Project Title: Functional Testing and Delivery of Pierce's Disease-Specific Promoters from Grape
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Principle Investigator: David Gilchrist (dggilchrist@ucdavis.edu); (530)-752-6614
Co-Principle Investigator: James Lincoln (jelincoln@ucdavis.edu) (530)752-6587
Department of Plant Pathology, University of California, Davis, CA 95616.
Cooperator: Douglas Cook (drcook@ucdavis.edu) (530)754-6561. Department of Plant Pathology, University of California, Davis, CA 95616

Objectives:

1. Evaluation of whole plant transgenics with stem inoculation under greenhouse conditions

This research will conclude with the release of the two promoters now in stable transgenic plant lines. Evaluation of the first 10 lines each of promoter G9353 and G7061 confirmed that they are *X.fastidiosa* (Xf)-inducible but also that there are substantial differences in level of expression among the individual transgenic lines, as expected. We have 10 additional independent transgenic lines of each promoter-GFP fusion to be inoculated and evaluated for timing and location of promoter-GFP response to Xf. Criteria for selecting the prime transgenic lines are that they express a specific, rapid and strong response to Xf infection with low background response.

2. Confirm the specificity of response of promoter G9353 to Xylella fastidiosa vs Xanthomonas campestris using intact stem inoculation under greenhouse conditions

It is essential that the response to *Xf* be specific. Initial observations are consistent with response to Xf but not to the related bacterium *Xanthomonas campestris* pv. *vesicatoria* . These experiments will be repeated on the population of plants from the two promoters that are being carried forward.

3. Promoter distribution to current researchers and long term storage

We will 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.

4. **Develop second generation expression cassettes** that control the specificity of gene expression and the subcellular destination of candidate proteins including, putative antibacterial peptides, PGIPs, quorums sensing inhibitors, or *Xf*-binding proteins. The functional activities to be incorporated include specific promoter, secretory leaders and possible RNA tethers for systemic movement of mobile proteins and RNA.

Description of activities conducted to accomplish each objective

1. ***Evaluation of whole plant transgenics with intact stem inoculation under greenhouse conditions***
   Previously, promoter-GFP fusions derived from three grape genes were introduced into the *V. vinifera* cultivar Thompson Seedless and subjected to preliminary testing with the detached branch and leaf assays. Transgenic grape plantlets, received from the UCD Transformation Facility were grown in the greenhouse for 2 months, 10 copy ramets were made before any inoculations but leaves of the primary transgenics are assayed for the presence of the transgenes by genomic PCR. Clones of the available transgenics were made to allow a variety of whole plant assays (Table 1).

2. ***Whole plant inoculations***
   The whole plant assays were conducted on full-sized (~1m tall) greenhouse grown plants. Inoculations were done by piercing the petiole of mature leaves with a 25G needle attached to a syringe of $2x10^7$ *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 large amounts of Xf being drawn into the xylem of the pierced petiole as detected by confocal microscopy (data not shown). Clones were inoculated by petiole puncture with *Xf* at 10^7cfu/ml and leaves were sampled at 1, 2, and 4 weeks for initial characterization by qPCR and confocal microscopy (Table 1 and Figure 5). This requires extensive sampling of stem, petiole and leaf tissue of all the transgenic plants. The same techniques will be used for inoculation with *Xanthomonas*.

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3. **Distribution of transgenic grape or plasmids bearing the promoters:** This project will generate intellectual property; The promoters that drive expression of grape genes due to the presence of *X f* will be made available to other PD projects through a standard material transfer agreement. 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.

4. ***Second generation expression cassettes***
   Three additional promoters XSP, F5H, and CMT have been isolated from grape and used to make binary vectors for inducible expression of GFP. These vectors are ready to be tested for expression in whole plant transgenics.

