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

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ABSTRACT
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 *Xylella 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. This project was initiated in July 2004 as a priority research area by the Pierce's Disease Research Board and will conclude in August 2009 with the delivery of two *Xf*-responsive promoters from grape to a) drive the site-specific expression of any candidate gene at locations where the bacteria reside and b) provide an induced reporter gene expression system that can be used as a powerful tool to study and characterize host responses to *Xf* and *Xf*-secreted effector molecules in intact xylem cells by observing fluorescence due to GFP. The promoters, G9353 and G7061 each will be available as GFP fusion constructs in transgenic Thompson Seedless clonally propagated lines. Illustration of the use of these promoter fusions as diagnostic tools for grape response to *Xf* EF-Tu infiltration can be found in the 2008 PD Symposium Proceedings report from Professor George Bruening's group.

INTRODUCTION
This project was initiated in July 2004 as a priority research area by the Pierce's Disease Research Board (3). The need for *Xylella fastidiosa (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 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 (5). An Affymetrix GeneChip was used to characterize the expression of ~15,000 *Vitis vinifera* genes in response to *Xf* infection and drought stress (1,2) (**Figure 1**).

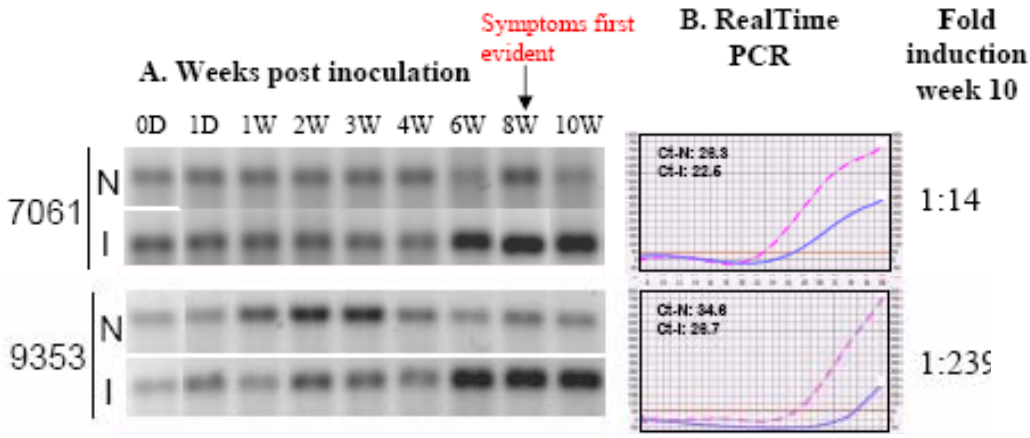

Figure 1. *Xylella fastidiosa* responsive promoters were obtained from genes found to be up-regulated in *X. fastidiosa* infected field grown plants but not up-regulated by water stress alone. Example data used to identify individual genes from which promoters were recovered as illustrated for G7061 and G9353.

Promoters for two of the *Xylella*-responsive vascular tissue localized genes, referred to 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 *Xf*.

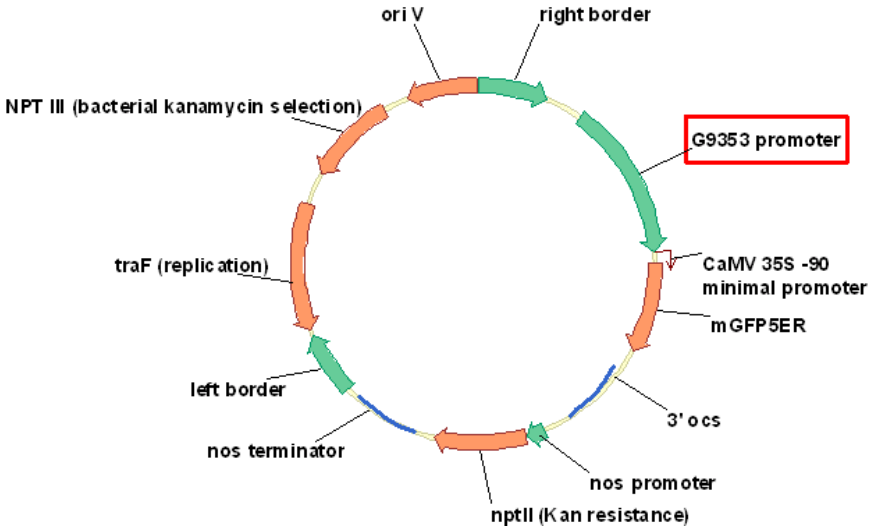

Figure 2. Binary plasmid containing the G9353 promoter sequence fused to GFP, which will be available to Pierce's Disease researchers upon request.

