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

Project Leaders:

David Gilchrist
Department of Plant Pathology
University of California
Davis, CA 95616
dggilchrist@ucdavis.edu

Douglas Cook
Department of Plant Pathology
University of California
Davis, CA 95616
drcook@ucdavis.edu

James Lincoln
Department of Plant Pathology
University of California
Davis, CA 95616
jelincoln@ucdavis.edu

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ABSTRACT

Several projects working toward understanding the genetic basis for susceptibility or resistance at the molecular level in grape to Pierce's disease need to test the ability of candidate genes to alter disease progression or activity of *Xylella fastidiosa (Xf) in planta*. A major limitation to developing assays to test candidate genes is the ability to regulate expression of these genes in time and space relative to the presence of the pathogen. Currently, we are able to express any candidate transgene constitutively and at high levels but cannot modulate gene expression in relation to pathogen presence. The goal of this research is to clone and characterize unique DNA sequences from grape that specifically regulate the expression of grape genes in tissues that are infected with *Xf*. The result will be the delivery of one or more *Xylella*-responsive promoters from grape to drive the bioassay of any candidate gene at locations where the bacteria reside or where bacterial signals extend. The promoters also would be capable of either increasing or suppressing the expression of a gene of interest. Grower acceptance as well as enabling the most subtle level of transgene expression requires that these genes be expressed only when and where they are needed.

INTRODUCTION

Among the potential solutions to Pierce's disease (PD) in grapes are approaches based on gene transfer technology. One research priority identified in 2004 by the PD/GWSS Program 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 *Xylella fastidiosa (Xf)*. This means the candidate genes driven by such promoters will be off (not expressed) and only on (expressed) when the bacteria or their secreted signals are present in the vascular system of the grape plant. Emphasis was placed on the urgency and practical utility of isolating promoters of PD responsive genes. Transgenic technology offers the possibility of modifying specific traits (e.g., PD susceptibility) based on the introduction of novel genes. One of the major bottlenecks in the genetic engineering of grape (or any plant) 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. By definition, constitutive promoters are expressed in all cells all the time. By contrast, *Xylella*-inducible promoters have the potential to confer transgene expression at the time and location of bacterial infection, thus delivering therapeutic proteins more precisely to their intended site of action. In addition to increasing the specificity of transgene expression, such promoters should reduce the possibility of unintended side affects in non-target tissues.

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 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. For example, we have recently determined that host gene expression is induced specifically in live cells of the phloem (Cook 2005). It remains uncertain, however, whether gene expression occurs only in phloem tissue that borders infected xylem elements, or whether bacterial infection can induce host gene expression at a distance. Promoter-GFP fusions being developed in this project should help answer such questions.

OBJECTIVES

1. Produce stable transgenic grape plants containing promoter-GFP fusions that respond to *Xylella* infection. At this point 4 constructs bearing promoters derived from genes induced in *Xf* infected grape but not in healthy grape tissue will be transformed into Thompson Seedless plants by the UC Davis Plant Transformation Facility. There are four genes, G8946, G9353 G7061and G7172 to initially be expressed transgenically. These genes were shown to be induced in the phloem of infected petioles and leaves, adjacent to sites of probable *Xf* infection in the xylem as indicated in the 2005 symposium report (Gilchrist et al. 2005). Expected delivery of the first set of transgenics is November 2006.
2. Develop a rapid *in planta* assay to characterize promoter-GFP expression in a series of independent grape transformants derived from each promoter fusion via detached leaf/branch bacterial uptake system. The goal is to identify a series of independent lines for each fusion where transgene expression is strong and reproducible. GFP expression will be monitored in both excised leaves and branches (described below), as well as in stem-inoculated whole plants. The progression of induction will be assessed qualitatively in relation to location bacteria monitored by RT-PCR of both the promoter expression and the presence of the bacterium.

3. Conduct detailed analysis of promoter-GFP expression, with the following specific goals in mind: (a) Determine the extent to which the transgenic promoter-GFP fusions reproduce the patterns of expression for the endogenous promoters. Using promoter-GFP fusion constructs, confocal imaging will be used to assess temporal and spatial aspects of G8946-GFP induction at the protein level, while Taqman RealTime PCR assays will be run in parallel to quantify transcript levels from both the native genes as well as from the introduced promoter-GFP constructs. (b) Define temporal and spatial aspects of promoter-GFP expression, especially as a function of the location and quantity of bacterial colonization in the vascular tissue. We already know that each of the native genes are induced strongly in the phloem of infected leaves and petioles, but it is uncertain, for example, if there is a requirement for bacterial colonization in the physically adjacent xylem or, alternatively, if the bacterium can induce host gene expression at a distance. Developing a detailed chronology of bacterial colonization and host gene expression will not only serve to characterize the transgenic promoters, it should also help with development of models for the mechanism by which the plant perceives the bacterium.
4. Continue validation of an additional 24 genes, which also appear to be expressed only in grape tissues infected with *Xf*. This will be done using the same procedures as described below for the first four genes.