Current accomplishments:

1. **Whole plant inoculation to determine strength, timing and location of promoter-GFP fusion induction as the final determinant of promoter response.** Two of the original 14 putative promoters, G9353 and G7061, have been advanced to the transgenic stage as GFP (green fluorescent protein) fusions and have been demonstrated to show a response to infection by *Xylella fastifiosa* (*Xf*) using confocal microscopic detection of GFP expression in the inoculated plants. A total of 42 plants were received (Table 1). The transgenic grape plantlets are grown in the greenhouse and each primary transgenic is 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.

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 4-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, ten 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 effector of pathogen secreted molecules as reported previously (1).

Immediate application: The activation of these two promoters following infection with Xf confirms the proof of concept that we can supply Xf-inducible promoters to PD researchers. 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, suggesting 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. We wish to continue investigating the recognition of Xf EF-Tu by grapevine, because the origins of Xf-induced PD symptoms are unknown, and EF-Tu appears to be an excellent candidate for such an inducer. The grapevine lines bearing transgenes for G9353- and G7061-driven GFP expression are proving to be valuable assets.

2. **Confirm the specificity of response of promoter G9353 to Xylella fastidiosa vs Xanthomonas campestris** using intact stem inoculation under greenhouse conditions. An important question is: are these promoters specific to *Xf* or do they also respond to closely related bacteria such as *Xanthomonas campestris* pv. *vesicatoria*? Inoculations of both Xylella and Xanthomonas were conducted on cloned copies of G9353 plant #2 in the greenhouse by petiole puncture (Figure 4) with 10^7 cfu/ml. GFP fluorescence, indicative of induced expression of G9353, was detected in leaves inoculated with *Xf* but not in cloned G9353 plants inoculated with *X. campestris* pv *vesicatoria* (reference 4). We are currently inoculating additional plants to acquire sufficient data to establish the level of variation in the population of transgenic plants in reference to lack of response to *X. campestris* pv *vesicatoria.*

3. **Promoter distribution to current researchers and long term storage**

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).

Research relevance statement to solving the PD/GWSS problem in California

Several research groups funded by the Pierce's Disease research program have been developing candidate transgenes with these attributes; the polygalacturonase inhibiting protein (PGIP) of Labavitch blocks PG activity, and the *rpf*F gene (DSF-synthesis) gene of Dr. Lindow appears to interfere with bacterial cell-cell communication. As requested, we will make our expression cassettes available to these and other investigators who have candidate genes that require testing in *V. vinifera*. We anticipate that the efficacy of such proteins will benefit from expression at sites of infection in response to the presence of the bacterium. Moreover, proteins that act directly on the bacterium, such as PGIP, should benefit from transport to the apoplast using an N-terminal signal sequence. Alternatively, enzymes that produce active metabolites, as may be the case for rpfF, may be most effective when retained in the cell. To accommodate these contrasting scenarios, we will produce expression cassettes with a range of promoters and with or with out signal sequences. 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 temperature unstable protein synthesis elongation factor EF-Tu.

In addition to their utility for engineering PD resistance in grape, the advent of X. fastidiosa-induced reporter gene expression would 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. Moreover, the recent development of X. fastidiosa-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. For example, we have recently determined that host gene expression is induced specifically in live cells of the phloem. 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 should help answer such questions.

Intellectual Property (IP): This project will generate intellectual property; specifically promoters that drive expression of grape genes due to the presence of *X. fastidiosa*. 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, 3) all materials will be made available to qualified researchers, including all those associated with the PD projects through a standard material transfer agreement.

References:

1. Goes da Silva, F., Iandolino, A., Al-Kayal, F., Lim, H., Bohlmann, M., Baek, J., Cushman, M., Leslie, A., Ergul, A., Xu, J., Figueroa, R., Kabuloglu, E., Osborne, C.,

Rowe, J., Tattersall, E., Cramer, G., Cushman, J. and Douglas R. Cook. 2005. Characterizing the Grape Transcriptome. Analysis of ESTs from Multiple Vitis Species and Development of a Compendium of Gene Expression During Berry Development. Plant Physiology 139: 574-597.

