Final Report for CDFA Contract Number 01-0712

Project Title: Functional Testing of Pierce's Disease-Specific Promoters from Grape

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Duration of Project: **7/1/2002 to 12/30/2009.** This project was initiated in July 2002, formal funding was ended in 2008 and the project was completed in 2009 with limited residual funds.

Keywords: Pierce's Disease, disease responsive promoters, Xf-specific expression of heterologous genes, targeted gene expression

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 manner. We set out to determine if promoter sequences that regulate gene expression in particular tissues (e.g., vascular tissue) or in response to particular situations (in our case, sharpshooter feeding or *Xylella fastidiosa [Xf*] present in the grape xylem*)* infection), could be indentified, cloned and tested in grape for their ability to drive expression of transgenes in the presence of Xf. In the absence of tissue or response-specific promoters, transgenic strategies for control of Pierce's Disease (PD) can use only so-called constitutive promoters wherein the transgene is expressed in all cells all the time, not just in the tissue or cells under threat by *Xf* where any gene conferring resistance is needed. Highly controlled gene induction is needed if the interest is in altering gene expression to avoid disease that is occurring in one or a few isolated cells and in real time. The isolation and characterization of *Xf*-responsive promoters has direct application to several current PD projects that are using the expression of transgenes as a method of PD resistance. 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 either a sense or an anti-sense construct of the gene of interest.

The objectives of this promoter isolation and analysis were to identify and characterize cis-acting DNA (adjacent) sequences that, when activated by the presence of *Xf,* would coordinately 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. Thus, the challenge in our studies is to demonstrate that any cis-acting sequences we identified have a unique functional role in PD symptom development. It was 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 7).

Objectives of Proposed Research and Path to Application:

Promoter identification*:* We first identified a set of plant genes whose expression is correlated with infection by *Xylella fastidiosa* as part of a study comparing expressed sequence tags (ESTs)from *Xf*-infected and healthy *V. vinifera* plants in the Napa Valley. The objective was to indentify genes that were 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.

The experimental approach 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 CA&ES Genomics Facility (http://cgf.ucdavis.edu/). We identified BAC clones of each of the three genes by hybridization with labeled gene fragments. Our specific interest was in sequences immediately 5' to the candidate genes (maximum 5 kbp), but sequencing the entire BAC is actually faster than isolating the specific promoter fragment to be sequenced. All templates were sequenced a minimum of both directions. From this data we identified 5' promoter regions in the sequenced genomic clones based on comparison to cDNA sequences for the three genes: PCR was then used to isolate and clone the potential 5' regulatory sequences into transformation ready vector constructs for three differentially expressed genes, referred to as G8946, G9353, and G7061 and used to produce promoter-reporter gene fusions (GFP) for transformation into grape. (Figure 7**)**.

Accelerated pathogen-response assay methods via branch uptake: To efficiently test the responsiveness of these three putative *Xf* –responsive elements, we developed a cut-branch assay that enables the bacteria to enter and spread though the vascular system of the cut grape stem in days to a few weeks. Briefly, branches of the PD susceptible root stock cv. Freedom are cut under water and allowed to take up *X. fastidiosa* cells for 2 hr from a bacteria suspension containing $2x10^7$ cfu/ml. Inoculated stems are then placed in water for 1-2 weeks, after which they were assayed for host gene expression by means of RT-PCR (3). The severed branch immersion in the bacterial suspension was followed by a time-course assay for appearance of the GFP marker fused to the putative promoter. The initial experiments used PCR to detect promoter activation while activation was subsequently characterized both by PCR and confocal microscopy using fully transformed plants carrying the promoter-GFP fusions. The net result was detection of the expression of the transformed promoter-reporter gene fusions several weeks prior to the expression of leaf symptoms. For example, the leaf scorching indicative of Pierce's Disease occurred within several weeks under these conditions, while the promoter G7061 was active within 5-7 days (Figure 2&3) (3). Genes G8946 and G7061 were strongly and reproducibly induced in stems inoculated with *Xf* well in advance of symptom

development (Figure 2). 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 confirmed by PCR to be expressed. These data indicated that the activation of these promoters was highly sensitive to the presence of bacteria themselves or to signals from the bacteria perceived by the plant at a distance from where the bacteria were easily detected (3). We concluded that the detached branch assay provided a simple and reliable method to monitor *Xylella*-induced gene expression, significantly shortening assay time compared to whole plant inoculation assays. These assays were then extended to and were confirmed with whole plants transgenic for respective promoter-GFP fusion constructs (Table 1).

