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

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ABSTRACT
Xylella fastidiosa (*Xf*) can persist as an endophyte or a pathogen depending on the host with which it is associated. We established that cell death symptoms in susceptible grapes result from the activation of programmed cell death (PCD) pathways with morphological markers of apoptosis. The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that are capable of suppression of Pierce's disease (PD) symptoms when expressed constitutively as transgenes. Using a functional cDNA screen, we identified several novel genes from grape and heterologous plants that suppressed PCD when expressed as transgenes. In 2007, we reported transgenes PR1 and CB456, expressed in the root stock cultivar Freedom, suppressed PD symptoms. In 2008, we reported comparable suppression of PD symptoms and a 4-6 fold reduction in the amount of bacteria present in inoculated transgenic Thompson Seedless plants expressing PR1 and CB456 fused to the 35S promoter when compared with untransformed control plants. All of the untransformed control plants died within two-three months after inoculation while the transgenic plants were asymptomatic up to 12 months, after which they were pruned, and cuttings made for a second inoculation. Current results from 2009 inoculations confirm the protection observed in 2008 and indicate a positive relationship between message level of CB456, a reduction in PD symptoms and a several fold reduction in bacteria titre in the inoculated plants. From the perspective of the grape-bacterial interaction, it appears that the anti-PCD genes tested to date suppress PD symptoms and functionally confine the bacteria to an endophytic ecology in the xylem equivalent to that seen in the related asymptomatic host *Vitis californica*. Experiments underway will determine if the protective effect of these genes is capable of being transferred across a graft union to protect a susceptible scion. In total, eight commercial wine varieties will be evaluated under controlled greenhouse conditions for susceptibility to PD when grafted to transgenic and to untransformed rootstocks as controls.

LAYPERSON SUMMARY
The mechanism by which *Xylella fastidiosa* (*Xf*) leads to death of susceptible grape plants is by activation of a genetically regulated form of programmed cell death (PCD), also known as apoptosis, which is functionally conserved in both animals and plants. Altered expression of known apoptosis-blocking animal and animal virus genes is a widely sought strategy for suppressing disease in animals where cell death is a basis for disease. We developed a functional screen for anti-PCD plant genes, identified six potential anti-PCD genes from cDNA libraries of grape, and transformed them into Pierce's disease (PD) susceptible plants. Two of these grape sequences (PR1 and CB456), when constitutively expressed as transgenes in susceptible grape lines, suppressed PD symptoms and reduced bacterial titer in the inoculated plants. These protective sequences are capable of being secreted outside the cell and will be evaluated for their ability to protect untransformed winegrape scions across a graft union. The current efforts will move the proof-of-concept protection strategy to potential application and quantify the effect of the transgenes on the bacteria resident in the plants. The eight inoculated untransformed winegrape varieties used as controls will simultaneously provide quantitative data on their relative susceptibility of to PD, a data set which at the present does not exist.

INTRODUCTION
Published information from our laboratory and others 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) (1,2,3,4,9). The induction of PCD results in an orderly dismantling of cells that includes maintaining integrity of the plasma membrane until internal organelles and potentially harmful contents including phenolics, reactive oxygen and hydrolytic enzymes have been rendered harmless to contiguous cells. However, when the cell contents are released in this manner they can serve as nutrients for microbial cells when they are present in the immediate environment (1, 7). Hence, bacteria, like *Xylella fastidiosa (Xf)* ,could receive nutrients from cells adjacent to the xylem that are triggered to undergo PCD and gradually releasing contents of the grape cell into the apoplastic space surrounding the xylem. These discoveries parallel investigations, now widely reported and accepted in human medicine, whereby genes, signaling pathways and chemical signals expressed by animal pathogens initiate infection by activating or blocking apoptosis through constitutive gene expression or signaling pathways present in all cells. Hence, this research on Pierce's disease (PD) is conducted within a global context in which the process of PCD with apoptotic morphologies is functionally conserved across the animal and plant kingdoms. Altered expression of known apoptosis-blocking animal and animal virus genes, or treatment with anti-apoptotic pharmacologically active peptides, and regulatory RNAs have been

shown to block PCD and suppress disease in plants where cell death is a symptom of disease, as is the case of PD (2,3,4,5,6,10).

