

FUNCTIONAL TESTING AND CHARACTERIZATION OF PIERCE'S DISEASE-INDUCED PROMOTERS FROM GRAPE

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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 (PD) 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 inability to regulate expression of these genes in time and space relative to the presence of the pathogen. We report here the initial characterization of transgenic grape plants with unique DNA sequences from grape (promoters) that specifically regulate the expression of a marker protein (GFP) in grape tissues that are inoculated with *Xf*, but not with a related bacterium, *Xanthomonas campestris* (*Xc*).

INTRODUCTION

Among the potential solutions to 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 *Xf*. This means candidate genes with the potential to suppress or block PD, when driven by *Xf* 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. In contrast, constitutive promoters are expressed in all cells all the time. *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 would reduce the possibility of unintended side effects in non-target tissues (Figure 1). The results displayed herein confirm that we have detected, cloned, expressed and validated the fact that two of the promoters tested to date are activated by the presence of *Xf* in the xylem of both detached branch and whole plant assays of plants expressing promoter-GFP fusions. Mock inoculation and inoculation with the related *Xc* under the same conditions do not activate the promoters.

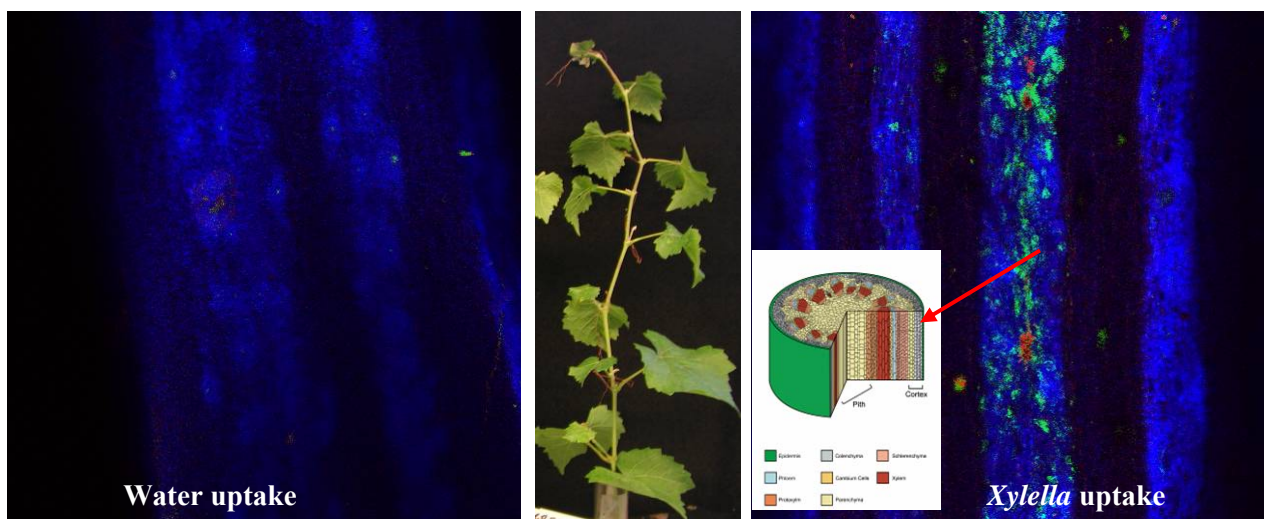
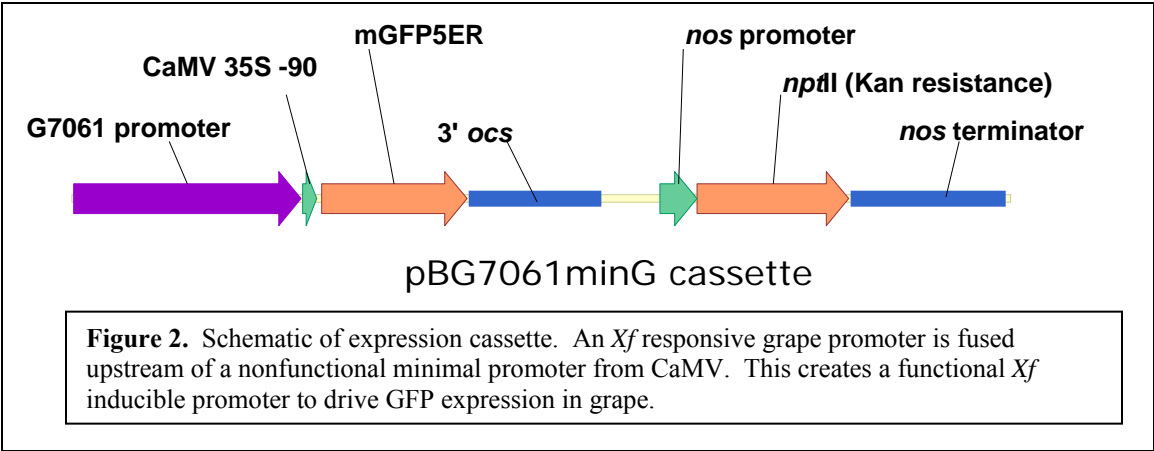


Figure 1. Expression of promoter G7061-11 fused to GFP detected after seven days in petioles of leaves attached to a detached branch into which the Temecula strain of *Xf* (10^7 cfu/ml) was taken up through the cut end of the branch. Water control and uptake of *Xc* shows no promoter activation under the same conditions.