RESULTS

Promoter identification

We have characterized approximately 25,000 grape genes (Cook 2005) and found a subset of 448 genes which are up-regulated specifically in response to *Xf* infection (Gilchrist et al. 2005). Four of these genes, G8946, G9353 G7061, and G7172 were shown by PCR to have expression patterns that are strongly correlated with *Xf* infection in both greenhouse and field-grown grapes (Figure 1). Studies with an Affymetrix GeneChip determined that susceptible *Vitis vinifera* responds to *Xf* infection with a re-direction (both up- and down-regulation) of gene transcription involving over 800 genes. Analysis of 24 genes (from the subset of 448) by *in situ* hybridization established that expression occurs coincident with the presence of *Xf* in the phloem of infected petioles and leaves, adjacent to sites of probable *Xf* infection in the xylem. The Cook lab has recently obtained sequence information 5' of the transcription start for an additional 200 genes (from the subset of 448) and these represent potential differentially expressed promoters.

Promoter isolation and binary vector construction

Bacterial Artificial Chromosome (BAC) clones of *V. vinifera* that contained the four *Xf*-inducible genes and their promoters were used to isolate and sequence the 5' promoter regions in genomic clones based on comparison to full-length cDNA sequences for the respective genes. PCR primers were designed to amplify and clone approximately 1300bp of sequence immediately 5' of the transcription start site, which was predicted based on proximity to the cDNA initiation codon (Cook 2005, Gilchrist et al. 2005).

Leaf and branch inoculation methods

Recognizing the value of having a rapid, laboratory-based assay for host gene expression, we sought to develop a cut-branch and a detached leaf assay that would enable the bacteria to enter and spread though the vascular system in days to a few weeks and then assay for both the location of the bacteria and the relative level of expression of the putative promoters. In these assays, the leaf scorching indicative of Pierce's disease occurs within several weeks in most cases. Briefly, branches of the PD susceptible root stock cv. Freedom were cut under water and allowed to take up *Xf* from 1 mL of a bacteria suspension containing $2x10^7$ cfu/ml for 2 hr. Stems inoculated in this manner were placed in water for 2 weeks, after which they were assayed for host gene expression by means of RT-PCR (Ref 4). As shown in Figure 2, genes G8946 and G7061 were strongly and reproducibly induced in stems inoculated with *Xf* well in advance of symptom development, consistent with our previous results using whole plants. Confocal imaging of GFP tagged *Xf* in these same tissues detected only very small amounts of bacteria in the stems (Figure 2 inset of stem cross-section) and none in the leaf lamina where the genes were detected as being expressed, suggesting that the up-regulation of these is highly sensitive to the presence of bacteria and at a distance from where the bacteria were easily detected. We conclude that this detached stem assay can provide a simple and reliable method to monitor *Xylella*-induced gene expression, significantly shortening assay time compared to whole plant assays. We are now attempting to extend the same assays with Thompson Seedless grape explanted tissue.

Branch uptake method

A terminal shoot approximately 60cm long is cut from greenhouse or growth chamber grown grape plants. The shoot is re-cut under water removing an additional 5cm. In a typical assay, approximately ten shoots are placed in a beaker of distilled water containing $2x10^7$ *Xf* cells/ mL. Shoots are allowed to uptake the bacteria suspension for 2 to 48 hrs depending on the experiment. During this uptake the beaker is placed in a laminar flow hood to increase transpiration. After the uptake period the shoots are transferred to individual 50 ml glass culture tubes containing distilled water for the remainder of the experiment. Shoots are incubated at room temperature under low intensity fluorescent lights for symptom development within 2-3 weeks. The two genes assayed in the experiment illustrated in Figure 2 were expressed in the stems and in the leaf lamina. GFP-tagged bacteria were visible by confocal microscopy in the cut stem cross-sections but not in the leaf lamina where the genes were expressed. This may reflect the low sensitivity of the confocal assay to detect the bacteria or that the genes are expressed in relation to systemic signals expressed by the bacteria. Clearly, the genes are expressed in asymptomatic tissue, which is extremely encouraging in terms of being able to activate these promoters before bacterial

populations build up and before PD symptoms appear. (Figures 2 and 3).

