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 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 12 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* and below that reported by Rodrigo Almeida (UCB) to be necessary for transmission.

INTRODUCTION

At the outset of this project in 2001, little was known about the mechanisms or genes involved in symptoms of Pierce's Disease (PD) or death of the grape plants infected with *Xylella fastidiosa* (*Xf*). In the course of these studies, previous and ongoing research in our lab: 1) established that the cell death leading to leaf scorch and cane death symptoms in Pierce's Disease (PD) is due to the activation of a programmed cell death (PCD) process, also known as apoptosis, 2) reconfirmed the well documented fact that several relatives of grape, including *Vitis californica*, and other host plants can harbor *Xf* without exhibiting PD symptoms. Our gene cloning studies began with the development of a functional screen (5), which we adapted to screening grape cDNAs that enabled us to identify six novel genes (out of ~200,000 screened) from grape that suppressed programmed cell death (PCD) in laboratory studies. Results obtained in 2008 (3) with two different anti-PCD gene sequences (P14LD and CB456), indicate that when introduced transgenically into the fruited PD-susceptible cultivar Thompson Seedless **(Figure 1)**, protected the vine against PD symptoms (**Figure 3)** and limited the bacterial titer four to six orders of magnitude below that reached in regular Thompson Seedless vines that are killed within two months after inoculation **(Table 2)**. Analysis of the disease level in transgenic and non-transformed control plants, based on a 5 point visual rating scale (**Figure 2**), coupled with quantitative qPCR, and visualization of the GFP-expressing *Xf* by confocal microscopy, established that protection by these anti-PCD transgenes

results in symptom suppression and limitation in bacterial multiplication within the xylem (**Table 2, Figure 3)**. A positive correlation between the qPCR data and the presence of live bacteria was established by plating stem extracts from a protected Thompson Seedless P14LD plant and untransformed control plants **(Figure 5**). Lastly, inoculation data indicate that the protective sequences are active only when secreted outside the cells suggesting they may function across a graft union .

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 (4,5) 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 under these conditions they can serve as nutrients for microbial cells when they are present in the immediate environment (2,4). Hence, bacteria like *Xf* could receive nutrients from cells adjacent to the xylem that are triggered to undergo PCD and gradually release contents of the grape cell into the apoplastic space surrounding the xylem. The fact that we measure bacterial titers 4-6 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.

Objectives of Proposed Research; 2008-2010:

1. Continue to evaluate recently obtained Thompson Seedless transgenic grape plants expressing the 8 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 *X.fastidiosa*.
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. Prepare transgenic plants for field evaluation and coordinate field site selection including the obtaining of necessary permits for field transfer. These steps will be coordinated by PIPRA
6. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape.

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 the 2008 Pierce's Disease Symposium and the results of inoculation of the first set of transgenic plants of Cv Freedom was reported in 2007 and 2008 (3). 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 270 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 2 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 are alive today at 10 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 results of two inoculations of the Thompson Seedless transgenics bearing the CBP14LD and the CB456 genes.

Table 1. Plant anti-PCD genes, isolated from a functional screen of cDNA libraries, transformed into *Vitis vinifera* Thompson Seedless.

	Construct	Putative Ortholog of Known Gene Based on BLAST Sequence Comparison	
	CB390	metallothionein	
*	CB456	Nematode induced gene	
	CBWG23	cupin-like	
	CBWG71	cytokine-like gene (MIF)	
	CB376	Mycorrhizal induced gene	
*	CBP14LD	P14 (homolog of PR1)	
*	CBP14B	P14 (homolog of PR1) secretory leader deleted	
	CBI35	Intron p35 (anti-PCD control gene)	

Thompson Seedless grape plants expressing anti-apoptotic genes.

Figure 1. Representative transgenic plants of Freedom and Thompson Seedless were morphologically and developmentally indistinguishable from the untransformed control plants. The Thompson Seedless plants were capable of bearing fruit, which also appeared normal.

All transgenic Thompson Seedless plants bearing either P14 or the CB456 sequences exhibited normal morphology, growth rates, were capable of bearing fruit, and were indistinguishable from the non-transgenic wild type Thompson Seedless control plants (Figure 1). After creating clones of these transgenic lines, the plants were trained to grow as 2 or 3 canes and maintained by periodic pruning of side and top branches (Figure 1).

Figure2. Five point Rating scale for PD symptoms on transformed grape plants, compared with untransformed plants and GFP-transformed controls, following inoculation with 20 µl 10^5 cells/ml of GFP-tagged X. *fastidiosa* by stem puncture. The plants pictured were rated 6 months after inoculation at which point all the inoculated control plants (transformed with GFP and untransformed) had lost their leaves and were dead to the base of the plant (category 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-December 2008 (second set), using a 5 point scale (1=dead and 5= asymptomatic) and

Figure 3 PD assay on transgenic Thompson Seedless plants inoculated in 2008 with 20µl of $2x10^5$ *Xf*/ml and photographed 6 month later. The 9353 control plant has a PD disease score of 1 and all others were scored as 5 on a 5 point scale.

photographed. Representative control (scored as 1) and transgenics from the second set (scored as 5) are shown in Figure 3).