OBJECTIVES

- 1. Characterization of the transgenic plants expressing promoter constructs, identified previously in this project, designated as G8946, G9353 and G7061.
 - a. Determine the extent to which the transgenic promoter-GFP fusions reproduce the patterns of expression for the endogenous promoters.
 - 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.
 - c. Assess the specificity of the promoters for activation by *Xf* compared with *Xc*.
- 2. Develop a new generation of expression cassettes that control the specificity of gene expression and the subcellular destination of candidate proteins.



RESULTS

With prior funding from this program, an Affymetrix GeneChip was used to characterize the expression of ~15,000 *Vitis vinifera* genes in response to *Xf* infection and drought stress. Promoters for three of the *Xylella*-responsive vascular tissue localized genes, referred to as G8946, 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 have received transformants (Table 1) from the UC Plant Transformation Facility within the past year from constructs of the initial promoters fused to GFP

Table 1. Transgenics received.

VARIETY: Thompson Seedless	# Lines or Independent Transformants
7061minGFP	22
8946minGFP	18
9353minGFP	9
Callus derived control	7
total	53

Each of the individual plants was verified by PCR of genomic DNA to contain the appropriate construct. The transgenic plants were then grown in the greenhouse and cuttings made to produce a small population of each transgenic plant (ramets). While the ramets of the transgenics were being made over a several months period preliminary assays were conducted using the cut-branch and detached leaf assays described in our 2006 PD-GWSS symposium report. Briefly, these assays enabled the bacteria to enter and spread though the grape vascular system of a cut branch or petiole in days to with a few weeks accompanied by PD symptoms. These short term pathogenicity tests were used to assess the relative level of expression of the transformed promoter-reporter gene fusions in the *Xf*- induced tissue (Figures 3 and 4). For the cut leaf assay, young, fully expanded, leaves are cut from greenhouse or growth chamber grown grape, the petioles re-cut under water and the leaves placed individually in 2ml plastic tubes containing 2×10^7 *Xf* cells per ml water. The detached branch uptake method is similar to the leaf method using individual branches with attached leaves are cut from the plant. Analysis of gene expression is by confocal microscopy showing evidence of the GFP-fused transgenes being expressed in the presence of *Xf* but not in the water control (Figure 1). RT-PCR of the endogenous genes in Thompson seedless indicated that this promoter was expressed

in the presence of *Xf*. This is confirmation that both the endogenous gene and the promoter gene fusion in transgenic plants are expressed in the presence of *Xf*. Comparable tests with *Xc* failed to show activation of the promoter and appeared the same as the water control.

The results to date indicate that in both assays methods, the promoter activation is *Xylella* responsive and appears near, but not directly in contact with the bacteria as indicated by preliminary analysis of the bacteria by RT-PCR. The PCR based method for detection and quantifying the bacterial presence in relation to promoter activation enabled both the bacteria and the promoter-GFP fusion transgene to be assayed in the same tissue. In comparison of the leaf and branch uptake methods, the results at this point suggest that the detached leaf assay is somewhat faster than the branch uptake with similar results but is more sensitive to activation of the promoter in the water control tissues than the branch uptake method. In both cases, it appears that, at least for the promoter assays done to date, that this method of placing the bacteria in the vascular system and detecting a response to the presence of the bacteria at the level of plant gene expression is valid. Interestingly, the two promoters illustrated in Figure 2 are both active in the presence of *Xf* but are expressed in different cells types within the cross-section of the petiole. These cell-specific locations are consistent with the previous *in situ* detection of RNA of these genes from the Cook lab (1), the original analysis that provided the basis for selection of the promoters used herein.