Evaluation of whole plant transgenics with intact stem inoculation under greenhouse conditions

The promoter-GFP fusions for the first three putative Xf-responsive promoters were introduced into the *V. vinifera* cultivar Thompson Seedless and subjected to preliminary testing with the detached branch (Figure 2 & 3) and later with the whole plant inoculations (Table 1 and Figures 4,5 and 6). Clonal copies of the available transgenic plants were then used to assess whether the isolated promoter elements were equivalent in expression pattern to the genes from which they were derived as previously found by Dr. Cook's Affymetrix Gene Chip analysis of a differential response to the presence of *Xf* but not other bacteria.

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 whole plants transgenic for G9353. To address this question, clonal copies of G9353 plant #2 were assayed in the greenhouse by petiole puncture (Figure 4) by inoculation with 10^7 cfu/ml of each of the two bacteria. GFP fluorescence, indicative of induced expression of G9353, was detected in leaves inoculated with *Xf*-infected plants but not the clonal G9353 plants inoculated with *X. campestris* pv *vesicatoria* (Figure 6, and reference 4). These data indicated that the response of G9353 was specifically responsive to the presence of Xyella capable of causing Pierce's Disease and not a related bacteria that also is pathogenic but does not cause Pierce's Disease.

Detailed Methods Used in this Project

BAC Sublibrary preparation and clone management. 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 were sequenced a minimum of both directions.

qPCR: qPCR was performed in an Applied Biosystems 7500 using SYBR greenER according to the manufacturer. The oligos used were:

Xf (5'AAAAATCGCCAACATAAACCCA, 5' CCAGGCGTCCTCACAAGTTAC);
Vitis vinifera actin (5' CTTGCATCCCTCAGCACCTT, 5'TCCTGTGGACAATGGATGGA); **cDNA9353** (5'AGGCATGAAGACCTCCTCACTGC, 5'TCCCTTCGATTGGACAAGCTGT)
cDNA7061 (5'GCTTAGAAATGTCCAATTTAATGAGTGGTG, 5'CCTCGTTACCTCTCTTCTTTCACCAA);
GFP (5'AATAAATCATAAGTTGGAATACAACTACAACTC, 5'AATAAATCATAAGTATTTTGTTGATAATGATCA)

***Confocal Microscopy*:** Real time, non-destructive images of the isolated promoters driving the expression of GFP in grape plants were obtained using a laser activated confocal microscope (BioRad MRC1024) by excitation at 488nm with a Krypton/Argon 15 mW laser. The use of the laser allows non-destructive GFP detection in intact plant leaves and roots. Three different fluorescent emissions were detected simultaneously with the following filters: (emission filter 578nm-618nm displayed as RED detects polyphenolics); (emission filter 506nm-538nm displayed as GREEN detects GFP); and (emission filter 664nm-696nm displayed as BLUE detects chlorophyll).

***Isolation of BAC clones containing the Xylella-induced genes by hybridization*:** Bacterial Artificial Chromosome (BAC) libraries of *V. vinifera* were obtained as high density filters for gene identification in grapes through the UC Davis CA&ES Genomics Facility (http://cgf.ucdavis.edu/). High-density filter sets of the library are used for hybridization-based clone identification. The hybridization pattern is used to determine clone identity based on a coordinate system.

***Sequencing the BAC clones to completion*:** Although our specific interest was in sequences immediately 5' and 3' to the candidate genes (maximum 10 kbp), we sequenced regions beyond where we believe the promoters to reside. The rationale derives from efficiencies and strategies of modern sequencing techniques; it is both faster and more cost effective to use the BAC shotgun strategy described below which automatically provides additional sequence information for less cost that if we were to attempt to focus on shorter regions immediately adjacent to either end of the candidate genes.