In the case of *Xf* and many other plant pathogenic bacteria, the bacteria live predominantly as endophytes or epiphytes but occasionally as pathogens. The relative susceptibility of the individual plant species is determined by unknown genetic factors. Presumably, sensitivity to the presence of the bacteria expressed as cell death-dependent symptoms is the result of signals expressed by the bacteria that lead to activation of PCD as appears to be the case with PD. With past funding we 1) developed a functional screen that enabled us to identify six novel genes (out of ~200,000 screened) from grape that suppressed programmed cell death (PCD) in laboratory studies (7, 11). We reported in 2008 (8) that the first of these genes tested involving two different anti-PCD DNA sequences (P14LD and CB456) were very effective in suppressing PD symptoms when introduced into the fruited PD-susceptible cultivar Thompson Seedless. Of equal importance to control of the disease was data indicating that the bacterial titer in the protected plants were reduced in amount by up to two to four orders of magnitude below that reached in untransformed Thompson Seedless vines that are killed within two months after inoculation. Analysis of the disease level in transgenic and non-transformed control plants was based on a five point visual rating scale, measurement of the bacterial titer by quantitative qPCR, and visualization of the GFP-expressing *Xf* by confocal microscopy (8). These results established that protection is related to suppression of symptoms (cell death) and suppression in bacterial multiplication. We have extended the analysis to include additional transgenes, quantitatively measured the amount of bacteria present by qPCR in plants expressing different transgenes, and measured the movement of bacterial in transgenic and control plants. Initial data indicates a positive correlation between the qPCR determined bacterial titre and the message level of CB456; high message, low bacteria titre. Lastly, the protective sequences are active in suppression of PD only when secreted outside the cells suggesting they may function across a graft union. This latter possibility is being tested with eight commercial grape varieties grafted to two different rootstocks expressing PR1 and CB456.

OBJECTIVES

1. Continue to evaluate recently obtained Thompson Seedless transgenic grape plants expressing the six candidate anti-apoptotic genes for blocking of PD symptoms (**Table 1 and Figure 1**).
2. Determine the bacterial movement within an inoculated susceptible grape stem by qPCR over time following inoculation (**Figure 1**).
3. Assess relationships between message level of CB456 and PR1 and the level of bacterial titre in transgenic plants (**Figure 2**).
4. Measure the effect, over a time course, of blocking PD symptoms with anti-apoptotic transgenes on *Xf* bacterial population levels and movement in the xylem by quantitative PCR (qPCR) and confocal laser scanning fluorescence microscopy to monitor GFP-tagged *Xf*. (Figures 2 and 2A)
5. Assess efficacy of protection against PD across a graft union by PR1 and CB456, first with Thompson Seedless. (**Figure 3**).
6. Perform parallel inoculations of the suite of eight winegrape varieties both on their own rootstocks and on untransformed Freedom and Thompson Seedless rootstocks. This objective addresses the research priority in the RFP regarding short term collection of quantitative data of the relative resistance (susceptibility) of commercial winegrape varieties.
7. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape.
8. Collaborate with PIPRA to obtain permits to enable field evaluation of transgenic PR1 and CB456 in a location providing for controlled inoculation.

RESULTS AND DISCUSSION

Genes identified as potential anti-PCD genes from the conditional life-death screens and cross graft protection potential.