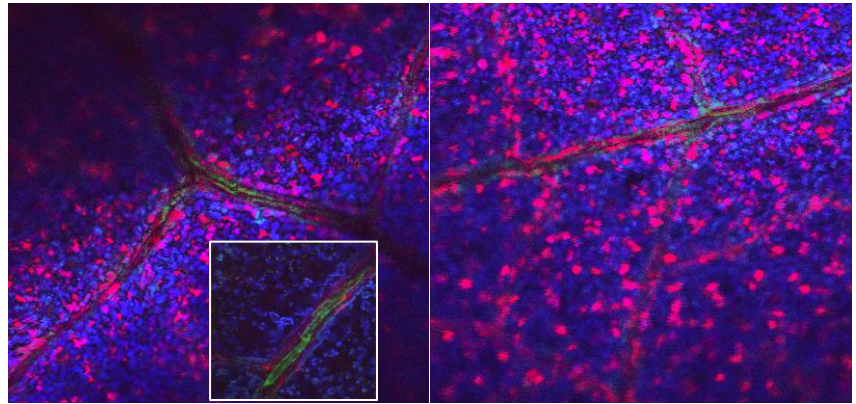


Figure 3. Confocal microscopic analysis at 4x of the activation of the G7061 *Xf* responsive promoter in the laminar tissue following uptake of the bacterium via the petiole in cut leaf assay. The promoter construct is fused to GFP for visualization of activation. The images above were captured two weeks after exposure to the bacteria. Green = GFP; Blue = chlorophyll autofluorescence; Red= polyphenolics released in dying cells. Insert shows an expanded view (25x) of a vascular element with intense GFP fluorescence indicating promoter activity.

As indicated earlier, the leaf scorching indicative of PD occurs within several weeks in the detached tissue assays. Confocal imaging of GFP tagged *Xf* in these same tissues detected only very small amounts of bacteria in the stems and none in the leaf lamina where the genes were detected as being expressed. This indicates that the activation of these promoters is highly sensitive to the presence of bacteria but is active at a distance from where the bacteria were easily detected. However, we also observed that these promoters were frequently activated in water controls of detached leaves making interpretation of expression patterns difficult with this system. Therefore, we are now focusing on whole plant assays (Figure 5).

The whole plant assays are conducted on full-sized (~1m tall) greenhouse grown plants. Inoculations are done by piercing the petiole of mature leaves with a 25G needle attached to a syringe of 2×10^7 Temecula strain of *Xf* per ml water. While still through the petiole, a small drop of *Xf* solution is expelled from the needle and the needle is gently withdrawn from the petiole leaving a 3-5ul drop of bacteria to be sucked into the petiole by negative vascular pressure. This method results in sufficient amounts of bacteria into the xylem of the pierced petiole to be detected by confocal microscopy to visualize the activated promoter (data not shown).

CONCLUSIONS

Transgenic technology offers the possibility of modifying specific traits (e.g., PD susceptibility) based on the introduction of novel genes. In addition to their utility for engineering PD resistance in grape, the advent of *Xf*-induced reporter gene expression provides an extremely powerful tool to study and characterize host response to *Xf* in intact tissue. 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. In the absence of site or response-specific promoters, transgenic strategies for control of PD can use only so-called constitutive promoters. The expression cassettes we are developing will allow precise regulation of gene expression, in particular tissues (e.g., vascular tissue) and/or in response to particular situations (e.g., sharpshooter feeding or *Xylella* infection), and direct secretion of transgenic proteins or small RNAs to the apoplastic compartment where the pathogen resides.

Results summarized in this report indicate that within the first set of three potential *Xf*-inducible promoters; at least two (G7061 and G9353) have been confirmed to be induced in detached tissue in the presence of the bacterium within one to two weeks after exposure to the bacterium. The detached tissue assays appear to have utility in assaying for bacterial-induced transgene expression. Whole transgenic plants expressing the first three promoter-GFP fusions have been inoculated and the

results show that one of the promoters (G9353) is induced within four days of inoculation. Further characterization of the biotic regulation of their expression, including proximity to *Xf*, is ongoing. A new set of potential promoters have been identified and are being assembled into a Gateway binary-based system for higher through put expression and analysis.