***Sublibrary preparation and clone management*:** BAC DNA was isolated using the Qiagen Large Construct kits with sheared fragments generated by HydroShear (Gene Machines, Inc.) and blunt-ended using a fill-in approach were cloned *en masse* into a SmaI-digested pUC18 sequencing vector. *Shotgun and finishing strategies:* Paired-end sequence reads were generated from the pUC18 subclone library, with two 384-well plates analyzed for each BAC clone. This yielded $1.05 \cdot 10^6$ bp of sequence, equating to 8.5X coverage of a typical 125 Kb BAC clone. All templates were sequenced a minimum of both directions. *Sequencing Methodology:* Our sequencing methodology used the Pharmacia Templiphi Kit (rolling circle amplification). We modified the

manufacturer's protocol to produce high quality sequencing templates using a 4X dilution of the commercial kit with reactions facilitated by a Packard Multiprobe II liquid handling robot and an Apogent Hydra 96 dispenser. We have complete cDNA sequences for each of the candidate genes that facilitated annotation of the BAC clones and identification of regions immediately upstream and downstream of the transcription units.

***Binary vector construction*:** Analysis of the sequence of the appropriate BAC clones allowed the design of PCR primers to amplify and clone the 5' promoter of the transcriptionally regulated grape genes into novel binary vectors. The binary used is a derivative of pBIN19 and uses the isolated grape promoter as an enhancer to activate a minimal CaMV 35S promoter sequence (-90 to +1) in front of a green fluorescent protein (GFP) coding sequence. The version of GFP coding sequence used is mGFP5ER designed by James Haseloff (Cambridge University) for plant expression. Details of the plasmids are available upon request.

***Agrobacterium tumefaciens transient assay*:** A modified version of the binary vector pBIN19 also was used to express the promoter-GFP constructs. These constructs were transformed into *A. tumefaciens* strains LBA4404, for whole plant transformation, or strain GV2260, for the transient infiltrations. *Agrobacterium* colonies containing the specific binary construct were selected by resistance to kanamycin.

***Infiltration to initiate the transient expression assay*:** A colony from a freshly streaked plate of GV2260 Agrobacterium is used to inoculate 0.5 ml of MG/L media plus kanamycin for selection of the binary vector. After overnight growth at 28^{o}C with shaking, 5 ml of MG/L plus kanamycin is added. The culture is incubated at 28^{o}C with shaking for an additional 4-6 hr and then diluted to an appropriate OD600 with water (assuming 1 OD600 = 10^9 bacteria per ml). Diluted *A. tumefaciens* was then infiltrated into a grape leaf using a needle-less syringe by first opening a small puncture in the leaf with a 26G needle, then immediately placing a needle-less syringe over the puncture and holding a finger under the back of the leaf. Sudden rapid pressure is applied to the syringe to infiltrate a small amount (<0.1 ml) of culture. We quickly learned that grape leaf tissues does not accept much infiltration solution due to the fact that the cells in a grape leaf are very densely packed together compared with a tobacco leaf for instance that readily takes up a pressure infiltration solution. However, we were able to introduce sufficient amount of bacteria to enable the assay to reveal the presence of the GFP marker. The plants are incubated in the light pending assessment of the leaf using confocal microscopy. The duration of a single assay was from 2 to 6 days but this assay, while providing confirmatory information, was not as efficient as the uptake assays to be described below.

Branch uptake method. A terminal shoot approximately 60cm long was cut from greenhouse or growth chamber grown grape plants. The shoot was 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 *X. fastidiosa* cells/ ml. Shoots are allowed to uptake the bacteria suspension for 2 to 48hrs 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 wks.

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. This assay is somewhat faster than the branch uptake but the results are similar.

Generation of transgenic grape plants. The U.C. Davis Plant Transformation Facility introduced the promoter-GFP fusions into the *Vitis vinifera* cultivar Thompson Seedless by *Agrobacterium tumefaciens*-mediated transformation of anther cultures. Embryos were then induced from the transformed tissue and transformed plantlets were regenerated.

