SYSTEMIC CONTROL OF PIERCE'S DISEASE BY ALTERED EXPRESSION OF ANTI-APOPTOTIC GENES OR THEIR RNA-BASED REGULATORY ELEMENTS

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ABSTRACT
Xylella fastidiosa (*Xf*) is both an endophyte and a pathogen. Cell death symptoms associated with the pathogenic state result from the activation of programmed cell death (PCD) pathways with morphological markers of apoptosis in the susceptible grape. 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 constitutively expressed as transgenes. We identified, using a functional cDNA screen, several novel genes from grape and heterologous plants that suppressed PCD when expressed as transgenes. We reported in 2007, several transgenes expressed in the root stock cultivar Freedom that suppressed PD symptoms. In addition, the level of bacteria in the vascular tissue are maintained four orders of magnitude lower than in untransformed control plants, all of which died. We now report that transgene expression in the fruited PD susceptible cultivar Thompson Seedless also affords protection against PD symptoms and limits the bacterial titer up to four to six orders of magnitude below that reached in untransformed plants that are killed within two months after inoculation. The protected plants have remained alive and asymptomatic nine months after inoculation. From the perspective of the grape-bacterial interaction, it appears that the anti-PCD genes tested to date suppress PD symptoms and functionally restore the bacteria to an endophytic ecology in the xylem equivalent to that seen in the asymptomatic host *Vitis californica*.

INTRODUCTION
At the outset of this project in 2001, little was known about the mechanisms or genes involved in symptoms or death of the grape plants infected with *Xylella fastidiosa* (*Xf)*. In the course of these studies, we established that the cell death leading to leaf scorch symptoms in Pierce's disease (PD) is the result of the activation of programmed cell death (PCD) with morphological markers of apoptosis. In addition, Dr. Tom Rost and we, independently, determined that PD symptoms can occur distal to sites where the bacteria are detected suggesting the presence of mobile signals from the bacteria. It also is documented that several relatives of grape, including *Vitis californica*, and other host plants can harbor otherwise lethal titers of *Xf* without exhibiting PD symptoms. The questions posed in this research were: a) are there genes in the plant that respond to *Xf* signals by triggering programmed cell death in certain grape genotypes, b) can this response be blocked genetically; and c) if so, does this then allow the bacteria to maintain the endophytic state, leaving the plant otherwise unaltered but free of disease symptoms; and/or d) does suppressing PD symptoms negatively affect the ability of the bacteria to colonize the vascular system.

Current literature and results from our laboratory indicate a number of plant diseases result from induction of PCD in the host cells in advance of microbial growth (2,11,12). 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, they can serve as nutrients for microbial cells when they are present in the immediate environment (2,9). Hence, bacteria like *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. The fact that we measure bacterial titers three-six orders of magnitude higher in symptomatic (ultimately dead) grape plants than in either asymptomatic wild grapes or the transgenic asymptomatic grape plants is consistent with enhanced nutrition in the xylem of infected symptomatic plants. The working scenario in this research is; blocking death, limits death dependent nutrient release, and thereby restricts bacteria multiplication but does not act as an antibiotic against the bacteria. If true, this scenario does not apply novel selection pressure on the bacteria any more than residing in *V. californica* or any other asymptomatic host.

Genetic strategies for disease suppression and development of a biological understanding of the bacterial-plant interaction are high priority areas in the PD/GWSS Research Program and the NAS report. The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when expressed in grape, will prevent colonization, systemic spread or symptom development due to the presence of *Xf* in the xylem. Recent published information from our laboratory 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 existing, genetically regulated, pathways leading to apoptosis or programmed cell death (PCD) (1,2,3,4,5,6). 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. These studies are the basis for extensive searches for apoptosis-based therapeutic approaches and agents in human medicine (7,18).

Hence, this research on 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 while sharing diagnostic markers of apoptosis including chromatin condensation and segregation into distinct masses referred to as pycnotic DNA bodies 10,13,14,15), oligonucleosomal DNA ladders, externalization of phosphatidylserine, and TUNEL-positive nuclei in the incipient plant disease lesions (2). Also, it is known that many proteins and several regulatory RNAs function in the induction or suppression of animal PCD and exhibit cross-functionality in the plant kingdom. Ectopic expression of known apoptosis-blocking animal and animal virus genes, or treatment with anti-apoptotic pharmacologically active peptides, has been shown to block PCD and suppress disease in plants where cell death is a symptom of disease, as is the case of PD (3,4,16,17).