Biological and genetic information related to P14LD and CB456

P14LD (VVP14LD).

P14LD is widely used as a marker for resistance, including the onset of systemic acquired resistance (SAR) but has no known function, although the gene was first identified more than 2 decades ago. Sequence comparison of VVP14LD from grape within genome databases using the Clustal W algorithm confirmed high conservation of several domains in orthologs of the gene analyzed from humans, dog hookworm, *Meloidogyne incognita*, tomato and alfalfa. In each case where expression is induced, they are associated with a situation in which apoptosis was blocked or suppressed. Specifically, in the case of plant response to disease, the presence of the P14LD message occurs in situations in which the induction of PCD during the compatible infection process is a dependent step and where blocking death is related to resistance. Localized expression of the protein at the point of PCD induction is consistent with the suppression of pathogen growth and confinement to a zone surrounding lesion, a situation that occurs in this and other plant-bacterial interactions as pointed out by Richael and Gilchrist (6). In the context of systemic movement of the protein, P14LD was discovered first as 15 Kda protein found in plant sap expressed from genetically resistant plants and the gene later cloned by reverse genetics (13). P14LD has a leader sequence targeted to the extracellular space and appears to be quite resistant to proteolytic cleavage due to the fact that it is recoverable in expressed plant sap (13). As a general principle secreted proteins are stable against proteolysis. Given that we have demonstrated that P14LD, constitutively expressed in grape confers protection against PD, the question of whether it can protect across a graft union is a next logical step in protection of cultivated grapes without altering the scion. In addition, P14LD and P14B transgenic Thompson Seedless plants are distinguished by the presence of the signal peptide directing the translated protein outside the cells in P14LD. The P14B plants express the coding sequence for the P14 gene but lack the signal peptide, thereby precluding secretion outside the cell.

Sets of both transgenic lines were inoculated in May 2008 with 20µl of 10^5 *Xf* cfu/ml and photographed 9 months later. The P14B#9 had lost all leaves within 3 months vs P14LD plants in which 17/23 independent transformants showed no PD symptoms from inoculation to date (December, 2008).

Figure 4 Dramatic illustration of the requirement for secretion by the P14 gene in suppression of PD symptoms. P14LD has the secretory leader vs P14B, which has the leader deleted.

qPCR values were: P14LD #9 = 1.0 X 10^2 cells per gram of stem, compared with P14B #23 = 1.2 X 10^7 . All 16 P14B primary transgenic plants were killed within 3 months post inoculation and carried an average of 10^6 bacterial cells per gram of stem. Consequently, this comparison indicates that suppressing death blocks bacterial proliferation but does not directly kill bacterial cells. Hypothesis now considered; food base acquisition is critical for bacterial multiplication and disease progression. Also, it should be noted in reference to the RFP, with the focus on PGIP and the postulated translocation of PGIP, that the VVP14LD gene codes for a protein that is approximately one-half the molecular size of PGIP and just as likely to be stable in the apoplast. We fused the P14LD leader to GFP and confirmed by confocal microscopy that this

leader functions to secrete the GFP protein to the apoplast (data not shown) and suggest that it could move within the plant tissue in which the gene is translated.

cDNA # 456

cDNA 456 is a 270 bp sequence that does not contain a protein coding sequence but does contain a stem/loop structure derived from the 3'UTR of an orthologous gene designated as p23 in potato and known as TCTP in animals. The p23 gene is reported to be up-regulated in *Meloidgyne incognita*-induced giant cells in tomato and we demonstrated a protective effect against apoptosis in yeast and tomato hairy roots prior to it being transformed into grape. The stem/loop structure appears to be quite interesting in the context of suppressing PCD. When we investigated the secondary structure of the sequence by folding analysis with the program mFOLD, the data revealed a striking conservation of the both the stem and loop sequence between cDNA 456 and *Bcl2* 3'UTR (data not shown). 3' UTRs have been shown to encode translational regulators. Dr. Martin Dickman and colleagues recently reported (10,11) protection against PCD in tobacco plants by expression of the 3'UTR of *Bcl2*, a widely studied apoptosis blocking gene in humans. The cDNA 456 protection in our experiments (15/25 independent transgenics fully protected after 10 months) as shown in **Table 2**, was due to RNA (presence confirmed by Northern analysis) and not a translated protein (lack of open reading frame based on comparison to the p23 gene). This indicates that the PD suppressive action is resident in the 32 bases comprising the cDNA 456 stem and loop. We hope to expand this project to determine if the cDNA 456 transformed Freedom and Thompson Seedless rootstocks that are protected against PD symptoms will transfer the protective effect across a graft to untransformed winegrape varieties.