Inoculation of greenhouse plants. Clones of the available transgenics were made to allow a variety of whole plant assays. 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 1,3, and 7 weeks for initial characterization by qPCR and confocal microscopy (Table 1 and like Figure 4).

Potential Impact and Relevance of the Research:

Among the potential solutions to Pierce's Disease in grapes are approaches based on gene transfer technology. Transgenic technology offers the possibility of modifying specific traits (e.g., PD susceptibility or resistance) based on the introduction of novel genes One of the research priorities identified by the 2003 PD/GWSS project reviews and at the August 2006 PD Workshop is the need to identify, clone and characterize unique DNA sequences that specifically regulate the expression of grape genes in tissues infected with *X. fastidiosa*. Emphasis was placed on the urgency and practical utility of isolating promoters of PD responsive genes and fusing these to candidate transgenes to block PD from several other projects. Several projects are working toward understanding the genetic basis for susceptibility or resistance at the molecular level in grape to Pierce's Disease. A major limitation to test candidate genes is the inability to regulate expression of these genes in time and space relative to the presence of the pathogen. Although researchers are able to express any PD-modulating candidate transgene constitutively and at high levels, it is not possible to specifically modulate gene expression in cells precisely where and when the pathogen is present. The goal of this research was to clone and characterize unique DNA sequences from grape that were able to specifically regulate the

expression of fused transgenes in tissues that are infected with *X. fastidiosa* or are receiving systemic signals of pathogen presence. The goal was to deliver one or more *Xylella*-responsive promoters from grape to drive the site-specific expression of any candidate gene at locations where the bacteria reside or where bacterial signals extend. Lastly, these promoters would likely be capable of either increasing or suppressing (through RNA interference) the expression of a gene of interest. The results displayed herein confirm that we have detected, cloned, expressed and confirmed that one of the promoters tested to date is activated by the presence of Xf in the xylem of both detached branch and whole plant assays of plants expressing promoter-GFP fusions (Table 1). Mock inoculation and inoculation with the related Xanthomonas campestris (Xc) under the same conditions do not activate the promoter (Figure 6).

The expression cassettes we developed have the potential to 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 may even direct secretion of transgenic proteins to the apoplastic compartment where the pathogen resides. In the absence of tissue or response-specific promoters, and proper protein targeting motifs, transgenic strategies for control of PD is dependent on generic constitutively active promoters that may not be optimal for purposes of controlling Xylella infection in xylem or even constructing transgenic plants. Xylella-inducible promoters have the potential to confer transgene expression at the time and location of bacterial infection; combined with apoplast-targeting peptides, these constructs are designed to deliver 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.

Linkage to ongoing PD research projects and application to the total research effort

Promoters derived from genes expressed in *Xf*-infected field grown grape plants were cloned and fused to GFP for characterization under controlled conditions. Results indicate that within the first set of three potential *Xf*-inducible promoters tested, at least two (G7061 and G9353) are induced in detached tissue in the presence of the bacterium within 1-2 weeks after exposure to the bacterium. Whole transgenic plants expressing the three promoter-GFP fusions were inoculated with *Xf* or *Xanthomonas campestris.* One of the promoters (G9353) was induced within 4 days of inoculation or 2 months before symptoms were first observed. *Mock inoculation and inoculation with X. campestris, under the same conditions, did not activate the promoters.* The expression cassettes we developed will allow 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) providing the opportunity for timely analysis of direct signal exchange between insects or bacteria, or enable direct secretion of transgenic proteins or small RNAs to the apoplastic compartments of surrounding cells where the pathogen resides. These promoter elements are available to the several PD researchers currently studying the effect of various transgene on suppression of PD symptoms in grape.

Summary and Conclusion of this Research Project

Promoters derived from genes expressed in *Xf*-infected field grown grape plants but not in healthy plants or plants under water stress were cloned and fused to GFP for analysis of expression induced by *Xf*. The first three promoter fusions were introduced into Thompson Seedless and subjected to preliminary testing with the detached branch and the whole plant inoculation procedures. Results confirmed that two (G7061 and G9353) are induced in detached tissue in the presence of the bacterium within 1-2 weeks after exposure to the bacterium and up to a month before symptoms appear (Figures 2 and 3). Mock inoculation and inoculation with *X. campestris* did not activate promoter G9353. The promoters G7061 and G9353 are available both in binary vectors and in fully transformed Thompson Seedless plants that we currently are maintaining under greenhouse conditions. In conclusion, we succeeded in accomplishing our objectives, which were to identify, isolate and analyze, and make available to the grape research community cis-acting DNA (adjacent) sequences that, when activated by the presence of Xf, would coordinately regulate PD-associated gene expression in grapes as tissue specific promoters of transgene expression.