However, mining of plant genome sequences in the available databases has not revealed plant sequence homologs of either the core pro- or anti-PCD pathway genes found in animals, even though the induction or suppression of PCD in transgenic plants by cross-kingdom expression of pro- and anti-apoptotic animal genes suggested that anti-death homologs likely exist in plant genomes (2,7,18). Consequently, identification of plant genome derived anti-death genes must be based on functional screens and is the premise that defined the direction of our research. Results presented this year and in 2007 (8) indicate that the approach has been successful.

OBJECTIVES

The objectives of the proposed research for 2008-2010 are as follows:

1. Continue to evaluate recently obtained Thompson Seedless transgenic grape plants expressing the eight candidate anti-apoptotic genes for blocking of PD symptoms **(Table 1).**
2. 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*.
3. Determine grape gene expression changes in transgenic compared with non-transgenic plants in response to *Xf* infection by differential transcriptional profiling using quantitative PCR.
4. Produce grape transgenic plants with modified candidate anti-apoptotic genes designed to enhance systemic movement *in planta*.
5. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape.
6. Graft the resistant transgenic Cv Freedom rootstocks of PR1 and cDNA 456 onto untransformed scions of Thompson Seedless and Chardonnay to monitor movement of either expressed proteins of these genes that contain a secretory leader on VVPR1 or the RNA derived from the 3'UTR from the ortholog of the potato p23 gene that shares stem and loop homology to the Bcl 2 3'UTR. The transformed grafted plants will be inoculated with *Xf* and scored for disease reaction in the untransformed scion.

Table 1. Thompson Seedless now available with putative anti-PCD genes under control of the 35S promoter.

Genotype	# Lines or Independent Transformants	# of Plants
TS – CBP14B	24	30
TS - CBP14LD	27	27
TS - CB376	28	29
TS - CB456	27	31
TS - I35	14	15
TS - CBMT	22	24
TS - CBWG23	23	25
TS - CBWG71	20	23
total	185	204

RESULTS

Genes identified as potential anti-PCD genes from the conditional life-death screens.

Previous funding on this project led to the development of a functional cDNA screen to identify plant genes, which when over-expressed as transgenes, suppress cell death triggered by chemical inducers of PCD. The genes in **Table 1** have been described in earlier reports to this symposium and the results of inoculation of the first set of transgenic plants of Cv Freedom was reported in 2007 (8). In summary of the 2007 Cv Freedom results, resistance against PD was observed in the susceptible grape rootstock by two anti-apoptotic transgenes (P14LD and MT) and one 350 bp DNA sequence associated with a nematode up-regulated gene designated p23. Furthermore, the expression of these three sequences, not only protected the

transgenic plants against PD symptoms and plant death but maintained the population of *Xf* at four orders of magnitude below the level observed in untransformed plants that died within two months (10^8 bacteria per gram of stem tissue) compared with the asymptomatic transgenic plants that carried a level of 10^4 cells/gm stem tissue in the most resistant lines that were alive at nine months. Interestingly, the 10^4 titer is equivalent to what we observed in the asymptomatic host *V. californica* 12 months after inoculation. In 2008, we began testing the anti-PCD genes expressed in Cv Thompson Seedless and report the early results of two inoculations of the Thompson Seedless transgenics bearing the CBP14LD and the CB456 genes.

Thompson Seedless grape plants expressing anti-apoptotic genes.
After creating clones of these transgenic lines, the plants were trained to grow as two or three canes and maintained by periodic pruning of side and top branches (**Figure 1**). Half of the transformed plants were individually inoculated November and December of 2007 and the second half in April and May of 2008. The inoculation method was the same for both sets however the concentration of *Xf* bacteria was 1000 fold less for the second set. The inoculation method was by needle puncture of the stem to allow uptake of 10-20 µl of *Xf* at $2x10^8$ cfu of the GFP-tagged *Xf* /ml for the 2007 inoculations and a 1000 fold less at $2x10^5$ cfu of the GFP-tagged *Xf* /ml for the 2008 inoculations. The plants were monitored visually for symptoms and by quantitative PCR (qPCR) for bacterial movement and multiplication. They were scored for disease severity in May 2007 (first set) and in October 2008 (second set), using a five point scale (1=dead and 5= asymptomatic) and photographed. Representative control (scored as 1) and transgenics from the second set (scored as 5) are shown in **Figures 1, 2, and 3**).