Leaf uptake method

Young, full-sized, mature leaves were cut from greenhouse or growth chamber grown grape, the petioles re-cut under water and the leaves placed individually in 2ml plastic tubes containing $2x10^7$ *Xf* cells/mL. The remainder of the uptake and incubation was similar to the branch method. Each petiole provides 20 sections for analysis by RT-PCR and confocal microscopy. Macroscopic leaf scorch symptoms appear on cv. Freedom within 2-3 weeks (Figure 4). In this case, the assay is used to detect the induction of G7172 along with detection of the pathogen. Regions m, b, and c were analyzed independently for both G7172 and *Xf*. RT-PCR is much more sensitive for detection of *Xf* than the GFP tagged visualization by confocal microscopy. The results to date indicate that, similar to cut branch uptake, in the detached leaf assay, the G7172 promoter is *Xylella* responsive and appears near where the bacteria could be detected by RT-PCR. This assay is somewhat faster than the branch uptake but the results are similar. In both cases, it appears that, at least for the promoter assays, this method of placing the bacteria in the vascular system and detecting a response to the presence of the bacterial at the level of plant gene expression is valid, whether the expression is uniquely induced or expression is up-regulated.

Whole plant transgenics

During the current project period, promoter-GFP fusions for first four genes are being introduced into the *V. vinifera* cultivar Thompson Seedless. Embryos have been induced from transformed tissue and we anticipate having transformed plantlets for initial assays by November 2006.

CONCLUSIONS

The only effective long term strategies for protecting grape against the impact of *Xf* resident in the vascular system of susceptible grape is to genetically alter the response of susceptible grape tissue to the death induced by plant response to the bacteria. This means expression of introduced resistance genes or genes that block the plant response. The most likely means by which either of these protective measures will take place is though transgene expression. The direct products of this research are the means to express any potential therapeutic transgenes in areas of infection when and only when the bacteria are present. These promoters are one critical tool necessary for genetic resolution of Pierce's Disease.

REFERENCES

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Gilchrist, G, J. Lincoln and D. Cook. 2005. Isolation and functional testing of Pierce's disease-specific promoters from grape. Proceedings Pierce's Disease Research Symposium, San Diego, CA, December 5-7. California Department of Food and Agriculture, Sacramento, CA.

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Figure 1. RT-PCR analysis of the G7172 transcript from greenhouse grown grape. RNA in lanes 1 and 3 are from healthy leaves and lanes 2 and 4 are from *Xf*-infected leaves. Actin specific primers used for lanes 1 and 2 as a control and G7172 specific primers in lanes 3 and 4 indicate the presence of the promoter transcript only in the infected plant in the greenhouse grown plants.