2. Cook, Doug. 2005. Functional genomics of the grape-Xylella interaction: towards the identification of host resistance determinants. Pierce's Disease Research Symposium. San Diego, CA December 5-7.
3. Gilchrist, G, J. Lincoln and D. Cook. 2006. Isolation and Functional Testing of Pierce's Disease-Specific Promoters from Grape. Pierce's Disease Research Symposium. San Diego, CA November 27-29
4. Gilchrist, G, J. Lincoln and D. Cook. 2007. Isolation and Functional Testing of Pierce's Disease-Specific Promoters from Grape. Pierce's Disease Research Symposium. San Diego, CA December 12-14.

Table 1. Promoter:GFP fusion constructs that have been tested in Thompson Seedless as transgenes. Testing is 50% complete.

Promoter name	Putative function of gene	# trans-formants	Xf uptake into detached leaf	Xf inoculation into attached leaf
G7061	unknown	22	GFP expression	GFP in initial experiments
G8946	unknown	18	No GFP	No GFP
G9353	Alpha-tubulin	10	GFP expression	GFP expression

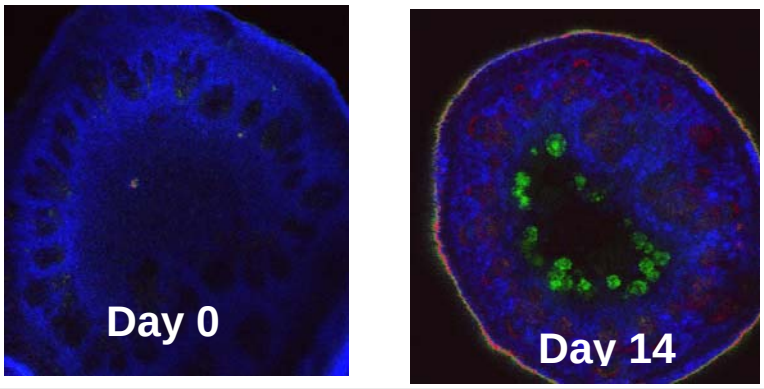

Figure 1 Example of the expression of promoter G9353 fused to GFP in response to the presence of *X. fastidiosa* in the vascular system of grape. Petiole inoculations at of 10^7 cfu/ml were followed by confocal assay of cross sections of the petiole at the point indicated. Blue color represents chlorophyll auto-fluorescence; Green color indicates the translation of GFP and, therefore, activation of the promoter 9353 in the presence of *X. fastidiosa.* Equivalent results were obtained by the Bruening laboratory using E. coli-expressed Xf EF-Tu demonstrating the value of these promoters as direct non destructive real time assays for response of grape to *X. fastidiosa* or *X. fastidiosa* associated signal molecules

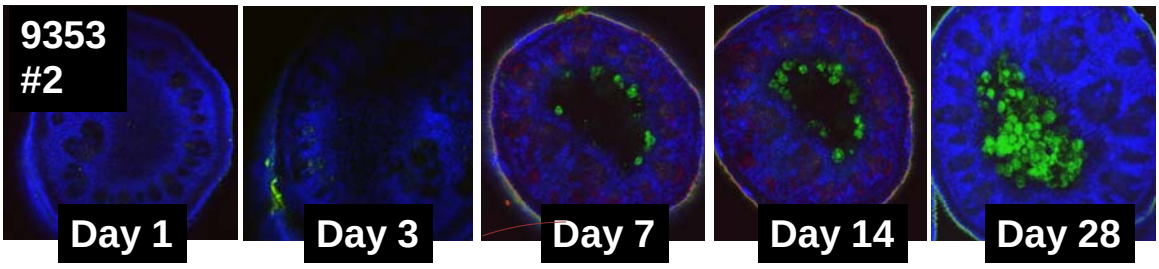

Figure 2. Time course illustration of the expression of promoter G9353 fused to GFP in response to the presence of *X. fastidiosa* in the vascular system of grape. Bacteria were introduced by puncture inoculation as illustrated in Figure 2. Analysis is by confocal microscopy. Blue color represents chlorophyll auto-fluorescence; Green color indicates the translation of GFP and, therefore, activation of the promoter 9353 in the presence of *X. fastidiosa.* Inoculation *with Xanthomonas campestris pv. vesicatoria* failed to induce expression of GFP by day 28.