Figure 1. Five point Rating scale for PD symptoms on transformed grape plants, compared with untransformed plants and GFP-transformed controls, following inoculation with 40 µl 10^8 cells/ml of GFP-tagged *Xf* by stem puncture. The plants pictured were rated six months after inoculation at which point all the inoculated control plants (transformed with GFP and untransformed) were dead (category 1). Stem death began to appear within four months in the GFP transgenic control plants. Plants rated in categories 4 and 5 at six months were unchanged at nine months, after which parent plants discarded. Ramets of all protected plants are maintained for future experiments.

9353 Control **P14LD-19** **456-1** **Uninoculated Control**

Figure 2. PD assay on the first set of inoculated Thompson Seedless plants transformed with anti-PCD transcripts P14 and 456 were inoculated with 20µl of $2x10^8$ *Xf*/ml and photographed three months later. The protected plants are compared with 9353, an *Xf*-inducible promoter fused to GFP, and an uninoculated untransformed Thompson Seedless plant.

The effect of anti-apoptotic transgenes on *Xf* bacterial populations was measured by RealTime quantitative PCR (qPCR) (**Table 2**). Analysis of *Xf* inoculated plants revealed that although bacteria can be detected everywhere in a infected plant, the inoculated cane samples are more consistent than the cane of the stem of primary branches. It is essential to determine the effect of blocking PCD-based symptoms in the transgenic plants on the bacterial multiplication and spread in terms of the overall impact of the transgenes. Based on initial experiments to ascertain which tissue to sample for *Xf* quantization, we sampled the stem of primary branches or petioles of individual plants. Although, this would allow repeated sampling of an individual plant over the course of the experiment, we found that it is not a reliable indicator of the overall bacteria level and could vary by as much as six orders of magnitude. These results indicate that equivalent results were obtained at the two inoculum concentrations. In both cases the mean bacterial load of unprotected control plants reached the same level (10^8) after two-three months at which point the plants began to die. The transgenic plants remained healthy appearing (categories 4-5) after assaying at six and nine months with bacterial titres ranging from 10^2 to 10^4 in the main canes of the inoculated plants (**Table 2**). Representative images of plants in the first inoculation with 20 µl at $2X10^8$ are shown in **Figure 2** with equivalent images of the second inoculation with 20 µl at $2X10^5$ are shown in **Figure 3**.

Table 2. Thompson Seedless Transgenics Genotype analyzed to date.

Thompson Seedless Transgenics Genotype analyzed to date	# of Lines evaluated to date	Percent of plants rated as Figure 1 categories 4 and 5 and protected as in Figures 2 and 3	Mean bacterial load per 0.1 gm of stem in each respective line
Inoculation 1 @ $2X10^8$ cfu			
TS - CBP14LD	4	3/4	10^4
TS - CB456	4	3/4	10^4
TS - 9353 (control)	6	0/6	10^8
Inoculation 2 @ $2X10^5$ cfu			
TS - CBP14LD	23	20/23	10^2
TS - CB456	26	23/26	10^2
TS – 9353 (Control)	6	0/6	10^8

Figure 3. PD assay on the second set of Transgenic Thompson Seedless plants inoculated in 2008 with 20µl of $2x10^5$ *Xf*/ml and photographed three month later. This inoculum is 1000-fold less than that used for the inoculations shown in **Figure 1**. The 9353 control plant has a PD disease score of 1 and all others were scored as 5 on a 5 point scale.

Bacterial plating for determination of bacterial viability in the control and transgenic plants.
The pathogenic *Xf* used to inoculate the plants shown in **Figures 1**, **2,** and **3** and **Table 2** were obtained from Dr. Steven Lindow. These bacteria-expressed GFP and were resistant to Kanamycin. Stems sections from the tissue used to generate the data in **Table 2** were further sectioned, incubated in water and centrifuged to pellet the bacteria, re-suspended in water and plated on *Xf* media containing Kanamycin. Bacteria expressing GFP were obtained from the control and transgenic protected plants as shown in **Figure 4**. The schematic illustrations indicate the relative amounts of bacteria estimated by qPCR and the color plates indicate representative fields on the media plates with colonies of GFP-expressing bacteria. These data confirm that many more bacteria were present in the control cane sections and that the bacteria recovered on the plates were viable progeny cells of the inoculated *Xf*. In summary, the qPCR and plating data indicate that the two anti-PCD genes analyzed to date suppress symptoms of PD, do not eliminate the bacteria from the tissue but do reduce the bacterial titre to a level that, while detectable, is orders of magnitude lower than the untransformed control plants.