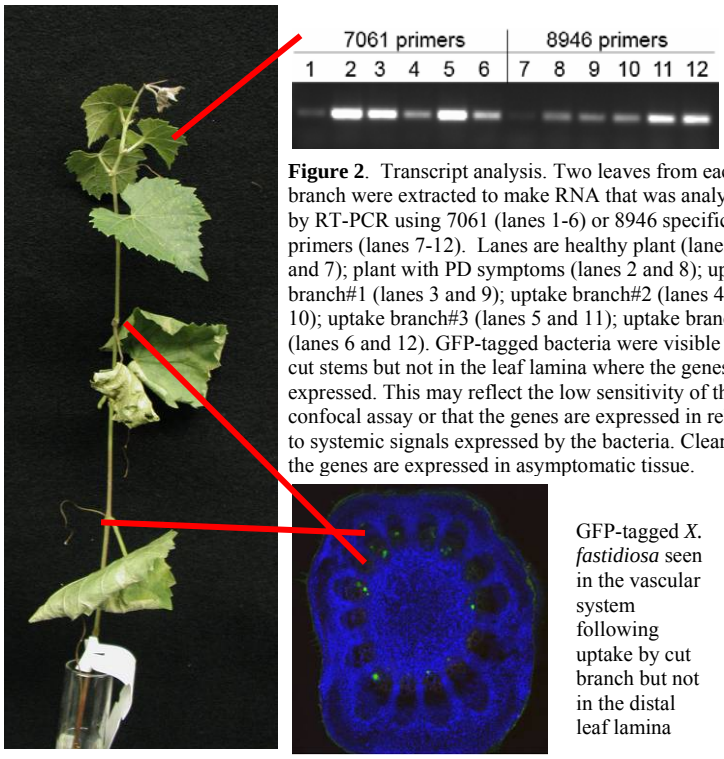

Figure 2. Transcript analysis. Two leaves from each branch were extracted to make RNA that was analyzed by RT-PCR using 7061 (lanes 1-6) or 8946 specific primers (lanes 7-12). Lanes are healthy plant (lanes 1 and 7); plant with PD symptoms (lanes 2 and 8); uptake branch#1 (lanes 3 and 9); uptake branch#2 (lanes 4 and 10); uptake branch#3 (lanes 5 and 11); uptake branch#4 (lanes 6 and 12). GFP-tagged bacteria were visible in the cut stems but not in the leaf lamina where the genes were expressed. This may reflect the low sensitivity of the confocal assay or that the genes are expressed in relation to systemic signals expressed by the bacteria. Clearly, the genes are expressed in asymptomatic tissue.

GFP-tagged *X. fastidiosa* seen in the vascular system following uptake by cut branch but not in the distal leaf lamina

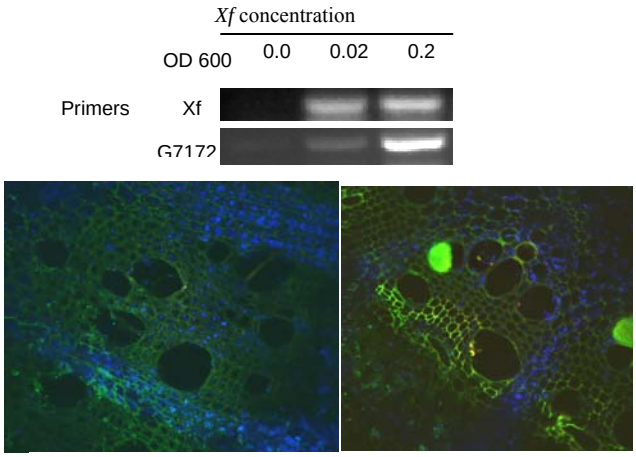

Figure 3. Branch uptake assay for expression of promoter G7172 in the presence and absence of *X. fastidiosa*, when sampled in leaf lamina. GFP-bacteria were visible 5-7 cm in the stem but only RT PCR detected the bacteria in petioles and leaf lamina in the entire branch by 3 days. Hence, the bulk of the bacteria were confined to the stem but cells had moved throughout the plant. The G7172 transcript was expressed in all tissues near where the bacteria could be detected by PCR but not in un-inoculated tissues. Expression of 7172 also appears to be positively correlated to the amount of bacteria in the tissue that was assay for the transcript.

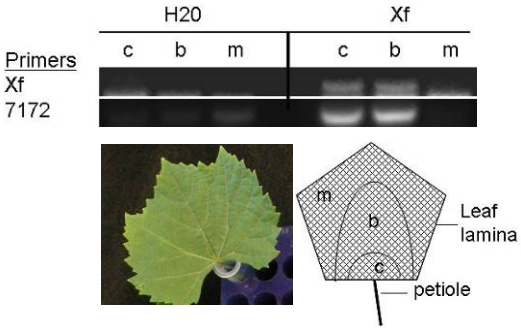

Figure 4. Example of detached leaf uptake assay for gene expression in the presence of *X. fastidiosa*. Bacteria at 10^7 CFU were introduced into the cut petiole followed by RT-PCR detection of targeted gene transcripts and the bacteria in the same tissue samples. In this case, the assay is used to detect the induction of G7172 along with detection of the pathogen in association with the bacterial but not in water control leaves. Regions m, b, and c were analyzed separately for both G7172 and *X. fastidiosa*; demonstrating that the G7172 promoter is Xylella responsive.