Intellectual Property (IP):

This project has generated intellectual property; specifically promoters that drive expression of grape genes due to the presence of *X. fastidiosa*. The issue of IP is being 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 promoter sequences, 3) all materials, including live plants 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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3. Gilchrist, D, 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
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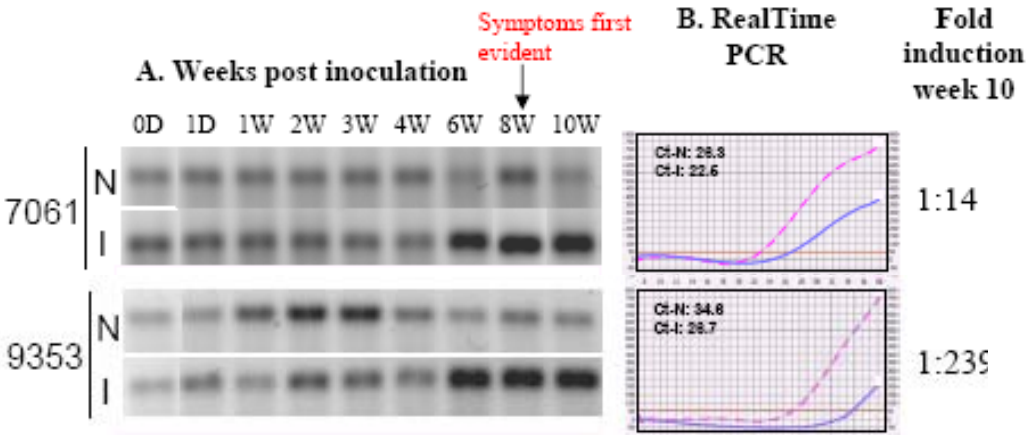
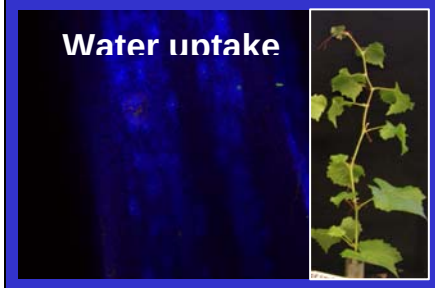


Figure 1. *Xylella fastidiosa* responsive promoters were obtained from genes found to be up-regulated in *X. fastidiosa* infected field grown plants but not up-regulated by water stress alone. Example data used to identify individual genes from which promoters were recovered as illustrated for # 7061 9353.



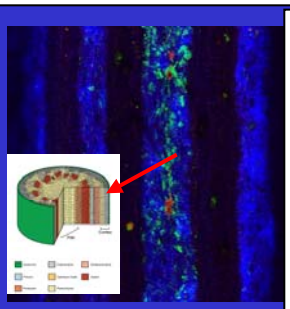


Figure 2 . Expression of promoter G7061 in plant #11 fused to GFP detected after 7 days in petiole of leaves attached to a detached branch into which *Xylella fastidiosa* was taken up through the cut end of the branch at 10 ml of 10^7 cfu/ml. Water control shows no promoter activation.

