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

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## ABSTRACT
We identified a set of plant genes whose expression is correlated with infection by *Xylella fastidiosa (Xf)* as part of a recent study of expressed sequence tags from *Xf*-infected and healthy *Vitis 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 prior to the occurrence of symptoms in both natural field infections and greenhouse inoculated plants. The transcriptional regulatory elements of these genes (i.e., promoters) hold great potential to fulfill a critical and, as yet, unmet need for control of Pierce's disease (PD) namely, the identification of gene promoters that can drive transgene expression only in *Xylella*-infected tissues both for basic research on PD responsive genes and for developing tactics for assessing potential genes conferring resistance to PD. We have focused on three promoters for grape genes whose expression analysis reveals a specific dramatic increase in expression in PD diseased grape compared to healthy or other inductions (called G8946, G9353 and G7061). The plasmid pBG8946minGFP has been transformed into *Agrobacterium tumefaciens* strain GV2260 and infiltrated using a needless syringe into both healthy and *Xf*-infected grape (Thompson seedless) leaves. After five days the leaves were imaged with a confocal microscope, which revealed the GFP fluorescence can be detected in *Agrobacterium*-infiltrated leaves of *Xf*-infected plants but not healthy plants.

## INTRODUCTION
A major limitation in using transgenes to study and alter the effect of pathogens on disease processes in plants is the absence of the ability to regulate the expression of the transgene in either a tissue or pathogen specific response. We and many other researchers of grape (or any plant) to assess the effect of a transgene on a specific trait (susceptibility to Pierce's disease [PD]) is the absence of suitable promoters, sequences that regulate gene expression 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 for control of PD 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. This can lead to unintended phenotypes and/or sickly transgenic plants. 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 *Xylella fastidiosa* (*Xf)*-responsive promoters has immediate and direct application to several current PD projects that are studying the 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.

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 (Figure 1). 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 is the test gene) that can be used to monitor the effect of the regulatory (promoter) sequences in the presence of *Xf*.

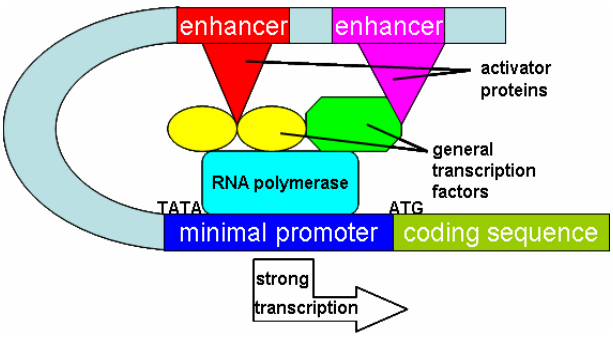


**Figure 1**. Diagram of a eukaryotic promoter showing a minimal promoter containing TATA and CAAT boxes. Activator proteins bind to enhancer elements for strong transcription.

We have identified a set of plant genes whose expression is correlated with infection by *Xf* 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 (G8946, G9353, and G7061) are induced early during disease development, prior to the occurrence of symptoms. The protein products of such genes are interesting in their own right, including what their predicted functions may suggest about the nature of host responses to this important pathogen. However, their transcriptional regulatory elements (i.e., promoters) hold great potential to fulfill a critical and, as yet, unmet need for control of PD - namely, the identification of gene promoters that can drive transgene expression only in *Xylella*-infected tissues. Identifying plant promoters is important if developing transgenic solutions to PD will have significant benefit, both in terms of public perception and transgene efficacy, if we use promoters that are expressed only in tissues that are infected by the pathogen (i.e., the transgene products should be spatially and temporally restricted to those times and places where the protein products are needed for disease resistance).

**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)
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.

**RESULTS**

The first step taken was to utilize a Bacterial Artificial Chromosome (BAC) set of libraries of *V. vinifera* on high density filters for gene identification in grapes through the UC Davis California and ES Genomics Facility (http://cgf.ucdavis.edu/). Our specific interest is in sequences immediately 5' to the candidate genes (maximum 5 kbp), but to be conservative we sequenced regions beyond where we believe the promoters to reside. We then proceeded with Sublibrary preparation and clone management, wherein BAC DNA was isolated using the Qiagen Large Construct kits, sheared fragments generated by HydroShear (Gene Machines, Inc.) and blunt-ended using a fill-in approach and cloned *en masse* into a SmaI-digested pUC18 sequencing vector. The next step was to generate paired-end sequence reads from the pUC18 subclone library, with two 384-well plates analyzed for each BAC clone. Theoretically this equated to 8.5X coverage of a typical 125 Kb BAC clone. To generate ordered contigs and facilitate the finishing phase of the project, we designed PCR primers from the ends of contigs using an automated Primer 3 software pipeline. All templates are to be sequenced a minimum of both directions.

Identify 5' promoter regions in the sequenced genomic clones based on comparison to cDNA sequences currently in hand for the three genes: We used PCR to isolate and clone the potential 5' regulatory sequences into transformation ready vector constructs (see below). These plasmids have been used to construct a collection of binary vectors containing grape 5' promoters 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.)