RESISTANCE TO PIERCE'S DISEASE BY TRANSGENIC EXPRESSION OF PLANT-DERIVED ANTI-APOPTOTIC GENES

Project Leaders:

David Gilchrist
Department of Plant Pathology
University of California
Davis, CA 95616
dggilchrist@ucdavis.edu

James Lincoln
Department of Plant Pathology
University of California
Davis, CA 95616
jelincoln@ucdavis.edu

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ABSTRACT

Several relatives of grape and other asymptomatic host plants can harbor high titers of *Xylella fastidiosa (Xf)* without exhibiting symptoms of Pierce's disease (PD). The basis of what is a genetic difference is unknown. We have established that leaf scorch PD symptoms in grape result from apoptosis or programmed cell death (PCD). Clearly, *Xf* does not have to kill in order colonize the vascular system leaving this endophytic association asymptomatic. We have identified from a cDNA library screen several grape genes that block PCD when over-expressed in grape tissue. Preliminary experiments indicate that one of these genes, VVPR1A, is expressed or up-regulated in situations in which PCD is blocked in humans, nematodes, hookworms and several plant species. This gene also is upregulated in the presence of *Xf*. We are testing the hypothesis that over expression of one or more of the 12 genes recovered in the anti-apoptotic screen, with an initial focus on VVPR1A, can block both PCD induced by *Xf* and disease symptoms associated with *Xf*. Preliminary results reported here indicate that grape plants over expressing VVPR1A, metallothionein, or a *Meloidogyne incognita* upregulated gene can block symptoms in a cut branch assay. Experiments with whole transgenic plants inoculated with *Xf* are in progress to assess the movement of bacteria, the induction of *Xf* responsive grape genes and if symptoms of PD are affected by the anti-apoptotic transgenes.

INTRODUCTION

Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/Glassy-winged Sharpshooter (PD/GWSS) Research Program and the National Academies report. Disease is defined as plants expressing several symptoms resulting from cell death (leaf scorch) or changes in tissue differentiation (green islands). The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when over expressed in grape, will prevent infection, spread or symptom development due to the presence of *X. fastidiosa* (*Xf*) in the xylem (Gilchrist and Lincoln 2004). Currently, several laboratories including our own have begun to carry out systematic studies of the molecular basis of susceptibility of plants to a range of pathogens including bacteria and fungi. The objective of these studies is to identify genetic or chemical approaches that have the potential to block susceptibility in grape to PD, thereby effectively creating cells that are refractory or insensitive to the signals expressed by pathogens that lead to susceptibility. Recent published information from our laboratory established that susceptibility of several plants to a range of pathogens depends on the ability of the pathogen to directly or indirectly trigger the activation of genetically determined pathways leading to apoptosis or programmed cell death (PCD) (Gilchrist 1998, Harvey et al. 2006, Lincoln et al. 2002, Richael et al. 2001). These discoveries parallel investigations now widely reported and accepted in human medicine whereby genes, signaling pathways and chemical signals expressed by animal pathogens initiate or block infection by activating or blocking apoptosis through constitutive genes or signaling pathways present in all cells. These studies are the basis for extensive searches for apoptosis-based therapeutic approaches and agents in plants as well as animals (Greenberg and Yao 2004, Nicholoson 2000).

Dr. Tom Rost reported, both in his 2005 PD Symposium address and his annual report (Rost et al. 2005) that *Xf* moves effectively and quickly through the plant following inoculation or uptake. However, the GFP-tagged bacteria have limited dispersal in the leaf lamina expressing the marginal leaf scorch symptoms of PD. These data, obtained in part using the confocal system in our laboratory, are consistent with our own observations using GFP-tagged *Xf* to visualize the bacteria in vascular elements connected to tissue showing the marginal scorch symptoms. These data suggest two key things. First, the cell death symptom is the result of mobile signals moving from the bacteria to cells distal to the bacteria and that strategies effective in blocking the death pathways will most likely consign the bacteria to an endophytic existence in the vascular system. Consistent with this hypothesis, Dr. Steven Lindow pointed out in his 2005 PD Symposium address that *Xf* is an effective endophyte in many asymptomatic plants and can be said to be "an endophyte gone bad in susceptible grape plants". It is a fact that several *Vitis* species, including wild grape, tolerate extremely high titers of *Xf* but remain asymptomatic, while many genotypes of cultivated grapes express PD symptoms at the same or lower titers. Clearly the presence of *Xf* in the xylem is not the single determining factor in disease. In PD and many other bacterial diseases, bacteria live predominantly as endophytes or epiphytes and only occasionally as pathogens. Susceptibility of the host tissues is determined by sensitivity to the presence of the bacterium and the signals expressed by the bacteria leading to PCD. Using genetic or chemical approaches to block PCD is an viable approach in both animal and plant disease prevention (Greenberg and Yao 2004, Harvey et al. 2006).