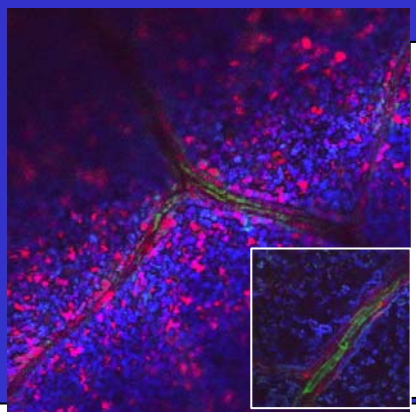


Figure 3. Confocal microscopic analysis at 4x of the activation of the G7061 *Xylella fastidiosa* responsive promoter in the laminar tissue following uptake of the bacterium via the petiole. The promoter construct is fused to GFP for visualization of activation. The images above were captured 2 weeks after exposure to the bacteria. The green color is GFP, while the blue color indicates chlorophyll autofluorescence and the red color is polyphenolics. The insert is an image of a vascular element with intense GFP fluorescence is taken at 25X indicating more precisely the location of the site and cells in which the promoter is active.

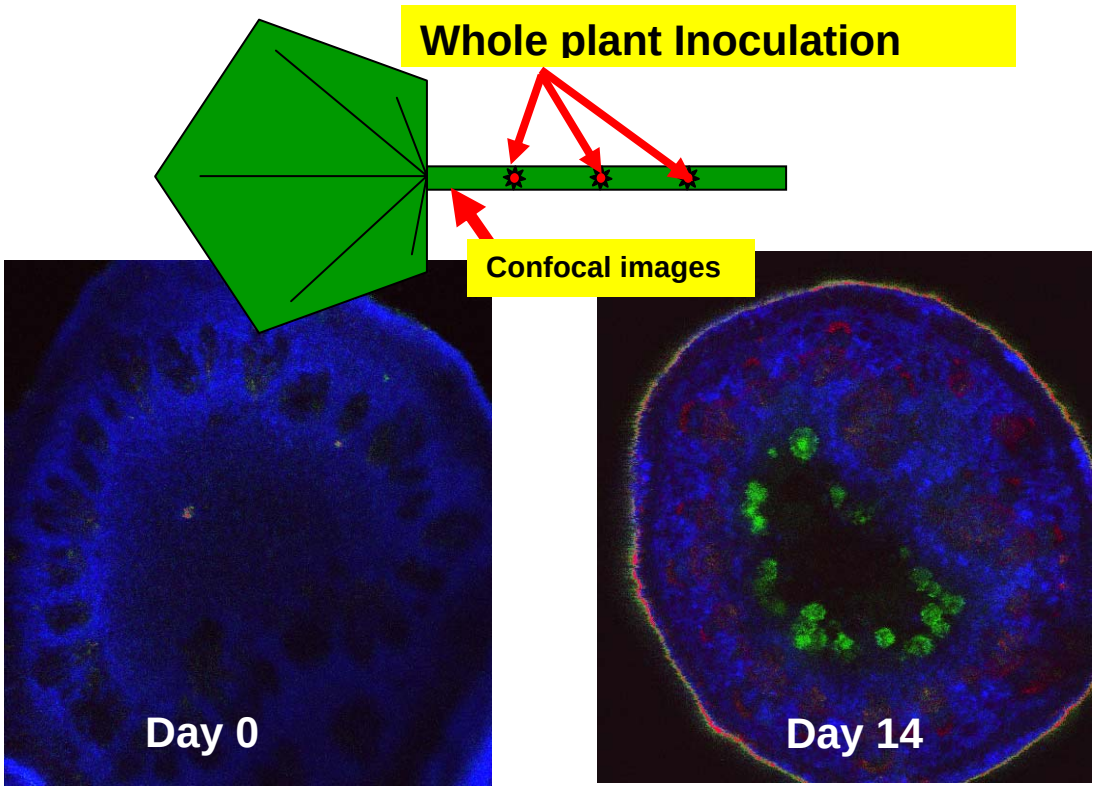


Figure 4 Analysis of the expression of promoter G9353 fused to GFP in response to the presence of *X. fastidiosa* in the vascular system of grape. Petiole inoculations at of 10^7 cfu/ml were followed by confocal assay of cross sections of the petiole at the point indicated. Blue color represents chlorophyll auto-fluorescence; Green color indicates the translation of GFP and, therefore, activation of the promoter 9353 in the presence of *X. fastidiosa*