The protective genes or DNA sequences, isolated by a functional anti-PCD screen, have been described in earlier reports to this symposium and the results of inoculation of the first set of transgenic plants of Cv Freedom and Thompson Seedless were reported in 2007 and 2008 (7,8). In summary of the previous results, resistance against PD was observed in the susceptible grape rootstock by the first two anti-apoptotic transgenes tested, P14LD and 350 bp DNA sequence associated with a plant pathogenic nematode up-regulated gene designated p23. The expression of these two sequences, not only protected the transgenic plants against PD symptoms and plant death but maintained the population of *Xf* at four or more orders of magnitude below the level observed in untransformed plants that died within 2 months (10^7 to 10^8 bacteria per gram of stem tissue) compared with the asymptomatic transgenic plants that carried a level of 10^2 to 10^4 cells/gm stem tissue. We are continuing to extensively evaluate the remaining four potential anti-PCD genes using the easily transformable susceptible test variety Thompson Seedless. These results have been extended to experiments designed to determine if the protection afforded by a rootstock expressing these transgenes can protect the scion of susceptible commercial winegrape varieties. Selected asymptomatic P14LD and CB456 transgenic Thompson Seedless and Freedom (commercial rootstock) plants were propagated and are being grafted to eight commercial winegrape varieties; Chardonnay, Pinot Gris, Sauvignon Blanc, Cabernet Sauvignon, Pinot Noir, Zinfandel, Syrah and Merlot. These combinations are being tested first under controlled greenhouse conditions. Plans are made to for field testing if the greenhouse results are positive. The first set of grafted plants have been inoculated with *Xf*. Data collection is scheduled to begin in November (**Figure 3**; example of micro-propagated grafted plant).

Controlled inoculation of Thompson Seedless grape plants expressing anti-apoptotic genes.
Clonal populations of transgenic lines bearing the genes indicated in **Table 1** and additional copies of transgenic PR1 and CB456, the plants were trained to grow as two or three canes and maintained by periodic pruning of side and top branches as illustrated in **Figure 1**. The transformed plants were individually inoculated April 29 through May 1 of 2009. The inoculation method was by needle puncture of the stem to allow uptake of 20 µl of GFP-tagged Xf at 2 x 10^7 cfu/ml. The plants were monitored visually for symptoms with each plant being photographed at four-five months post-inoculation (**Figure 1**). The level of *Xf* bacteria was monitored by qPCR for bacterial movement and multiplication. Plants were initially scored for disease severity in October 2009, using a five point scale (1=dead and 5= asymptomatic). Representative control (scored as 1) and transgenics (scored as 5) are shown in **Figure 1**. Data collection and qPCR analysis of all the plants in **Table 1** is continuing. We anticipate completion of this extensive data set by the end of 2009.

The effect of anti-apoptotic transgenes on *Xf* bacterial populations measured by qPCR
The effect of blocking PCD-based symptoms in the transgenic PR1 and CB456 plants on the bacterial multiplication and spread is not known but is critical to establish the limiting parameters of protection against PD. The first experiment in this series was to stem-inoculate control and transgenic CB456 plants with *Xf*. Inoculated canes were sampled at 1 cm sections from the point of inoculation to 20 cm above the inoculation site (**Figures 2 and 2A**). Bacterial movement in the susceptible control plant was limited to the first 10 cm at three weeks post inoculation with an average titre of 500 to 1,000 cells per cm of stem but by three months had progressed to the 20 cm distance with a relatively uniform distribution in all sections of 10^6 to 10^7 cells per cm of stem (**Figure 2**). In contrast, the CB456 had no detectable bacteria (<100 cells) in any of the stem sections at the three week interval but cell numbers increased by the three month while the transgenic plants remained asymptomatic. At three months, the distribution of cells in the CB456 transformed plants was uniform over the 20 cm length at a titre of 5x10^3 to 10^4 cells per cm. The net effect of the CB456 transgene under these conditions is to limit bacterial titre but not distribution/movement of bacteria in the asymptomatic plants.