The effect of anti-apoptotic transgenes on symptom development and Xf bacterial populations were measured by RealTime quantitative PCR (qPCR) (Figure 3 and 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 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

Table 2 Thompson Seedless Transgenics Genotype evaluated at 9 months post inoculation (November, 2008)	# of Lines evaluated falling into each category from 1-5 See Figure	Bacterial load per 0.1 gm of stem in each respective line. Determined by qPCR.
Inoculation with $2X10^5$ cfu		
TS - CBP14LD Category 5	17/23	$2.3 X 10^2$
TS - CBP14LD Category 4	0	
TS - CBP14LD Category 3	1	$1.0X 10^1$
TS - CBP14LD Category 2	2	$1.3 X 10^8$
TS - CBP14LD Category 1	3	$2.6 X 10^8$
TS – CB456 in Category 5	15/26	$2 X 10^2$
TS – CBP14 –B (leader deleted) All 14 plants in category 1 (dead)	14	$6.1 X 10^9$
TS – (Control) all 6 plants in category 1 (dead)	6	$1.8 X 10^8$
Vitis californica Category 5, no visible symptoms	2	$1.0 X 10^4$

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 6 orders of magnitude. Our 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 titers ranging from $10^2$to 10^4 in the main canes of the inoculated plants (Table 2). Representative images of plants in the second inoculation with 20 µl at $2X10^5$ are shown in Figure 3.

Bacterial Plating for determination of bacterial viability in the control and transgenic plants.

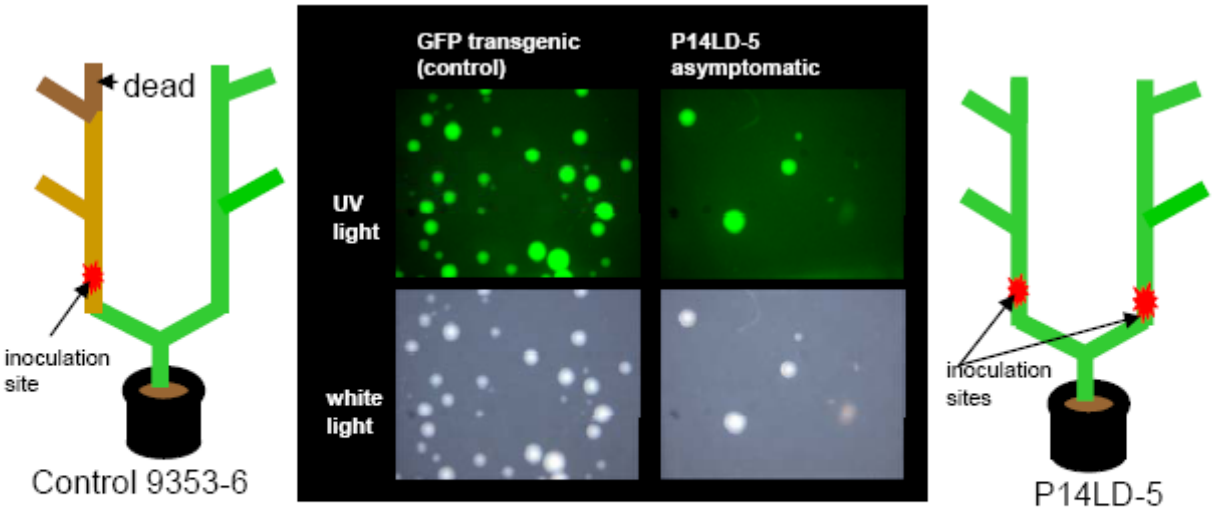

Figure 5. Live GFP-tagged *Xf* bacteria isolated from extracts of infected transgenic Thompson Seedless plants that did and did not carry an anti-PCD gene. Shown are micrographs of *Xf* growing on PD3 plates supplemented with 30µg/ml kanamycin.

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 in the control and transgenic protected plants as shown in Figure 5. The relative amounts of bacterial estimated by qPCR and the color plates showing representative fields on the media plates with colonies of GFP-expressing bacteria 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*, not dead cells. Lastly, 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 titer to a level that, while detectable, is orders of magnitude lower than the untransformed control plants but below the level capable of causing disease. The plating data also confirm the quantitative detection of live bacteria by qPCR and not a skewed estimate of bacterial populations in the respective plants by amplification of DNA from dead bacteria.

Conclusions:

In the past year we successfully demonstrated resistance against PD in the susceptible grape variety Thompson Seedless by one anti-apoptotic transgene (PR1) one 270 bp DNA sequence homologous to the 3'UTR of a nematode up-regulated gene designated p23. Both cDNAs were recovered anonymously from the plant-based cDNA screen and have functional links with conserved domains to anti-apoptotic orthologs in the animal kingdom (1,12). We further demonstrated that expression of these sequences, not only protected the transgenic plants against PD symptoms and plant death but maintained the population of *Xf* at four to six orders of magnitude below the level observed in untransformed plants that died within 2 months (10^8 bacteria per gram of stem tissue) following controlled inoculations in the greenhouse. One 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. In summary, our 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. Lastly and most importantly, viable plants with resistance to Pierce's Disease have been produced, the proof of concept validated, and plans being prepared for field evaluation of the material.

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