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 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. Please see article by Dr. Bruening in these Proceedings for specific information on the EF-Tu assay.

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 petiole inoculated and analyzed
G7061	unknown	22	12	9 of 12 show GFP expression	4 of 10 show GFP expression	12/22
G9353	Alpha-tubulin	20	10	3 of 10 show GFP expression	3 of 10 show GFP expression	10/20

OBJECTIVES

1. Evaluation of whole plant transgenics G9353 and G7061 with stem inoculation under greenhouse conditions.
2. Confirm the specificity of response of promoter G9353 to *Xf* vs *Xanthomonas campestris* using intact stem inoculation under greenhouse conditions.
3. Promoter distribution to current researchers and long-term storage.

RESULTS AND DISCUSSION

Two of the original 14 putative promoters, G9353 and G7061, have been advanced to the transgenic stage as GFP (green fluorescent protein) fusions (4) and show a response to infection by *Xf* as visualized by RT-PCR (**Figure 3**) and GFP expression in the inoculated plants using confocal microscopy (**Figure 4**). A total of 42 plants were received (**Table 1).** Prior to any functional analysis, the transgenic grape plantlets were grown in the greenhouse and each primary transgenic was assayed for the presence of the transgenes by genomic PCR prior to any further experimentation. Currently we have tested 50% of the primary transgenic plant lines. While all plants tested to date contain the transgene not all are 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.

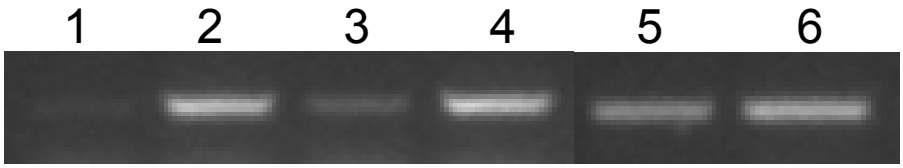

Figure 3. RT-PCR analysis of the G9353 transcript from greenhouse grown grape leaves. RNA in lane 1 healthy cv. Freedom; lane 2 *Xf*-infected cv.Freedom; lanes 3and 5 are healthy Thompson Seedless; and lanes 4 and 6 are *Xf*-infected Thompson Seedless. Lanes 1-4 were amplified with G9353 specific primers and lanes 5-6 were amplified with actin specific primers as a control. Lanes 2 and 4 indicate the induction of the G9353 transcript in the infected plants in the greenhouse.

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 four-fold to be visualized as differential by confocal fluorescence microscopy. The remaining plants will be subjected to final testing with whole plant inoculations (**Table 1**). Clonal copies of each line will be made before evaluation by inoculation. Following clonal propagation, 10 transgenic lines of each promoter remain to be inoculated with *Xf* under greenhouse conditions, assessed by confocal microscopy and qPCR for timing and location of response of the respective promoters to the presence of the bacteria. These studies will develop a sufficiently detailed picture of the temporal and spatial aspects of *Xylella*-induced gene expression during bacterial colonization in stems, leaves and petioles of grapes as a base line for use as a diagnostic tool for the effect of pathogen secreted molecules as indicated earlier. Criteria for selecting the prime transgenic lines are that they express a specific, rapid and strong response to *Xf* infection with low background response. 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*.