Relationship between the level of *Xf* bacteria in the grape stem to the quantitative expression level of transcript CB456. A question of biological importance is the relationship between the amount of message, bacterial titre in the stem and PD symptoms. **Figure 4** shows the results of the first experiment using CB456 as the target wherein there is a strong positive relationship between high message level and suppression of Xf bacterial titre in the inoculated transgenic CB456. The transcript level and bacterial titre in the stem were measured by simultaneous qPCR (**Figure 4** with representative plants). Another point is apparent from these data; there is a wide range of expression levels of the CB456 transcript seen in independent transformants. This underscores the need to assay multiple transformants as in any genetic screen. The net result is that, given the observed relationship between message level and protection level, it is important to establish the level of transcript expression when selecting individual transformant plants to propagate and carry forward as rootstocks or clonal populations of potential protected plants. These analyses will continue until all current populations of plants bearing the respective transgenes have been characterized.

Assessment of potential protect of untransformed scions from transformed rootstock expressing PR1 and CB456
Micro-grafting is conducted in sterile Magenta GA-7 Plant Culture Boxes (3 x 3 x 4") containing 50 ml media under a 16 h light, eight h dark photoperiod at 25°C. The rootstocks and scions for micro-grafting are selected from established, actively growing plants in agar culture. Rootstock plantlets obtained *in vitro* are allowed to grow until several leaves are produced (six–eight weeks) and divided into three–four explants, each containing a single node. The apex of the rootstock nodal explants arr then cut longitudinally with a new, sharp scalpel blade, producing a small (two–four mm) longitudinal cleft, and placed on the medium. A scion with a single node and a leaf was selected to match the size of the rootstock. The basal part of the stem of the scion was cut into a wedge to match the cleft of the rootstock and was carefully fitted on to the cleft of the rootstock on the medium. After four weeks incubation healing in a magenta box, the rooted plantlet is transferred to sterile soil and covered with an inside out plastic bag to avoid desiccation. The bag is slowly removed over a one week period and the grafted plant is ready to be transferred to the greenhouse for assays. Success rate is greater than 90% using this procedure. The first set of grafted plants was moved to the greenhouse June 2009.

CONCLUSIONS
The experiments and results described herein indicate progress toward identifying DNA transcripts of grape which, if regulation of the natural transcripts is altered in transgenic plants, result in the suppression of symptoms of PD with an associated limitation in bacterial titre to levels generally associated with a benign endophytic association. A natural example of such an asymptomatic endophytic relationship is given by *Vitis californica* where the bacterial titre (~10^2 to 10^4 cells per cm stem) is similar to the asymptotmatic PR1 and CB456 transgenic plants. In contrast, untransformed control plants exhibiting PD death symptoms had bacteria levels four orders of magnitude higher. Further, we observed a positive relationship between high message levels of CB456 with suppressed symptoms of PD and suppressed bacterial titre in the protected plants. To date the most effective of the anti-PCD transgenes are a pathogenesis related gene designated PR1 and a non-coding 350 bp DNA sequence associated with a nematode up-regulated gene designated p23. Rootstocks derived from transgenic PR1 and CB456 plants, shown to be protected against PD symptoms, have been grafted to eight different susceptible commercial winegrape varieties to be tested under controlled greenhouse conditions for the possibility of movement of the protective effect across a graft union. In summary, the current experiments indicate that the effect of the

anti-PCD genes leads to suppression of symptom expression but exert a bacterial growth limiting activity but are not lethal to the bacteria. The symptom suppressive genes do not act as antibiotics and do not affect the non pathogenic endophytic ecology of the bacteria in the xylem.

