being subject to random shotgun sequencing.

B. Sequence the BAC clones to completion.

Although our specific interest is in sequences immediately 5' and 3' to the candidate genes (maximum 10 kbp) we will sequence regions beyond where we believe the promoters to reside. The rationale derives from efficiencies and strategies of modern sequencing techniques; it is both faster and more cost effective to use the BAC shotgun strategy described below which automatically provides additional sequence information for less cost that if we were to attempt to focus on shorter regions immediately adjacent to either end of the candidate genes.

C. Identify 5' promoter regions in the sequenced genomic clones based on comparison to cDNA sequences currently in hand for the four genes.

We have complete cDNA sequences for each of the candidate genes that will facilitate annotation of the BAC clones and identification of regions immediately upstream and downstream of the transcription units. As described below, we will use PCR to isolate and clone these 5' and 3' regulatory sequences into transformation ready vector constructs (see below). Generally, we anticipate using conventional 3' terminators, such as that from the *Agrobacterium* octopine synthase gene (*ocs*). However, one of the candidate genes (a small auxin upregulated, *saur,* mRNA homolog) is predicted to confer post-transcriptional regulatory properties that may be involved in *Xylella*-specific RNA levels. Thus, we will clone the 3' region of this candidate gene and incorporate its structure into a subset of the transgene constructs described below.

II. Construct transformation-ready vectors systems containing *Vitis* promoters fused to GFP.

A set of plasmids has been constructed previously that allows the rapid assembly of novel binary plasmids in E. coli. One is a low copy backbone plasmid with elements from Agrobacterium; the second is a high copy E. coli plasmid containing a cassette of T-DNA elements; and the third is a high copy E. coli plasmid comprised of a linker and many unique restriction sites for ease of cloning the several classes of sequences to be recovered and tested. These plasmids will be used to construct a collection of binary vectors containing grape 5' promoters and 3' sequences for expression of GFP genes. Analysis of the sequence of the appropriate BAC clones will allow the design of PCR primers to amplify and clone the 5' promoter and 3' sequences of the transcriptionally regulated grape genes into novel binary vectors. (Details of the plasmids are available upon request.)

III. Production of transgenic plants and plant tissues of grape and application of transient assay of promoters

We will employ three different but functionally related approaches to testing and characterizing the isolated promoter regions indicated above. All three of the approaches described below will be initiated simultaneously in the interest of time. Each of the promoters of the four genes will be assembled in several different configurations with the reporter gene (GFP) and will be evaluated in conjunction with a constitutive promoter (CaMV 35S or FMV 34S) giving a total number of 40 transgene constructs. Total costs will be minimized by terminating any of the whole plant transformants bearing promoter constructs that are demonstrated by the transient or *A. rhizogenes* assays to be unresponsive to the presence of *Xf*.

A. Stable, full-plant grape transformation will be provided on a recharge basis by the Ralph M. Parsons Foundation Plant Transformation Facility at the rate of $2,000 per construct. This facility is located at UC Davis as a service oriented facility dedicated to providing cost effective plant transformation services for the University of California system and outside academic and industrial partners.

B. Transient and root-specific stable transformations will be used for rapid identification of promoter specificity and relative strength. The intent is to decrease the number of whole plant transformations that need to be conducted -- because whole plant transformation is labor intensive, time consuming and expensive. The transient assays using *Agrobacterium tumefaciens* and the root transformations by *A. rhizogenes*, bearing the test promoters and marker genes, will be conducted by techniques that have used successfully for several years in the Gilchrist Lab.

C. *A. rhizogenes*-derived root transformations will be used for initial assay of the expression of transgenes in differentiated tissue with vascular connections to *Xf*-infected stem sections. *A. rhizogenes* effects stable transformation of plant tissues by transferring genes of interest to intact plants under controlled conditions. The inoculation with A. rhizogenes bearing a gene of interest leads directly to the formation of transformed roots, which appear within 2-3 weeks and at which point the pathogen can be introduced into the assay system. Our procedure will be to introduce the putative promoter sequence, coupled to GFP, into grape roots via transformation as indicated above. Our recent data obtained with the *Xf*-GFP indicates that the bacteria can move both up and down from the site of infection. Hence, the presence of the bacteria, either directly placed in the transformed tissue with the putative promoter constructs have a chance of responding to the direct presence of *Xf* (in the roots) or to distal signals from bacteria present in the stem. Not only will these assays indicate *Xf* responsive promoters, some information on the strength of the promoters but whether they are responsive to distance signals also. These are all procedures that have been developed in our lab with grape as recipient host tissues.

IV. Characterization of GFP expression during *Xylella* infection and leafhopper feeding to identify desired promoter specificities.

Confocal Microscopy. Real time, non-destructive images of the isolated promoters driving the expression of GFP in grape plants will be obtained using a laser activated confocal microscope (BioRad MRC1024) by excitation at 488nm with a Krypton/Argon 15 mW laser. The use of the laser allows non-destructive GFP detection in intact plant leaves and roots. For stem imaging, hand sectioning will be used. Three different fluorescent emissions can be detected simultaneously depending on the filter set used. Current configuration is with the following three filters: (emission filter 578nm-618nm); (emission filter 506nm-538nm); and (emission filter 664nm-696nm).

The first characterized promoters are expected to be available beginning in February 2006 with the final characterization and methods for expression completed by May of 2006. All promoters and characterization details will be available for research purposes at the conclusion of the two-year project.

CONCLUSIONS

The research envisioned will be accomplished by combining expertise and materials from two laboratories, active in PD research, to isolate and characterize PD-responsive promoters from grape. The current project led by Dr. Cook has already identified several genes that are expressed strongly in *Xylella*-infected tissues, but not in healthy counterparts. The project led by Dr. Gilchrist has developed both a transient leaf-based and a stable root-based grape assay and has identified putative anti-PD genes from grape. We are poised to isolate the promoters of the PD-responsive genes from BAC genomic DNA libraries of Cabernet Sauvignon in the Cook lab and functionally test them by techniques used in the Gilchrist lab.

FUNDING AGENCIES

Funding for this project was provided by the American Vineyard Foundation and the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.