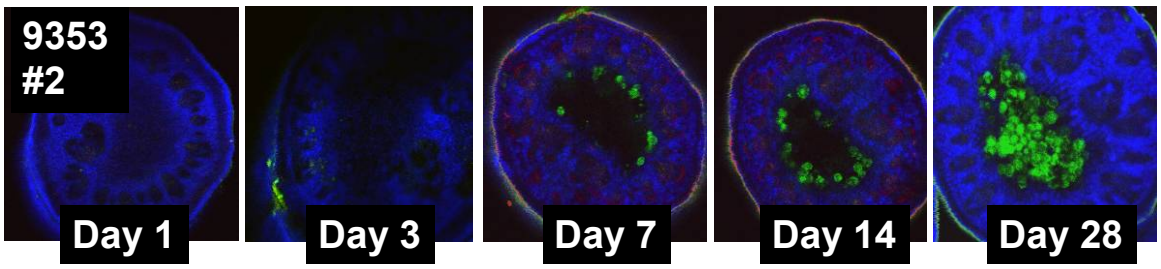

Figure 4. Specificity of the response of promoter G9353 fused to GFP in response to the presence of *Xf* in the vascular system of grape compared with *Xanthomonas campestris.*Blue color represents chlorophyll auto-fluorescence; Green color indicates the translation of GFP and, therefore, activation of the promoter 9353 in the presence of *Xf*.

In addition to the *Xf*-activated response of these promoters, it is essential that the response to *Xf* be specific to this bacterium. To assess the specificity, *Xanthomonas campestris* pv. *vesicatoria (Xcv,* another xylem dwelling bacteria that closely resembles *Xf* but is not pathogenic on grape served as the bacterial test case. Transgenic G9353 was inoculated with *Xcv* and compared to clonal plants inoculated with pathogenic *X f* at the same cell density and evaluated by confocal microscopy after 21 days. The presence of Xcv in the xylem did not trigger the expression of G9353-GFP whereas the promoter driven GFP was activated by *Xf,* as shown previously. These observations indicate that the promoter was responsive to *Xf in planta* but not to the related *Xcv* (**Figure 5).** In addition, recent results from collaborative efforts between the Gilchrist laboratory and the Bruening laboratory have demonstrated that promoter G9353 expression is activated by injection into the grapevine petiole of *Escherichia coli* cells expressing a single *Xf* protein, the temperature unstable protein synthesis elongation factor EF-Tu. The same *E. coli* strain, not induced to generate *Xf* EF-Tu, and another strain, not bearing the EF-Tu construction, both failed to induce GFP accumulation, indicating that the effect is due to *Xf* EF-Tu and not, for example, to endogenous *E. coli* EF-Tu. In other systems, examples of plant recognition of the EF-Tu from specific plant pathogenic bacteria are well documented.

Please see the report in this volume of the Proceedings of the 2008 Pierce's Disease Research Symposium by George Bruening entitled "Exploiting *Xylella fastidiosa* Proteins for Pierce's Disease Control" for additional research description of the diagnostic application of G9353 transgenic plants.

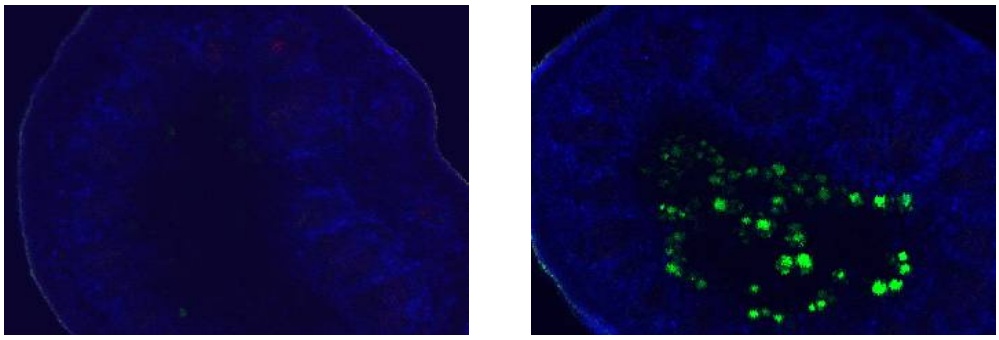

Figure 5. Time course analysis of the expression of promoter G9353 fused to GFP in response to the presence of *Xf* in the vascular system of grape. Bacteria were introduced by puncture inoculation as illustrated in **Figure 2**. Analysis is by confocal microscopy. Blue color represents chlorophyll auto-fluorescence; Green color indicates the translation of GFP and, therefore, activation of the promoter 9353 in the presence of *Xf*.

CONCLUSIONS

The activation of these two promoters following infection with *Xf,* but not *Xcv,* confirms the proof of concept that we can supply *Xf*-inducible promoters to PD researchers. Our intention is to 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 (**Figure 1**).

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

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