REFERENCES CITED

1. Gilchrist, D. G. 1998. Programmed cell death in plant disease: the purpose and promise of cellular suicide. Ann. Rev. Phytopathology 36:393-414.
2. Richael, C., Lincoln, J., Bostock, R., and Gilchrist, D. G. 2001.Caspase inhibitors reduce symptom development in compatible plant-pathogen interactions and limit pathogen multiplication *in planta.* Physiol. and Mol. Plant Pathol. 59(4) 213-221.
3. Lincoln J.E., Richael, C., Overduin, B., Smith, K., Hall, B.D. Bostock, R.M., and Gilchrist D. G. 2002. Expression of the anti-apoptotic baculovirus p35 gene in tomato results in inhibition of cell death and a decreased susceptibility to a variety of pathogens. Proc Natl Acad Sci. 99: 15217-15221.
4. Harvey, J. JW, J. E. Lincoln, K. Zumstein and D. G. Gilchrist 2007. Programmed cell death suppression in transformed plant tissue by cDNAs identified from an Agrobacterium rhizogenes-based functional screen. Molecular Genetics and Genomics, 279, 509-521.
5. Greenberg, J.T. and Yao, N. 2004. The role and regulation of programmed cell death in plant-pathogen interactions. Cellular Microbiology. 6:201-211.
6. Nicholoson, D.W. 2000. From bench to clinic with apoptosis-based therapeutic agents. Nature. 407:810-816.
7. Gilchrist, D.G., and J.E. Lincoln 2007 Resistance to Pierce's Disease by transgenic expression of plant-derived anti-apoptotic genes. San Diego, CA December 12-14.
8. Gilchrist, D.G., and J.E. Lincoln 2008 Systemic Resistance to Pierce's Disease by transgenic expression of plant-derived anti-apoptotic genes. San Diego, CA December 15-17
9. Li, C, S J. Barker, D G. Gilchrist, JE. Lincoln and W A. Cowling. 2008.*Leptosphaeria maculans* elicites apoptosis co-incident with lesion formation and hyphal advance in *Brassica napus.* Molecular Plant Microbe Interactions, 21:1143-1153.
10. Reed, J. C. (2002). "Apoptosis-based therapies." Nature Reviews Drug Discovery 1(2): 111-121.
11. Harvey, J. JW, J. E. Lincoln, K. Zumstein and D. G. Gilchrist 2007 Programmed cell death suppression in transformed plant tissue by cDNAs identified from an *Agrobacterium rhizogenes*-based functional screen. Molecular Genetics and Genomics 279:509-521.

FUNDING AGENCIES

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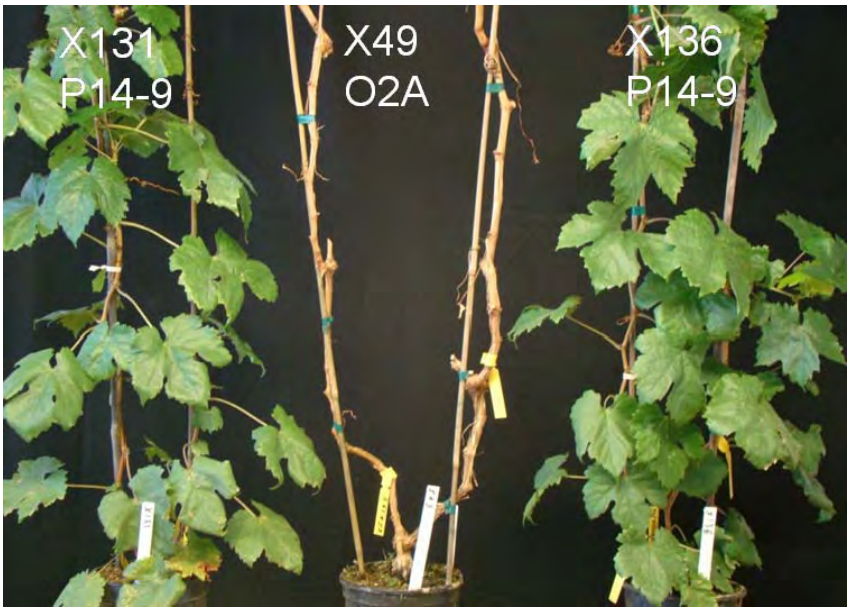

Figure 1. Illustration of two independent transgenic plants expressing the PR1 gene compared with the mock-transformed O2A control. The plants were inoculated with ~2,000 bacterial cells into the stem at the location marked with the yellow tags. This photo was taken 4 months after inoculation.. Bacteria levels determined by qPCR were 10^2 for the PR1 plants vs 10^5 for O2A.