**Systems for analysis of the PD responsiveness of the isolated promoters**

We are using three different but functionally related approaches to testing and characterizing the isolated promoter regions derived above. These include transient assays on infected and healthy leaves, transgenic hairy roots and whole plant transgenics. All three of the approaches will be initiated simultaneously in the interest of time. Each of the promoters of the three genes have been 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).

**Identify and determine sequence of promoters**

We have focused on three promoters for grape genes whose expression analysis reveals a specific dramatic increase in expression in PD diseased grape compared to healthy or other inductions (Figure 2). These three genes (called G8946, G9353 and G7061) have each been used to isolate by hybridization a BAC clone of grape genomic DNA containing the gene. These BAC clones were then subjected to shotgun sequencing. The resulting sequence, once assembled and annotated for the location of the hybridizing cDNA, were used to make PCR primers for approximately 1200bp of sequence just 5' of the cDNA start codon.

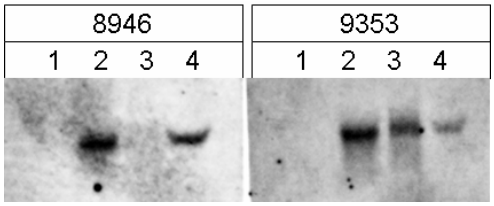


**Figure 2.** Northern analysis of *Xf*-inducible gene expression. RNA isolated from leaves of healthy Thompson seedless (lane 1), *Xf*-infected Thompson seedless (lane 2 and 3), and *Xf*-infected Chardonnay (lane 4) were hybridized with labeled 8946 cDNA or 9353 cDNA.

**Construct transformation-ready vectors**

PCR primers were used to amplify grape (Chardonnay) genomic DNA. We readily obtained the promoter of G8946 with a single PCR reaction. However G9353 and G7061 are proving to be more difficult. The promoter regions of G9353 and G7061 are very AT-rich and PCR efficiency is poor for AT-rich sequences. Repeated attempts to PCR the entire promoter region have failed. Therefore, we have divided the promoter regions of G9353 and G7061 into three smaller overlapping regions for PCR. So far, for both promoter regions of G9353 and G7061, two of the three fragments have been successfully cloned. After isolation and sequence verification, these three fragments will be put back together by overlap extension PCR to recreate the whole promoter region. The promoter of G8946 has been cloned upstream of a GFP reporter gene in a transformation ready vector and called pBG8946minG (Figure 3).

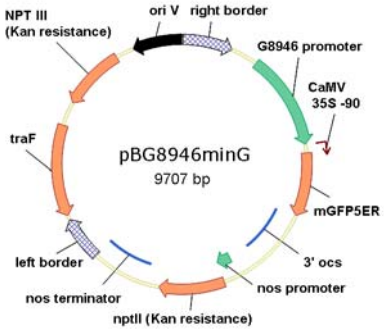


**Figure 3.** Map of binary vector pBG8946minG. This vector uses the grape promoter G8946 to drive expression of a GFP gene. In Agrobacterium this vector will transfer DNA into grape cells.

**Transient functional assays of the promoter-GFP fusions**

The plasmid pBG8946minG has been transformed into *A. tumefaciens* strain GV2260 and infiltrated using a needless syringe into both healthy and *Xf*-infected grape (Thompson seedless) leaves. After five days the leaves were imaged with a confocal microscope. We find that GFP fluorescence can be detected in *Agrobacterium*-infiltrated leaves of *Xf*-infected plants but not healthy plants (Figure 4).

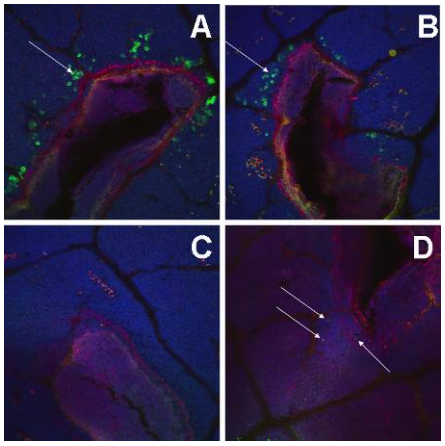


**Figure 4.** *Agrobacterium*-mediated transient gene expression in grape leaves. *Agrobacterium tumefaciens* GV2260 containing pBG8946minG (panels C and D) or a control plasmid, pCB5minG, using the CaMV 35S promoter (panels A and B) were infiltrated into Thompson seedless healthy (panels A and C) and *Xf*-infected (panels B and D) leaves. After 5 days, confocal microscopy was used to detect GFP expression. In these micrographs, green is GFP expression; blue is chlorophyll auto-fluorescence; and red is polyphenolics accumulation. Arrows show individual cells expressing GFP.

**Produce stable transgenic grape plants**

The plasmid pBG8946minG has also been transformed into *A. tumefaciens* strain LBA4404 and is currently being used by the UCD Transformation Facility to create transgenic Thompson seedless plants.

**CONCLUSIONS**

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.

**FUNDING AGENCIES**

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