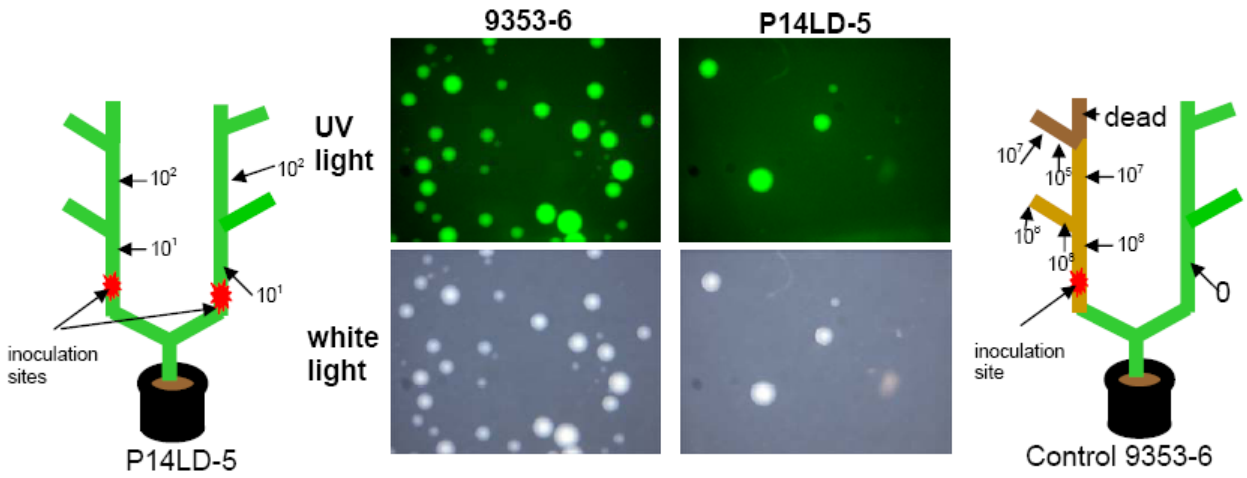

Figure 4. Live GFP-tagged *Xf* bacteria can be isolated from extracts of infected Thompson Seedless plants. Shown are micrographs of *Xf* growing on PD3 plates supplemented with 30µg/ml kanamycin. Consistent with the qPCR data, the control plate has a lot more bacteria. In fact, many of the plates for the protected transgenics had no colonies on plates. This data also provides evidence that the qPCR is not just amplifying DNA of dead *Xf* cells in the protected transgenic plants.

CONCLUSIONS
In the past year, we successfully demonstrated resistance against PD in the susceptible grape rootstock cv. Freedom by two anti-apoptotic transgenes (PR1 and MT) and one 350 bp DNA sequence associated with a nematode up-regulated gene designated p23. All three cDNAs were recovered anonymously from the plant-based cDNA screen and all have functional links with conserved domains to anti-apoptotic orthologs in the animal kingdom (1,18). We further demonstrated that expression of these three sequences, not only protected the transgenic plants against PD symptoms and plant death but maintained the population of *Xf* at four orders of magnitude or greater below the level observed in untransformed plants that died within two months (10^8 bacteria per gram of stem tissue) following controlled inoculations in the greenhouse. The key point is that altered expression of the anti-apoptotic transgenes does not kill the bacteria but does restrain the titer in the asymptomatic transgenic plants from a lethal level of 10^8 to a level of 10^4 to 10^2 cells/gm stem tissue in the most resistant lines; the 10^4 titer is equivalent to that which we measured in the asymptomatic host *V. californica* 12 months after inoculation. Interestingly, the 10^4 cells/gm stem tissue titer level in the asymptomatic transgenic plants and *V. californica* is equivalent to that observed by Dr. Lindow in his *rpfF* transformed plants that also are asymptomatic suggesting that susceptible grape plants can tolerate a bacterial population at the 10^4 without showing PD symptoms. Hence, the current experiments indicate that the effect of the anti-PCD genes suppresses symptom expression but does not exert a direct inhibiting effect on the bacteria. The symptom suppressive genes do not act as antibiotics and do not affect the natural endophytic ecology of the bacteria in the xylem. In essence, an endophyte gone bad has been returned to the state of a benign endophyte.

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