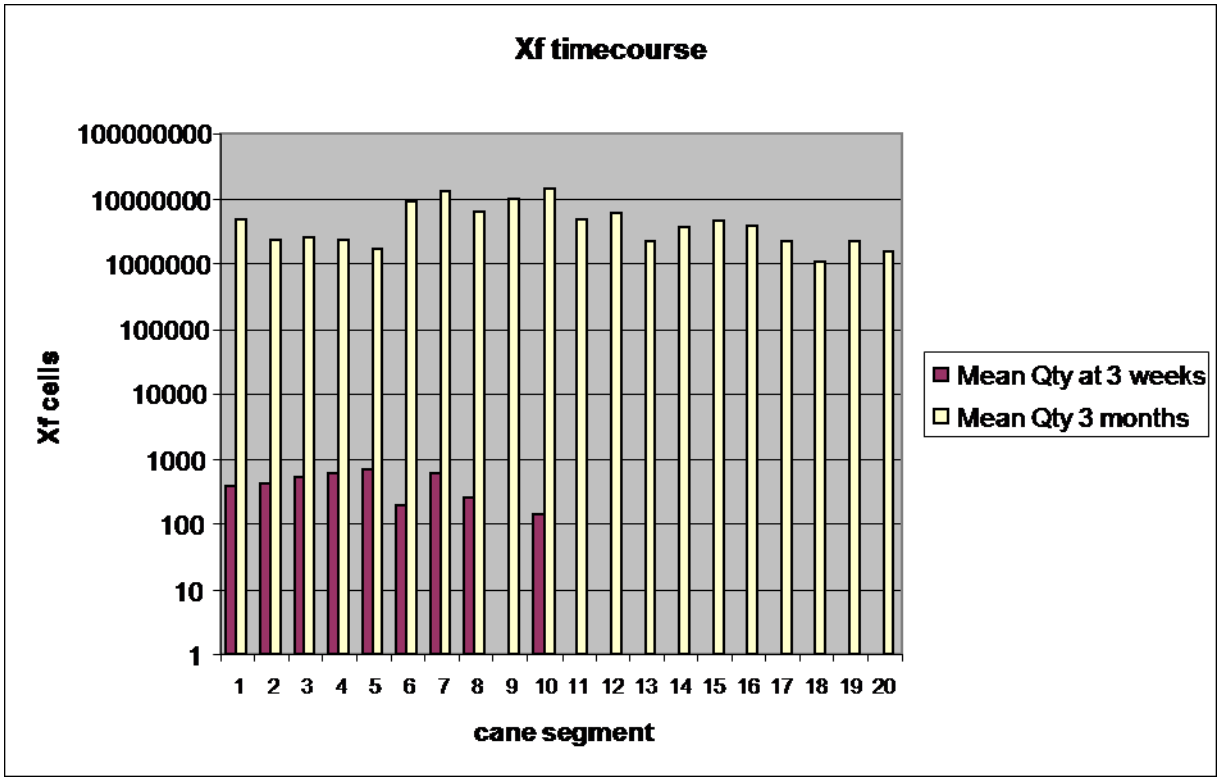

Figure 2. Distribution of bacterial cells in a susceptible grape stem following inoculation of the stem with $2x10^3$ cells of *Xylella fastidiosa*. Sampling of consecutive cane sections was at 3 weeks and 3 months followed by qPCR determination of bacteria concentrations.