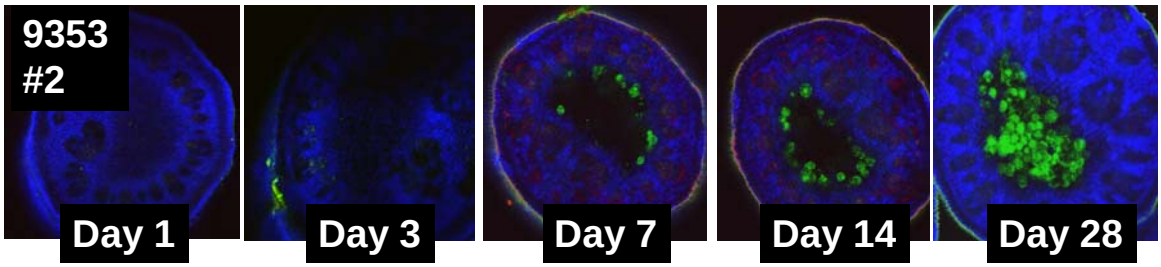


Figure 5. Time course analysis of the expression of promoter G9353 fused to GFP in response to the presence of *X. fastidiosa* in the vascular system of grape. Bacteria were introduced by puncture inoculation as illustrated in Figure 2. Analysis is by confocal microscopy. Blue color represents chlorophyll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

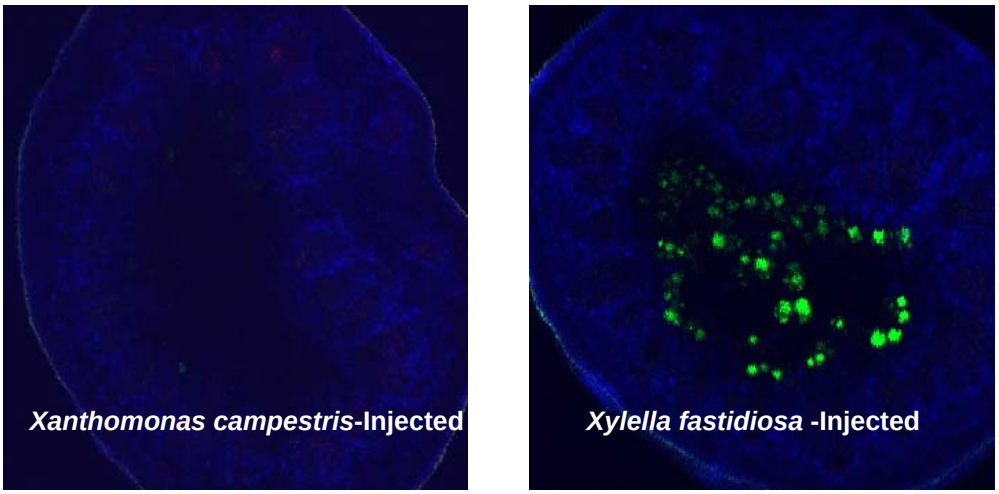


Figure 6. Specificity of the response of promoter G9353 fused to GFP in response to the presence of *X. fastidiosa* in the vascular system of grape compared with *Xanthomonas campestris.*Blue color represents chlorophyll auto-fluorescence; Green color indicates the translation of GFP and, therefore, activation of the promoter 9353 in the presence of *X. fastidiosa*

Table 1. Promoter:GFP fusion constructs that have been tested in Thompson Seedless as transgenes.

Promoter name	Putative function of gene	# transformants	Xf uptake into detached leaf	Xf inoculation into attached leaf
G7061	unknown	22	GFP expression	No GFP
G8946	unknown	18	No GFP	No GFP
G9353	Alpha-tubulin	12	GFP expression	GFP expression

Table 1. Results of inoculation experiments on transgenic Thompson Seedless plants bearing the first three promoters derived from Xylella fastidiosa induced genes fused to GFP. Plants were inoculated with 10^7 cfu/ml of X. fastidiosa, maintained under adequate moisture in the greenhouse and assayed for GFP expression with confocal microscopy at 14 days