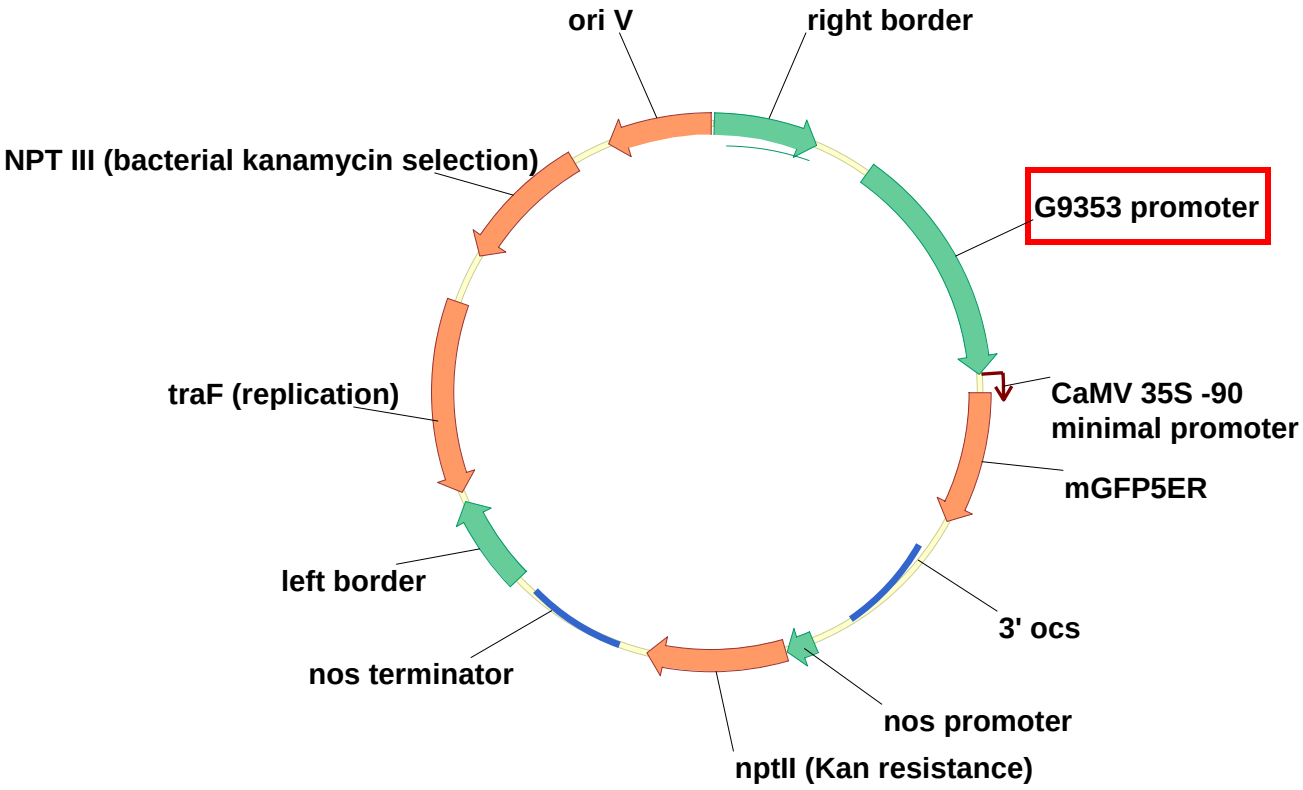

Figure 3 Binary plasmid containing the G9353 promoter sequence fused to GFP, which will be available to Pierce's Disease researchers upon request.