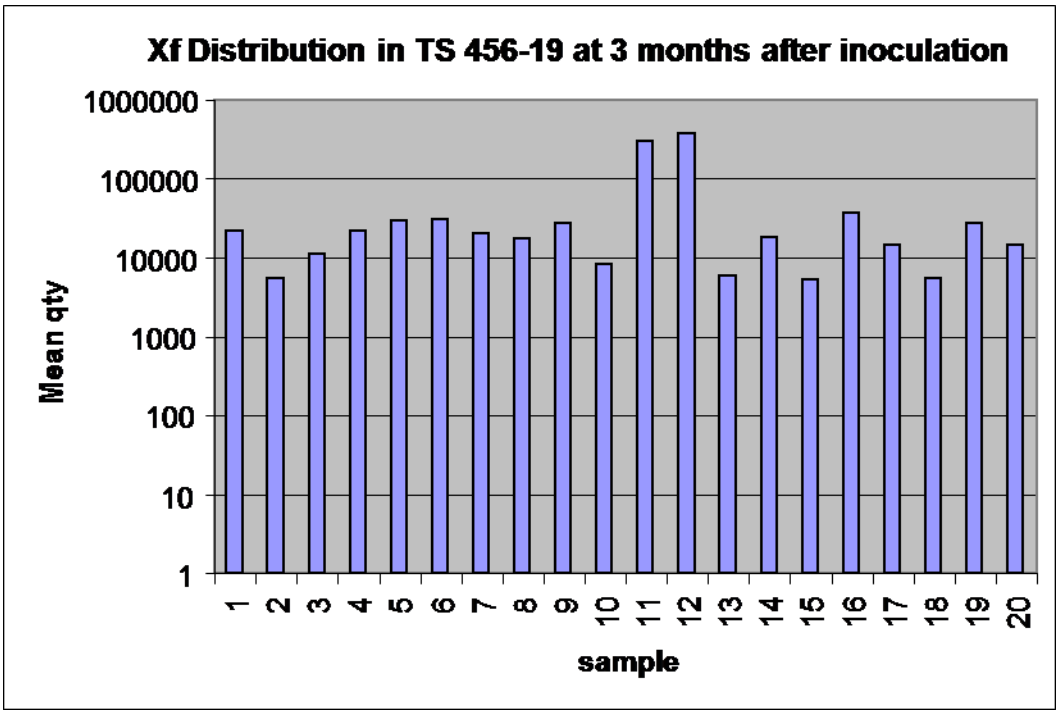

Figure 2A. Distribution of bacterial cells in a CB456 transgenic grape stem following inoculation of the stem with $2x10^3$ cells of *Xylella fastidiosa*. Sampling of consecutive cane sections from the inoculation site (1) in 1 cm sections up to 20 cm (20) was at 3 months followed by qPCR determination of bacteria concentrations. Compare these levels with Figure 2 determination in untransformed Thompson Seedless.

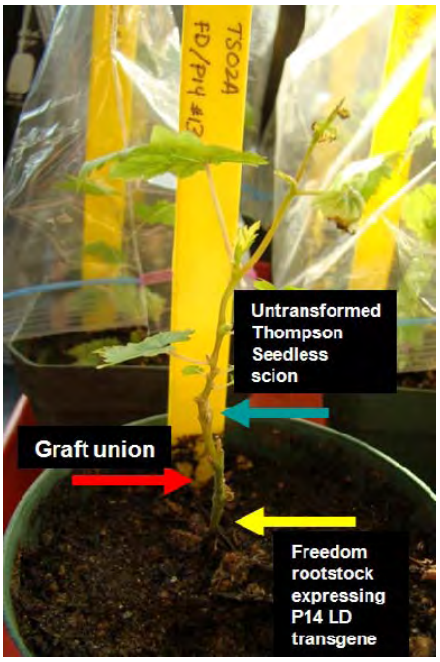

Figure 3. Illustration of grafting of micro-propagated rootstock and scion under sterile conditions

Table 1. Thompson Seedless plants transformed with anti-PCD genes under control of the 35S promoter being tested for susceptibility under controlled greenhouse conditions. Plants, each with two canes, were inoculated in April and May, 2009. First cane assays using qPCR were initiated at 5 months post inoculation (August 2009). © = control plants.