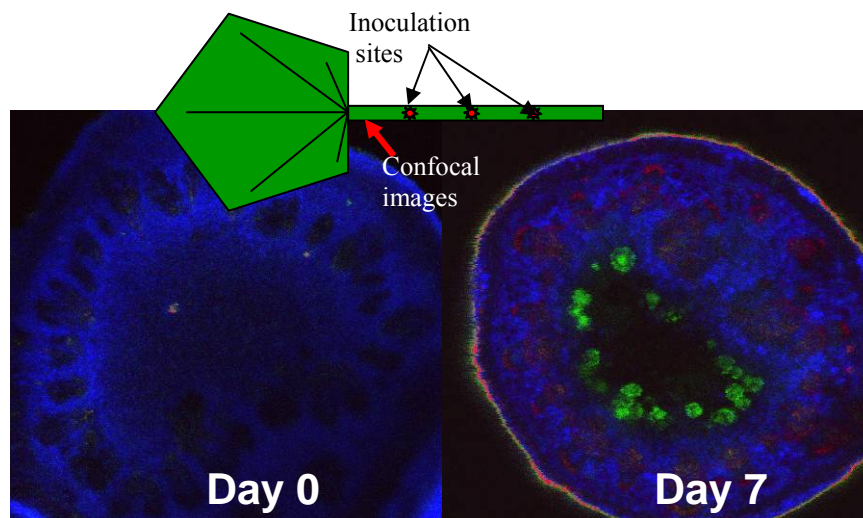
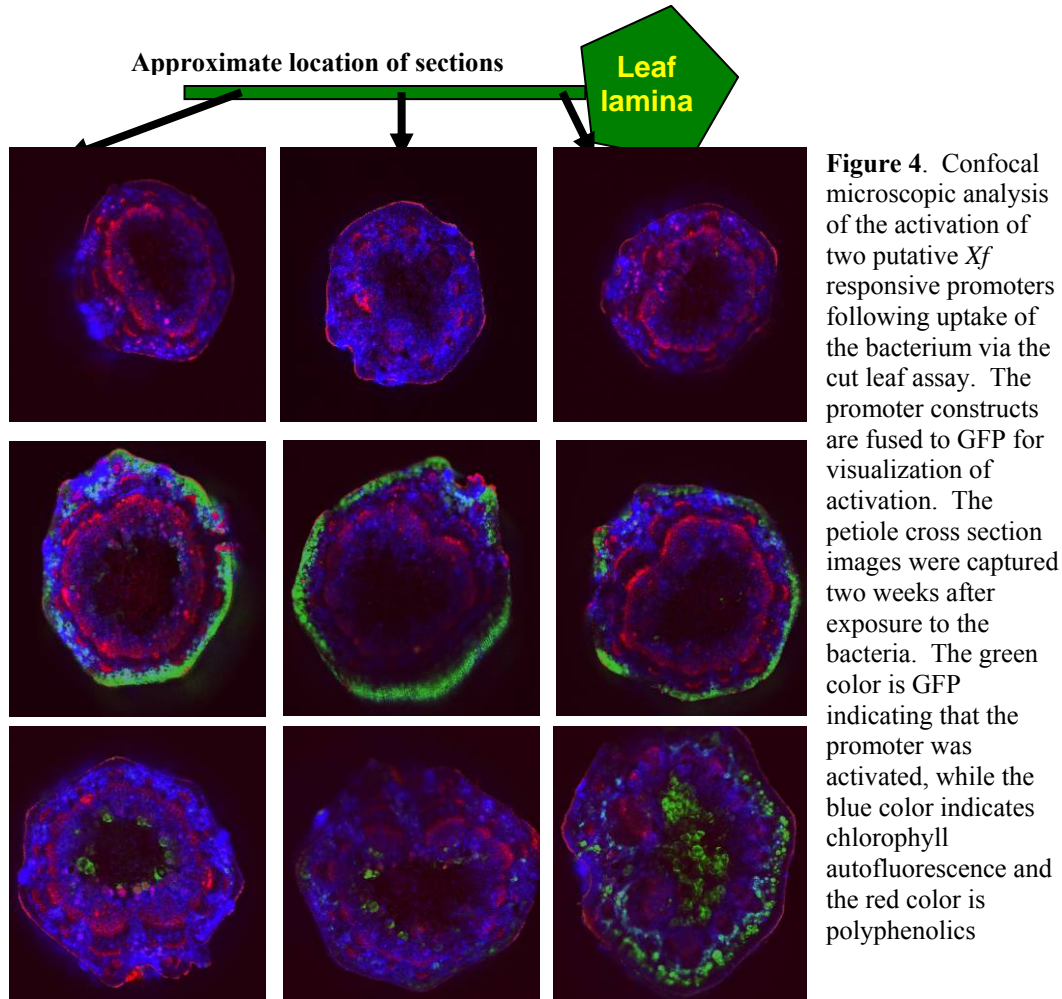


Figure 5. *In planta* activation of promoter G9353. Leaves of the transgenic plant 9353#2 were inoculated with *Xf* by petiole piercing while still attached to the plant. No detectable activation of the promoter is seen just prior to inoculation (Day 0). While after 7 days cells near the xylem can be seen expressing GFP. Water control and *Xc* were negative.

REFERENCES

1. Cook, Doug. 2006. Functional genomics of the grape-*Xylella* interaction: towards the identification of host resistance determinants. Pierce's Disease Research Symposium, San Diego, CA November 27-29.
2. Gilchrist, G, J. Lincoln and D. Cook. 2006. Functional Testing of Pierce's Disease-Specific Promoters from Grape. Pierce's Disease Research Symposium. San Diego, CA November 27-29.

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