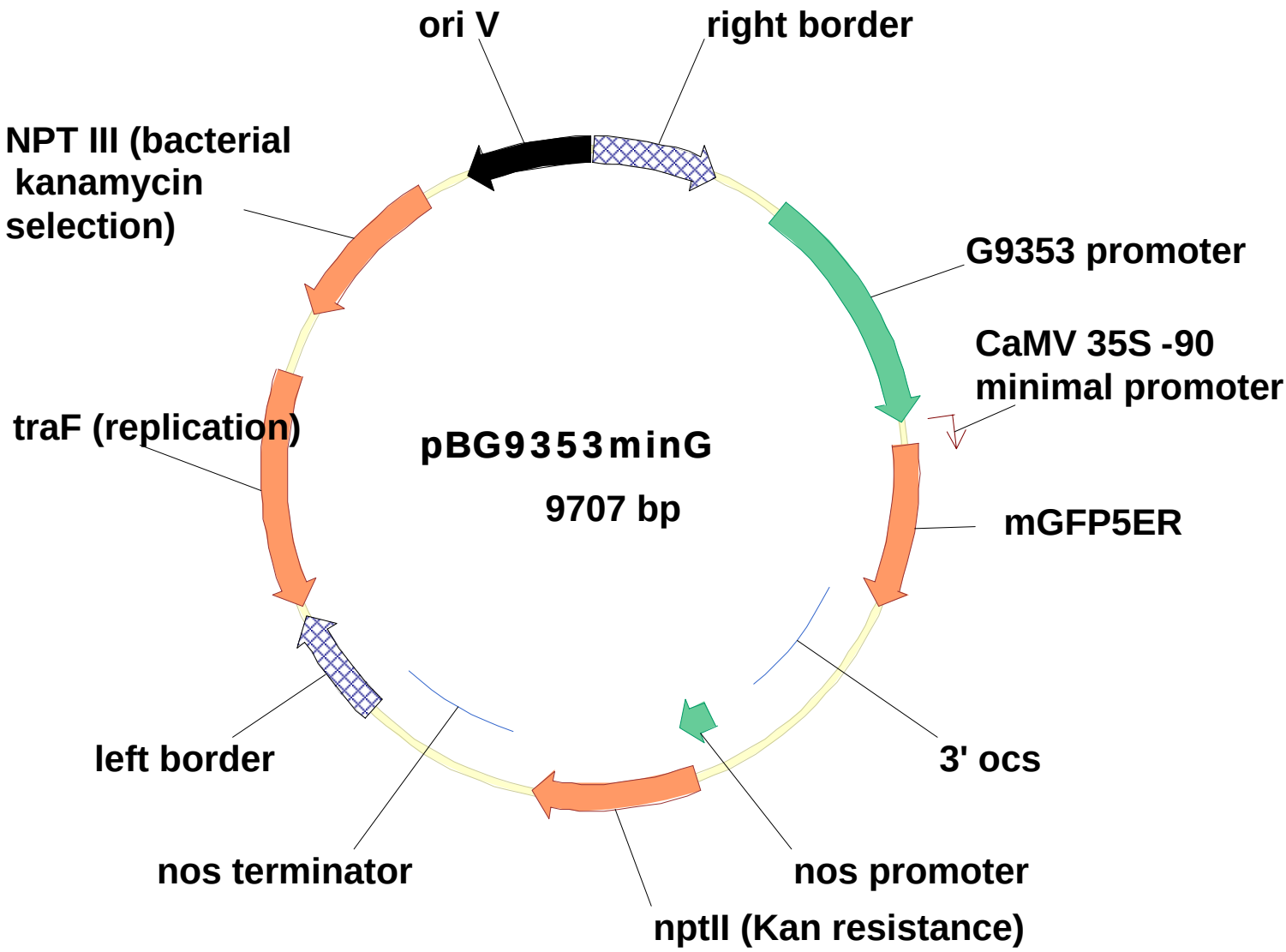


Figure 7 . Map of binary vector pBG9353minG. In *Agrobacterium*, this plasmid will transfer DNA from the right border clock-wise through the left border into grape cells. This vector uses the grape promoter G9353 which contains putative *X. fastidiosa* sensitive enhancers to drive expression of a CaMV 35S minimal promoter (-90bp) fused to a GFP reporter gene (mGFP5ER). A control construct replaces the G9353 promoter with a full CaMV35S promoter.