Genotype	# Independent transformants	# of Plants
TS – CBP14B ©	5	5
TS - CBP14LD	18	38
TS - CB376	7	12
TS - CB456	13	28
TS - I35 ©	4	6
TS - CBMT	3	3
TS - CBWG23	5	8
TS - CBWG71	4	6
TS- control ©	7 different genotypes	20
total	66	126

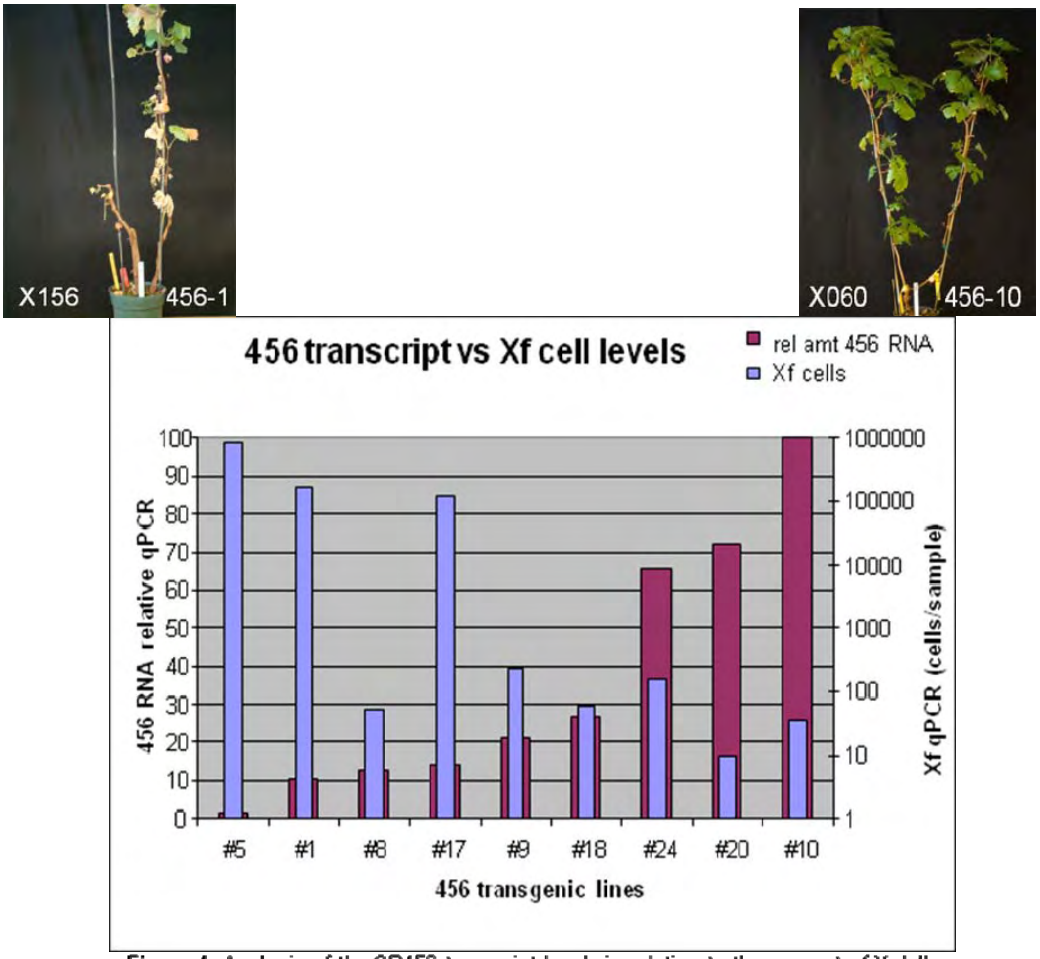

Figure 4. Analysis of the CB456 transcript levels in relation to the amount of Xylella fastidiosa cell in the stems of individual primary transgenic lines stem-inoculated with $2x10^3$ bacterial cells four months prior to sampling. Note there is an inverse relationship between the message level and the amount of bacteria residing in the stem tissues. #1 and #10 are shown as representative plant phenotypes.